THE EFFECTS OF METFORMIN ON THE VASCULAR SYSTEM

Helen Elizabeth Archer
Doctor of Philosophy

Aston University
September 2003

Summary

Aston University

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Metformin is a commonly used antidiabetic drug. In addition to its glucose and lipid lowering effects, it appears to have independent effects on the vascular system, which may increase the quantity and quality of life of diabetic patients by reducing macrovascular complications associated with diabetes. This thesis aims to address some of the mechanisms through which metformin may exert its vascular effects.

Macrovascular contraction and relaxation effects of metformin were measured using a Mulvany Halpern myograph. Mouse aortic ring sections were treated for 1 and 4 hours in vitro with metformin at 10^{-5}M, and for 2, 4 and 8 weeks in vivo with metformin at 250mg/kg/day. The rings were contracted with increasing concentrations of noradrenaline (10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M) in the absence and presence of metformin. Maximally contracted tissue was then relaxed using increasing acetylcholine concentrations (10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M). Metformin increased the sensitivity of the aorta to noradrenaline-induced contraction. The maximal effect in vitro was seen after 4 hours giving a 221% increase in contraction after 4 hours at noradrenaline 10^{-6}M. Acetylcholine-stimulated relaxation via endothelium also increased with metformin after 4 hours by 36.85%. The maximal effect of metformin treatment in vivo was seen on aortic contraction after 8 weeks: the effect of metformin treatment on relaxation was less marked at this time. Metformin also increased passive tension generated by the aortic vessel wall after 4 hours, which was reversed by administration of papaverine, which acts directly on vascular smooth muscle.

Metformin was shown not to alter nitric oxide production by the mouse aortic wall after 1 and 4 hours in vitro. Metformin lowered basal calcium concentrations, as measured by FURA/2AM, generating a slow sustained increase in calcium release induced by noradrenaline during contraction.

This research programme has shown that metformin can increase both the contraction and relaxation capabilities of aortic sections treated both in vitro and in vivo with therapeutic concentrations of metformin at 10^{-5}M. Metformin has been shown to act directly in the vascular wall to alter vascular contractility via effects on both vascular smooth muscle and endothelium, and to influence calcium movements independently of nitric oxide.

For Mum and Dad, Marion, Ian and Chris.
Dedicated to the memory of Gran.

Acknowledgements

This thesis would not have been possible without the valuable advice of my supervisors Dr Clifford J Bailey (Life and Health Sciences, Aston University) Dr Harry Howlett and Dr Nicolas Wiernsperger (Merck-Sante). I would also like to thank Dr Oliver Downing, Dr David Poyner and Dr Nick Hartell and the late Dr John Smith (all Aston University) for their advice and assistance.

A special mention of thanks for Mrs Susan Turner (Aston University) for her excellent technical advice, encouragement throughout the course and her friendship, which has helped me through some difficult times.

I would also like to thank Mr Brian Burford, Mr Melvin Gamble, Mr Alan Richardson, Mr Derek Stirling and Mr Kevin Hughes (all Aston University) for their expert assistance and technical advice, without which this project would not have been possible.

Finally, I would like to thank all my family and friends for their unfailing support and encouragement through thick and thin, especially Mum and Dad, Marion and Ian, Auntie Janet, and last but by no means least Chris.

This project was sponsored by Merck-Sante

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Posters presented

- **Temporal co-ordination of insulin-stimulated release of nitric oxide from vascular endothelium**
- **Effect of metformin on aortic contractility**
- **Direct effect of metformin on vascular contractility**

Chapter 1: - Introduction

1 Introduction

This thesis investigates the vascular effects of the antidiabetic drug metformin. The investigation arose from circumstantial evidence suggesting that metformin might reduce the development and progression of macrovascular disease, which is an underlying cause of coronary heart disease, independently of its effects on glycaemic control in patients with type 2 diabetes. Principal studies focus on the direct and indirect effects of metformin on myograph parameters of vascular contractility and relaxation using isolated mouse aorta. Cellular mechanisms were also studied with the aid of mouse aortic tissue and cultured A7r5 vascular smooth muscle cells, focusing on the nitric oxide pathway and the role of calcium.

1.1 The prevalence of diabetes

The prevalence of type 2 diabetes is increasing at an alarming rate. It is estimated that worldwide the prevalence of diabetes was 135 million people in 1995 and it is projected by the World Health Organisation (WHO) global study figures that there will be 300 million people diagnosed with diabetes worldwide by 2025 (King et al 1998). In the UK, the current figure stands at 1.4 million people, a prevalence of 2.5%. There are over a further 1 million estimated to have type 2 diabetes, who do not know they have the condition, and are yet to be diagnosed (Day 2001). These people are also at risk of developing complications as raised blood glucose concentrations cause extensive tissue damage, particularly to the microvascular

system and the longer these individuals remain uncontrolled the greater the degree of damage, particularly to the eyes, nerves and kidneys. It has been shown that intensive treatment over a 6 year period effectively delays the onset and slows progression of retinopathy, nephropathy and neuropathy in type 2 patients (Diabetes control and complications trial research group 1993). Diabetes is also associated with premature and advanced macrovascular disease, which is largely responsible for the reduced life expectancy. Type 2 diabetic patients with no history of myocardial infarction (MI), have the same risk of infarction as non-diabetic patients who have had a prior MI (Haffner et al 1998). It is well documented that in type 2 diabetes cardiovascular complications are the leading cause of death and are responsible for 70% of patient deaths. (Marso 2002). It was estimated that in 1989 diabetes cost 4-5% of the total UK healthcare budget (Laing and Williams 1989) and this is still rising. In 2000 Diabetes UK put the cost at £4.9 billion pounds, which is 9% of the entire NHS budget (Diabetes in the UK-Fact sheet 18). The social, medical and economic cost of managing diabetes and its secondary complications could have a profound impact on society.

Type 2 diabetes is more prevalent in affluent societies and is therefore often referred to as a disease of the western world. There is also a higher incidence of type 2 diabetes among different socio-economic groups, with those from poorer backgrounds or inner city areas showing a higher incidence in both male and females Figure 1-1: - Prevalence of type 2 diabetes in different socio-economic groups in England and Wales. It accounts for >90% of all diabetic cases. Type 2 diabetes mainly develops in the middle aged and the elderly, being diagnosed by high circulating blood glucose concentrations (random >11.1mmol/l, fasting >7.0

mmol/l, WHO, Geneva, 1999). It is usually attributed to the combined effects of insulin resistance (the inability of insulin to act with normal potency on target tissues such as the muscle) and islet β-cell dysfunction. Although type 2 diabetes is most common among the elderly (Wahl et al 1998) 65 years and older, excess dietary calories, obesity and lack of exercise can lead to cases of type 2 diabetes in younger generations. Indeed there has been a recent increase in diagnosis of type 2 diabetes in children and teenagers (Fagot-Campagna 2001). Certain ethnic groups are also more prone to developing type 2 diabetes see Figure 1-2:- Prevalence of adult type 2 diabetes in different ethnic groups in England., e.g. Asians, Afro-Caribbeans (Marks 1996). It is therefore important that improvements are made in the prevention as well as the detection of diabetes; if the present situation remains unchanged, there will be a danger of healthcare systems becoming bankrupt by the problem (Day 2001).

Figure 1-1: - Prevalence of type 2 diabetes in different socio-economic groups in England and Wales.

Information from: - the office for National Statistics 2001 based on type 2 patient statistics taken from general practice. Adapted from Day 2001.

Figure 1-2:- Prevalence of adult type 2 diabetes in different ethnic groups in England.

Data sourced from Marks et al 1996 and the office for national statistics 2001. Adapted from Day 2001.

1.2 Types of diabetes

The prevalence of diabetes throughout the world varies, but in the UK in 2000 the figure stands at about 2.5% of the population (Holmes 2000) and this figure is constantly rising. There are two main types of diabetes, and their main features are summarised below in Table 1-1: - Comparing type 1 and type 2 diabetes: -

Table 1-1: - Comparing type 1 and type 2 diabetes

Table adapted from Lauralee Sherwood, Human physiology, from Cells to Systems, West publishing company (1993), 676.

Type 1 diabetes usually presents in childhood. It is an autoimmune condition in which antibodies attack pancreatic insulin secreting cells, the beta cells in the islets of Langerhans. The triggers for this condition involve a combination of genetic predisposition and environmental factors. For example, early exposure to certain viruses, wheat or cows milk and an increased dietary nitrate and nitrite content appear to cause hypersensitivity to insulin (Zimmet et al 2001). Research from Australia also suggests that infected vegetables may be a source of chemicals, which damage the pancreas, e.g. soil bacteria such as *Streptomyces* produce toxins that may initiate an autoimmune attack (Myers et al 2002). In type 1, insulin is no longer naturally secreted, so regular subcutaneous injections of the hormone are given to sustain life and control blood glucose concentrations, in an attempt to mimic the natural endocrine action of the pancreas.

Type 2 diabetes mellitus (T2DM), which is the focus of this study, usually presents in middle age and later. The mean age of people with type 2 diabetes in the T^2ARDIS (Type 2 Diabetes: Accounting for a major resource demand in society) study in the UK is 67 years and a mean time since diagnosis of 8 years (Bottomley 2001). In T2DM insulin is still secreted in modest amounts and occasionally larger amounts than normal, but the target tissues become less sensitive to insulin (Kahn et al 2003). There is typically a genetic predisposition to the disease, but there are many complex contributory factors that add to the long pathogenic process of this condition. These contributory factors include lifestyle, obesity, overeating and under-activity, long infections and various drugs e.g. glucocorticoids can also impair glucose action, which all contribute to developing diabetes (Davidson 1995). Long periods of chronic stress can also elevate blood glucose concentrations by

counteracting insulin action or reducing beta cell functions. Reduced insulin action can also occur as part of the natural ageing process, where a deterioration of homeostatic control increases the risk of developing the condition.

There is increasing concern in Europe and North America in particular that the occurrence of type 2 diabetes is rising inordinately. In 1997, it was estimated 124 million people worldwide had diabetes, 97% of these had type 2 diabetes (Amos et al 1997). This indicates that western lifestyles appear to increase the risk of developing type 2 diabetes and the average age of diagnosis appears to be decreasing. Studies in different countries of north America and Europe have suggested that the average age of diagnosis for T2DM is around 60 years of age, and it is estimated that these individuals will have been experiencing pathological hyperglycaemia prior to diagnosis due to the presence of established complications at diagnosis (U. K. Prospective Diabetes Study Group. 1995). There are more young adults and even children are now presenting with Mature-onset diabetes of the young the onset occurs before the age of 25. Research suggests these children and adolescents may have a primary defect in β cell function in the pancreas, caused by a mutation in any one of six genes identified (Fajans et al 2001).

It is estimated that nearly 50% of type 2 diabetic patients before diagnosis have undetected diabetes for many years (probably 10-12 years) (Davies and Gray 1996). This means that about 1.4 million people in the UK are unaware they have the condition. These patients are not receiving treatment and are increasing the risk of diabetic complications in later life. Indeed, about 35% of T2DM patients already have a complication of diabetes at diagnosis (Diabetes Update, Winter 2000). This

is a major health care issue, since early detection and effective treatment are key elements to reduce the degenerative complications of the disease. The following are warning signs that indicate a person may have type 2 diabetes: -

Increased thirst and an increased desire to urinate

Extreme tiredness and blurred vision

History of being overweight and unexplained weight loss

Genital itching/ regular episodes of thrush and wounds slow or fail to heal

Dyslipidaemia, hypertension, and other existing cardiovascular diseases

First-degree relatives with T2DM also persistent infections

Overweight babies and known intolerance to glucose during gestation.

All of the above suggest that individuals should be screened for type 2 diabetes. (The above list was adapted and taken from a Diabetes UK promotional leaflet, and Krentz and Bailey 2001.)

The American Diabetic Association (ADA) screening for diabetes has recommended that all patients of 45 years of age and over who are at risk of diabetes, should be regularly screened for diabetes, with a repeat screening every 3 years to highlight problems earlier than normally detected (ADA, Diabetes care 2002). It is also suggested patients who are obese 120% of desired body weight or BMI 27kg/m^2, and those with first-degree relatives with diabetes. Some ethnic groups such as African-Caribbean or Asian people are more prone to diabetes and are 4 - 5 times more likely to develop diabetes than the white caucasian (europid) population. It is therefore recommended that screening should be started much earlier and take place more frequently in these groups to improve lives and save scarce health resources (Davidson 2001). The United Kingdom Prospective

Diabetes Study (UKPDS) was a study that started in 1977 and ran for over 20 years: it was a randomised trial specifically concentrating on the treatment of type 2 diabetes. The results confirmed that complications were significantly reduced if blood glucose and blood pressure were tightly controlled. For a 1% reduction in HbA_{1c} there was an associated reduction in risk of 21% for any diabetes related endpoint over 10 years (UKPDS 35, Stratton et al 2000). Recent evidence also suggests that reducing the rise in postprandial glucose is also beneficial to lowering the development of complications in particular lowering cardiovascular risks (Banora and Muggeo 2001).

1.3 Current methods of diagnosis and treatment of diabetes

There is an urgent need to ensure that the detection of type 2 diabetes is made early. Substantial evidence suggests that the earlier the implementation of tight glycaemic control the better the prevention of associated diabetic complications (Campbell 2000). The World Health Organisation has recently revised the target for fasting plasma glucose (FPG), lowering it from 7.8 mmol/L to 7.0 mmol/L as the diagnostic boundary for diabetes (WHO, Geneva 1999). Alternatively a random plasma glucose concentration >11.1 mmol/L is diagnostic of diabetes or 75g of glucose is given and the plasma concentration is taken after 2 hours for an oral glucose tolerance test. HbA1c tests are only currently recommended to monitor the control of known diabetics (Barr et al 2002). The HbA1c expresses a strong correlation between the concentration of glycated haemoglobin and the mean concentration of glucose concentrations over the previous 1-3 months (Nathan et al 1984). The UKPDS found that if intensive therapy lowers HbA1c concentrations by 0.9% compared to conventional treatment over 10 years, this substantially reduces

mortality and morbidity (UKPDS 33, Lancet 1998). For example a later study by the UKPDS showed a 1% fall in plasma glucose over 10 years caused a 37% reduction in microvascular complications (Stratton et al 2000). So vigorous treatment is important, and may require the use of several agents to treat the condition effectively. Blood pressure control is also important showing a 15% reduction in deaths related to diabetes (Adler et al 2000) with a reduction in systolic blood pressure of 10mm Hg per 10 years. A basic if somewhat outmoded diagnostic test for diabetes is glucose being present in the urine. When the concentration of glucose in the plasma exceeds the renal threshold of 10 mmol/l glucose appears in the urine (Davidson 1995). It is important that this is spotted as soon as possible, as this makes diabetic patients susceptible to urinary tract infections and in the long-term kidney damage.

The pathophysiology of type 2 diabetes is often separated for convenience into two stages: - The first is the development of insulin resistance often accompanied by an increase in insulin secretion, eventually followed by a decrease in beta cell function. The second stage is due to a more severe insulin secretory defect (a fall in the amount of insulin secreted is insufficient to compensate for insulin resistance). This new technique by the WHO of "clinical staging" replaces the previous system of "clinical classes" (Bennett 1999). The new diagnostic criteria should increase the number of diabetic patients identified, establishing earlier, more effective treatment to reduce the incidence of complications. The widening of diagnostic techniques should ensure that symptoms are treated before obvious complications have set in. Approximately 35% of type 2 diabetic patients show complications at diagnosis, as the onset of diabetes can occur up to 20 years prior to diagnosis, meaning

considerable damage has already occurred before the condition is recognised. Macrovascular complications associated with myocardial infarction can begin up to 20 years prior to type 2 diagnosis. Atherosclerotic changes that predispose patients to stroke can begin up to 12 years before diagnosis, (Diabetes Update, winter 2000) this is illustrated in Figure 1-3: - Calculating the onset of vascular Complications.

Figure 1-3: - Calculating the onset of vascular Complications

Adapted from the Diabetes UK Winter 2000 issue. The figures are calculated by using the average yearly increase in complications and by extrapolating backwards, it is possible to determine the time when the damage began to occur.

1.3.1 Dietary Measures

At the diagnosis of type 2 diabetes there are various treatment options available. The first approach is to attempt to control the glucose concentration by diet and other non-pharmacological measures. Most of these diets aim for the diabetic patient to achieve and maintain their ideal body weight. There are also unmeasured diets that have a list of foods to be avoided and items that can be eaten when desired (Davidson 1995). Research by Chandalia (2000) gave subjects a high fibre diet (24g, of which 8g was soluble) showed an absence of weight loss but a decrease in plasma glucose concentrations, confirming earlier work by Wursch and Pi-Sunyer (1997) that a high fibre diet benefits diabetic patients. The basis of dietary advice is an intake of 55% of the total energy content of the diet should

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promoting better glycaemic control. There are few side effects; the most common adverse effect is episodes of hypoglycaemia. Sulphonylureas cause increases in body weight if they are used at high doses and for prolonged periods and they are not recommended for patients already suffering from obesity (Davidson 1995).

1.3.3 Biguanides

Biguanides act by increasing the sensitivity of certain tissues to insulin such as the liver, and peripheral tissues such as the skeletal muscle and adipose tissue (Campbell 2000). They act in a manner that is complementary to treatment with sulphonylureas as they allow the insulin secreted to act more effectively on tissues. There were 3 main antidiabetic biguanides were introduced clinically in the late 1950s and early 1960s: -buformin, metformin and phenformin. Phenformin was withdrawn in the 1970s due to a high number of cases of lactic acidosis (Nattrass and Alberti 1978), and buformin, which has limited use in a few countries. Metformin is now considered the biguanide of choice and takes precedence among this group of drugs (Bailey 92). Metformin is the most commonly used biguanide: it became available in the UK in 1960 and in the US in 1995 (DeFronzo 1995), it counters insulin resistance in target tissues and reduces plasma glucose, and this form of treatment especially with metformin rarely causes hypoglycaemia (Garber et al 1997). Biguanides were first discovered in the 1920s and then rediscovered in the 1950s. Metformin is now used worldwide and is now the most widely used oral antidiabetic agent, and is the only biguanide still used in most countries as other biguanides were withdrawn as they caused lactic acidosis. However metformin rarely causes this problem when used in patients with normal renal function (Davidson 1995), but it can cause side effects associated with the gastrointestinal

tract this can limit the use of high doses in some diabetic patients. The benefits of metformin will be addressed in more detail later, as this drug is the main focus of this study due to its reported ability to lower mortality and the reduction of micro- and macrovascular complications in obese diabetic patients (UKPDS 34, Lancet 1998).

1.3.4 Alpha – glucosidase inhibitors

An example of this type of inhibitor is acarbose, which was introduced, in the early 1990s; other drug members of this group include Voglibose and Miglitol. It acts on the alpha– glucosidase enzymes in the brush border of the small intestinal cells and slows down the digestion of oligosaccharides to monosaccharides by direct reversible enzyme inhibition (Nattrass and Bailey 1999). This reduces the rate of which glucose is absorbed and this in turn lowers postprandial hyperglycaemia. Alone it is less successful at lowering hyperglycaemia than the previous two groups of agents. Alpha glucose inhibitors act mainly on postprandial hyperglycaemia, which reduces this postprandial rise in glucose after eating, reduces complications (Lebovitz 1999), since it has little effect on basal hyperglycaemia. Acrabose can also increase hepatic transaminases, which is reversible if treatment is withdrawn, but liver transaminase enzymes should be monitored for the first 12 months of treatment (Wallace and Matthews 2002). The potential side effects of abdominal discomfort, flatulence and diarrhoea mean that the dose used in some patients is limited (Bailey 2000). These side effects and limited efficacy, limits this treatment to an average of three years. In the UKPDS the discontinuation rate was high compared to other treatments, with a 49% non-compliance rate after 1 year and 39% after 3 years (UKPDS 44, Holman et al 1999).

1.3.5 Thiazolidinediones

Thiazolidinediones have a similar effect to biguanides, but they act through a different mechanism to biguanides to reduce insulin resistance. Thiazolidinediones mainly act in the peripheral tissues by acting as insulin sensitisers (Smith 1999) through effects on adipose tissue as well as increasing glucose uptake by skeletal muscle and decreasing gluconeogenesis in the liver, and TNFα-induced insulin resistance is partially reversed. They also have the added benefit of reducing free fatty acid concentrations in the plasma (Bailey 2000). Their mechanism of action is mainly by binding to the nuclear peroxsome proliferator-activated gamma (PPARγ) receptor on the nuclear membrane as described by Desvergne and Wahli 1999, which is expressed predominantly in adipose tissue. They also act to improve insulin sensitivity in muscle, where PPARγ is expressed to a lesser extent (Rocchi and Auwerx 1999). It increases insulin sensitivity by increasing fatty acid (FA) clearance from adipose tissue, while not increasing FAs in muscle. In essence, the FAs become trapped in the fat tissue decreasing its availability and therefore its uptake by muscles improving insulin sensitivity (Randle et al 1963). Thiazolidinediones act on adipose tissue to reduce adipose tissue components that circulate (such as free fatty acids, tumour necrosis factor α which inhibit the insulin signalling cascade, and the hormone Adiponectin is decreased during insulin resistant states), this increases insulin sensitivity (Lebovitz 2002). They stimulate insulin sensitive proteins involved in the metabolism of lipids and glucose including acyl CoA synthase, malic acid, GLUT 4 transporters; it also enhances synthesis of lipoprotein lipase and fatty acid transporter proteins (Bailey 1999). However there are also adipose-independent mechanisms of action of thiazolidinediones as they retain their insulin sensitising activity in transgenic mice

lacking adipose tissue (Burant et al 1997). There are 3 main Thiazolidinediones: - Troglitazone, Rosiglitazone and Piolitazone. One of the first agents discovered in this group Troglitazone, was withdrawn after it caused severe hepatotoxicity (Campbell 2000). In addition, two other thiazolidinediones Ciglitazone and Englitazone did not progress to clinical development as they had adverse effects on the liver. Darglitazone did not have these adverse liver effects and was safer for clinical trials (Day 1999). Newer agents such as Rosiglitazone and Pioglitzazone do not appear to show this adverse effect. They do not cause hypoglycaemic episodes, but some of their side effects include an average of 3 - 4kg increase in weight in the first year alone. However there is a redistribution of fat away from the visceral region, which is closely linked to insulin resistance (Montague and O'Rahilly 2000). Oedema is also a problem in 5-10% of patients treated with a thiazolidinediones (Bailey and Day 2003).

1.3.6 Meglitinides

Two frequently used members of this group repaglinide and nateglinide act to transiently increase the secretion of insulin. They act in a similar way to sulphonylureas but for a much shorter duration and bind to a different site on the sulphonylurea receptor (Fuhlendorff et al 1998). They initiate insulin secretion by closing the K^+ ATP channel (Dornhorst 2001). The rapid "on-off" action of meglitinides helps to prevent hypoglycaemia especially between meal times and at night (Campbell 2000). They are usually taken just before meals to reduce post-prandial hyperglycaemia. The commonest side effect with meglitinides is weight increase. Mitiglinide is a new meglitinide analogue from this group and currently undergoing phase II trials in Europe.

1.4 Treating the progressive deterioration of glucose control

All types of monotherapy gradually fail as the natural history of T2DM progresses. After 3 years of monotherapy ~50% of patients in the UKPDS failed to achieve HbA1c values in the desired range of <7% (UKPDS 49, Turner et al 1999). This requires addition of a second oral antidiabetic agent. Here two differently acting types of agents from the above classes can be used in combination to offer the patient the optimum glycaemic control. This can be very effective when each agent treats one of the two defects commonly associated with diabetes, e.g. insulin resistance and impaired insulin release (Campbell 2000). Effective combination therapy should improve the patients' quality of life and reduce clinical symptoms, restoring glucose within normal concentrations in a fasted and postprandial state. It is essential combination therapy be introduced earlier to aim for better metabolic control and reduce or delay the development of long-term vascular complications (Van Gaal and De Leeuw 2003). Unfortunately if inadequate glycaemic control persists, this indicates beta cell failure, which is likely to be due at least in part to a reduction in β cell mass (Bailey and Day 2003). In such cases in order to provide adequate glycaemic control, insulin therapy is introduced. Treatment with insulin cannot always achieve ideal glycaemic control due to the risk of hypoglycaemia and weight gain. It should be appreciated though that insulin injections do not completely return glycaemic control to normal, and the detrimental cycle of hyperinsulinaemia and insulin resistance still continues.

Early treatment and effective control of type 2 diabetes is very important due to the degenerative nature of the disease. Hyperglycaemia is one of the most significant risk factors, and by implementing stricter control and a more aggressive approach

to treatment (not deciding treatment by failure), is a much more effective approach for preventing long term complications. As illustrated by the UKPDS 35 (Stratton et al 2000), just a 1% reduction in HbA_{1c} reduces the risk of macrovascular complications by 14% and microvascular complications by 37%. Combination therapies are able to target both insulin resistance and impaired insulin secretion. This is why combination therapy used early in the treatment process has potentially significant benefits and gives the patient the greatest chance of avoiding the long-term complications.

1.4.1 Obesity treatments

Obesity is one of the risk factors that cause diabetes and it can aggravate treatment of the condition. Most therapies apart from metformin actually cause weight gain, so it is important to control the weight of the patient as this can complicate treatment regimens. Modest weight reduction improves insulin sensitivity as well as glycaemic control and reduces blood lipid concentrations (Hollander et al 1998). Orlistat also decreases total LDL cholesterol reducing the cardiovascular risk of these patients (Kelley 2002). Two new anti-obesity agents are available to reduce and keep weight constant during treatment. Orlistat is a gastrointestinal lipase inhibitor, which binds to the active site of the digestive enzyme lipase: this reduces hydrolysis of lipids. As a result, Orlistat causes impaired lipid absorption, but can alter the absorption of other medications also. On average, it causes a loss of 2–4 kg beyond that achieved with a restricted diet (Nattrass and Bailey 1999). The other anti-obesity drug is Sibutramine, which is a serotonin and noradrenaline re-uptake inhibitor and enhances the satiety response after eating. It also increases thermogenesis by acting on brown adipose tissue. On average it causes 3–6 kg

weight loss (Day and Bailey 2002), additional to that achieved with an energy-restricted diet. There is a significant reduction in waist size, reducing visceral obesity and (potentially) the risk of coronary heart disease (CHD).

1.4.2 Insulin treatment

In T2DM, insulin is only used when all other forms of treatment fail to achieve adequate glycaemic control, and about 25% of diabetic patients are treated with insulin (Edelman and Henry 1995). There are two possible courses of action; the first is a once daily injection of isophane insulin normally given alongside an oral antidiabetic agent such as a sulphonylurea or metformin as a combination therapy. Insulin is normally given in the morning and is best suited to elderly patients. The most common treatment is a multiple injection regimen with twice-daily injections of premixed insulins, a rapid action 30% and 70% isophane an intermediate acting insulin. Insulin treatment causes weight gain and therefore with this treatment oral antidiabetics can cause additional weight gain (such as sulphonylureas) should be contraindicated. It is still safe to use metformin, as weight gain is not associated with its use (Wallace and Matthews 2002).

Type 1 diabetics follow a stricter regime of taking preprandial soluble insulins and nocturnal isophane to maintain basal glucose. This treatment is not currently used in T2DM, but evidence suggests postprandial glucose control is crucial in glycaemic control. Similar regimes to type 1 diabetes may be introduced in the future.

1.5 Insulin resistance

Insulin resistance is defined as decreased tissue sensitivity to the actions of insulin. About 25% of the population with normal glucose tolerance show a degree of insulin resistance (Reaven 1994). People with insulin resistance diagnosed using the homeostasis model assessment (HOMA) developed by Matthews et al (1995) but who were non-diabetic showed an increase incidence of myocardial infarction and death (Hedblad et al 2002). There are several different causes of insulin resistance. The commonest predominant presentation of insulin resistance in T2DM appears to result mainly from genetically inherited and environmentally acquired abnormalities in the function of intracellular signalling intermediates for insulin. In some individuals with T2DM there is a decrease in insulin receptors on the target tissues, which is not rate limiting. However, a contributory defect to this resistance is the malfunction of the insulin receptor, for example a problem with the receptor signalling and coupling, such as inadequate tyrosine kinase activity. For example in muscle the main defect is a block in the glucose transport/ phosphorylation step, but defects in muscle alone can not account for the degree of hyperglycaemia in T2DM (Reaven 1995). Defects at key stages further down the signalling pathway may also contribute to insulin resistance. These defects can have both a genetic and environmental contribution (American diabetes association 1997). A rare case is a defective insulin molecule or receptor.

Another possibility is high levels of a circulating antagonists causing de-phosphorylation of a key signalling molecules such as tyrosine kinase. These factors all contribute to the development of T2DM. As a result, less glucose is stored in the body tissues as fewer glucose transporters are recruited to stimulate

glucose uptake into muscle and fat. In an attempt by the body to compensate for the elevated glucose concentration (hyperglycaemia), more insulin is secreted so hyperinsulinaemia often results in an attempt to compensate (DeFronzo 1997). This can further aggravate the development of diabetes and its complications due to the down regulation of receptors. Increased insulin exposure induces internalisation and degradation of insulin receptors and impairs some steps of postreceptor signalling. In some individuals, the beta cells are unable to maintain a sustained increase in insulin concentration and deterioration occurs leading to the progression of T2DM (DeFronzo, Bonadonna and Ferrannini 1992). Figure 1-4: - The pathogenesis of T2DM. When the insulin concentration declines, there is an increase in free fatty acids (FFA) and an additional decrease in glucose uptake in the muscle. There is also an increase in hepatic free fatty acid oxidation and gluconeogenesis, the elevation of FFA and glucose further aggravate ß- cell function. Insulin resistance is usually associated with metabolic disorders, but it can be present with no warning signs (Bonora et al 1998).

Figure 1-4: - The pathogenesis of T2DM

Krentz A. J and Bailey C. J (2001) *Type 2 Diabetes in Practice*. 14. Original source from DeFronzo (1988) *Diabetes*. **37: 667-687.**

There is an increase in insulin resistance and a fall in beta cell function, in type 2 diabetes. This is shown in Figure 1-5: - The gradual progression of insulin resistance and beta cell failure. In severe insulin resistance, a small decline in beta cell function triggers diabetes (thick line Figure1.5). There is a more dramatic fall in beta cell function in people who are insulin sensitive (thin line Figure 1.5).

Figure 1-5: - The gradual progression of insulin resistance and beta cell failure

Adapted from O'Rahilly S and Saville J (1997) *British Medical Journal*. **314:** 955-959.

There is also an elevation in total triglyceride, VLDL cholesterol and lowered HDL cholesterol concentrations associated with insulin resistance, aggravate the condition in key tissues such as the liver and muscle. They also put insulin resistant individuals at greater risk of developing atherogenic cardiovascular conditions (Haffner et al 1999). Impairment of insulin action also causes elevated glycogenolysis and gluconeogenesis increasing already elevated concentrations of glucose. Evidence from Misra et al (1997) has shown that total and intra-abdominal body fat contribute to insulin resistance. Subcutaneous fat made the largest contribution to the degree of insulin resistance (Groop 1999).

1.6 Current long term complications in diabetes

There is an abnormally high mortality rate amongst type 2 diabetic patients in all causes, with 75 - 80% of these deaths resulting from premature macrovascular disease associated mostly with CHD (Panzram 1987). The prevalence of cardiovascular disease in diabetes compared to the general population was discussed as early as 1979 in the Framingham study, and the particular risk to women diabetic patients was highlighted (Kannel et al 1979). Type 2 diabetes is increasing in incidence as the population in most western countries begins to age. As a result, multiple pathologies are more common, and cardiovascular disease is often already present at diagnosis. The condition in diabetic patients is generally more extensive and severe than seen in the general population. Adults with diabetes have an annual mortality of 5.4%, and their life expectancy is reduced on average by 5-10 years compared with the general population (Donnelly et al 2000).

In the Munster Heart Study (Standl 1999) 9 variables were identified which independently contribute to coronary heart disease; - age, smoking history, personal history of angina pectoris, family history of myocaridal infarction, high systolic blood pressure, high levels of low-density lipoprotein (LDL), low levels of high-density lipoprotein (HDL), high triglyceride level and the presence of type 2 diabetes

The following risk factors tend to be higher in diabetic patients: - obesity, higher plasma lipid concentrations (dyslipidaemia), hypertension, atherosclerosis and a procoagulant condition all increasing their predisposition to CHD. These disorders are linked to insulin resistance and are usually referred to as the "Insulin Resistance

Syndrome," "Metabolic Syndrome," "Dysmetabolic Syndrome" or "Syndrome X," it is even known as Reaven's Syndrome. The Hoorn Study (Gerritsen et al 2000) showed that 8/10 problems with cardiovascular autonomic function were linked with glucose intolerance, others were moderately associated e.g.: - age and an increased waist to hip ratio (indicates central adiposity stores).

There are two main types of blood vessel damage caused by hyperglycaemia. The first is microvascular disease, which affects the small blood vessels (especially capillaries) causing thickening of the endothelial basement membrane with disruption of the protein matrix through glycation. This alters the structural integrity of the membrane, which increases the permeability of these vessels (Davidson 1995). Examples of the consequences include: - retinopathy the commonest cause of blindness in people 30-69 years of age, with a 3 fold increase in visual impairment in diabetic patients (Hayward et al 2002). There are 4 main categories of eye disease: - background retinopathy caused by micro-aneurysms and haemorrhages not associated with visual loss, diabetic maculopathy the commonest cause of vision loss, proliferative diabetic retinopathy causes vision loss due to its complications and finally advanced diabetic eye disease (Kohner and Barry 1984). Nephropathy which is diagnosed by the presence of microalbuminuria greater than 300mg/ 24 hours, these patients are likely to progress to end-stage renal disease over a period of a few years (Gall et al 1997). Finally neuropathy, affects 40-50% of all patients with diabetes (Donnelly et al 2000). Neuropathy is caused by hypoxia and hyperglycaemia, which leads to anaerobic glycolysis in diabetic nerves; this causes alterations in K^+ conductance (Thomas 1999).

The second is macrovascular disease, which affects the larger blood vessels causing atherosclerosis, and contributes to heart disease, which is the main cause of death in over 70% of diabetic patients in 2002 (Nicolaides and Jones 2002). Atherosclerosis is best described in 3 separate stages, these are: -initiation, progression and complications of the plaque (Ouriel 2001). Hyperglycaemia and oxidative stress associated with insulin resistance (Wheatcroft et al 2001), along with the receptor for advanced glycation end products; they activate factors that effect the expression of mediators of atherosclerosis. These include factors that attract monocytes and lymphocytes to the vascular cell wall and proinflammatory mediators and adhesion molecules on the endothelial surface, which all can be stimulated. Hyperinsulinaemia is the body's attempted compensatory mechanism to reduce hyperglycaemia, by the hypersecretion of insulin from the beta cells in the pancreas. However, it is not yet known how hyperinsulinaemia contributes to atherogenesis. The second stage occurs when the T- lymphocytes and monocytes reach the intima of the blood vessel wall. At this point, they secrete cytokines that trigger lesion formation. At the endothelial space monocytes ingest oxidised low-density lipoprotein (LDL) and become foam cells, as a result LDL is deposited in the walls of these large vessels causing atherosclerosis (Beckman et al 2002). The elevation of plasma fatty acids is due to a fall in lipid synthesis or increased lipolysis (linked with insulin resistance). The liver also synthesises more very-low-density lipoprotein (VLDL) and fewer high-density lipoprotein (HDL). The clearance of VLDL is reduced by insulin resistance, prompting dyslipidaemia. Vascular smooth muscle proliferation increases and fibrin is also deposited in the arteries, contributing to the formation of thrombic inclusions overlying unstable atherosclerotic plaques. Fibrin also stimulates endothelial cell proliferation and

induces binding and accumulation of LDL (Palumbo 1998). It is thought this is all brought about by damage to the endothelium, causing endothelial dysfunction, reducing the production of nitric oxide (NO). It is NO that inhibits vascular smooth muscle growth and adhesion of inflammatory cells to the endothelium (Monacada and Higgs 1993).

Elevated insulin concentrations cause reabsorption of tubular sodium mainly in the proximal tubule. Raised insulin also causes increased catecholamine concentrations, which increases sympathetic activity. Several characteristics of Syndrome X, such as hypertension may affect intracellular calcium distribution, favouring vasoconstriction in cardiac and in the peripheral smooth muscle. Insulin acts both alone and in combination with insulin-like growth factors (e.g. IGF-1) and stimulates proliferation of smooth muscle in the arterial walls, which can trigger or aggravate existing macrovascular disease (Palumbo 1998). The changes to blood vessels include an increase in the amount of connective tissue e.g. collagen, fibronectin and glycoproteins causing a loss of elasticity.

The British Diabetic Association (BDA) cohort study (1972 – 1993), and followed up until 1997 (BDA cohort study Q & A) showed high fatality rates from cardiovascular disease (CVD) of type 1 diabetic patients of 30+ years of age. It also indicated that the sex hormones in premenopausal female diabetic patients give no additional protection against CHD. The multiple risk intervention trial (MRFIT) showed that serum cholesterol, blood pressure and smoking are the major factors influencing coronary heart disease (CHD), and that these factors can be manipulated to prevent CHD (Stamler et al 1998). The Diabetic control and

Complications Trial 1993 (DCCT), provides compelling evidence of the benefits of intensive therapy in type 1 diabetes (Keen 2001). It showed that intensive insulin therapy reduces long-term complications by 35-76%. The UKPDS 33 provided similar evidence for the benefit of improved glycaemic control in type 2 diabetes.

It has been shown in several studies (e.g. DCCT and UKPDS) that the closer the blood glucose concentration is kept to normal, the fewer and less severe these abnormalities, and there is a lower incidence of both forms of vascular disease. The Wisconsin study carried out over 16 years showed a strong consistent relationship between hyperglycaemia and the incidence/ progression of micro- and macrovascular complications in type 1 and type 2 diabetic patients (Klein 1995). The Diabetes Intervention Study, 11 year follow up Hanefeld et al 1996, has evidence from the type 2 (non insulin dependent diabetes mellitus, NIDDM) UKPDS Policy Group that suggests the parameters for blood glucose, triglycerides and blood pressure are significant factors in indicating the risk of CHD and death. Therefore, better control of these three factors reduces the incidence of CHD and death.

1.7 Focus on macrovascular complications

In the present study the focus will be on macrovascular complications, as these cause the largest risk to premature death by contributing to CHD. In addition to this there are other treatments available that may lower the risks of macrovascular complications (Laakso and Lehto 1997). These include lowering total triglycerides, LDL cholesterol or total cholesterol and increasing HDL cholesterol and reducing hyperinsulinaemia, these predict the development of coronary artery disease, which are all disturbed during type 2 diabetes (Haffner 2002). At present there is evidence to support the theory that physical activity is a preventative measure, but further research is required to determine how intensive this should be. Dietary evidence suggests a diet low in saturated fats, may reduce the risk of CHD Rudel and Kelley et al (1998). Donnelly et al (2000) indicates that the commonest macrovascular disorders are ischaemic heart disease, stroke and peripheral vascular disease. These complications occur at a much higher incidence than in the general population. For coronary heart disease and stroke the increased incidence is between a 2-4 fold and 2-3 fold respectively, and there is a 2-3 times increase in peripheral vascular disease. The incidence of these conditions is well documented (Howlett and Bailey 1999). Microalbumin is also a well-established risk factor for cardiovascular disease and could be the most useful predictor of cardiovascular risk in diabetic patients (Fonseca 2000). Cardiovascular disease develops much earlier in diabetic patients and occurs in men and women equally unlike the general population. To reduce cardiovascular risk, 5 main factors are targeted in diabetic patients, these are: - blood pressure <130/80 mm Hg, LDL cholesterol <2.6 mmol/l, Triglyceride <1.7 mmol/l, HDL cholesterol >1.1 mmol/l, glycosylated haemoglobin <7% (American diabetes association 2003).

1.8 Therapeutic benefits of Metformin

Metformin dimethylbiguanide is made up of two guanidine molecules and its structure is illustrated in Figure 1-6: - The structure of metformin.

Figure 1-6: - The structure of metformin

The diagram was adapted from Bailey C. J (1992) Biguanides and NIDDM. Diabetes Care. 15. 6: 755.

Biguanides were first used to treat diabetes in medieval times, when *Galega officinalis* (goat's rue or French lilac) was used in Europe (Bailey and Turner 1996). It was only in 1918 that guanidine was proven to be a hypoglycaemic agent (Bailey and Krentz 2001). The late Dr Sterne discovered metformin and he first published articles on metformin in the UK in 1957 (Sterne 1957), but only in the last decade at the end of 1994, was metformin approved in the States and first introduced for use in 1995. Metformin not only lowers blood glucose, but also

inhibits lipolysis and reduces circulating fatty acids and VLDL production (Witters 2001). It also increases plasma HDL, so metformin improves glucose control and lipoprotein metabolism (Johnston et al 1990). This benefits the patient due to its ability to carry out multiple actions, and may be why it has vascular protective benefits.

Metformin is a white crystalline compound with a molecular weight of 165.63, the pKa of metformin is 12.4 and it is freely soluble in water (Kirpichnikov et al 2002). The pH of a 1% aqueous solution is 6.68, and at doses of 500-1500mg metformin has an absolute oral bioavailability of 50-60% (Pentikainen et al 1979).

The maximum effective dose is about 2000mg, with the starting dose of 500mg, which begins to lower basal plasma glucose within 3 - 5 hours. The dosage is then increased every 2-weeks until it meets the required targets for lowering glucose. 50-60% of the drug is absorbed from the small intestine, and it has an absorption time of between 0.9-2.6 hours (Bailey 1993), which depends upon the dosage. It is not metabolised and is excreted in the urine with nearly 90% being eliminated over a 12-hour period (Bailey and Turner 1996). Metformin's duration of action is typically 6-12 hours. The concentrations of therapeutic levels detected in the human blood range between 0.1-3 μg/ml, which is about 10^{-6}-10^{-5} M (Cusi and DeFronzo 1998). Its main action is the enhancement of the action of insulin in peripheral tissues, particularly skeletal muscle and creating a reduction in hepatic gluconeogenesis. It enhances insulin sensitivity by both direct and indirect effects on tissues.

1.8.1 Glucose Stabilisation

At the level of the cell, metformin acts in several ways to improve the action of insulin, especially in the liver and to a lesser effect in skeletal muscle. The dominant effect of metformin is on the liver; this has been investigated in real using nuclear magnetic resonance. Metformin improves fasting hyperglycaemia in patients by reducing hepatic glucose production, due to a decrease in gluconeogenesis (Roden et al 2001). In diabetic subjects there is glycogen cycling which accounts for 25% of glucose production and gluconeogenesis was three times higher in diabetic patients compared to control subjects. Metformin reduced gluconeogenesis by 36% in diabetic patients lowering glucose production (Hundal et al 2000). One way meformin operates is by increasing tyrosine kinase activity (Stith et al 1998), and the production of 1, 4, 5-inositol trisphosphate shown in the in the Xenopus oocyte (Stith et al 1996). In the presence of insulin the uptake of glucose by muscle is increased, causing increased glucose oxidation and the formation of glycogen (glycogenesis). The number and activity of GLUT1 and GLUT4 transporters (Bailey and Turner 1996) is elevated in different types of tissue, and so is the synthesis of glycogen. However Thomas et al (1998) was able to show that by inducing insulin resistance, metformin was still able to stimulate glucose uptake without effecting GLUT 1 and GLUT 4 glucose transporters. Therefore, metformin must be able to act independently via another pathway to stimulate glucose uptake into muscles. The primary receptors that metformin acts upon in the liver and muscles still remain unidentified. The concentration of metformin varies between tissues with a high concentration accumulating in the liver and kidneys (Wilcock and Bailey 1994). The highest concentrations of metformin are found in the intestinal walls and the salivary glands. It is in the

intestine where metformin increases glucose utilisation and lactate production (Bailey, Wilcock, Day 1992). As general rule metformin concentrations in other tissues are similar to the plasma concentrations.

Several studies particularly the UKPDS 36 (Adler et al 2000) have shown that metformin is consistent in its action of reducing fasting plasma glucose concentrations by 3.3 – 3.9 mmol/L. The HbA1c value is also reduced by a maximum of 1.5 – 2.0% in type 2 diabetic patients. The decrease was independent of the following factors: - age, ethnicity, duration of diabetic condition, BMI body mass index, fasting and glucose–stimulated plasma insulin or C-peptide. This was confirmed by meta-analysis on the data as documented by DeFronzo (1999).

1.8.2 Effect on Lipid concentrations

Metformin is one of the few drug treatments that are able to reduce or stabilise body weight, this action may be due to increased glucose utilisation in the splanchnic bed. The action of the drug also increases the sensitivity of adipose tissue to insulin; by reducing fatty acid oxidation by 10 - 20% (Cusi and DeFronzo 1998). This at least partially corrects the imbalance in the glucose-fatty acid cycle. Metformin also improves the lipid profile of the blood by causing a fall in hepatic production of VLDL (Zavaroni et al 1984), and is associated with a fall in postprandial lipemia concentrations due to an increase in chylomicron extraction (Jeppesen et al 1994), which reduces the triglyceride concentration by 20-25%. There is also a 5 - 10% fall in total blood cholesterol, caused by a reduction in the LDL fraction due to the fall in VLDL, there is relatively no change in HDL. There were also claims by Scott and Tomkin 1983 that metformin caused a fall in

cholesterol production in the intestine due to an increase in intestinal 3-Hydroxy-3-methylglutaryl-CoA reductase. Metformin may also have anti-thrombotic and anti-atherogenic actions. This is thought to be due to the increased fibrinolytic activity and a reduction in fibrinolytic inhibitor plasminogen-activator type 1.

1.8.3 Contraindications

Side effects of metformin include gastrointestinal problems such as abdominal discomfort and diarrhoea this is evident in 20-30% of patients (DeFronzo and Goodman 1995). These problems are usually mild and in some cases are minimised by a slower titration and taking the drug with meals. There are some patients who cannot tolerate metformin: - a study by DeFronzo and Goodman 1995 showed 1% of patients taking metformin alone and 4 - 5% of diabetic patients treated with metformin and glyburide are intolerant to it. Metformin can have an effect on the absorption of some nutrients in the diet, for example vitamin B12 and folate (DeFronzo and Goodman 1995), but this is not usually significant enough to display adverse symptoms. However, if low plasma concentrations of vitamin B12 and folate are detected supplements can be given or metformin use discontinued. An advantage of metformin is the rare occurrence of hypoglycaemic attacks.

Unlike other biguanides lactic acidosis with metformin is rare, only in 1-5 cases per 100, 000 patients per years of treatment (Brown et al 1998). If lactic acidosis does occur in such cases, it is usually due to a concurrent predisposing disorder (e.g. hypoxaemia or septic shock) or due to use of the drug in a contraindicated condition such as renal impairment, which allows excess accumulation of the drug as excretion is reduced. Excess accumulation of the metformin increases lactate

production leading to an acidosis. If creatinine levels exceed about 124μmol/L in females and 133μmol/L in male patients then metformin should be withdrawn from the patients' treatment regimen (DeFronzo 1999). It is crucial that the plasma creatinine is periodically measured particularly in elderly patients, and the patients muscle mass and protein turnover are considered. 150μmol/L serum creatinine is considered as the cut off point for renal failure (Jones et al 2003). Metformin should not be given to patients over 80 years of age if their creatinine clearance is not normal, as this indicates impaired renal function an important contraindication to metformins use. Other contraindications include patients with congestive heart failure and patients suffering from sepsis (Misbin et al 1998). In many cases, it is unclear if lactic acidosis is due to metformin or other underlying medical conditions.

Research carried out by Emslie-Smith et al 2001 shows that from their study 24.5% of people who receive metformin had contraindications to its use. This translates to 6.4% of all type 2 diabetic patients. Even though patients develop contraindications this rarely causes discontinuation of metformin therapy, despite this fact lactic acidosis still remains rare.

1.9 Current Evidence and speculation on the effects of Metformin on the vasculature

There is very little solid evidence about receptors that metformin might act upon to activate its cellular actions (Wiersperger and Bailey 1999) see Figure 1-7: - Metformins cellular actions. What is indisputable is the therapeutic benefits it exerts not only to reduce hyperglycaemia as shown earlier, but also its ability to reduce factors that have a cumulative effect and together cause an increased incidence of macrovascular complications. The UKPDS compared metformin with other drug treatments and with a conventional (diet) group treatment. Metformin was only selected for overweight and obese patients as metformin does not promote weight gain. Initial treatment with metformin caused a 30% reduction in risk for all macrovascular diseases (myocardial infarction, sudden death, angina, stroke and peripheral disease) over a median duration of 10.7 years compared with the conventional (diet) group. It was also found that fewer patients showed diabetes-related endpoints, the risk reduction being 19% (UKPDS 34, Turner et al 1998).

C. J Bailey (Personal communications)

Tight glycaemic control reduces the glycation of proteins in the endothelium preventing them from increasing their permeability, and allowing them to still act as effective membrane filters. This prevents blood vessels from losing their elasticity as glycation of the vascular smooth muscle fibres causes them to stiffen. Methylglyoxal is an intracellular metabolite its formation is documented in detail by Thornally (1996), it is thought to be involved in the pathogenisis of diabetic complications, either as a direct toxin or by enhancing the production of advanced glycation end products (AGEs). Recent research suggests that methylglyoxal was the major precursor of AGE in endothelial cells (Westwood 1997). There is also a report to suggest that metformin reduces plasma methylglyoxal a reactive α-dicarbonyl via the glyoxalase pathway, and methylglyoxal is detoxified to D-lactate (Beisswenger 1999). Metformin has several other reported favourable effects in the vasculature. These include: -

- Decreasing proliferation of vascular smooth muscle in vitro (Koschinsky et al 1988)
- Increases GLUT1 protein production and glucose transport in vascular cells (Sasson et al 1996)
- In hypertensive rat models it lowers blood pressure, blunting an increase in intracellular calcium. Intracellular calcium is responsible for vasoconstriction in vascular smooth muscle cells in response to a variety of vasoconstrictors, e.g. platelet derived growth factor and angiotensin II (Sharma and Bhalla 1995).
- Reduces thrombus formation in experimentally injured arteries (Massad et al 1988).
- Protects ischaemic myocardial tissue after coronary artery ligation

(Charlon et al 1988).

- Prevents neovascularization after experimental hypoxia of the cornea (Kissun 1988).

(The above list was created from Cusi K and DeFRonzo R. A (1998) Metformin a review of its metabolic effects.)

One study by Marfella et al (1996) demonstrated that over an 8 week test period on newly diagnosed type 2 patients, metformin improved endothelium-dependent vasodilatation reducing blood pressure in response to L-arginine, which is the precursor of nitric oxide an important vasodilator chemical in the vasculature. Metformin has also shown an ability to reduce PAI-1 concentrations and is documented in the UKPDS 34 (Turner et al 1998). This may be due to its ability to cause a reduction in the synthesis of PAI-1, which was illustrated in a human hepatoma cell line. Metformin also increases fibrinolysis in type 2 patients. Another important factor in reducing vascular complications is the ability of metformin to decrease platelet aggregation and reduce blood viscosity all documented by Palumbo (1998).

It was shown by Bouskela et al (1997) that metformin helped to maintain the arteriolar vasodilatation stimulated by insulin in cases of diabetes that did not exhibit severe hypoglycaemia. The vasodilator effect of metformin has been shown to stimulate rat tail arteries to relax by hyperpolarization Chen et al (1997). It is postulated that this effect is due to insulin altering intracellular calcium in the arteries. These results were not reflected in the carotid arteries of swine.

Improvement in the lipid profile has often been noted among those treated with metformin, typically in patients who have hyperlipidaemia (DeFronzo 1999). Metformin reduces the circulating total cholesterol, triglyceride and LDL cholesterol (Dailey et al 2002) in a 52 week study, so that there is less atherogenic lipid to act in the formation of atherosclerotic plaques. Small increases in HDL concentrations have been reputed in some studies with metformin. The overall changes to the lipid profile help to reduce cardiovascular risk.

Hypertension may also benefit from metformin treatment. Metformin has been shown to lower BP in hypertension, and there are clinical reports of improvements lasting up to two months after treatment is stopped in diabetic patients (Palumbo 1998). Similar effects were demonstrated in non diabetic hyperlipidemic patients giving a decrease in both systolic blood pressure of 11.3% and diastolic blood pressure of 13.3% after 6 months (Descovich et al 1978). Other studies show a fall in systolic blood pressure of 40mmHg and diastolic of 24mmHg (Landin et al 1991, Landin-Wilhelmsen et al 1992, Tengborn et al 1990). However many more studies have failed to show a significant effect of metformin on blood pressure (Nagi and Yudkin 1993, Dornan et al 1991, DeFronzo and Goodman 1995, Snorgaard et al 1997 to name just a few) and it is generally considered that metformin has little significant effect on BP.

Another significant advantage seen with metformin, but not with other antidiabetic drugs, is the maintenance or reduction in body weight. The reduction or stabilisation in weight is probably determined by the increased utilisation of glucose into oxidation and anaerobic pathways. For example instead of being stored

as excess glucose in adipose tissues, glucose turn over is increased and splanchnic anaerobic glucose metabolism is increased by 9.5% in the presence of metformin (Bailey et al 1992).

Metformin is able to stimulate glucose uptake into a variety of tissues e.g. diaphragm muscle, epididymal fat, skin, brain, and renal medulla via anaerobic metabolism. Only fat demonstrated an effect on aerobic metabolism with metformin. The degree of effectiveness of metformin to stimulate glucose uptake in different tissues varies depending on the following factors: - inherent bias of the tissue for aerobic or anaerobic glucose metabolism. The concentration of metformin accumulates in the tissue, as it is not metabolised (Wilcock and Bailey 1994).

The focus of this thesis is the reduction of diabetic vascular complications by using the antidiabetic drug metformin. This drug is a widely used biguanide, which has additional properties besides its glucose lowering action. However little information is available on how it exerts these additional cardiovascular protective effects, but this project will attempt to address some of these aspects.

1.9.1 Aims of Study

Evidence by the United Kingdom Prospective Diabetes Study (UKPDS 34, Turner et al 1998), has shown that metformin reduces premature macrovascular disease during long-term treatment of obese T2DM, those patients most at risk of developing macrovascular complications. In particular metformin is associated with reduced morbidity and mortality from ischaemic heart disease in obese patients (BMI 30+), this may be due to yet unreported effects of metformin. The aim of the present programme is to address the question of whether metformin can act directly or indirectly on the vascular system to improve vascular function, and by what mechanisms the drug might exert its apparent vasoprotective effect.

Recent research by Dale Carter (personal communications 2000) in this laboratory has shown an increase in arterial compliance in mice chronically treated with metformin, but little is known about the underlying mechanisms, the dosage or period of treatment before these effects become noticeable. Therefore, these issues will be examined during the current study. In the previous study, cholesterol had little or no effect on the structure of the aortic walls of mice, establishing metformin must act via another pathway.

Metformin is an oral-antidiabetic agent, one of the drugs most commonly used worldwide to treat type 2 diabetes. It controls blood glucose levels by decreasing hepatic glucose output and increasing glucose utilization particularly in the skeletal muscle and intestinal wall. In the UKPDS 34 (Turner et al 1998) metformin has been shown to increase the survival of diabetic patients. This could not be attributed solely to an improvement in blood glucose control, because patients

treated with metformin showed fewer myocardial infarctions (MIs) and strokes, and increased survival, compared to patients achieving similar glycaemic control with other antidiabetic agents. However little is known about the mechanisms responsible for this effect of metformin: this study will address this issue. There are four commonly used "predictors" of vascular mortality in patients with diabetes. These are the presence of coronary heart disease, overt proteinuria, raised glycated haemoglobin and hypertension (Donnelly et al 2000), although many other risk markers have been considered. These risk factors are all prevalent in poorly controlled diabetes, and they should be appropriately targeted by therapeutic interventions. Patients should also be made aware of the possible prevention of vascular disease, by compliance with these interventions. The continuing high prevalence of vascular disease in patients with diabetes, especially type 2 diabetes, emphasises the importance of antidiabetic therapy that can independently reduce vascular disease. Cardiovascular disease is the leading cause of death in all types of diabetes (Morrish 2001). The multifactorial intervention trial (Gaede 2003) demonstrated long-term intensive intervention, which involved multiple risk factors, reduced both micro- and cardio- vascular episodes by 50% in T2DM patients. A European study by William et al (2002) showed that the cost of treating diabetic complications in patients with both micro and macrovascular complications was 250% more than those with no complications. Metformin is not only used to treat diabetes but has also been studied in prevention trails showing a 31% reduction in the incidence of diabetes (Diabetes prevention program research group 2002).

The main focus of this research is to investigate the potential vasoprotective effect of long-term treatment of type 2 diabetes mellitus (T2DM) with metformin. Therefore, the aim of the present study is to investigate the actions of metformin on the vascular system in terms of contractility, and localisation of the mechanism to specific sites and actions within the blood vessel wall. Particular focus has been diverted towards the involvement of nitric oxide and studies to investigate if calcium plays a role in this pathway of action. Segments of intact blood vessels (mouse aorta) will be used along with A7r5 cultured cells of vascular smooth muscle to investigate the insulin signalling pathways, in an attempt to define and understand the mechanism of action of this agent.

1.9.2 Organisation of thesis

The background, rationale and aims of the research program have been detailed in this chapter. Suppliers of materials and a detailed account of the methods used for the research are described in chapter 2. Characterisation of the A7r5 cell line will be discussed in chapter 3, along with its growth in the presence of metformin. Chapter 4 addresses the time period it takes for metformin to exhibit its changes on contractile and relaxation functions of the aortic vessel, which is an extension of the work completed by Dale Carter 2000. Chapter 5 examines the changes in length-tension relationship of the aorta in the absence and presence of metformin. The cellular mechanisms are considered in chapter 6, which notes the release of nitric oxide, by various experimental methods. The final experimental chapter 7 considers the potential role of calcium in the contractile response, which is normally increased in the presence of metformin. Chapter 8 is the overall discussion, which will evaluate and interpret the main findings and provide a perspective on the main actions and pathways through which metformin appears to exert its vasoprotective effects, which could account for increased survival rate of patients who are treated with metformin.

Chapter 2: - Materials and methods

2 Materials and Methods

2.1 In vivo studies

2.1.1 Animal care and tissue usage

Normal male and female homozygous (+/+) lean mice were used from the Aston colony (Bailey et al 1982). The mice used were 4 – 8 months of age and weighed between 40 - 60g. Male and female obese (ob/ob) mice were also used. The ob/ob mouse is a model of obesity and non-insulin-dependent diabetes mellitus. The ob/ob mice were used at a similar age of 3 - 6 months of age and weighed between 70-120g, see Appendix 1. The animals were fed a standard rodent dry pellet diet (supplied by SDS Economy Rodent Breeder, Special Diet Services, Witham, Essex, UK.) and normal tap water *ad libitum*. The conditions within the animal unit kept the air at the optimum temperature range for mice 19-23°C, accompanied by a lighting schedule of 12 hours of simulated daylight and 12 hours of darkness, to regulate circadian rhythms and stimulate and synchronise breeding cycles. The humidity was also tightly controlled and for rodents this was in the range of 55% ±15 which is the accepted range and complies with the Home Office Animals, Scientific Procedures Act 1986.

The animals were weighed and humanely killed by concussion followed by cervical dislocation, both schedule one methods. The thorax was opened and the aorta was removed by placing watchmaker forceps underneath the vessel to aid the dissection. Once removed the vessel was approximately 2 – 3cm in length. This was then placed

into a physiological salt solution (PSS), modified from a study by Palmer et al (1998), see Appendix 2. The surrounding excess connective and fatty tissue was removed using watchmaker forceps and butterfly scissors, with the aid of a dissection light microscope. It is Important that the vessel is not over cleaned, and all manipulations are carefully performed with minimum movement of the vessel, so as not to disturb the inner endothelial cell layer or tear the tissue. Then approximately 1 cm lengths were taken from the aorta to form aortic ring sections. The region of the aorta was always noted because the upper region of the aorta does not stretch to the same degree as the lower thoracic region. This was taken into consideration during testing, so only identical regions were compared.

2.2 Arterial studies

2.2.1 Pre-incubation with metformin

Tissue samples were mounted onto two horizontal stainless steel wires, approximately 150μm in diameter. These wires were passed through the lumen of the aorta. One wire was attached to an immovable arm the other to a strain gauge for isometric tension recording, see Appendix 3a. This enabled the isometric contraction and relaxation to be assessed immediately after the incubation period. The tissue was placed into the organ bath containing a physiological salt solution (PSS) a modified Krebs solution, pre-warmed to 37°C and continuously gassed throughout the experiment. The PSS was also gassed prior to usage with 5% (carbon dioxide) CO_2, 95% (oxygen) O_2 mixture. The contraction and relaxation of control samples was first determined. Then aortic samples were incubated for 1 or 4 hours, with a concentration of metformin at 10^{-5}M. This concentration of metformin mimics the typical maximum circulating concentration of the drug during therapeutic use in man. The same circulating

concentration of metformin is achieved 24 hours after administration to mice at a dose of 250mg/kg/day, see Appendix 4, Lord et al (1983)

2.2.2 Mulvany and Halpern Myograph

The model used to assess isometric tension consisted of two separate chambers. This allowed two experiments to be run simultaneously, see Appendix 5. This myograph was used in all studies and was recently characterised by Angus and Wright 2000, to measure the effect of both contractile and relaxant agonists on the aorta. The transducer used to measure the displacement was 'Small vessel contraction equipment' supplied by Pioden Controls Ltd, Canterbury, England; its sensitivity and accuracy are addressed in Appendix 3b. The deflection was pre-calibrated before the study began, e.g. 10 squares deflection on the flatbed recorder (BBC Goerz) was adjusted to be equal to 1g: this was also equivalent to 9.8mN, which is the optimal resting tension of the tissue (Rossoni et al 2002). The aorta was incubated for 1 or 4 hours with metformin. After the initial incubation the vessel was subjected to a 1g tension: this was readjusted every 15 minutes over a 45 minute equilibration period, before noradrenaline and/or acetylcholine were administrated.

2.2.3 Determination of contractile response

An organ bath and standard compliance apparatus (DC bridge amplifier, transducers and a BBC Goerz chart recorder) were used to assess the response of arterial vessels. The viability of the thoracic aorta was determined prior to each experiment. Each experiment was initiated by adding potassium chloride- 1M KCl, until reproducible wall tension was recorded. After using KCl the aorta section was then washed three times by changing the PSS at 10 minute intervals (O'Brien et al 2001). The vessels

were contracted using noradrenaline, (bitartrate salt, at 1 x 10^{-9} – 1x10^{-6}M final concentrations) by constructing a cumulative concentration-response curve (Sheykhzade et al 2000). 100µl of noradrenaline was added directly into the organ bath, containing 10ml of PSS (modified Krebs), using a graduated Gilson pipette. The contractile response after prior exposure to metformin was expressed as a percentage contractile response of the control. Noradrenaline was stabilised for storage at -20°C and by a trace amount of ascorbic acid, which was added in all test solutions during the study.

The relaxation capability of the noradrenaline-contracted tissue was determined using acetylcholine (at 1 x 10^{-9} – 1x10^{-6}M final concentrations), by constructing a cumulative concentration-response curve (Sheykhzade et al 2000). The relaxation response was calculated as a percentage relaxation, after the maximal 10^{-6}M noradrenaline concentration. This was determined separately for the control and metformin treated samples. Acetylcholine was stored at -20°C. Noradrenaline and acetylcholine once thawed were kept on ice prior to use, to prevent deterioration of the drugs.

2.3 Long term studies

Four separate in vivo studies were carried out, in which mice were treated with metformin for different periods of time: - 24 hours, 2 weeks, 4 weeks, 8 weeks. Each study comprised of a control group and a treatment group. Treated mice were given 250mg/kg/day of metformin in their drinking water (Bailey et al 1992), lean mice drink on average 5ml a day see Appendix 6. Each group contained six male mice. Blood samples were taken at the start of each study; approximately 75µl from the cut

tail tip and then each following week, approximately 50µl of blood (Flatt and Bailey 1981) was collected in heparinised microfuge tubes, kept on ice. The samples were centrifuged at approx 1000rpm for 30 seconds; the plasma was then removed and frozen for later insulin and triglyceride determination. At the end of the study the aortas were removed and contractile response was determined as detailed earlier in this chapter (2.2.2, 2.2.3), and their distensibility was determined as described in 2.4.2.

2.3.1 Insulin determination (ELISA)

The determination of mouse insulin was undertaken by modification of the procedure for a rat insulin enzyme linked immunosorbant assay (ELISA) kit from Mercodia Ltd, Mercodia AB, Sylveniusgatan 8A, Uppsala, Sweden. The Mercodia insulin ELISA kit is based on a solid phase two-site enzyme immunoassay. It uses the direct sandwich technique, where two monoclonal antibodies are directed against separate antigens on the insulin molecule. The insulin in the sample reacts with the peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the wells of the 96 well plate. Washing of the wells removes any unbound enzyme labelled antibody. The bound conjugate is detected by the reaction with 3,3', 5,5'-tetramethylbenzidine. The reaction is stopped by adding acid, giving a colorimetric endpoint that can be read spectrophotometrically. (This information is as supplied in the directions booklet by Mercodia.)

20µl instead of the usual 25µl of plasma samples and standards (0.15, 0.4, 1.0, 3.0, 5.5 µg/l) were placed into wells of a 96 well plate coated with mouse anti-insulin antibody. In accordance the other solutions were scaled down in equal proportions.

Therefore 40µl of conjugate solution (formulae not disclosed by Mercodia) was placed into each sample/standard containing well. The wells were incubated at room temperature for 2 hours with gentle agitation using a laboratory shaker (Model RS500, Luckham, Recipro-shake major, Luckham Ltd, Sussex, England). After the incubation period each well was washed six times, using 280µl of washing solution (formulae not disclosed by Mercodia). After the final wash the plate was inverted onto absorbent paper and tapped to dislodge any remaining excess liquid. Then 160µl of peroxidase substrate was added to each well, and incubated at room temperature for 15 minutes. Then 40µl of stop solution (1M H_2SO_4) was applied to each well and the plate placed on the shaker for 5 seconds to ensure the solutions were mixed. The absorbance of the content of each well was then measured at 450nm using a plate reader. (Anthos 2001, Anthos Labtec instruments, Austria, Salzburg). The concentration of insulin in each sample was determined from a standard curve generated from the standard samples.

The lower detection limit of this rat insulin assay was calculated to be 0.07 µg/l (two standard deviations above zero), and at the upper limit samples of up to 576 µg/l can be measured without giving false results, as indicated by linearity of the standard curve on a log scale. The precision of the Mercodia kit shows ~2.0-2.5% coefficient of variation within the same assay and ~3.0-4.0% coefficient of variation between assays, this was calculated using 4-replicates. The kit also has a recovery rate of 97-98%. All of the results are expressed in µg/l and can be expressed as pmol/l by multiplying by the conversion factor of 174, (1 µg/l is equivalent to 174 pmol/l). The Mercodia rat insulin ELISA gives highly reproducible results obtained in a few hours, with little specialised equipment and without the limitation of radioactive material.

2.3.2 Triglyceride determination

This was carried out using the INFINITY™ triglycerides reagent kit from Sigma Diagnostics (Poole, UK). The procedure was adapted to use 10µl of sample and 1000µl (1ml) of reagent in cuvettes (for details of reagent contents see Appendix 7a). This method allows rapid, precise plasma triglyceride concentrations to be determined. The principle of the method is to measure glycerol yielded from the hydrolysis of triglyceride. The glycerol is phosphorylated in the presence of glycerol kinase by adenosine 5'-triphosphate. The product is glycerol 3-phosphate, which is oxidised to yield hydrogen peroxide and is catalysed by the enzyme L-alpha-glycerophosphate oxidase. Peroxidase allows hydrogen peroxide to react with 4-aminoantipyrine and 3,5 dichloro-2-hydroxybenzene sulfonate generating a red coloured dye. This sensitive red chromogen allows small samples to be used (McGowan et al 1983 and Fossati P et al 1982). The chemical reactions involved in the triglyceride assay procedure are summarised in Appendix 7b. A glycerol standard at a concentration of 195mg/dl (2.2 mmol/l) was used. The samples and standards were incubated in a waterbath at 37°C for 5 minutes, after which the cuvettes were dried and the absorbance measured at 520nm using a spectrometer (Ultraspec 1000E, Pharmacia Biotech, Cambridge, England).

The concentration of triglyceride was determined by the calculation in Appendix 8. The colour of the reagent was stable for approximately 15 minutes. The sensitivity range of samples detected is linear up to 800mg/dl (9 mmol/l) of triglyceride when the assay is run as documented above. All of the samples tested were within this range. The correlation coefficient of the assay was 0.999 (Sigma diagnostics, INFINITY™ Triglycerides reagent performance characteristics).

2.4 In vitro studies

2.4.1 Length – tension curves

The length-tension relationship allows us to examine how the change in length of a muscle affects the tension that the muscle is able to generate. One end of the tissue is attached to an immovable arm, while the other attaches to a force transducer. The change in length causes a corresponding change in tension (Klabunde 2002). Factors, such as muscle tension and reduced compliance can cause a resistance to stretching or an increase in the tension generated, at a given length in a vessel.

2.4.2 Length – tension curves and the effect of metformin

The compliance was determined in terms of length and tension; these factors can be easily measured using the MYO-01 Small Vessel Myograph System, (Experimetria Ltd, Budapest, Hungary see Appendix 9, Part A and B). The length is adjusted using the micromanipulator arm and is the controlled variable. The tension is determined by the deflection of the pen, on a BBC Goerz chart recorder. The tension deflection is precalibrated, e.g. 5 squares per 1g equivalent to a tension of approximately 10mN.

The aortic tissue was incubated in the presence and absence of metformin at 10^{-5}M concentration: 10^{-5}M metformin approximates to the plasma metformin concentrations during clinical therapy, and this concentration can be achieved in mice with a dose of 250mg/kg/day, as described earlier. The incubation times were for 1 and 4 hours. Some of the metformin treated samples were treated with papaverine 10^{-5} and 10^{-6}M for 10 minutes (Pöch and Kukovetz 1971), which is a direct smooth muscle relaxant, and the effect was observed. The determination of tissue tension was also carried out at the termination of the *in vivo* studies when animals had been treated for

2, 4, 8 weeks respectively with metformin (250mg/kg/day). Some aortic samples had their endothelial cell lining removed by scraping the wire vigorously around the inside of the lumen, to compare the tension in the absence and presence of intact endothelium.

The aortic tissue was threaded onto 2 separate wires, each attached to a tissue holder; refer to Appendix 9, Part C. These were attached to the MYO-01 Small Vessel Myograph (characterised by Angus and Wright 2000). Once they were secured in position in the tissue chamber containing 3ml of PSS, and the recorder was activated, the biting point of the tissue needed to be determined. This was done by adjusting the length, so that there was initially zero tension on the tissue, indicated by no deflection. This was used as point zero length and tension; all subsequent changes in length should also affect the tension. The tissue was stretched by a gradual increase in length. The tissue continued to be stretched until there was no further increase in tension, i.e. tension stabilised; this was considered the maximum tension the tissue could sustain.

2.5 Nitric oxide determination

2.5.1 The release of nitric oxide determined by spectrofluorimetry

Nitric oxide (NO) is involved in vascular relaxation: this was confirmed independently by Furchgott and Ignarro in 1986 (Vallance 2001), who proposed that endothelium derived relaxing factor EDRF was the simple inorganic molecule NO. Therefore NO may be a potential mediator of the effects of metformin on vascular compliance. For example metformin may affect the release of NO from endothelial cells, hence the present studies to measure NO release. Small sections of aorta,

approximately cuboid pieces of wall comprising of endothelium and underlying vascular muscle, weighing approximately 5mg, were cut using butterfly scissors. The sections were placed into wells of 96 well plates, six pieces of tissue per column of wells from each animal used. To each well 10µl of 100µM fluorescein dye was added. The dye, called 4,5-diaminofluorescein diacetate (DAF-2 DA), was added to produce a final concentration of 10µM in 100µl volume of the well (Blute et al 2000). The dye reacts with NO and the increase in the fluorescence of the dye is proportional to the release of NO from the tissue in the well. The dye was incubated for 30 minutes to allow it to be taken up into the tissue. Then the tissue was washed in PSS buffer to remove free dye (extracellular) in the solution. DAF-2DA is a diacetate form of the DAF fluorescein probe, which can easily permeate the cells in the tissue. It is hydrolysed by intracellular esterase enzymes to generate DAF-2 (Kojima et al 1998), which is the active form of the dye that will fluoresce on reaction with NO.

The sections of tissue were treated with metformin concentrations of 10^{-3}M and 10^{-2}M, at time periods of 1 and 4 hours. After this time the tissue degenerated rapidly and it was not sufficiently functional for further studies. The release of NO was determined using a dual scanning microplate spectrofluorimeter (Gemini SpectraMax XS, Molecular Devices Corporation, 1311 Orleand Drive, Sunnyvale, California, 94089) using Soft Max Pro- programme as an operating system for the machine. The dye was excited at 490nm and the emission was detected at 515nm. Static readings were taken at determined time spans from each well.

2.5.2 Total nitric oxide release. Griess reagent

An indirect way of measuring NO is to determine the amount of nitrite and/or nitrate released into the solution bathing the tissue. The total amount of NO is determined as the change in the sum of the nitrate and nitrite content of the solution. This can be measured using a cell suspension or a cross section of aorta incubated in PSS (these are later digested to determine NO/mg of tissue, see section 2.5.4) and placed in 96 well plates.

The Griess reaction was undertaken by first generating a standard reference curve to quantify the total amount of NO in each of the samples. The standard concentration range of 0-100μM for total NO (nitrite and nitrate) were generated from standards of nitrite and nitrate at 0.1M. These were solutions of sodium nitrite and sodium nitrate respectively. To measure total nitrite in the tissue samples a range of 8 different standard concentrations 100, 50, 25, 12.5, 6.25, 3.125, 1.565, 0 μM were achieved by serial dilution, all samples/ standards were carried out in triplicate in a 96 well plate. It is important to note the reference curves were generated in the same medium used to sustain the cells and nourish the tissue. Each well contained 50μl of either a standard or sample.

To each well being assayed for nitrite content, 40μl of assay buffer containing 14mM sodium phosphate was added at pH 7.4, see Appendix 10a. For each well assayed for total NO, 40μl of assay mix was added, see Appendix 10b. 10μl of NADPH was then added to all wells at a concentration of 10μM (prepared in 14mM phosphate buffer pH 7.4. The plates were protected from the light and gently agitated using a shaker for 45 minutes at room temperature.

The next step is known as the Griess Reaction, and its use on biological fluids is outlined by Green et al (1982). The reaction measures the nitrite content. A 1% sulfanilamide (or sulfanilic acid) solution in 5% concentrated phosphoric acid H_3PO_4 (once the 2 parts were mixed they were kept refrigerated and used within 12 hours) and 0.1% NED solution, (see Appendix 10C) were left to equilibrate to room temperature for 15 – 30 minutes prior to use. Using a multichannel pipette 50µl of 1% sulfanilamide solution was added to all wells. The plate was then incubated for 5 – 10 minutes at room temperature and protected from the light. 50µl of 0.1% NED was added as before to all wells. This gave each well a final volume of 200µl. Plates were then incubated for a further 5 – 10 minutes at room temperature avoiding exposure to light. This developed a stable purple/ magenta colour almost immediately. The absorbance of the solution was measured within 30 minutes in a plate reader (Anthos 2001-see 2.3.1) using a filter at 550nm. After 30 minutes the reagent colour started to fade, so all readings were made within 30 minutes.

2.5.3 Fluorometric assay to measure nitrite using 2, 3 diaminonaphthalene (DAN)

The aorta was removed, dissected longitudinally, and cut into 6 sections. Each section was placed into a 96 well plate, containing 200µl of Ca^{2+} free PSS, as calcium can interfere slightly with the fluorescence (Damiani and Burini 1986). The tissue was allowed to equilibrate for 1 minute, then 20µl of drug sample of either acetylcholine 10^{-5}, 10^{-6}, 10^{-7}M, or insulin 10^{-5}, 10^{-6}, 10^{-7}M, or noradrenaline 10^{-5}, 10^{-6}, 10^{-7}M was added and then the reaction was allowed to proceed for 10 minutes.

After 10 minutes the tissue was removed, and placed in 2.8M NaOH for digestion and later determination of protein concentration by bicinchonic acid (BCA) determination. The 200µl content of the well was then mixed and split into 2 separate and identical 100µl samples. In a 96 well plate two sets of standards curves were produced, one a nitrite and the other a nitrate standard, both of 100µl aliquots in the following concentrations 200, 100, 50, 25, 12.5, 6.25, 3.13, 0 µM.

To all wells 10µl of 40µM NADPH was added and left for 2 minutes before 50µl of 14nM nitrate reductase was added to one set of standards and one half of the 100µl samples. To the other half of the samples and nitrite standards an equal amount (50µl) of PSS was added. After 5 minutes 50µl of distilled water was added to all wells to stop the nitrate reductase reaction.

The fluorometric determination was carried out by adding 50µl of DAN at a concentration of 0.05µg/ml in 0.62M HCl. Then the plate was incubated at room temperature for a period of 10 minutes; at the end of this period 10µl of 2.8M Sodium hydroxide (NaOH) was added to each well to stop the reaction. The concentrations of DAN and NaOH and the incubation period were selected as described by Misko et al (1993.) Who previously validated and characterised the technique, where DAN (2,3-diaminonaphthalene) combines with nitrite ($NaNO_2$) in acidic conditions produced by hydrochloric acid (HCl), to form the highly fluorescent product 1(H)-naphthotriazole, see Appendix 11. The final volume of each well was 270µl. The plate was then read at an excitation of 365nm and an emission of 450nm.

2.5.4 Protein determination

The aortic tissue (~ 1-10mg) was digested in 500µl of 2.8M NaOH at 60°C until there was no solid material visible. 50µl samples were removed after protein digestion and placed into 96 well plates. Each sample was determined in triplicate. Bovine serum albumin was used to produce a standard curve of concentrations 1000, 800, 600, 400, 300, 200, 100 50, 0 µg/ml. The working reagent consisted of bicinchonic acid (BCA) solution and 4% copper sulphate solution in the ratio 50:1 v/v; 200µl of the working reagent was added to each well. The protein concentration was determined using the bicinchonic acid method adapted from Smith et al (1985). The plate was incubated in a shallow water bath at 60°C for 20 minutes and then read at 570nm using the Anthos 2001 plate reader.

2.6 Cell culture

2.6.1 The A7r5 smooth muscle cell line

The A7r5 smooth muscle cell line was purchased from the European Cell and Culture Collection (ECACC). The cells are a smooth muscle cell line obtained from the thoracic aorta of the DB1X rat, A7r5 are sub-clones of a cell line derived from the medial layer of fetal rat aorta (Sato et al 1994). The cells appeared flat and like ribbons during the early stages of growth, and at confluence the cells align themselves in parallel strips. Kimes and Brandt (1976) have described the characteristics and properties of the A7r5 cell line. These authors document that the cells form into a spindle shape and give the impression of being thinner than previously seen.

2.6.2 A7r5 smooth muscle cell media and supplements

The cells were grown in 75cm^3 flasks containing 40cm^3 of Dulbecco's modified Eagle's medium (DMEM). This contained 0.11g/l of sodium pyruvate and 1000mg/l glucose, additionally supplemented with 2 mmol/l L-glutamine, 1% Penstrep solution (10,000 units/ml penicillin, 10,000 µg/ml streptomycin and 25 µg/ml of amphotericin B in 0.85% saline) covering the spectrum of bacteria, fungi and yeasts, obtained from Gibco, and 10% bovine foetal calf serum (FCS).

2.6.3 A7r5 smooth muscle cell maintenance and propagation

The A7r5 cell line was passaged at approximately 80% confluence. To passage the cells the medium was removed and the cells washed with 3ml of PBS-EDTA (2.5g EDTA per litre in Ca^{2+}, Mg^{2+} free PBS) for 30 seconds. This step was repeated before adding 2.5ml Trypsin-EDTA (1 x solution of 2.5g/L Trypsin, 0.38g/L EDTA, 4Na/L in HBSS- Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} from Gibco) for 30 seconds. 0.3ml of this solution was left remaining in the flask and incubated at 37°C for about 3 minutes to aid enzyme action. Any remaining cells were dislodged with a sharp tap of the flask. 10ml of fresh DMEM medium was added to the flask to produce a new cell suspension. 4ml of this cell suspension was removed and added to 36ml of fresh medium in a 75cm^3 flask. The flasks were pre-incubated in 5% CO_2 and 95% O_2 at 37°C. It took approximately 3 days for the cells to reach confluence.

2.6.4 A7r5 smooth muscle cell seeding

After the process of trypsinisation 5ml of the cell suspension was added to 45ml of medium. 3ml was then placed into each of the 6 well plates and the plates were incubated until confluent. These cells were then used for calcium imaging studies.

2.6.5 A7r5 cell growth in the presence of metformin

Cells were seeded into each flask at the same concentration (~ 1 x 10^4 cells/ml). A separate flask was required for each time period determination at 24, 48, 72 and 96 hours. The metformin concentrations investigated were 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}M and a control (containing no metformin).

Cells were removed after each set period using 2.5ml of 0.25% trypsin EDTA for 1.5 minutes then removing 2.2ml of the trypsin and incubating at 37°C for a further 3 minutes.

2.7 Calcium studies

Tissue samples were incubated with metformin 10^{-5}M for 1 and 4 hours and confluent A7r5 cells in 6 well plates were incubated for longer periods of 24 hours. After incubation with metformin the cells were incubated in the fluorescent dye FURA/2AM for approximately 30 minutes in Ca^{2+} and Mg^{2+} free PSS solution. The reaction that occurs between the cells and the FURA/2AM dye is shown in Appendix 12. It is important to appreciate that by incubating the cells with FURA/2AM toxic products are generated (formaldehyde and acetic acid), and therefore any studies being undertaken must be for a limited period, to avoid functional deterioration of the cells/ tissue. The family of calcium indicators such as FURA-2 developed by Tsien 1980 has limited sensitivity above 1 μM and a dissociation constant of 0.14 μM, by ratioing the results the effects of uneven dye loading, leakage and photobleaching can reduce the effects associated in cells of unequal thickness. (Fura and Indo Ratiometric Calcium Indicators, Molecular Probes). After removing the excess dye by washing, the starting concentration of calcium was determined using a standard curve of

calcium concentrations 0-200μM, incubated with the dye. Once this had been determined the tissue/ cells were subjected to a set submaximal concentration, 10^{-5}M noradrenaline. The intracellular free calcium ion concentration induced by noradrenaline was then determined and any changes in calcium intracellular concentration were calculated

2.8 Intracellular imaging

2.8.1 Intracellular nitric oxide/ calcium image analysis

Thin slices of aorta (approx 0.5mm) were produced as follows: - by taking a cleaned section (see earlier studies section 2.1.1) of aorta ~10mm in length and placing it onto a hard cutting surface tile. The aortic section was then cut in half longitudinally using a sharp razor blade to open the aorta, with the luminal surface exposed. The two halves of the aorta were placed onto a nitro-cellulose membrane with the endothelial side facing upwards. These sections attempt to recoil: this is prevented by adhesion to the nitrocellulose membrane.

A glass stage or slide was prepared by lightly smearing it with silicon grease, producing two parallel single tracks, approximately 1cm apart on which to mount the samples. The nitro-cellulose membrane containing the two halves of the aortic section were placed onto a silicon grease track prepared on the cutting board. Thin horizontal slices (~0.5mm wide) are then produced using the "Mickle Way" Chopper. The slices were carefully mounted onto the silicon grease tracks on the stage/ slide with the aid of forceps, avoiding contact with the endothelial surface. About four slices could be mounted at one time onto the stage/ slide.

The chamber was flooded with 10μM DAF-2 DA dye and the tissue left to incubate at room temperature for 60 minutes to ensure the cells could take up the maximum amount of dye (Appendix 13 shows an example of how the dye is cleaved inside the cell). The advantage of the dye in its cleaved form of DAF-2T is not only its specificity, but its sensitivity at low NO concentrations operating in a range of 2-1000nM (Nakatsubo et al 1998). After this time the excess dye that had not been taken up into the cells on tissue samples was removed by washing the sample several times with Krebs solution (see Appendix 14).

There are two types of reading that can be recorded with the DAF-2DA dye. The first is a comparison of $\Delta F/F_0$, indicating the change in fluorescence. This is proportional to the amount of nitric oxide present in the medium. The area beneath the graph is equivalent to the total amount of NO in the medium. The second type of reading is DAF Δ rate sec-1. This indicates the change in rate and concentration of NO released over any given time period. The working range of the fluorescence of the dye was: - maximum excitation of DAF-2 DA at 495nm and the maximum emission of the dye at 515nm.

2.8.2 Intracellular calcium imaging

To enable calcium imaging of the same tissue as the NO imaging, the tissue was simultaneously incubated with the calcium sensitive dye FURA-2AM at 10μM concentration (Jaconi et al 1990) for approximately 30 minutes (Broillet et al 2001). The concentration and time period were chosen for the reasons considered previously in section 2.7 and the reaction details are given in Appendix 12. The fluorescence of the dye was determined at two separate wavelengths: - at 350nm the dye is excited but

is insensitive to calcium. The fluorescence at this wavelength was divided by the decrease in fluorescence observed at 380nm on the release of calcium. The information from the known calcium concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125, 0 μM) was determined to produce a standard curve, from which the unknown concentrations can be calculated.

2.8.3 Drug perfusion

DAF-2 DA and FURA-2AM preloaded aortic samples were placed under the digital-imaging camera. A suitable region of each tissue sample was selected showing an aortic section with both endothelial cells and vascular smooth muscle (region selection is illustrated in Appendix 15a). Specific areas were then selected and highlighted. The equipment has reservoirs (see Appendix 15b for equipment set up), which contain determined amounts of modified Krebs solution. This is used for tissue perifusion at a constant rate of ~2ml/min. To the reservoirs determined amounts of drugs and hormones were added to bathe the cells. The concentration of test substances placed in the reservoir were ~ 10^{-6}M insulin and 10^{-6}M acetylcholine (Ach) which were used separately. The volume of these solution had to be sufficient to covered the period of incubation at a perfusion rate of ~2ml/min to continuously supply the tissue chamber with liquid which bathes the tissue. Note that the final concentration of the drugs was identical to that in the reservoirs, as this directly perfuses the tissue.

2.8.4 Apparatus required for real time fluorescence

During the perfusion a single image was focused upon with the aid of a 10 bit cooled digital CCD Camera (Hammamatsu C4880-81). This camera was attached to an epifluorescence microscope (Olympus Bx50WI), which was programmed to the specified emission wavelengths of the DAF-2 DA dye and FURA-2AM. There was an x40 water immersion objective was used with a 0.8 numerical aperture. This system has been characterised by Dr N. Hartell (Hartell 2001) (now at London University School of Pharmacy). The experiments performed here were carried out in collaboration with Dr Hartell. The data collected were displayed using OpenLab software and analysed by software programs written and custom made by Dr Hartell (Reynold and Hartell 2000). To ensure the system was stable a base line was first obtained before administering any drug to the system. Readings were taken of NO and Ca^{2+} in real time at 6 second intervals before and during administering the test solutions of ~ 10^{-6}M insulin and Ach. Real time sampling was addressed by Lipp (2001): it is considered a useful tool for studying cell-signalling molecules, such as calcium.

2.8.5 Fluorescence appearance

Readings were recorded from the selected areas of interest as the emission of the dye was tracked and a graph was generated from these results. The still images displayed were those observed from the DAF fluorescence. Colour differences in the image were used to semi-quantitate the changes in fluorescence $\Delta F/F_0$, blue indicating a cold image with little NO present, through green and yellow to red that indicates high concentrations of NO.

2.8.6 Wavelength determination

When taking fluorescent measurements the excitation and emission wavelengths must be sufficiently different to ensure the excitation does not contribute towards the output being recorded (Bashford 2000). Therefore it was important to scan and observe the excitation and emission spectra of the fluorescent agent. Fluorescein dyes tend to have overlapping excitation and emission spectra. Hence the selected excitation wavelength was towards the blue region of the maximum excitation and the emission wavelength was towards the red emission maximum to minimise any interference.

2.9 Chemicals

The chemicals and specialised kits used throughout the experiments and their suppliers are listed in Appendix 16.

2.10 Statistics

When only two sets of data were compared from different animals unpaired parametric t-test was performed, with an appropriate post-test. When there were two or more sets of data compared, an unpaired Analysis of Variance (ANOVA) was performed. This test identifies the differences between sets of data, but in order to determine the significance between specific subsets of these results a post-test was required; - a Tukey test was performed for this purpose. When comparing values to the control a post-hoc Dunnetts test was performed. The exact type of statistics performed in each case is stated in each of the legends. The Instat computer software package was used to undertake all statistical tests, and 'Elements of statistics' (Daley et al 1995) was consulted, for selection of the appropriate test.

Chapter 3: - Characterisation of vascular smooth muscle cells

3 Characterisation of vascular smooth muscle cells

3.1 Introduction

3.1.1 Vascular smooth muscle

Vascular smooth muscle cells (VSMC) provide vital structural support and they help to regulate internal pressure within the blood vessels of humans and other vertebrates (Graves and Yoblonka-Reuveni 2000). VSMC normally exist in the blood vessel wall in a dormant and differentiated state. They express the essential profile of contractile proteins, ion channels and the normal complement of membrane receptors and signalling molecules that develop during differentiation of adult human VSMCs; these are necessary for control of membrane potential and the characteristic contractile function (Gollasch and Haas el al 1998). Intimal VSMC also play a key role in the development of chronic vascular disease and atherosclerotic plaques, caused by phenotypic changes and partial loss of its properties once differentiated (Owens et al 1995), this issue will be addressed in greater detail in chapter 4.

Most VSMC lines that are readily available are of rat origin: there are several different cell lines available and a few of these are briefly outlined here. There are 3 main vascular cell lines, these are A9, A10 and A7r5 cells, these were 3 individually derived clonal cell lines from 14-17 day old embryonic BDIX rats. Kimes and Brandt discovered and first characterised these cells in 1976 and they are now well-established and utilised cell lines.

3.1.2 Origin of A7r5 Cells

A7r5 VSMCs were selected for experimentation. They are derived from the thoracic aorta in this study from rats (*Rattus norvegicus*). Although A7r5 VSMC are embryonic smooth muscle cells, they have adult-like cell characteristics (Firulli et al 1998). In this thesis they are used to provide a model for investigation to illustrate the effects of antidiabetic drugs such as metformin and various inhibitory drugs on vascular tissue, fluorescent dyes were used to monitor the release of nitric oxide and calcium. The cells were obtained from the European Cell and Culture Collection (ECACC). For details on the A7r5 cells, the cell culture methods of propagation and subculturing the cell line see sections 2.6.1-2.6.5.

The A7r5 Cell line was originally established by harvesting the thoracic aortas of 14-17 day old embryonic BDIX rats, and removing the adventitia layer (Kimes and Brandt 1976). The remaining media and intimal layers of the aorta were used for cell culture. Approximately 10 aortas were used and minced up, this was then placed into tissue culture flasks containing 5ml of modified Eagle's medium supplemented with 20% foetal calf serum and 2% chicken embryo extract (CEE). The cultures were incubated at 37°C in 12% CO_2 and 88% air. Once this culture was confluent the cells were dissociated using 0.25% Viokase, cells were then centrifuged for 5 minutes at 150g and resuspended. After 40-90 minutes approximately 90% of the cells had reattached. Only non-detached floating cells were aspirated and re-cultured, any attached cells were discarded. This step was repeated several times (selective serial passage). The method relied on the fact that muscle fibroblasts have a faster rate of attachment than myoblasts. Kimes and Brandts cell lines are defined as a population of cells adapted to continuous culture conditions (about 8 passages). Once the cell line

was established CEE was removed from the medium and foetal calf serum was reduced from 20 to 10%. Three independently derived clonal cell lines were then established A7r5, A9 and A10. The chromosome number was also investigated by Kimes and Brandt (1976). The chromosome number obtained for A7r5 cells was 84, indicating that the cell were tetraploid. The A7r5 cell line is the one that has been selected by our laboratory for further experimentation as these cells mimic adult cell behaviour, even though they are of embryonic origin.

3.2 Characterisation of the A7r5 Cell line

3.2.1 Maintaining and cessation of growth

The medium of the A7r5 cells was changed every 2-3 days after subculturing to prevent the build up of metabolites, and to maintain growth. The split ratio for subculturing was no greater than 1:2 – 1:3 as recommended by ATCC (Rockville, MD). If the split is any greater than this cell growth can be very slow and the cells are prone to infections. If regular medium changes (every 2-3 days) take place over longer periods of time (such as a month or longer). Cells were not subcultured until they reached 80-90% confluence to ensure the highest number of cells was seeded into the next passage. Once the cells had been used and experimental studies completed they were frozen for storage to be used at a later date. When freezing the cells a medium containing 95% culture medium and 5% DMSO was used as recommended by ATCC (Rockville, MD), 1ml aliquots of cells were frozen at –70°C for 24 hours and then transferred into liquid nitrogen.

3.2.2 Description and morphology of A7r5 cells

The most detailed studies carried out on the A7r5 cell line were performed by Kimes and Brandt (1976). They documented the establishment of the cell line as summarised at the beginning of this chapter, which is not addressed here. The appearances of the cells are oblong, flat and sometimes ribbon-like during proliferation. When growth ceased the morphology of the cells appeared quite different: they became thinner and spindle shaped lining up in parallel arrays radiating from numerous centres.

3.2.3 Cell growth and enzymatic changes

A7r5 cells proliferate as myoblasts and then develop into cells, which resemble the phenotype of smooth muscle. The stages of growth can be identified by characteristic enzymatic changes. Figure 3.1 illustrates the findings that Kimes and Brandt (1976) made in terms of two key characteristics of differentiated muscle cells, which are changes in the enzymes myokinase (MK) and creatinine phosphokinase (CPK).

Figure 3-1: - Enzymatic changes accompanying A7r5 cell growth

Adapted from Figure 2 by Kimes and Brandt 1976.Characterisation of two putative smooth muscle cell lines from rat thoracic aorta. Exp Cell Res 98. 353.

The activity of the enzymes occurs as a function of growth and differentiation in the smooth muscle cell line. The increase in the enzymes MK and CPK occurs as the A7r5 cell culture reaches the stationary phase (Kimes and Brandt 1976), this has been observed separately by Shainberg et al (1971) and Turner et al (1974) in primary cell cultures. Similar enzyme secretion patterns have been seen in established cell lines and are documented by Shainberg et al (1971) and Tarikas et al (1974). The enzyme CPK is only synthesised by the A7r5 cells after the cessation of cell division (ATCC).

3.2.4 Genetics of vascular smooth muscle

The cell type has been characterised by the expression of smooth muscle isoforms of structural genes, which are involved in contraction of this muscle type. A7r5 cells express all SMC markers studied including contractile and cytoskeletal proteins such as: SM α-actin, SM myosin heavy chain (SMMHC), SM calponin, SM22, tropoelastin, this work was carried out by Firulli et al (1998). These markers show a decreased expression in dedifferentiated cells (by being grown in the medium M199) as confirmed by western blotting (Gollasch and Haase 1998). The presence of retinoic acid can also induce differentiation, when added as a supplement to medium.

3.2.5 Electrophysiology

An important characteristic of muscle cells is their ability to produce action potentials. In smooth muscle neighbouring cells are electrotonically tightly coupled to one another (Harris et al 1971). The A7r5 cells are able to produce spontaneous action potentials during the stationary phase of growth. Electrical coupling was strongly indicated by the synchrony of the membrane depolarisation of nearby cells. The cells were placed in buffered saline with a high calcium concentration during the recording

of the action potential readings, which spreads across a monolayer culture (Kimes and Brandt 1976).

3.2.6 Ultrastructural analysis

Low-resolution microscopy was also performed on the A7r5 cell line by Kimes and Brandt (1976), and this confirmed that the cells were of smooth muscle origin. The major characteristics of a smooth muscle cell line, which confirmed their authenticity, are the presence of 60-80nm surface vesicles. The presence of thin filaments 6-8nm orientated parallel to cells close to the surface membrane, accompanied by a well-developed rough endoplasmic reticulum with a sac like appearance covering 1-20% of the cell volume, which lies close to the plasma membrane. Thick filaments 15-18nm were also occasionally observed. The ratio of thin to thick filaments was as high as 20:1. All of the normal cell organelles associated with smooth muscle such as mitochondria, golgi apparatus, vacuoles, microtubules occasional lysosomes and lamellar bodies, were all present and observed in the ultrastructure of the A7r5 cell line. Visual inspection under a high-powered light microscope confirmed the general features noted by Kimes and Brandt (1976) after ~4-7 days in the present A7r5 confluent cultures.

3.2.7 Calcium channel expression in vascular smooth muscle

Little information is available pre-1998 about the presence of ion channels in cultured vascular smooth muscle cells (VSMC) during differentiation. However there was evidence available that L-type calcium (Ca^{2+}) channels are influenced by differentiation in other cell lines. The A7r5 VSMC line was shown to express functional L-type Ca^{2+} channels. Gollasch and Haase et al (1998) tested the hypothesis

that: - the L-type (C class) channel is a specific differentiation marker of VSMC, and expression of the channels depends on the stage of cell differentiation.

Gollasch and Haase et al (1998) determined the differentiation state of the cells by immunohistochemistry and Western blotting to identify the presence of Smooth muscle (SM) α -actin and SM-myosin heavy chains (MHC). Their studies suggested that the number of functional Ca^{2+} channels was lower in dedifferentiated VSMC and that the number increased upon differentiation with retinoic acid. The function of the Ca^{2+} channels was confirmed using a patch-voltage clamp amplifier technique.

By further analysis of the Ca^{2+} channel Gollasch and Haase et al (1998) discovered that the Ca^{2+} channels α_1 subunit in particular, directly correlated to the expression of SM α –actin and SM-MHC. Therefore Gollasch and Haase et al 1998 concluded the expression of the L-type Ca^{2+} channel α_1 subunit, was important for a functional Ca^{2+} channel, and this was accompanied by the expression of SM-specific proteins required for SM cell function. This makes the L-type Ca^{2+} channel a conventional marker of differentiated VSMC: this may be of physiological importance and influence their behaviour under certain pathophysiological circumstances (Gollasch and Haas et al 1998).

3.3 Typical growth and appearance of A7r5 cells

The above section addresses the observations from other studies, mainly by Kimes and Brandt (1976) and more recently by Firulli et al (1998) characterising the A7r5 cell line. The following section details comparable experiments and observations made in our laboratory.

3.3.1 Experimental details

The A7r5 cells were seeded into flasks as previously detailed in section 2.6.5. It is important to note that the initial cell concentration seeded into all flasks was constant. Varying concentrations of metformin (10^{-5}-10^{-2}M) were placed directly into the culture medium and the cells were then counted after 24, 48, 72, 96 hours respectively described below.

3.3.2 Construction of a growth curve

Cells were removed by trypsinisation as detailed in section 2.6.5. Once the cells were detached they were resuspended into 10ml of DMEM (Dulbecos modified Eagle's medium). These cells were then prepared for counting using a haemocytometer as detailed in the next section 3.3.3. Cell counts were taken over time and this information was then used to construct a growth curve.

3.3.3 Cell counts

Once the cell suspension had been prepared (i.e. trypsinisation and resuspension detailed above) the cells were ready for counting. The haemocytometer was prepared by placing a cover slip over the haemocytometer chambers. To ensure the cover slip was attached properly to the haemocytometer the appearance of Newtons rings was required at the edges of the cover slip to ensure it was securely fixed in place. Using a Pasteur pipette some of the cell suspension was transferred into both of the haemocytometer chambers. The pipette tip was placed to touch the edge of the cover slip; the cell suspension was allowed to enter the haemocytometer chamber by capillary action. Care was taken to ensure that the chamber was not under or over filled, and that there was an even distribution of the cell suspension.

Cells were counted initially in the centre/ middle square of the haemocytometer and then counted in the four corner squares, see Figure 3.3. This was done for both haemocytometer chambers on the slide, (Note: - only one chamber is illustrated in Figure 3.3, diagram 1). This ensured that a total of 10 squares were counted. Only the cells touching the top and left outline of the square were included in the counting, and this had to be consistent throughout the experimental study, see Figure 3.3. If more than approximately 10% of the cells appeared clustered, the slide was cleaned and the whole process repeated. To ensure that cells were not clumped or clustered in the cell suspension, vigorously pipetting of the original cell suspension was undertaken. This separated and dispersed any cell clumps. Each square of the haemocytometer, when the cover slip is in place contains a volume equivalent to $0.1mm^3$ or 10^{-4} cm^3. The number of cells per ml (taken as $1cm^3$) was calculated as shown in figure 3.2: -

Figure 3-2: -Cell count calculation

Cells per ml = average cell count per square (count 10 squares) x dilution factor x 10^{-4}

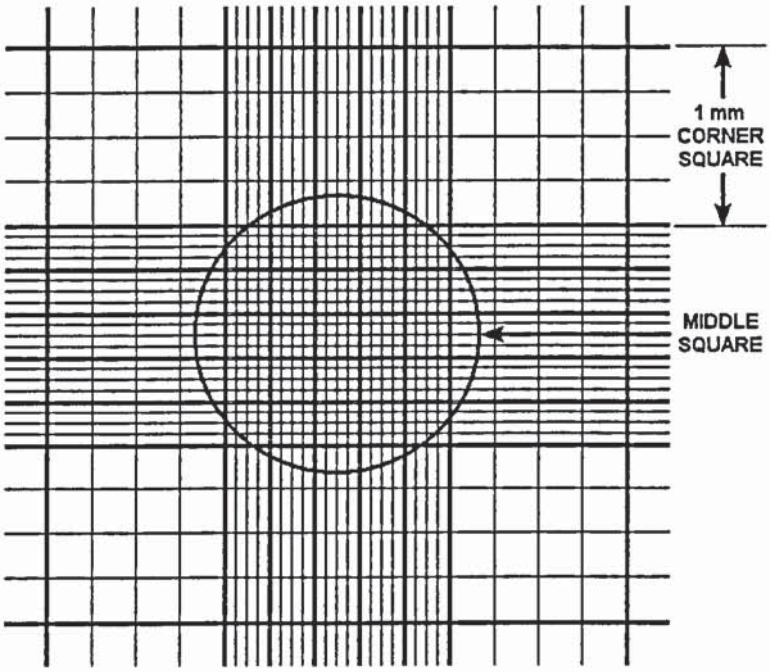
Once the cells have been determined per ml this information can then be translated into a growth curve as shown in the following section.

3.4 Aims

This study aimed to address if changing the medium significantly increased A7r5 cell growth, and to determine if metformin inhibits vascular smooth muscle growth over 96 hours, and at what concentration of metformin inhibition is significant if any.

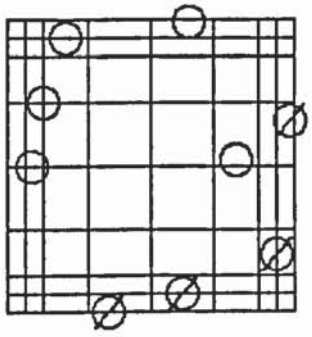
Figure 3-3: - The haemocytometer and cell counts

Diagram 1
Standard Haemocytometer chamber



The circle indicates the approximate area covered at 100X microscope magnification (10X ocular piece and 10X objective). Cells were included if they crossed the upper or left region of the square (O). Exclude if they touched the bottom or right region of the square (Ø). Count 4 corner squares and middle square in both chambers (one chamber represented here).

Diagram 2
Corner Square (Enlargement)



Count cells on top and left touching middle line (O). Do not count cells touching middle line at bottom and right (Ø).

3.5 Growth curve

The A7r5 cells are grown in two separate conditions. The first requires the medium being changed every 2 days; the other involves no change of medium through out the experiment. This experiment was performed to compare the conditions required for growth.

Figure 3-4: - Growth curve of A7r5 VSMC.

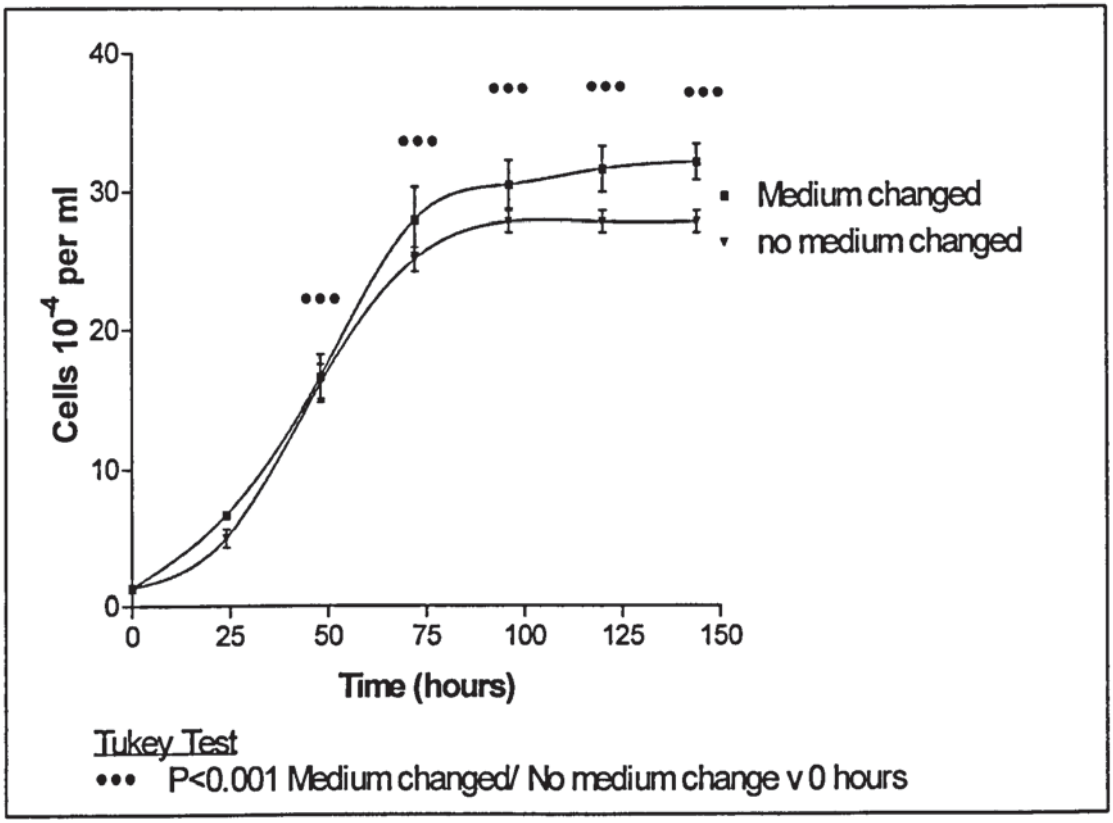


Figure 3-4: - The A7r5 cell line was grown in 2 separate conditions one involved the medium being regularly changed every 48 hours, the other involved no medium change. There was no significant change in growth between the 2 conditions, though the medium that wasn't changed did show a slight fall in cell growth. ±SEM (n=6)

Both growth curves shows that cells were initially slow to grow and did not show a significant increase in cell number until 48 hours after seeding ($P<0.001$). It is also important to note that the medium of one set of cells was changed every 2-3 days as recommended by ATCC (Rockville, MD). The growth of the cells when the medium was not changed was inhibited slightly, but this inhibition was not significant.

However the metabolites that build up in the medium are visible under the light microscope and give the medium a slightly grainy appearance, which becomes evident after 48-72 hours.

3.5.1 Appearance of cells

The appearance of normal A7r5 cells after inoculation with 1 x 10^{-4} cells is shown in Figures 3.5-3.10 after 24, 48, 72, 96, 120, 144 hours of growth (medium changed every 2 days).

Figure 3-5: - A7r5 cells after 24 hours

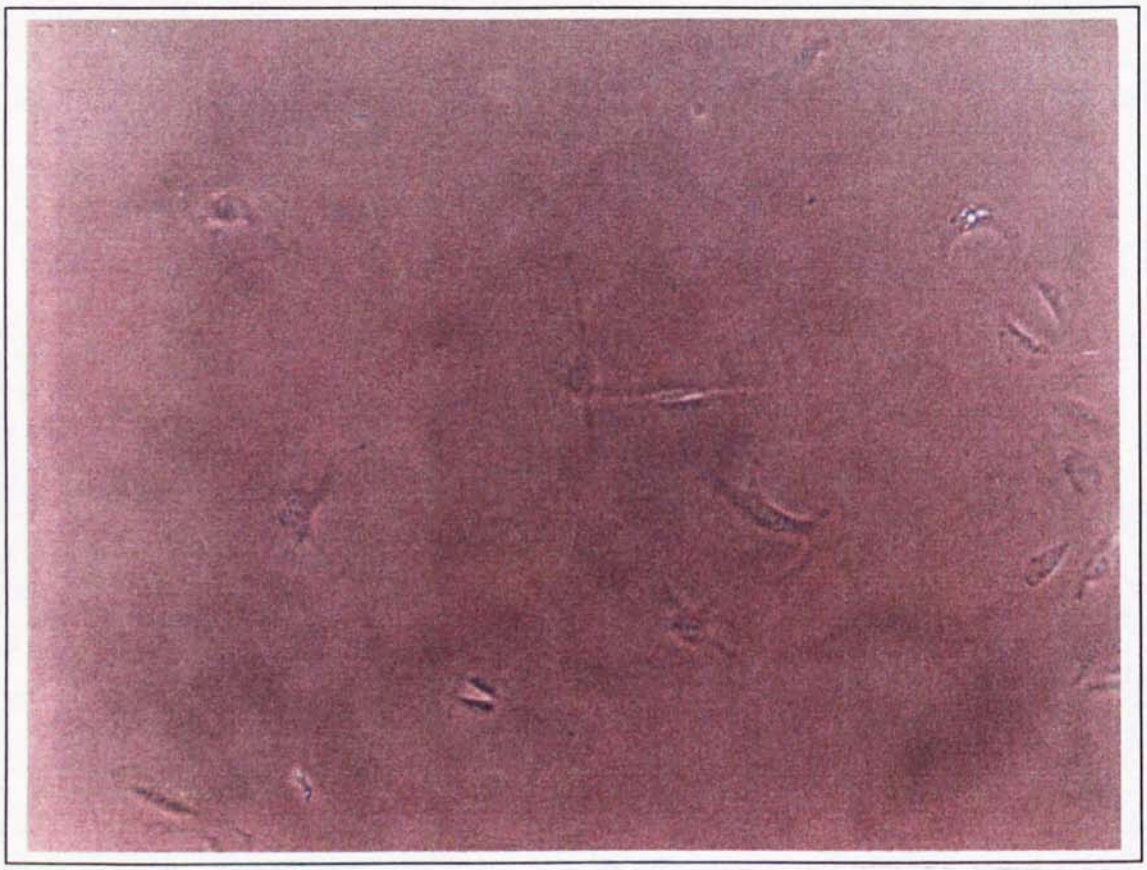


Figure 3-6: - A7r5 cells after 48 hours

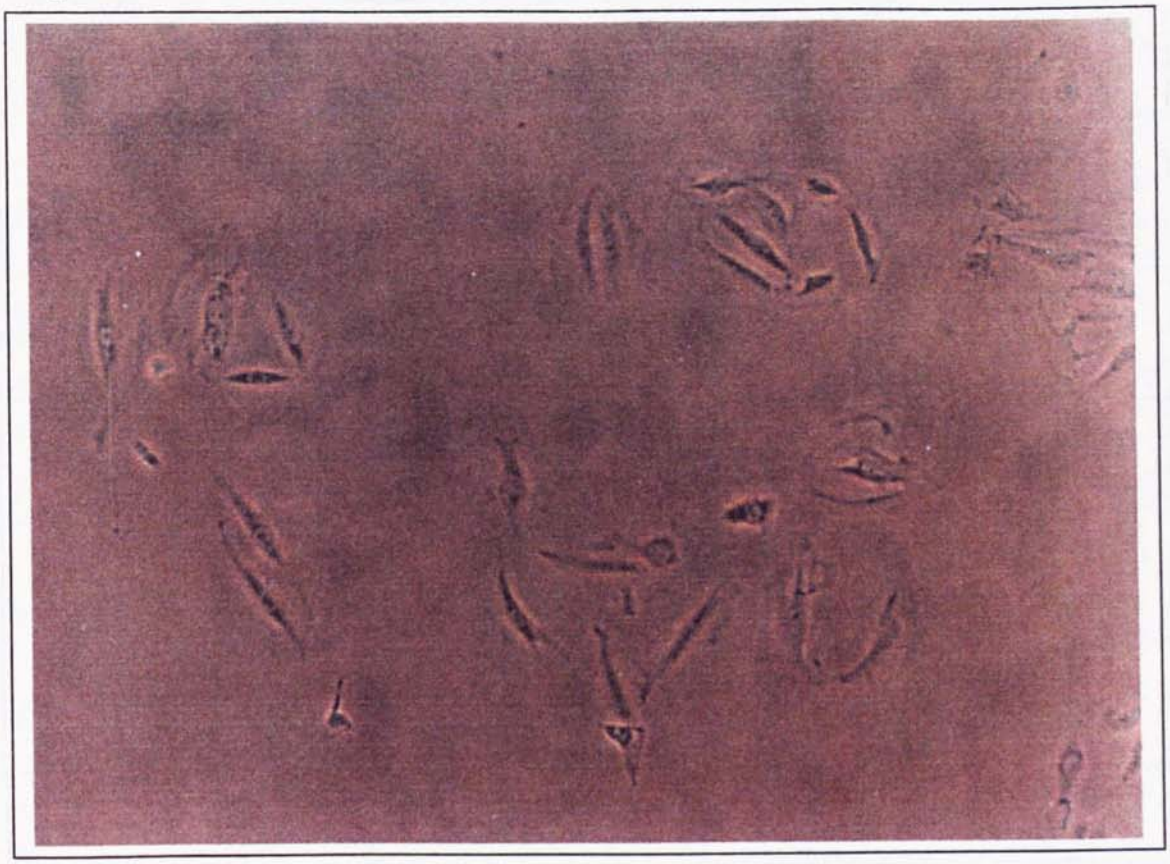


Figure 3-7: - A7r5 cells after 72 hours

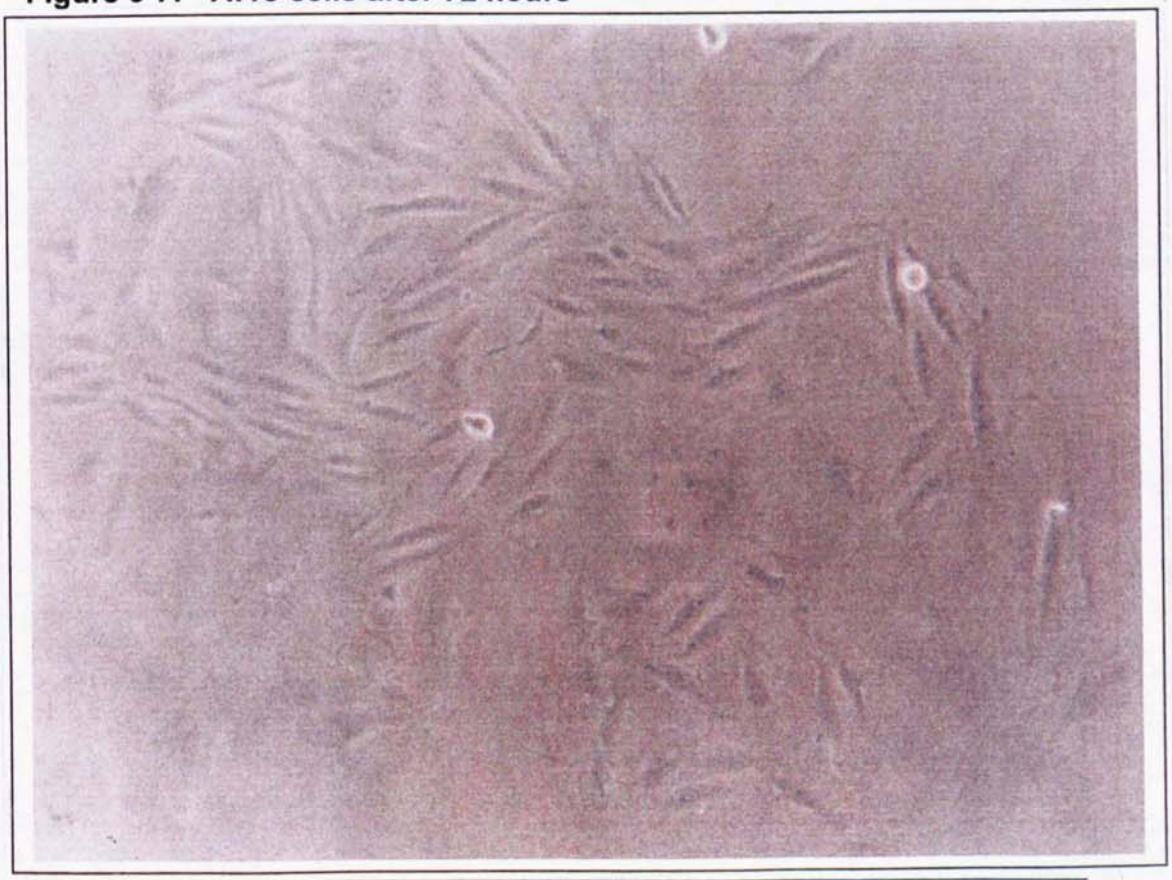


Figure 3-8: - A7r5 cells after 96 hours

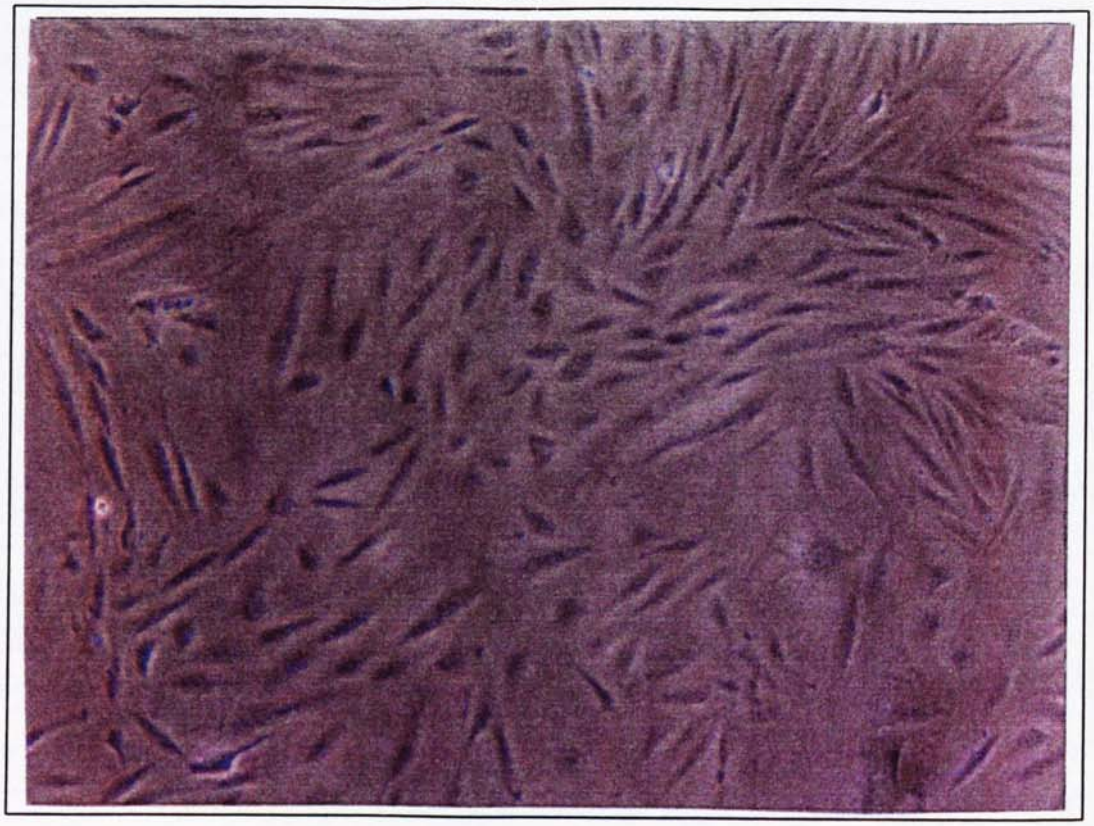


Figure 3-9: - A7r5 cells after 120 hours

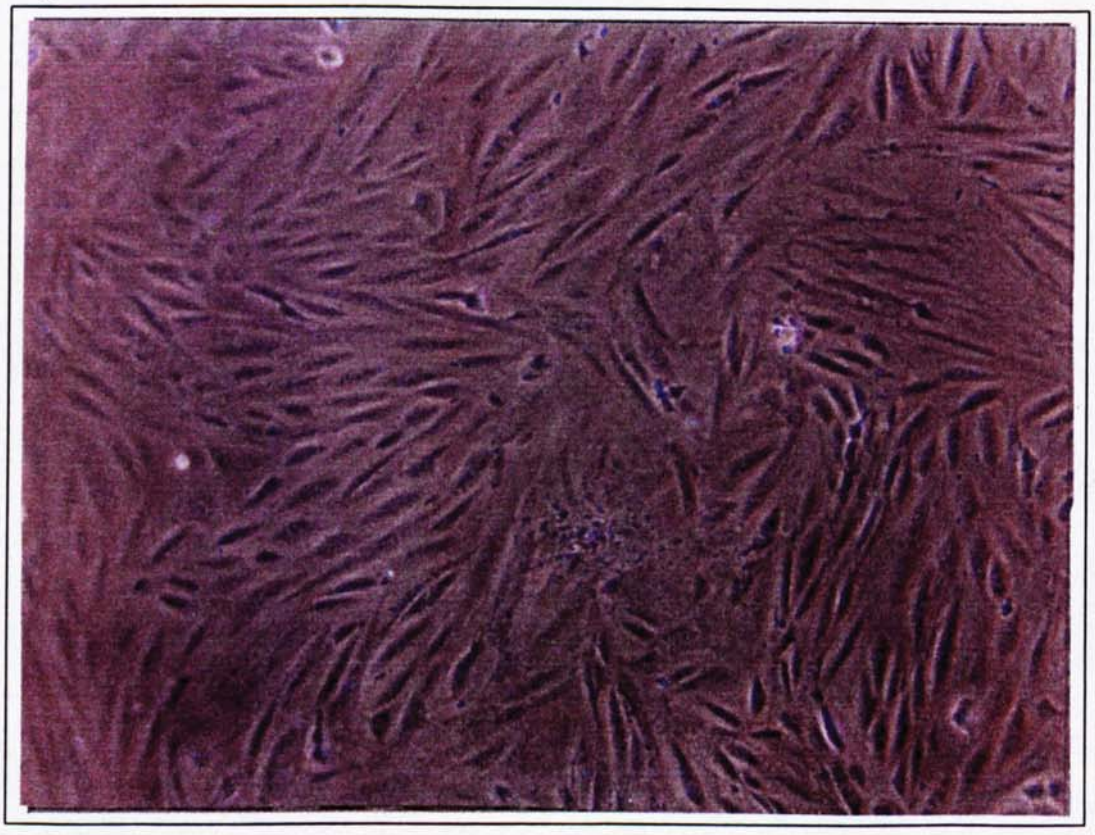
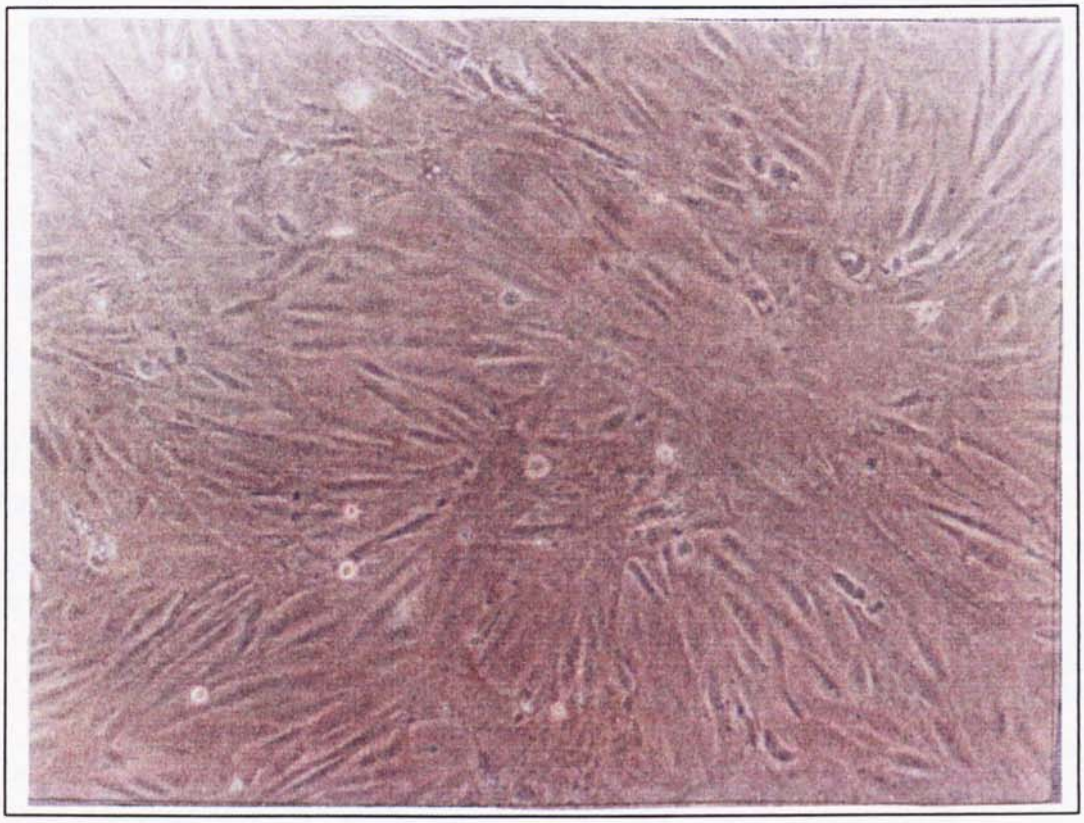


Figure 3-10: - A7r5 cells after 144 hours



3.6 The effect of metformin on the growth of A7r5 cells

A7r5 cells were grown in the presence of increasing concentrations of metformin. The growth of the vascular smooth muscle cells were monitored every 24 hours for 96 hours.

Figure 3-11: - Growth of A7r5 cells in the presence of metformin

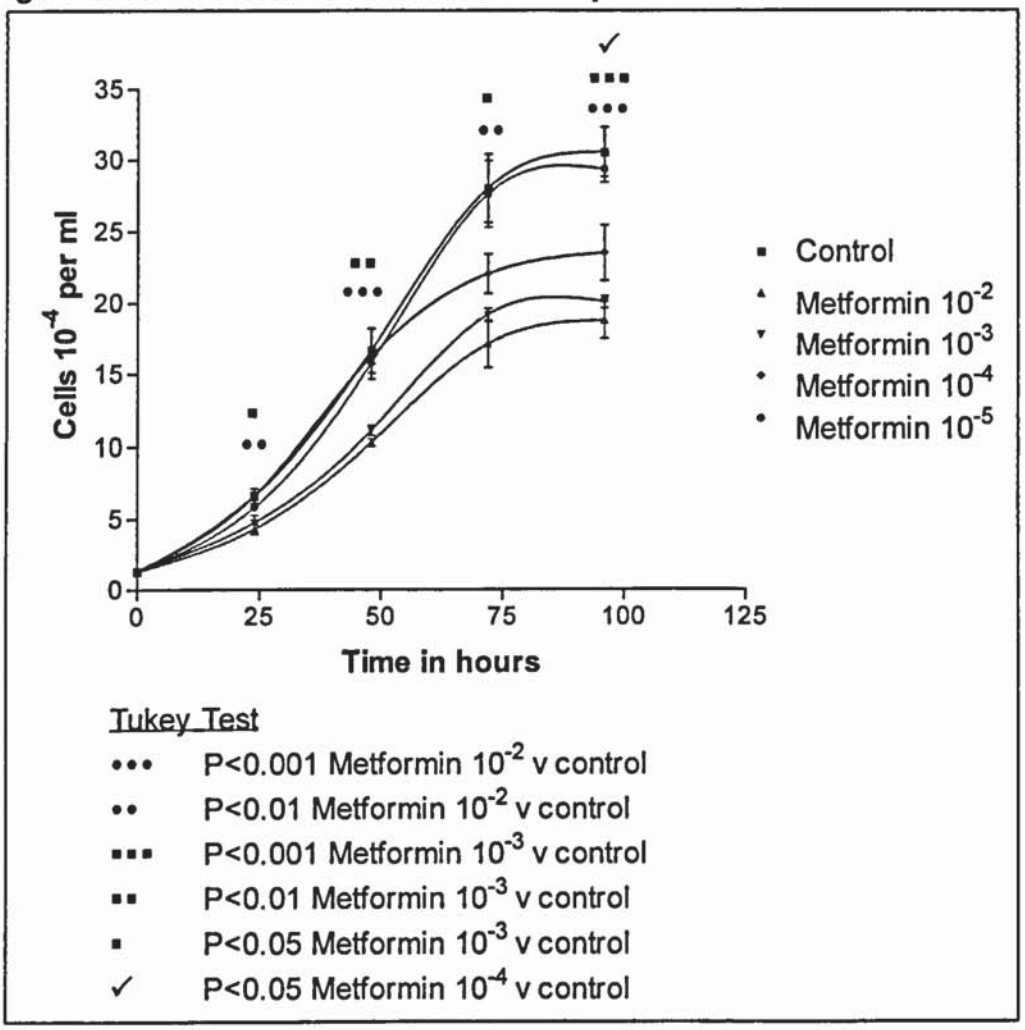
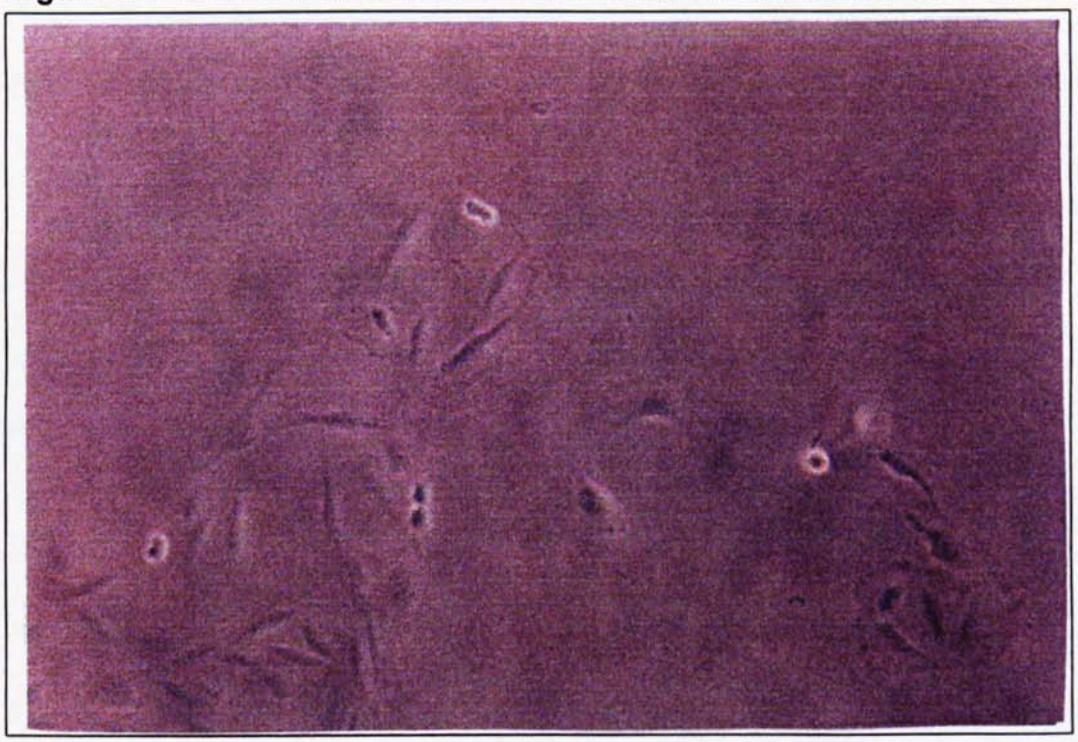


Figure 3-11: - The A7r5 cells were grown in increasing concentrations of metformin 10^{-5}M-10^{-2}M, and cell growth was monitored every 24 hours for 96 hours. The growth of the cells was inhibited by the increasing concentration of metformin. Maximum inhibition is seen with metformin 10^{-2}M, minimum inhibition is seen with Metformin 10^{-5}M. ±SEM. (n=6).

When the cells were incubated with metformin the growth of the A7r5 cells were reduced in a concentration dependent manner. The highest concentrations of

metformin 10^{-3}M and 10^{-2}M were the most effective at inhibiting cell growth ($P<0.001$) in comparison to the control and their greatest effect was seen after 96 hours. The most effective inhibition was exerted by metformin 10^{-2}M at all time periods 24 ($P<0.01$), 48 ($P<0.001$), 72 ($P<0.01$), 96 ($P<0.001$) compared to the control. Metformin 10^{-3}M also exerted an inhibitory effect but this was to a much lesser extent after 24 ($P<0.05$), 48 ($P<0.01$), 72 ($P<0.05$) hours compared to samples containing no metformin, but the greatest effect was exerted after 96 hours as described earlier. Lower concentrations of metformin, such as 10^{-4}M were only effective after longer time periods. The lowest concentration of metformin tested 10^{-5}M, which is closest to the natural circulating concentrations of metformin seen in the blood, had no significant impact on VSMC growth over the time periods studied.

Figure 3-12: - A7r5 cells incubated for 96 hours with metformin 10^{-2}M



On growth of A7r5 cells the inhibitory effect of metformin 10^{-2}M after 96 hours can easily be seen visually by comparing the growth seen above (Figure 3.12) with that seen in normal cells, shown in Figure 3.8.

3.7 Insulin-stimulated 2-deoxy-[^{3}H]-glucose uptake on A7r5 cells

This work was undertaken in detail by Carter (2000), His preliminary findings on these cells grown in petri dishes up to passage 18, showed that no significant stimulated glucose uptake (prior to serum starvation) could be detected in this cells line. For this reason obviously they can not be used to measure the glycaemic lowering properties of metformin on the vascular smooth muscle cells in blood vessels. This may be due to the lack of insulin sensitive receptors in this particular cell line. As often cell lines used for the study of antidiabetic drugs are selected for glucose uptake properties based on the expression of insulin receptors such as the L-6 skeletal muscle cell line. This matter therefore, will not be addressed in this thesis, as it is investigated in more detail by Carter (2000).

3.8 Calcium studies on A7r5 cells

The release of calcium in the presence and absence of meformin was studied in both aortic tissue segments and A7r5 cells in detail later in chapter 7. Also the effects of inhibitors on the insulin pathway were tested on the A7r5 cell line, to see if calcium release was affected by changes in the insulin-signalling pathway. Therefore these issues will not be dealt with here in this chapter, but later in chapter 7.

3.9 Discussion

The first important observation made in the present series of experiments with A7r5 cells was that the medium used for culturing the A7r5 cells needed to be changed every other day. If the medium was older than 2 days cell growth was not optimal. Even though not changing the medium had no statistically significant effect on A7r5 cell growth, it could be clearly seen that maximal growth was prevented.

The study observing the growth of A7r5 cells showed that only high concentrations of metformin 10^{-3}-10^{-2}M, were able to significantly inhibit cell growth within 1 day. Lower concentrations such as 10^{-4}M, exerted an effect after prolonged exposure of the cells. The concentration closest to that seen in circulation, metformin 10^{-5}M had little effect on A7r5 VSMC growth. Over a longer period however, this concentration may be effective enough to reduce excessive VSMC proliferation seen in the development of atherosclerosis, but long-term studies cannot be undertaken with A7r5 cell cultures without passaging (as cell numbers do not permit this). Incidents of atherosclerosis and VSMC proliferation associated with vessel damage in diabetic patients are reduced when they are treated with metformin (Koschinsky et al 1988), this may be due to metformin inhibiting VSMC growth in the long term.

This work was consistent with similar studies carried out by Peuler et al (1996) who only used low concentrations of metformin. The maximum concentration used in their studies was 100 µmol/l, which is approximately 10^{-8}M metformin. Our studies confirm that concentrations lower than 10^{-4}M does not cause VSMC inhibition of growth.

The original studies carried out by Bűnting et al 1986 used much higher concentrations 10^{-6}-10^{-2}M of metformin, similar to the study carried out in our laboratory. Their studies showed some degree of inhibition exerted by concentrations 10^{-2}M-10^{-5}M after the time periods 4 and 7 days studied. The work carried out in our laboratory shows that the inhibitory effect begins early (within 24 hours) and is most prominent in the higher metformin concentrations. Our studies however, showed that after 96 hours metformin ($\leq 10^{-4}$M) had a significant inhibitory effect. Maybe if we had been able to test for a longer period of time we would have seen a similar effect develop with metformin 10^{-5}M. This would possibly help to explain the reduction in macrovascular complications seen with long-term treatment of metformin, due to metformin reducing VSM proliferation associated with atherosclerosis.

Chapter 4: - Vasomotion of aorta, and the effect of metformin

4 Vasomotion of aorta, and the effect of metformin

Arteries are the main blood vessels that carry blood away from the heart and most carry oxygenated blood and vital nutrients around the body. The principal exception this is the pulmonary artery, which supplies the lungs with deoxygenated blood. The main artery that carries oxygenated blood via distal direction is the aorta; its structure and function will be considered in detail, as this was the blood vessel chosen for examining the effects of metformin on macrovascular function.

The main rationale for choosing the aorta is that this vessel allows direct observation of the effects of metformin as it has been reported to increase its vasomotion, increasing both contraction (Carter 2000) and relaxation (Katakam et al 2000). The model chosen for the experiment is the lean Ob/Ob (also referred to as +/+) mouse, and the vessel needs to be large enough to manipulate on the equipment, and obtain a clear detectable reading. Therefore as the aorta is the largest artery and is fairly straight in the thoracic region, it was the vessel of choice.

4.1 The healthy aorta

The human aorta originates from the left ventricle (see Figure 4-1: - The aorta and its main branches); it then arches backwards and to the left forming the aortic arch, it passes from this point down through the thorax on the left side of the spinal column. It opens into the abdominal cavity through a small orifice in the diaphragm known as the aortic hiatus, beyond this point it is called the abdominal aorta. The aorta ends at

the lower section of the fourth lumbar vertebra where it divides into the common iliac artery (Watson 1998). The aorta of the mouse shows a similar anatomy, although the organisation of the left carotid-subclavian complex is a single vessel at its junction with the aorta (Greene 1955).

Figure 4-1: - The aorta and its main branches

Adapted from: - Watson R. (1998) Anatomy and physiology for nurses, 10th edition. Chapter 17. The Circulation. 227.

The section used in this study is the thoracic aorta, as it is the straightest section of aorta, it has no major emanating vessels and therefore will be easier to manipulate on the Mulvany-Halpern and myograph equipment. The aorta is probably not representative of a typical artery as it contains a large amount of elastin, however it is the largest and easiest vessel to manipulate from the lean Ob/Ob mouse.

4.1.1 Structure of the aorta

Both arteries and veins comprise of the same basic layers in cross section of the vessel wall, however the layers are present in different proportions. The main focus of this thesis is on arteries, taking the aorta as the tissue for experimentation. There are 3 distinct layers (see Figure 4-2: - The layers of the artery wall): the outer layer is the tunica adventitia consisting of connective tissue rich in collagen and elastic fibres. The central layer is the tunica media, made up of connective tissue with some elastic, collagen fibres and smooth muscle cells. The innermost layer is the tunica intima and this lines the blood vessel, it mainly consists of endothelium, which creates a smooth surface to reduce friction and prevent clotting. Below the endothelium is a single layer of elastic fibres in arteries. The aorta is unique amongst arteries for its high proportion of elastin and its ability to change in diameter between systole and diastole. It is also a major site of atherosclerotic disease and an important site of action of vaso-active agents as discussed in later sections of this thesis.

Figure 4-2: - The layers of the artery wall

The above diagram was adapted from: - A Marshall visual guide. (1998). The human body; a comprehensive atlas of the structures of the human body. p128.

The thick layer of muscle and elastic fibres in the arterial wall help it to absorb and withstand the pressure wave that is hydraulically transmitted through the blood. The elastic fibres allow the arterial wall to stretch with the force of the systole, the recoil of the vessel wall helps to propel the blood further during diastole. The cardiovascular system is constantly under high tension created by two key forces. The first is the internal pressure generated by the heart, which is transmitted by the force exerted by the blood on the vessel wall. The pressure in the aorta averages around 100mm Hg and the blood flow rate is 30cm per second. The second is the external pressure exerted by the muscle surrounding the artery; this produces the peripheral resistance, which is offered mostly by the smaller arteries and arterioles (Marshall 1998).

4.1.2 How the vasculature is controlled

The high proportion of smooth muscle in the walls of arteries facilitates vasoconstriction and vasodilatation to change the radius of the artery lumen. Arterioles contain fewer elastic fibres but they have a thick smooth muscle layer, which contains concentric layers of mainly circular smooth muscle surrounding the arteriole (Solomon et al 1993). Detailed analysis of blood pressure control is beyond the remit of this thesis and the topic has been reviewed in several recent articles (Lewington et al 2002, Chobanian et al 2003).

Contraction

The smooth muscle is innervated by post-ganglionic sympathetic fibres, which release noradrenaline, increase vascular tone, generate contraction, reduce vessel radius; decrease blood flow and increase vascular resistance. Vasoconstriction is also under myogenic control: here the membrane potential fluctuates spontaneously causing contraction (Sherwood 1993b). Vessels typically constrict when the concentration of

oxygen increases and the concentration of carbon dioxide decreases, signifying a fall in tissue metabolites such as adenosine, lactate and K^+. This thesis does not cover the control of the microcirculation; detailed reviews of this topic are available in the literature (Wiernsperger et al 2003, Kollros et al 1997). Other conditions also cause vasoconstriction such as a cold environment and hormones such as vasopressin (an antidiuretic hormone) and angiotensin II (is a potent constriction agent only requires $1x10^{-6}$g to cause an increase of 50mm Hg in arterial pressure).

It is the vasomotor centre in the brain that controls vasoconstriction through out the blood vessels in the body (see Figure 4-3: - Brain regions involved in regulating the circulation). The vasomotor control centre lies in two locations in the brain: - the reticular substance of the medulla, and the lower third of the pons. From this control centre parasympathetic impulses are carried through the vagus nerve to the heart. Then sympathetic impulses are passed through the spinal cord to the peripheral sympathetic nerves, which innervate almost all the blood vessels of the body (Guyton and Hall 1996b). The function of the vasomotor centre is still not fully understood but currently there are considered to be 3 distinct regions: -

- Vasoconstrictor area known as C-1, it is in the upper medulla, here there are fibres that release noradrenaline. The fibres neural effected here for part of the sympathetic nervous system.
- Vasodilator area known as A-1, this is located in the lower region of the medulla. The neural fibres in this area project upwards into the C-1 vasoconstrictor region. By doing this they are able to inhibit vasoconstriction.
- Sensory area A-2 this is located in the post lateral areas of the medulla and the lower pons. This provides a reflex control over circulatory function such as the baroreceptor reflex, which acts to control arterial blood pressure.

Figure 4-3: - Brain regions involved in regulating the circulation

Regions of the brain that play an important role in nervous regulation of the circulatory system. The dashed lines are the inhibitory pathway. Guyton & Hall. (1996). Textbook of medical physiology, 9th edition. p211.

Humoral and neural routes therefore mainly control contraction.

Dilation

During vasodilatation the circumference and radius of the vessel increase due to the relaxation of the smooth muscle layer in the arterioles. This causes an increase in blood flow and a reduction in the resistance. Factors that trigger vasodilatation include heat, which allows additional heat loss to the surrounding environment. Other factors such as a fall in myogenic control also trigger vasodilatation along with local intrinsic changes in the environment. If there is a fall in oxygen in the surrounding tissue or an increase in carbon dioxide caused by exercise, dilatation can increase the blood supplying the tissue by as much as ten fold (Solomon et al 1993) to meet its demands. This is an example of active hyperemia. Other metabolites released by metabolically active tissue can also trigger vasodilatation such as an increase in

acidity induced by carbonic and lactic acid. There can also be increase in osmolarity as cells release metabolites that alter the osmotic potential around them.

In the same way that an increase in sympathetic nervous stimulation causes vasoconstriction, a reduction or inhibition of vasoconstriction takes place by reducing sympathetic nervous impulses leading to vasodilatation. There are also chemicals released locally from a tissue, which affect the blood vessels and induce relaxation (Rehman 2001) one of the most well known was formally known as endothelial derived relaxation factor (EDRF), but is now more commonly known as nitric oxide (NO), (this will be addressed in detail in chapter 6, as NO is a key control factor in vascular relaxation and the main focus of this thesis). Other substances derived from the endothelium cause smooth muscle relaxation, the endothelin action on ET_B receptor is a common example. Another local chemical mediator is histamine released from mast cells (Bockman and Zeng 2002). It mainly operates in injured tissues increasing the blood flow as part of the inflammatory response.

4.1.3 Resistance in the vascular system

Arterioles are the major site of peripheral resistance and the larger arteries are the main sites at which constriction and blockage occurs, in order to limit tissue perfusion in diabetic macrovascular disease. The contractile state of arteriole smooth muscle is under both nervous and local chemical control. The endothelium is made up of a single layer of endothelial cells that line the lumen of the blood vessels. These cells are responsible for the release of the chemical mediators involved in local arteriole regulation.

These chemicals are released in response to changes in the local interstitial environment such as a reduction in oxygen tension or stretching of the blood vessel wall. These chemicals then act on the underlying smooth muscle to alter its state of contraction. One of these mediators is endothelial-derived relaxing factor (EDRF), which induces vasodilatation, by relaxing the surrounding arteriolar smooth muscle. EDRF has been identified as the chemical nitric oxide (NO), one of the most important vaso-relaxant signals in the circulatory system. The relevance of NO and the effect of metformin are investigated as the main focus of chapter 6 later in this thesis.

Other important chemicals are released such as endothelin, which has the opposite effect causing vasoconstriction when acting on the ET_A receptor. Other chemical mediators are also released in response to chronic changes in blood flow. These chemicals cause long-term changes to the blood flow in a particular area of the body. The main factor that affects blood flow to an area is the radius of the blood vessel. The factors that affect the flow rate through a blood vessel are summarised in Poiseuille's law, which can be represented by the following equation Figure 4-4: - Poiseuille's law: -

Figure 4-4: - Poiseuille's law

$$\text{Flow Rate} = \frac{\pi \Delta P r^4}{8 \eta L}$$

KEY
ΔP= change in pressure
r^4= radius
η = viscosity
L = length of vessel

If the radius of a blood vessel is halved the resistance to the flow of blood increases by sixteen times, decreasing the blood flow to one sixteenth. This factor is illustrated in Figure 4-5: - The relationship of flow and resistance to vessel radius.

Vasodilatation is an important factor in delivering blood to vital organs. If blood flow is severely restricted by increased constriction it can be associated with vascular disease and major organ damage e.g. persistent coronary vessel constriction is a major contribution to CVD.

Figure 4-5: - The relationship of flow and resistance to vessel radius

Table taken from Lauralee Sherwood (1993b.) Human physiology. From cells to systems. Table 10.4 Functions of endothelial cells p304.

4.1.4 Effect of insulin on the vasculature

Insulin also has several effects on the vascular system. These include causing mild vasodilatation Yki-Jarvinen (1999) showed that insulin is a hormone able to act directly on the vascular system. In her conclusion she lists the effects of insulin on the peripheral flow of blood as: -

- Physiological concentrations of insulin do not change peripheral blood flow.
- Insulin is a weak vasodilatator compared with classic endothelium-dependent vasodilators such as acetylcholine and with stimuli such as exercise.
- Glucose uptake in skeletal muscle is not significantly altered in normal or insulin-resistant subjects by altering blood flow.

The effects of insulin on vessel stiffness

- Physiological concentrations of insulin rapidly decrease vessel stiffness in normal subjects.
- The ability of insulin to diminish stiffness is severely blunted in insulin resistant subjects
- A blunted effect of insulin on stiffness might contribute to the association between insulin resistance and systolic hypertension.

Figure 4-6: Haemodynamic actions of insulin

"Insulin signalling pathways related to production of nitric oxide" is taken from Montagnani M and Quon M. J (2000) Diabetes, Obesity and Metabolism. Vol.2 p288

4.2 Macrovascular disease

Macrovascular disease is probably the most important complication to consider in diabetes, as it is 2-3 times more prevalent in the diabetic patients compared to the normal population (Stamler et al 1993). It also causes approximately ~70-75% of all type 2 diabetes related deaths. Therefore it is important to ensure that type 2 patients receive prevention and management of these complications as part of their treatment (Panahloo and Yudkin 2002). Some risk factors that appear to predispose diabetic patients to vascular disease are raised such as arterial pressure, cholesterol and fasting glucose these are key but reversible predictors of this disease (Lee et al 2001).

Cardiovascular disease especially coronary heart disease (CHD) is the commonest complication associated with type 2 diabetes in westernised societies and accounts for a high mortality rate in these patients (Elkeles et al 2002). The risk of CHD is approximately 2-5 times greater in type 2 diabetic patients than in the normal non diabetic population (Pyorala & Laakso 1983). There appears to be a much greater relative risk for women (2.58) of coronary related deaths then there is in men (1.85) (Lee et al 2000).

There are several risk factors associated with macrovascular disease. The factors that will be considered in more detail in this chapter include glycaemic control; hyperlipidaemia; hypertension; microalbuminuria; insulin; pro-insulin like molecules; homocysteine; coagulation and fibrolysis factors; smoking and obesity.

4.2.1 Glycaemic control

The impact of hyperglycaemia is still difficult to determine and is often the most controversial risk factor that is discussed. The impact of hyperglycaemia is believed to have a knock on effect on other substances produced and released in cells. For example hyperglycaemia may affect endothelial cells and trigger changes that lead to atherosclerosis. The production of irreversible terminal products known as advanced glycosylation end product (AGE) proteins, where cross-linkage between collagen and other proteins occurs in the vascular wall can accumulate over time, which also contribute to atherosclerosis, the development of diabetic complications and the aging process (Beisswenger and Szwergold 2001). Products produced by AGE such as cytokines and can induce cell proliferation further aggravating the development of an atherosclerotic state. Hyperglycaemia can also cause glycosylation of LDL and HDL changing their function. LDL becomes likely to be oxidised and HDL transportation of cholesterol is impaired: together these promote atherosclerosis (Panahloo & Yudkin 2002).

The UKPDS was started in 1976 to assess the long-term outcomes of diabetic patients. However the 10-year follow up may still have been too short to assess any changes in atheroma. There was little difference between the types of glycaemic control and the individual therapies. There is a link between impaired glucose tolerance (post-glucose load hyperglycaemia) and CHD (Haffner 1998). The post-glucose load (postprandial) hyperglycaemia may simply be a marker of insulin resistance or hyperinsulinaemia, which are associated with CHD. The cardiovascular events and mortality increase with increasing HbA_{1c} (Kuusisto et al 1994). An increase in microalbuminuria and an albumin excretion rate above 8mg/24 hours and poor glycaemic control are all predictors of cardiovascular disease (Gall et al 1995).

There is also evidence that hyperglycaemia may independently contribute to cerebrovascular disease risk such as stroke. One of the newest markers significantly associated with cardiovascular events is a reduction in small artery elasticity, which is independent of age (Grey et al 2003).

AGE formation is elevated in diabetes as it can be produced by glucose via a number of pathways there are four of these main pathways, which will be discussed: -

- The first is an increase in glucose metabolism via the sorbitol pathway, see Figure 4-7: - Sorbitol pathway. Glucose is converted to sorbitol by the aldose reductase enzyme. Sorbitol is then converted to fructose by sorbitol dehydrogenase. However antioxidants such as glutathione reductase (GSH) involved in insulin metabolism require NADPH. As the NADPH is used in the sorbitol pathway instead this decreases GSH activity, resulting in an increase in oxidative stress. This reaction also alters nitric oxide synthesis, as NADPH is also required in this process. Oxidative stress markers become elevated which promotes an atherogenic state (Chappey et al 1997).

Figure 4-7: - Sorbitol pathway

Adapted from Duckworth W. C. (2001). Hyperglycaemia and cardiovascular disease. Current Atherosclerosis Reports. **3:** 383-391.Figure 1. p386.

- There is also an increase in free radicals when glucose concentrations are elevated and this promotes AGE formation as it subjects the cell to oxidative stress. It is important to appreciate the AGE formation increases with age, as they accumulate over many years. Their formation is promoted by transition metals and decreased by reducing compounds such as a fall in GSH (Duckworth 2001).
- Other products such as methylglyoxal (ME) and 3-deoxyglucosone (3DG) are increased by the postprandial rise in glucose. They are very reactive precursors that generate AGEs. Therefore complications may be attributed to repeated hyperglycaemic fluctuations rather than an average raised glucose concentration (Beisswenger et al 2001).
- During hyperglycaemia glucose and other reducing sugars can react with the amino groups in the proteins and lipids and even nucleic acids. These reactions are known as Maillard reactions: they create Schiff bases and lead to Amodori products. These are then converted into AGEs. This affects enzymes and structural proteins, which become glycosylated and this causes acute and chronic complications (Duckworth 2001).

The four main pathways are summarised below in Figure 4-8: - Pathways of advanced glycation endproduct (AGE) production from glucose.

Figure 4-8: - Pathways of advanced glycation endproduct (AGE) production from glucose

Adapted from Duckworth W. C. (2001). Hyperglycaemia and cardiovascular disease. Current Atherosclerosis Reports. 3: 383-391.Figure 2. p387.

There are AGE receptors on various tissue cells such as smooth muscle cells, monocytes, macrophages and endothelial cells. These receptors are increased in the kidneys and blood vessels. Vascular damage due to AGE formation has been associated with factors such as free radicals, reduced nitric oxide, vascular thickening and cross linkage of structural proteins an increase in vascular permeability and smooth muscle cell proliferation. By reducing AGEs the number of micro and macrovascular complications can be reduced (Singh 2001).

4.2.2 Hyperlipidaemia

In type 2 diabetes lipid and lipoprotein abnormalities are common. Poor glycaemic control seems to lead to disturbances increasing triglyceride containing lipoproteins, VLDL and chylomicrons, which can be easily reversed by using insulin therapy. Dyslipidaemia seems to develop in type 2 diabetes, as it is strongly associated with insulin resistance. The characteristic changes that take place are high triglyceride concentrations ($\geq$ 2.65mg/dl), low concentrations of HDL cholesterol ($\leq$ 0.80mg/dl). However LDL and total cholesterol are usually normal. The risk of vascular disease is associated with small LDL particles that are predisposed to oxidation (Panahloo & Yudkin 2002).

The Paris prospective study (Fontbonne et al 1989) has shown a link between high triglyceride concentrations and coronary heart disease (CHD). There is also a link between cholesterol and cardiovascular disease (CVD). For every unit rise in serum cholesterol the risk was much greater in diabetic patients. There is a clear relationship between triglyceride and LDL cholesterol; triglyceride concentrations are proportional to small LDL and a fall in HDL cholesterol.

There are several courses of action for modifying this dangerous blood lipid profile. The first lines of action include lifestyle changes such as exercise and dietary modification, the blood lipid profile should also be checked annually. Pharmacological measures should only be considered as a secondary prevention. Cholesterol concentrations have been lowered by treatment with 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor reduces CHD related mortality and morbidity (Shephard et al 1995). The Scandinavian survival study (Pyörälä et al 1997), showed that simvastatin reduced coronary events more effectively than in those who were non-diabetic. The British Diabetic Association (Joint British recommendations for prevention of CHD in clinical practice 2000) have made recommendations for targets of certain lipid concentrations in order to prevent CHD, total cholesterol less than 5.0mmol/l and LDL cholesterol of 3.0mmol/l or less. The statin drugs are those recommended to prevent coronary risk. Fibrate drugs are also believed to lower circulating triglyceride concentrations. Both fibrates and statins can be combined but liver transaminase and creatine kinase (CK) need to be monitored as a precaution.

4.2.3 Hypertension

About 50% of all diabetic patients have hypertension, compared to 15-20% of the normal population (Geiss et al 1995). Diabetic patients between the ages of 20-44 years of age are 2-3 times more likely to have hypertension. Hypertension is a significant risk factor for stroke, 2.47-ratio risk for diabetic patients who have hypertension (Davis 1999). Hypertension does not only cause an increase in the cardiovascular risk but also increases the risk of developing other conditions such as retinopathy and microalbuminuria.

It is important that hypertension is treated aggressively in diabetes as hypertension aggravates micro and macrovascular disease. The UKPDS 38 treated 2 groups one that received tight blood pressure control (144/82) and a group with less tight control (154/87). The tight blood pressure control caused a 24% reduction in all diabetes related endpoints, 32% reduction in diabetes related deaths (2/3 due to cardiovascular deaths), 37% reduction in microvascular endpoints and 44% reduction in strokes. However this treatment of tight blood pressure control was fairly costly and on average 20% of patients required 3 or more pharmacological treatments to achieve the desired blood pressure control.

4.2.4 Microalbuminuria and proteinuria

Both microalbuminuria and proteinuria are markers of cardiovascular risk in diabetes patients. Microalbuminuria is an independent risk factor for cardiovascular disease. It is suggested that microalbumin is a marker of renal (or glomerular) as well as general endothelial injury, which initiates the atherothrombotic process (Deckert 1989).

4.2.5 Insulin

Insulin resistance is believed to affect about 25% of the population (Reaven 1994). The cluster of atherogenic and thrombotic risk factors is known to be associated with the underlying ailment of insulin resistance. Therefore insulin resistance may be a risk factor associated with atherothrombotic vascular disease even in people who do not have type 2 diabetes (Mills & Grant 2002).

Insulin may be directly be related to atherogenesis (Stout 1990), studies in animals have shown that high insulin concentrations stimulate smooth muscle cells and macrophages in the arterial wall to synthesise cholesterol. High insulin concentrations can also cause abnormalities in smooth muscle causing proliferation and the binding of LDL at this site and in macrophages. However there does not appear to be any evidence that insulin causes harm to the arterial wall in humans. In the UKPDS there was no excess risk of cardiovascular morbidity or mortality compared to those on other treatments (Turner et al 1998).

There is evidence that raised plasma insulin may be a risk factor by itself for cardiovascular disease (Jarrett 1994). Insulin resistance may be linked to apolipoprotein B for cardiovascular risk (ischaemic heart disease) to be increased. There was a study carried out over 5 years by Desprēs et al 1996, which discovered a strong association between cardiovascular disease and insulin concentrations in the serum. However there was a further substantial increase in risk of cardiovascular disease with both an elevation of serum insulin and apolipoprotein B.

4.2.6 Pro-insulin like molecules

Circulating immuno-reactive insulin consists of approximately 30% of proinsulin-like molecules, the concentration of proinsulin correlates with cardiovascular risk (Zethelius et al 2002), just as insulin itself. It is due to this fact that clinical trials of proinsulin-like molecules were discontinued. It is suggested that proinsulin (31 and 32) is linked to the production of PAI-1 independently of insulin this has been proven *in vitro* (Panahloo & Yudkin 1996).

4.2.7 Homocysteine

There is an association between vascular disease and raised concentrations of homocysteine, which indicates a decline in renal function (indicated by reduced creatinine clearance) or albuminuria in diabetic patients (Gutowski et al 1997). Homocysteine is a novel risk factor for cardiovascular disease. It has been shown a fall in total homocysteine concentrations is associated with an improvement in even modest improvements in HbA1c blood glucose concentrations (Passaro et al 2003). Patients with complications such as nephropathy were treated with folic acid supplements (2mg), which reduce plasma homocysteine concentrations by 5 μmol/l from e.g. 18 to 13.9 μmol/l.

4.2.8 Coagulation and fibrinolysis

Diabetes is connected to several defects in the coagulation and fibrinolytic pathways. A star in Figure 4-9: -The coagulation and fibrinolytic pathway. highlights the common defects. These procoagulation changes contribute to plaque thrombosis causing an acute myocardial infarction (AMI). One key molecule plasminogen activator inhibitor-1 (PAI-1) is critical in regulating fibrolysis; its concentration is elevated in type 2 diabetic patients. PAI-1 is produced by a number of cells including endothelial cells and hepatoctyes. The plasma concentration of PAI-1 closely correlates with insulin like molecules and triglyceride concentrations. Diabetic patients with AMI have higher concentrations of PAI-1than non-diabetic patients; this indicates a reduction in the ability to break clots, showing a fall in the efficiency of thrombolytic therapy received. Any rise in cardiac enzymes also suggests impaired infusion.

Figure 4-9: -The coagulation and fibrinolytic pathway.

The coagulation and fibrinolytic pathway. Defects that favour coagulaion and impair fibrolysis are indicated with a star. Adapted from Panahloo and Yudkin (2002). Macrovascular disease and diabetes Oxford textbook of endocrinology and diabetes. 1814.

PAI-1 becomes elevated in type 2 diabetic patients, due to an increase in platelet release and storage. PAI-1 is elevated as it is released in greater amounts from endothelium due to hyperglycaemia modified LDL and VLDL and cytokines. Plasminogen may also become glycated and as a result this can reduce clot lysis (Brown 2000). There is also a strong association between glycaemic control and fibrinogen (Bruno et al 1996). Even after adjustments for age and sex there is still a significant increase in fibrinogen and this may be a possible link to the increase in cardiovascular risk. There are also changes in platelet aggregation and adherence, which causes the intrinsic coagulation system to become active, which might act to convert fibrinogen into fibrin. Studies have shown in diabetic patients there is an elevation in platelet aggregation and β-thromboglobulin and plasma factor 4, platelet derived growth factor and increased platelet turnover. High numbers of these factors

are rectified by intensive insulin therapy and returns some of the normal platelet function (Colwell 1994).

4.2.9 Smoking and obesity

Smoking is well known to increase cardiovascular mortality in patients without diabetes, therefore the risk in diabetes is even more important. The MRFIT trial (Stamler et al 1993) showed that as the number of cigarettes smoked increased, so too did the cardiovascular death rate. The death rate was much higher in males with diabetes that smoked compared to those without diabetes who also smoked. Therefore getting diabetic patients to stop smoking is a very important prevention of cardiovascular disease.

The nurse health study (Colditz et al 1995) showed there was an association between diabetes and obesity. The risk of diabetes increases as the BMI (Body mass index) increases. It is central obesity that is associated with diabetes risk of vascular disease (Licata et al 2003). Therefore appropriate measures should be taken to reduce central obesity by taking appropriate exercise and altering their diet to reduce their weight.

4.3 Atherosclerosis

4.3.1 Aetiology and extent of atherosclerosis

Atherosclerosis develops much earlier and at a much quicker rate in diabetic patients leading to widespread lesions developing through out the arterial system (Grant and Davis 2003). The thickening of the intima layer is often an early sign of atherosclerosis (Makimattila et al 2002) and is often linked to insulin resistance (Haffner 1999), and thickening of the muscle in the arterial walls contributes to hypertension and calcification of the vessels. The lesions of atherosclerosis are mainly

of the large and medium sized arteries containing a higher quantity of elastic and muscular tissue (Ross 1999). Lesions can start in very young patients and fatty streaks are often even evident in infants and young children (Ross 1986).

The atherosclerotic plaques that arise in diabetic patients are not vastly different than those seen in non-diabetic patients (Drouet 1999). However the extent of the disease is often greater in diabetic patients and involves both the coronary and peripheral arterial circuits (Vigorita et al 1980). Some factors that predispose diabetic patients to arterial disease such as atherosclerosis include hypertension and dyslipidemia, which accounts for the increased load of atheroma. However these factors do not fully explain the extent of the degenerative changes or the pattern of damage seen in diabetic vessels. Newer identified risk factors for atherosclerotic disease such as C-reactive protein, lipoprotein, fibrinogen and homocysteine may be potential screening targets to highlight early signs of the disease (Hackam and Anand 2003).

4.3.2 Structural abnormalities

There are several characteristic changes seen in atherosclerosis and the first of these is a thickening of the intima, which increases with the duration of diabetes and extreme hyperglycaemia. This thickening can be prevented or inhibited with the use of angiotensin-converting enzyme inhibitors (Hosomi et al 2001).

The media layer often develops a hyaline appearance, where it takes on an amorphous, ground glass like look, caused by the break down of structural proteins such as collagen and the uptake of glycated plasma proteins. This is naturally part of the aging process and is associated with hypertension. Hyaline material contains glycoprotein and this stains using a periodic acid-Schiff (PAS) reagent. The media is

also likely to calcification in the coronary and peripheral arteries. The structural changes and loss of elasticity lead to hypertension and may contribute to the increased macrovascular risk seen in diabetic patients (Grant & Davis 2003).

4.3.3 The start of atherosclerosis

Atherosclerosis is often considered to be an inflammatory disease, as it results from a response to injury usually of the endothelium, which is the first step in the disease (Ross & Glomset 1973). More recent evidence suggests that it is probably more likely to be endothelium dysfunction and not injury that is the key issue leading to atherosclerosis. Whichever case is true it is evident that lesions in atherosclerosis represent different stages in chronic inflammation of the artery (Ross 1999).

Atherosclerosis is no longer considered just a disease of lipid deposition in the arteries, but as an inflammatory process, involving highly specific responses (Rifai & Ridker 2002). As cholesterol deposition cannot fully explain the extent of the disease involving endothelial dysfunction and proliferation of smooth muscle cells. It is important to establish inflammation is involved, as atherosclerosis is the cause of most CHD. Inflammation markers such as C-reactive protein (CRP) may play a key in highlighting inflammation and therefore individuals at risk of atherosclerosis and CHD (Ridker et al 1998). CRP is involved in activating complement as it induces the production of the monocyte chemo attractant protein-1 (MCP-1) (Pasceri et al 2001), and the upregulation of intracellular and vascular cell adhesion molecule-1 and E-selectin (Pasceri et al 2000). It also promotes macrophage uptake of LDL and monocyte tissue factor production. CRP is also present in atherosclerotic plaques and myocardium after an infarction, where it may have joined with the terminal complement complexes (Shakdi et al 1999).

4.3.4 Types of atherosclerotic plaques

In the early stages of atherogenesis LDL (low-density lipoprotein) starts to gather in the subendothelial space (Diaz et al 1997) and this is what contributes to endothelial dysfunction. The LDL becomes oxidised by various types of material including smooth muscle cells, endothelial cells and macrophages. LDL induces the chemotaxis of monocytes to the area where they become trapped, it is then that they ingest the underlying LDL molecules and become foam cells. Foam cells eventually deteriorate and necrotic debris and lysosomal enzymes accumulate. This is how plaques begin to form (Colwell 1991). The processes that take place that lead to atherosclerosis are summarised in the diagram below Figure 4-10: - The pathogenesis of atherosclerosis.

Figure 4-10: - The pathogenesis of atherosclerosis

Slide obtained from Dr C. J. Bailey personal communications.

Myocardial infarctions are often the result of a rupture of an atherosclerotic plaque. The rupture is most often associated with vulnerable plaques; rupture is much less likely with a stable plaque (Libby 1995). Stable plaques are characterised by the presence of a lipid core covered with a thick fibrous cap and there is also an over expression of vascular smooth muscle cells. It is the fibrous cap that prevents the plaque rupturing and causing a thrombus. Vulnerable plaques on the other hand are characterised by a large lipid core with a thin fibrous cap. There is also an increase in inflammatory cells seen at these sites, these contribute to the plaque rupturing as they degrade the fibrous cap and reduce the number of smooth muscle cells. These changes all act to reduce the stability of the plaque, making it more likely to rupture. See Figure 4-11: - Types of atherosclerotic plaques

Figure 4-11: - Types of atherosclerotic plaques

This diagram was adapted from: - Colwell 2000. Pathogenesis of vascular disease. Diabetes, Obesity and Metabolism. **2.** 2: S19-24. From the original article: - Libby 1995. Molecular bases of the acute coronary syndromes. Circulation. **91**: 2844-2850.

4.4 Active tension

The tension generated by contractile elements of muscle is called active tension (Witteman 2003). The actual process of smooth muscle contraction is dealt with in greater detail in the next chapter, chapter 5. When the α receptors on the smooth muscle walls of the large blood vessels are challenged with nordrenaline contraction is initiated by the release of calcium, which induces the shortening of the sacromere and causes vasoconstriction. The tension generated in the tissue can be determined in grams or as a force in Newtons, to illustrate the tension generated in the blood vessel wall. The ability of smooth muscle to become partially contracted in blood vessels is very important in maintaining and regulating changes in blood pressure (Cothran 2003).

In contrast the relaxation of the smooth muscle in blood vessels can be triggered by acetylcholine. Acetylcholine has an indirect effect as it triggers the release of EDRF, which is NO. The release of NO is addressed in greater detail in chapter 6.

4.5 Experimental detail and aims

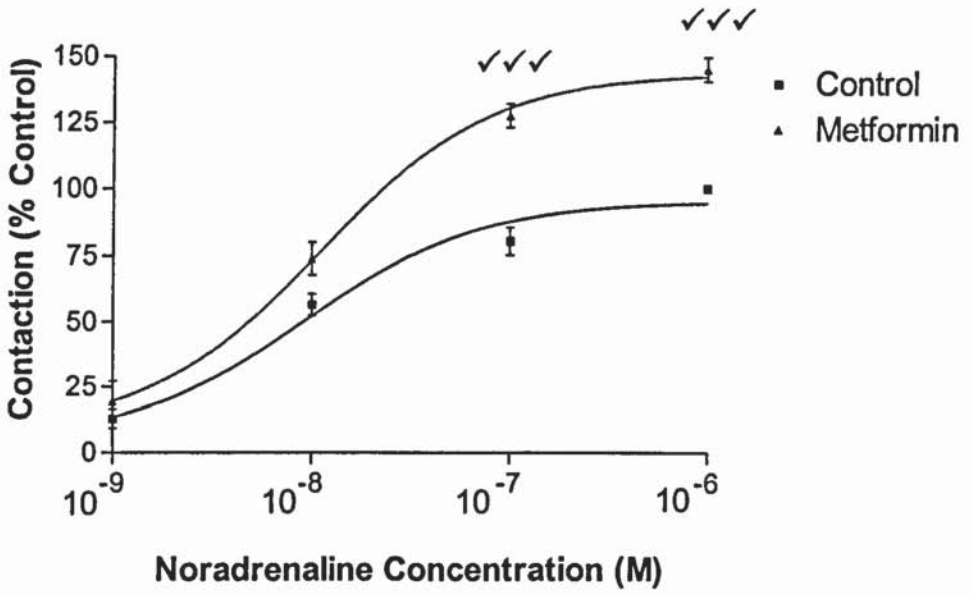
Studies were carried out as outlined in section 2. Two separate studies were undertaken, one involved in vitro treatment of the aorta the other in vivo treatment and ex vivo studies on the aorta with metformin. The aim of doing this was to see if the effects of metformin were dependent or independent of the internal environment, and the time period taken for metformin to have an impact on the contraction and relaxation properties of the arteries. All experimental procedures involving aortic contraction and relaxation are outline in greater detail in section **2.2-2.3.**

4.6 Results

In vitro (1 and 4 hour exposure to metformin)

These are the results obtained from the in vitro and in vivo studies involving metformin and the challenge of noradrenaline and acetylcholine. The first set of studies performed were the in vitro studies where aortas were incubated with metformin for 1 and 4 hours. The 1-hour studies were performed using the Mulvany Halpern myograph equipment are shown below.

Figure 4-12: - Contraction of thoracic aorta in lean male mice, in the absence and presence of metformin 10^{-5}M after a 1 hour incubation



Tukey Test
✓✓✓ P<0.001 Metformin v control

Figure 4-12: - The tissues were incubated for 1 hour, the control without metformin and the experimental sample with metformin 10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of noradrenaline 10^{-9}–10^{-6}M. The contraction is expressed as percentage of the control 10^{-6}M noradrenaline. The tissue sample incubated with metformin 10^{-5}M showed a 49% greater contraction at 10^{-6}M noradrenaline compared to the control. ± SEM. (n=6)

Table 4-1: - Tension generated by contracting tissue in grams after 1 hour

	NA 10^{-9}	NA 10^{-8}	NA 10^{-7}	NA 10^{-6}
Control	+0.082	+0.354	+0.504	+0.627
Metformin	+0.122	+0.462	+0.800	+0.910

The units of tension are in grams, additional to the original 1g stretch.

Table 4.1: - This shows the exact tension in grams generate by the contracting tissue of the control and the tissue treated with metformin 10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is much greater in the metformin 10^{-5}M treated tissue due to the larger contraction. (n=6)

Figure 4-13: - Relaxation of thoracic aorta in lean male mice, in the absence and presence of metformin 10^{-5}M after a 1 hour incubation

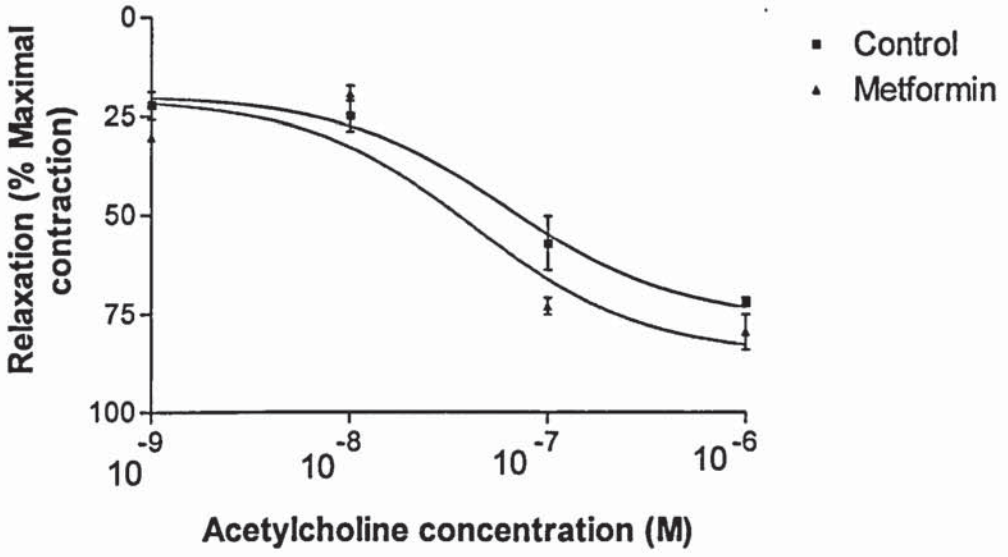


Figure 4-13: - The tissues were incubated for 1 hour, the control without metformin and the experimental sample with metformin 10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of acetylcholine 10^{-9}–10^{-6}M. The relaxation is expressed as percentage of the control 10^{-6}M noradrenaline seen in the control of the previous experiment Fig 4-12. The tissue samples incubated with metformin 10^{-5}M showed 8.45% greater relaxation at 10^{-6}M acetylcholine compared to the control. ± SEM. (n=6)

Table 4-2: - Tension generated by relaxing tissue in grams after 1 hours

	Ach 10^{-9}	Ach 10^{-8}	Ach 10^{-7}	Ach 10^{-6}
Control	+0.488	+0.471	+0.269	+0.176
Metformin	+0.477	+0.408	+0.169	+0.129

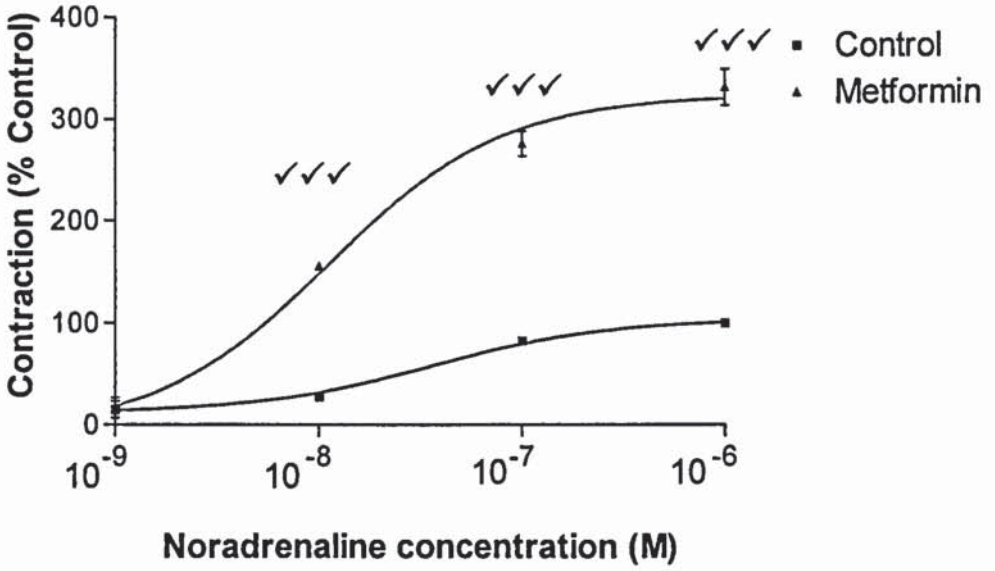
The units of tension are in grams, additional to the original 1g stretch.

Table 4.2: - This shows the exact tension in grams generate by the relaxing tissue of the control and the tissue treated with metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is less in the metformin 10^{-5}M treated tissue due to the larger relaxation. (n=6)

In vitro studies showed that the upper section of aorta after a 1-hour incubation with 10^{-5}M metformin, showed an increased aortic contraction by a maximum of 49% ($p<0.001$) compared to control at 10^{-6}M noradrenaline. At 1 hour the effect of acetylcholine was not significant and only caused an additional 8.45% relaxation.

The 4 hours studies were performed in a similar manner, with metformin incubation for 4 hours. Before being subjected to increasing and accumulating concentrations of noradrenaline to determine the tissues ability to contract. In figure 4-15, relaxation is determined in a similar manner in maximally contracted aortic rings using accumulating and increasing concentrations of acetylcholine.

Figure 4-14: - Contraction of thoracic aorta in male lean mice, in the absence and presence of metformin 10^{-5}M after a 4 hour incubation



Tukey Test
✓✓✓ P<0.001 Metformin v control

Figure 4-14: - The tissues were incubated for 4 hour, the control without metformin and the experimental sample with metformin 10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of noradrenaline 10^{-9}–10^{-6}M. The contraction is expressed as percentage of the control 10^{-6}M noradrenaline. The tissue sample incubated with metformin 10^{-5}M showed a 221% greater contraction at 10^{-6}M noradrenaline compared to the control. ± SEM. (n=6)

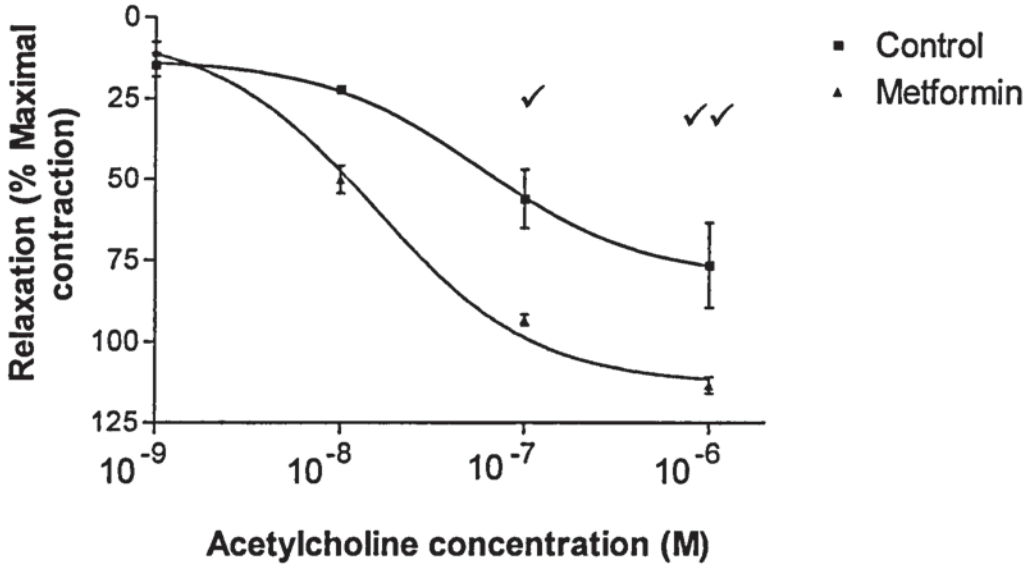
Table 4-3: - Tension generated by relaxing tissue in grams after 4 hours

	NA 10^{-9}	NA 10^{-8}	NA 10^{-7}	NA 10^{-6}
Control	+0.113	+0.206	+0.612	+0.739
Metformin	+0.125	+1.155	+2.039	+2.454

The units of tension are in grams, additional to the original 1g stretch.

Table 4.3: - This shows the exact tension in grams generate by the contracting tissue of the control and the tissue treated with metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is much greater in the metformin 10^{-5}M treated tissue due to the larger contraction. (n=6)

Figure 4-15: - Relaxation of thoracic aorta in male lean mice, in the absence and presence of metformin 10^{-5}M after a 4 hour incubation



Tukey Test
✓✓ P<0.01 Metformin v control
✓ P<0.05 Metformin v control

Figure 4-15: - The tissues were incubated for 4 hour, the control without metformin and the experimental sample with metformin 10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of acetylcholine 10^{-9}–10^{-6}M. The relaxation is expressed as percentage of the control 10^{-6}M noradrenaline seen in the control of the previous experiment Fig 4-14. The tissue samples incubated with metformin 10^{-5}M showed a 36.85% greater relaxation at 10^{-6}M acetylcholine compared to the control. ± SEM. ((n=6)

Table 4-4: - Tension generated by contracting tissue in grams after 4 hours

	Ach 10^{-9}	Ach 10^{-8}	Ach 10^{-7}	Ach 10^{-6}
Control	+0.630	+0.575	+0.324	+0.173
Metformin	+0.658	+0.367	+0.049	-0.100

The units of tension are in grams, additional to the original 1g stretch.

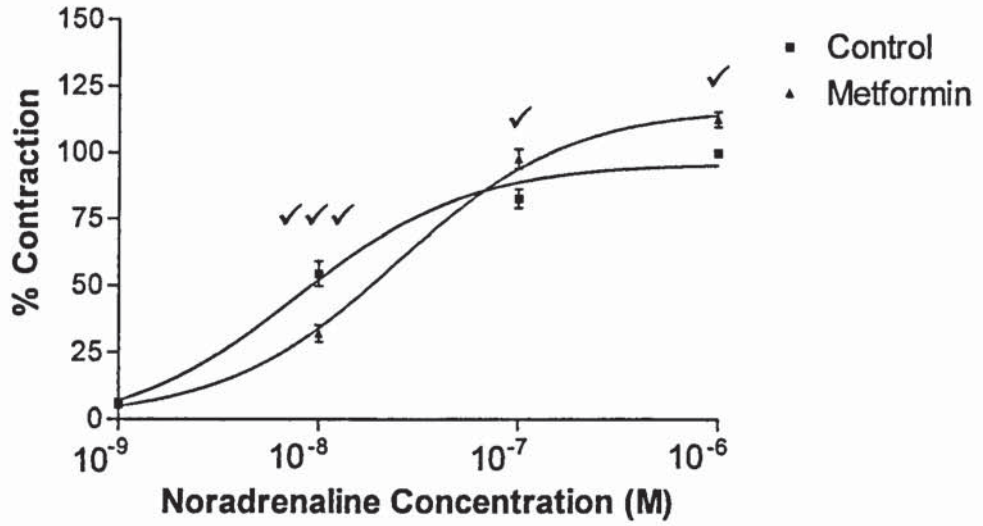
Table 4.4: - This shows the exact tension in grams generate by the relaxing tissue of the control and the tissue treated with metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is less in the metformin 10^{-5}M treated tissue due to the larger relaxation. (n=6)

This effect was further enhanced after a 4-hour incubation with 10^{-5}M metformin, giving an increase of 221% ($p<0.001$) compared to the control. At 4 hours a maximum noradrenaline contracted aorta showed an increased relaxation in response to acetylcholine, increasing the relaxation response by 36.85% to 10^{-6}M Ach ($P < 0.01$) compared to the control.

In vivo treatment (ex vivo aorta)

In vivo the effects took much longer to be displayed. There was no effect after 24 hours of treatment with metformin (250mg/kg/day) in their drinking water; these results are therefore not shown here. As a result the mice were treated for longer periods of time before they were tested for any changes in vasomotion. The contraction and relaxation in the 2, 4 and 8-week studies were performed in the same manner as previously documented in both figures 4-14 and 4-15.

Figure 4-16: - Contraction of thoracic aorta in lean male mice, in the absence and presence of metformin 10^{-5}M after 2 weeks



Tukey Test
✓✓✓ P<0.001 Metformin v control
✓ P<0.05 Metformin v control

Figure 4-16: - The animals were treated for 2 weeks, the control without metformin in their drinking water and the experimental group was treated with 250mg/kg/day in their drinking water, which produces a circulating plasma concentration of metformin 10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of noradrenaline 10^{-9}–10^{-6}M. The contraction is expressed as percentage of the control 10^{-6}M noradrenaline. The tissue sample incubated with metformin 10^{-5}M showed a 21% greater contraction at 10^{-6}M noradrenaline compared to the control. ± SEM. (n=6)

Table 4-5: - Tension generated by contracting tissue in grams after 2 weeks of treatment

	NA 10^{-9}	NA 10^{-8}	NA 10^{-7}	NA 10^{-6}
Control	+0.034	+0.294	+0.448	+0.541
Metformin	+0.030	+0.173	+0.528	+0.609

The units of tension are in grams, additional to the original 1g stretch.

Table 4.5: - This shows the exact tension in grams generate by the contracting tissue of the control and the tissue treated with 250mg/kg/day equivalent to metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is much greater in the metformin 10^{-5}M treated tissue due to the larger contraction. (n=6)

Figure 4-17: - Relaxation of thoracic aorta in lean male mice, in the absence and presence of metformin 10^{-5}M after 2 weeks

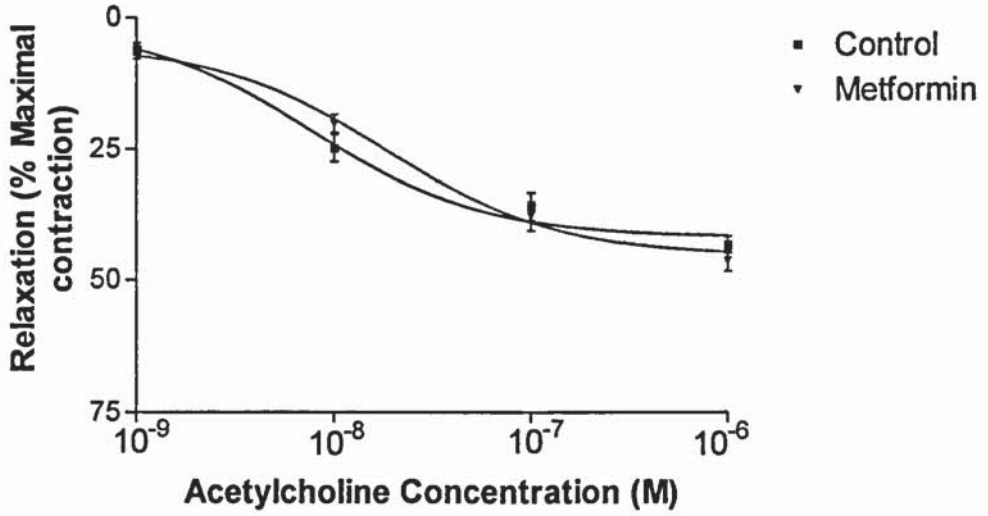


Figure 4-17: - The animals were treated for 2 weeks, the control without metformin in their drinking water and the experimental group was treated with 250mg/kg/day in their drinking water, which produces a circulating plasma concentration of metformin10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of acetylcholine 10^{-9}–10^{-6}M. The contraction is expressed as percentage of the control 10^{-6}M noradrenaline in Fig 4-16. The tissue sample incubated with metformin 10^{-5}M showed no significant increase in relaxation when treated with metformin 10^{-5}M. ± SEM. (n=6)

Table 4-6: - Tension generated by relaxing tissue in grams after 2 weeks of treatment

	Ach 10^{-9}	Ach 10^{-8}	Ach 10^{-7}	Ach 10^{-6}
Control	+0.509	+0.407	+0.346	+0.307
Metformin	+0.503	+0.432	+0.337	+0.290

The units of tension are in grams, additional to the original 1g stretch.

Table 4.6: - This shows the exact tension in grams generate by the contracting tissue of the control and the tissue treated with 250mg/kg/day equivalent to metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. There is no major change in tension as metformin does not significantly increase relaxation. (n=6)

The first response to treatment of 250mg/kg/day with metformin in the drinking water was displayed at 2 weeks when there was an increase in maximum noradrenaline (10^{-6}M) induced contraction by 21% ($p<0.05$). However at lower noradrenaline concentrations metformin does not increase the percentage contraction, so after 2 weeks it is only the highest concentration (10^{-6}M) of noradrenaline that increased contraction. However there was no significant change after 2 weeks of metformins on acetylcholine-induced relaxation.

The body weight of the mice was monitored through out the study, there were relatively no change in body weight in any of the 2 groups over a 2 week period.

Figure 4-18: - Mean increase in body weight over 2 weeks

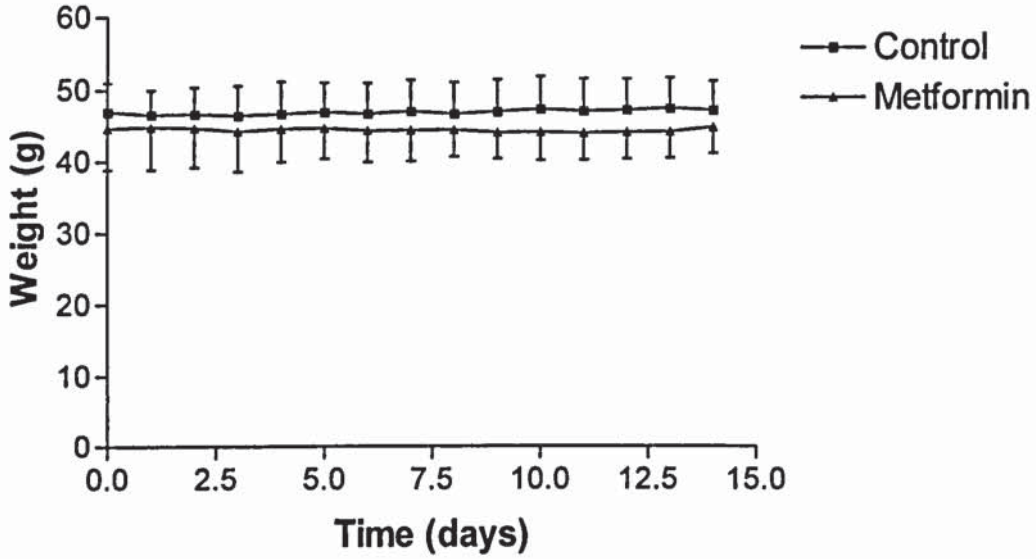


Figure 4-18: - The body weight of the lean male mice was taken at the start of the study and monitored every day for 2 weeks there is no significant change in body weight in any of the groups. ± SEM. (n=6)

As well as no change in weight, the food consumption of the mice was also monitored, in case metformin altered feeding habits. There was however no change in feeding habits between control and metformin treated mice in the 2-week period.

Figure 4-19: - Food consumed g/mouse/week during 2 weeks

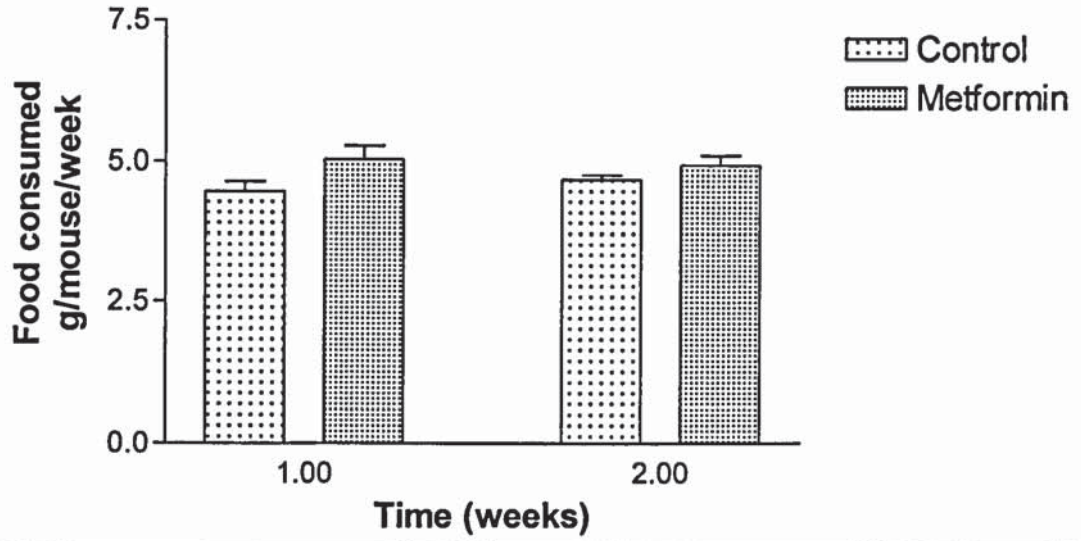


Figure 4-19: - The food consumed by each mouse was calculated every day. Then an average was taken for each day over weeks 1-2, an average amount is plotted on the graph. There is no difference in the amount of food consumed each day over the 2 week period by the control and metformin treated groups. ± SEM. (n=6)

Blood glucose concentrations were monitored from tail blood at the start of the study and then every following week. There was no significant change in blood glucose over the 2-week period of treatment with metformin.

Figure 4-20: - Blood glucose concentrations per week over 2 weeks

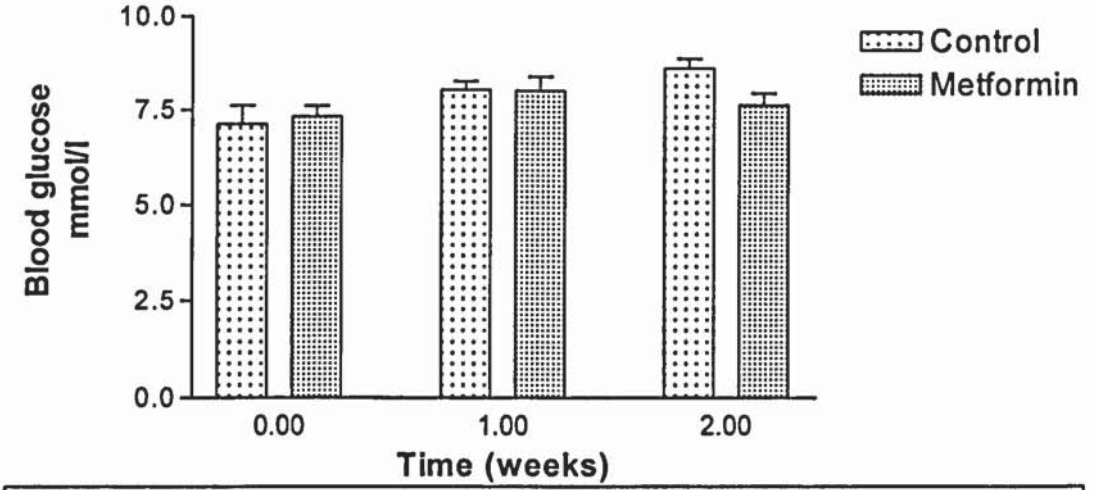


Figure 4-20: - Blood samples were taken from the tails of lean male mice at the start of the study and weekly thereafter. The blood glucose was taken using a blood glucose monitor and average values for each group were plotted. There is no difference in the blood glucose concentrations between the control and metformin treated groups over this 2 week period. ± SEM. (n=6)

Serum triglyceride concentrations were monitored over the 2 week study, there was no significant difference between control and metformin treated mice.

Figure 4-21: - Change in serum triglyceride profile after 2 weeks in the presence and absence of metformin

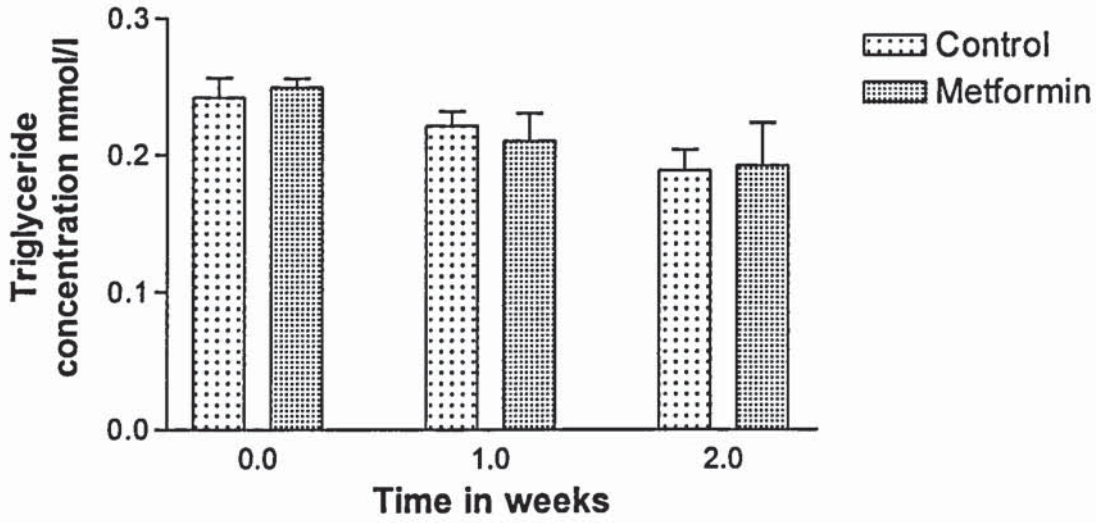


Figure 4-21: - Blood samples were taken from the tails of the lean male mice at the start of the study and then weekly thereafter. The blood triglyceride was determined using the INFINITY™ triglyceride reagent kit from Sigma and the average concentration of each group was plotted. There is no difference in the triglyceride concentrations between the control and metformin treated groups over this 2 week period. ± SEM. (n=6)

The serum was also tested for any changes in insulin concentration, again as in previous studies there was no significant changes between control and metformin treated mice.

Figure 4-22: - Changes in insulin profile after 2 weeks treatment with metformin

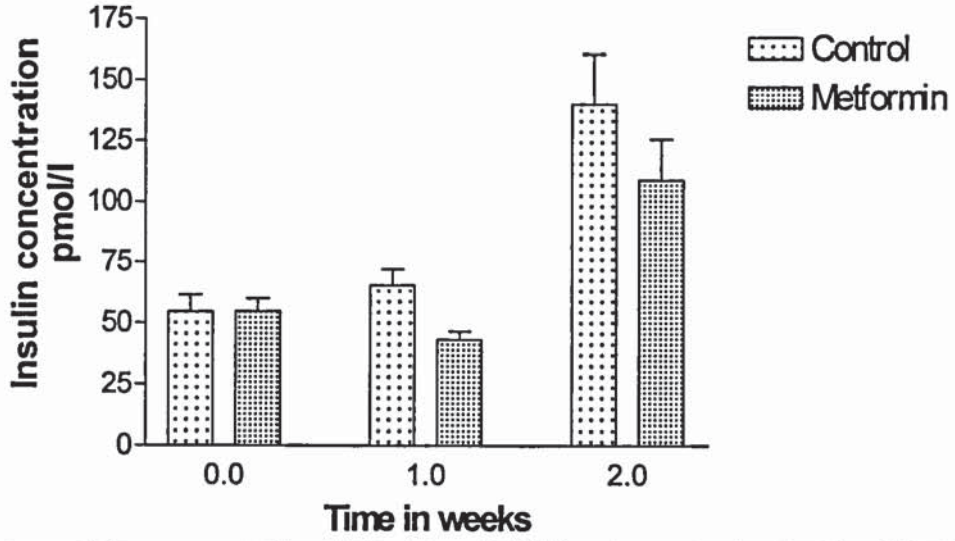
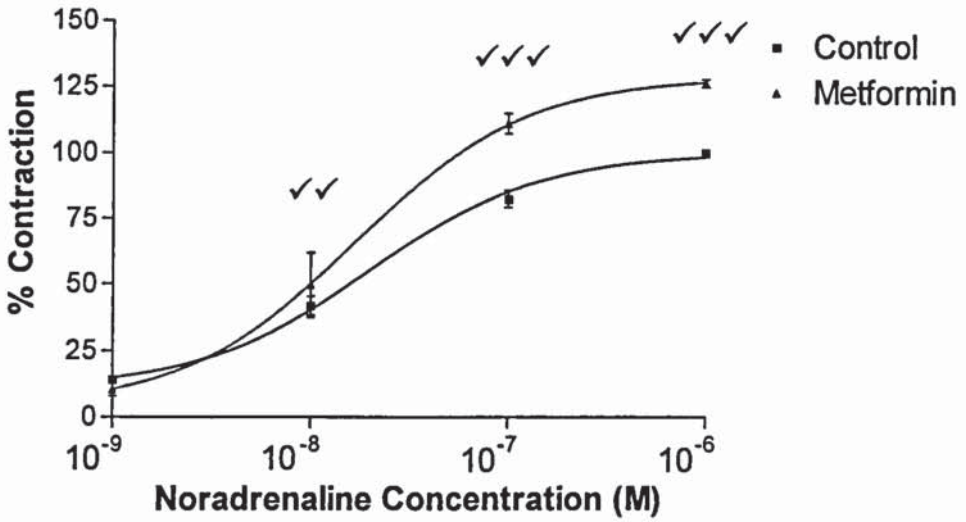


Figure 4-22: - Blood samples were taken from the tails of the lean male mice at the start of the study and then weekly thereafter. The blood triglyceride was determined using the ELISA insulin kit from Mercodia and the average concentration of each group was plotted. There is no difference in the insulin concentrations between the control and metformin treated groups over this 2 week period. The insulin values are non-fasting. ± SEM. (n=6)

There were also no significant changes in bodyweight, food intake, blood glucose, serum triglyceride and serum insulin concentrations in the mice, over a 2 week period of treatment with metformin.

The time period of treatment with metformin was increased to 4 weeks in vivo. Similar experiments were performed as seen in the 2-week studies. The contraction and relaxation studies are performed after 4 weeks in a similar manner to studies in figures 4-14 and 4-15.

Figure 4-23: - Contraction of thoracic aorta in lean male mice, in the absence and presence of metformin after 4 weeks



Tukey Test
✓✓✓ P<0.001 Metformin v control
✓✓ P<0.01 Metformin v control

Figure 4-23: - The animals were treated for 4 weeks, the control without metformin in their drinking water and the experimental group was treated with 250mg/kg/day in their drinking water, which producing a plasma concentration of metformin 10^{-5}M. The tissues were calibrated with 1g for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of noradrenaline 10^{-9}–10^{-6}M. The contraction is expressed as percentage of the control 10^{-6}M noradrenaline. The tissue sample incubated with metformin 10^{-5}M showed a 29% greater contraction at 10^{-6}M noradrenaline compared to the control. ± SEM. (n=6)

Table 4-7: - Tension generated by contracting tissue in grams after 4 weeks of treatment

	NA 10^{-9}	NA 10^{-8}	NA 10^{-7}	NA 10^{-6}
Control	+0.096	+0.284	+0.560	+0.679
Metformin	+0.072	+0.337	+0.753	+0.857

The units of tension are in grams, additional to the original 1g stretch.

Table 4.7: - This shows the exact tension in grams generate by the contracting tissue of the control and the tissue treated with 250mg/kg/day equivalent to metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is much greater in the metformin 10^{-5}M treated tissue due to the larger contraction. (n=6)

Figure 4-24: - Relaxation of thoracic aorta in lean male mice, in the absence and presence of metformin after 4 weeks

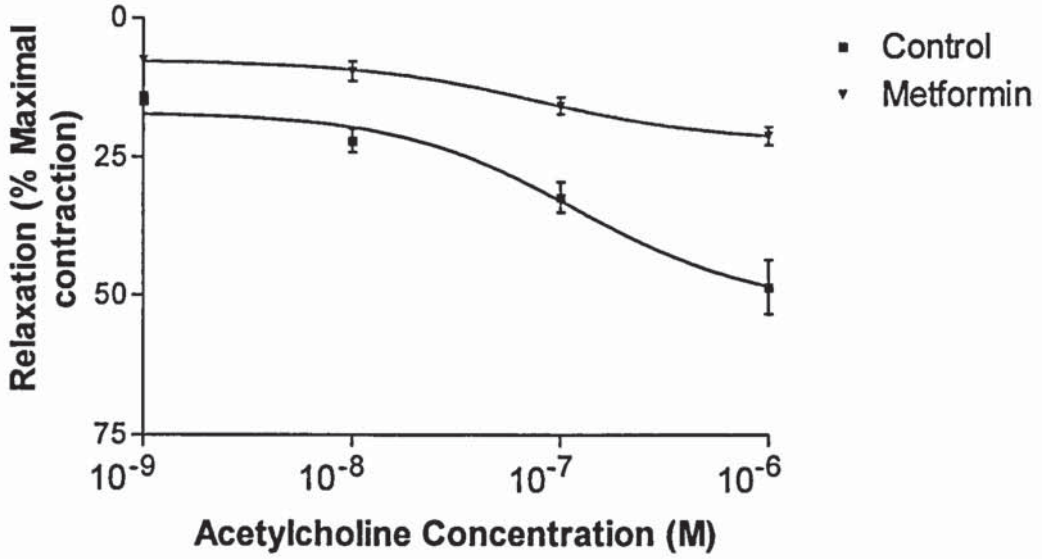


Figure 4-24: - The animals were treated for 4 weeks, the control without metformin in their drinking water and the experimental group was treated with 250mg/kg/day in their drinking water, which produces a circulating plasma concentration of metformin10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of acetylcholine 10^{-9}–10^{-6}M. The contraction is expressed as percentage of the control 10^{-6}M noradrenaline in Fig 4-23. The tissue sample incubated with metformin 10^{-5}M showed no significant increase in relaxation, in fact less relaxation is seen in this case when treated with metformin compared to the control. ± SEM. (n=6)

Table 4-8: - Tension generated by relaxing tissue in grams after 4 weeks of treatment

	Ach 10^{-9}	Ach 10^{-8}	Ach 10^{-7}	Ach 10^{-6}
Control	+0.580	+0.529	+0.460	+0.349
Metformin	+0.627	+0.614	+0.572	+0.535

The units of tension are in grams, additional to the original 1g stretch.

Table 4.8: - This shows the exact tension in grams generate by the contracting tissue of the control and the tissue treated with 250mg/kg/day equivalent to metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is greater in the metformin treated sample as it does not increase relaxation in this case. (n=6)

After 4 weeks in vivo treatment the effect of metformin was more enhanced with noradrenaline 10^{-6}M than that seen after 2 weeks giving a 29% greater contraction ($P<0.001$). However the relaxation effect of acetylcholine was not increased after treatment in vivo with metformin. This may indicate a sign of adaptation by the tissue, where it is unable to relax as much as previously seen.

The body weight and food consumption were again monitored through out the 4-week period. Body weight remained unaltered, and there were no changes in the average food consumption over this time.

Figure 4-25: - Mean increase in body weight over 4 weeks

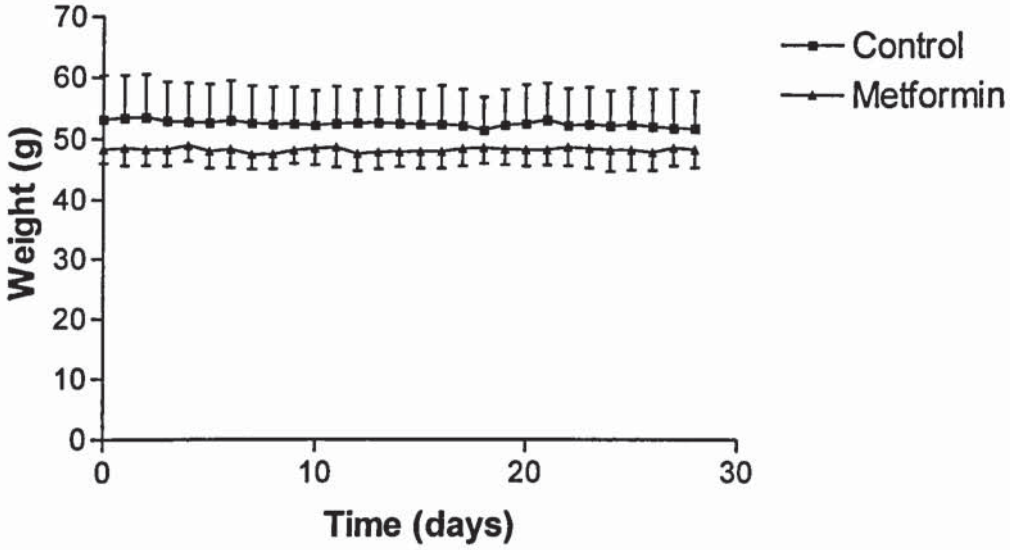


Figure 4-25: - The body weight of the lean male mice was taken at the start of the study and monitored every day for 4 weeks there is no significant change in body weight in any of the groups. ± SEM. (n=6)

Figure 4-26: - Food consumed g/mouse/week during 4 weeks

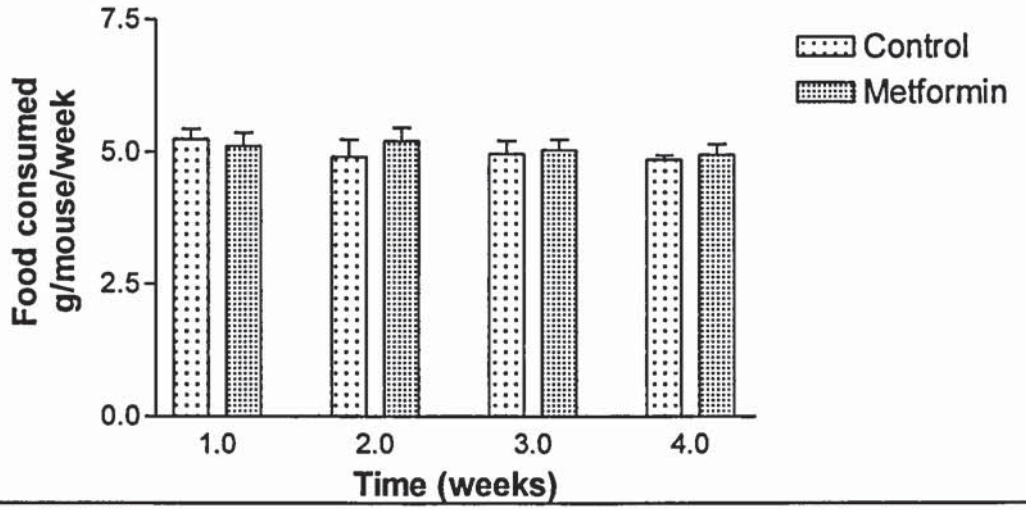


Figure 4-26: - The food consumed by each mouse was calculated every day. Then an average was taken for each day over the first, second, third and fourth weeks, this is average amount that is plotted on the graph. There is no difference in the amount of food consumed each day over the 4 week period by the control and metformin treated groups. ± SEM. (n=6)

The blood glucose and triglyceride concentrations of tail blood taken from mice were monitored. There were no significant changes in these measurements over 4 weeks.

Figure 4-27: - Blood glucose concentrations for per week over 4 weeks

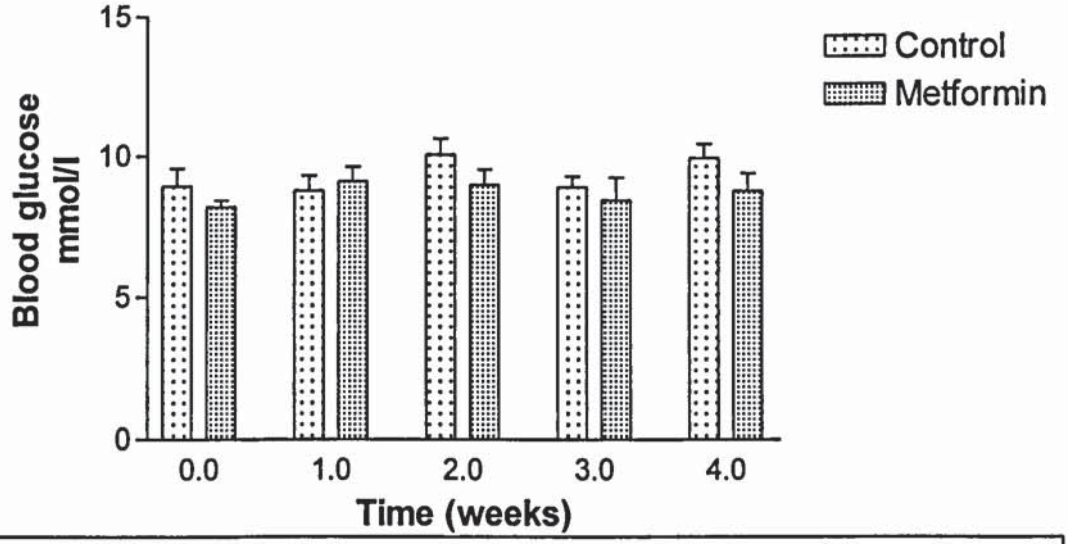


Figure 4-27: - Blood samples were taken from the tails of the lean male mice at the start of the study and then weekly thereafter. The blood glucose was taken using a blood glucose monitor and the average value of each group was plotted. There is no difference in the blood glucose concentrations between the control and metformin treated groups over this 4 week period. ± SEM. (n=6)

Figure 4-28: - Change in serum triglyceride profile after 4 weeks in the presence and absence of metformin

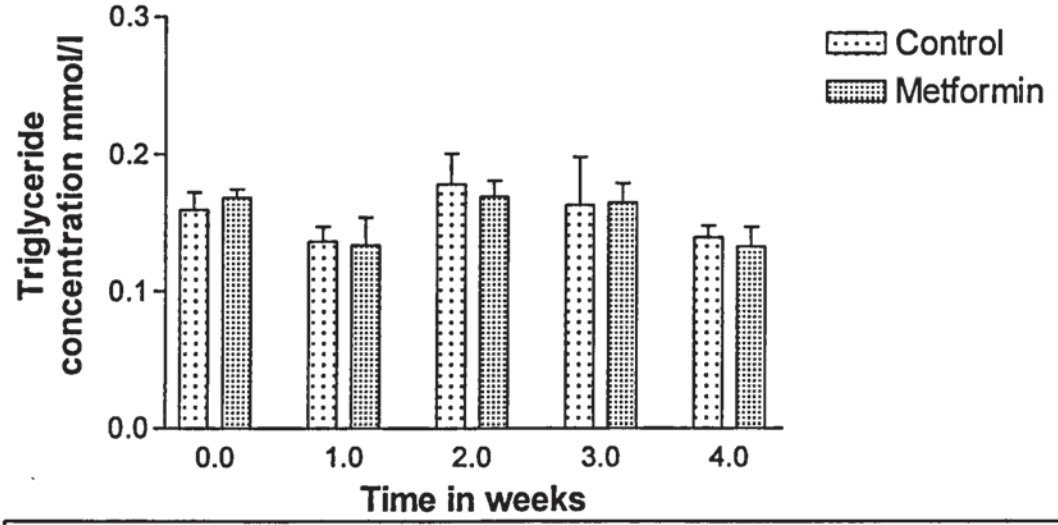


Figure 4-28: - Blood samples were taken from the tails of the lean male mice at the start of the study and then weekly thereafter. The blood triglyceride was determined using the INFINITY™ triglyceride reagent kit from Sigma and the average concentration of each group was plotted. There is no difference in the triglyceride concentrations between the control and metformin treated groups over this 4 week period. ± SEM. (n=6)

The serum concentrations were also taken from weekly samples of tail blood. There were no significant differences between the control and metformin treated mice.

Figure 4-29: - Changes in insulin profile after 4 weeks treatment with metformin

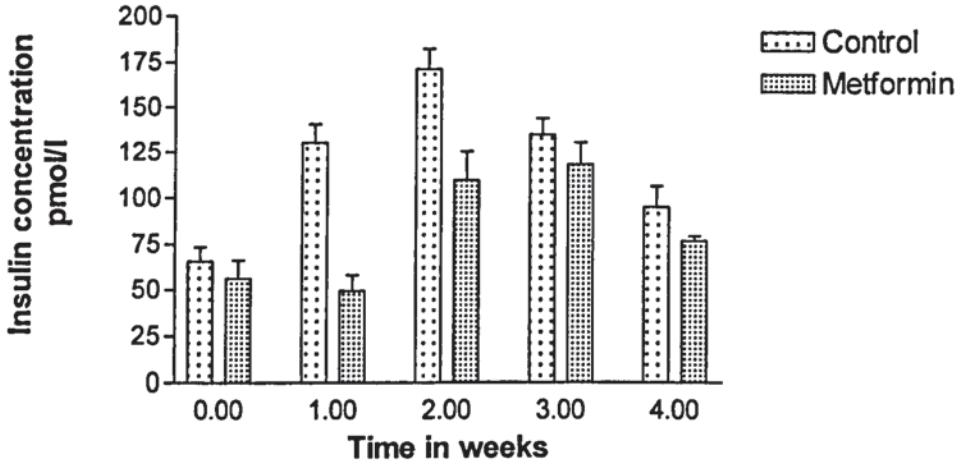
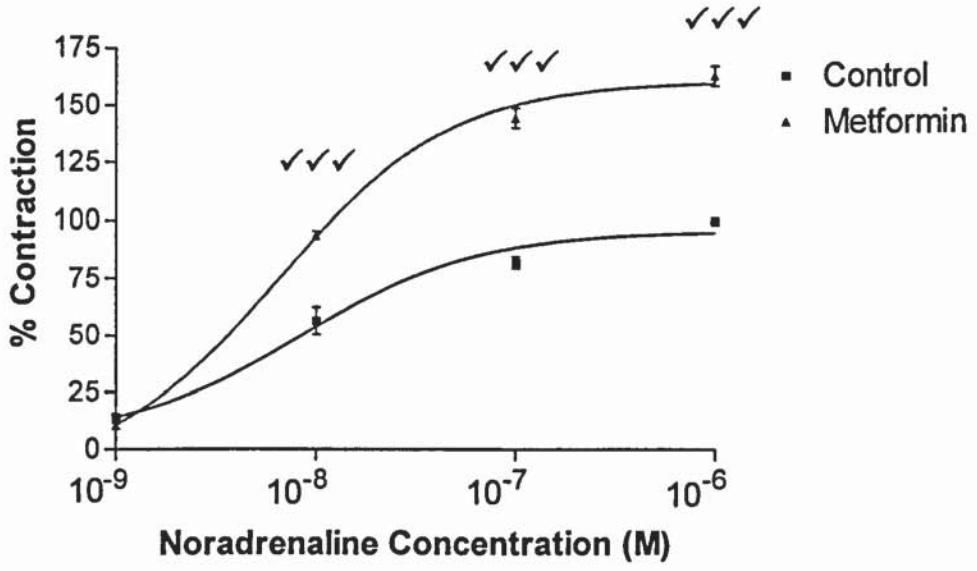


Figure 4-29: - Blood samples were taken from the tails of the lean male mice at the start of the study and then weekly thereafter. The blood triglyceride was determined using the ELISA insulin kit from Mercodia and the average concentration of each group was plotted. There is no difference in the insulin concentrations between the control and metformin treated groups over this 4 week period. The insulin values are non-fasting. ± SEM. (n=6)

Once again there were no statistically significant changes in the mice regarding body weight, food in take, blood glucose, triglyceride or insulin serum concentrations over a 4 week period of treatment with metformin.

The final sets of studies performed were carried out for 8 weeks. Again contraction and relaxation studies were performed as documented earlier, along with additional studies on the serum from tail blood samples.

Figure 4-30: - Contraction of thoracic aorta in lean male mice, in the absence and presence of metformin after 8 weeks



Tukey Test
✓✓✓ P<0.001 Metformin v control

Figure 4-30: - The animals were treated for 8 weeks, the control without metformin in their drinking water and the experimental group was treated with 250mg/kg/day in their drinking water, which produces a circulating plasma concentration of metformin 10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of noradrenaline 10^{-9}–10^{-6}M. The contraction is expressed as percentage of the control 10^{-6}M noradrenaline. The tissue sample incubated with metformin 10^{-5}M showed a 65% greater contraction at 10^{-6}M noradrenaline compared to the control. ± SEM. (n=6)

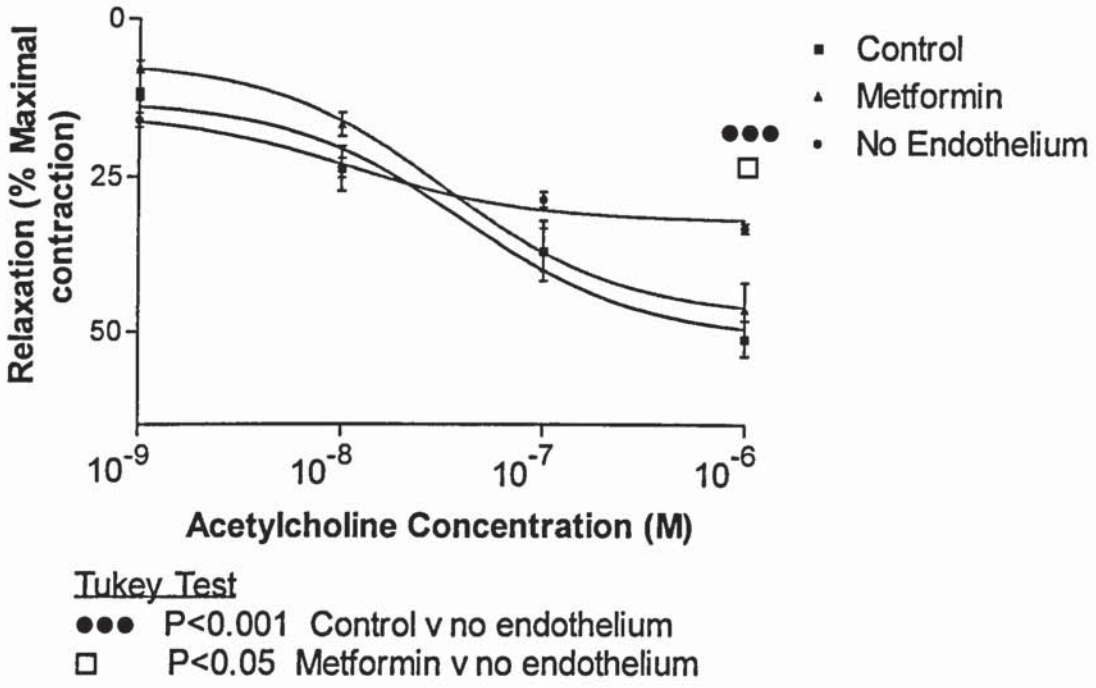
Table 4-9: - Tension generated by contracting tissue in grams after 8 weeks of treatment

	NA 10^{-9}	NA 10^{-8}	NA 10^{-7}	NA 10^{-6}
Control	+0.131	+0.550	+0.801	+0.972
Metformin	+0.103	+0.913	+1.407	+1.585

The units of tension are in grams, additional to the original 1g stretch.

Table 4.9: - This shows the exact tension in grams generate by the contracting tissue of the control and the tissue treated with 250mg/kg/day equivalent to metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is much greater in the metformin 10^{-5}M treated tissue due to the larger contraction. ± SEM. (n=6)

Figure 4-31: - Relaxation of thoracic aorta in lean male mice, in the absence and presence of metformin after 8 weeks



Tukey Test
●●● P<0.001 Control v no endothelium
□ P<0.05 Metformin v no endothelium

Figure 4-31: - The animals were treated for 8 weeks, the control without metformin in their drinking water and the experimental group was treated with 250mg/kg/day in their drinking water, producing a circulating plasma concentration of metformin10^{-5}M. The tissues were then calibrated with a 1g weight for 45 minutes to maintain a constant tension. After this period the tissue was exposed to cumulative concentrations of acetylcholine 10^{-9}–10^{-6}M. The contraction is expressed as percentage of the control 10^{-6}M noradrenaline in Fig 4-30. The tissue sample incubated with metformin 10^{-5}M showed no significant increase in relaxation, in fact less relaxation is seen in this case when treated with metformin compared to the control. The impaired relaxation of the metformin tissue is not due to the removal of the endothelium, as the tissue sample with the endothelium removed shows less relaxation. ± SEM. (n=6)

Table 4-10: - Tension generated by relaxing tissue in grams after 8 weeks of treatment

	Ach 10^{-9}	Ach 10^{-8}	Ach 10^{-7}	Ach 10^{-6}
Control	+0.855	+0.742	+0.612	+0.475
Metformin	+0.897	+0.810	+0.615	+0.521
No endothelium	+0.816	+0.743	+0.693	+0.649

The units of tension are in grams, additional to the original 1g stretch.

Table 4.10: - This shows the exact tension in grams generate by the contracting tissue of the control and the tissue treated with 250mg/kg/day equivalent to metformin10^{-5}M. Note that the tension generated is additional to the 1g weight exerted for the calibration of the tissue. The tension is greater in the samples with no endothelium and metformin treated tissue respectively as they do not increase relaxation. (n=6)

In the final in vivo study after 8 weeks of treatment with metformin there was a 65% ($P<0.001$) rise in the maximal contraction seen with metformin at 10^{-6}M noradrenaline. There was still no significant increase in the relaxation of the tissue treated with acetylcholine. To see if the reduction in relaxation was due to loss of endothelium or its damage a wire was dragged around the interior of the lumen to remove the endothelium and then relaxation was retested with acetylcholine. When the endothelium was disrupted there was a marked reduction in the relaxation.

The body weight of the mice was monitored again over the 8-week period, and the food consumption of the mice also did not significantly change over the 8-week period.

Figure 4-32: - Mean increase in body weight over 8 weeks

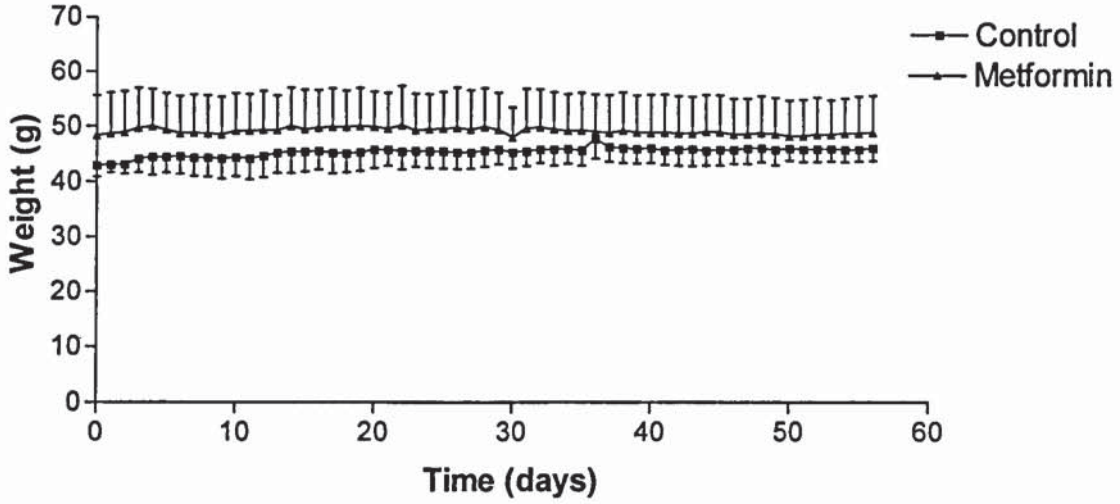


Figure 4-32: - The body weight of the lean male mice was taken at the start of the study and monitored every day for 8 weeks there is no significant change in body weight in any of the groups. ± SEM. (n=6)

Figure 4-33: - Food consumed g/mouse/week during 8 weeks

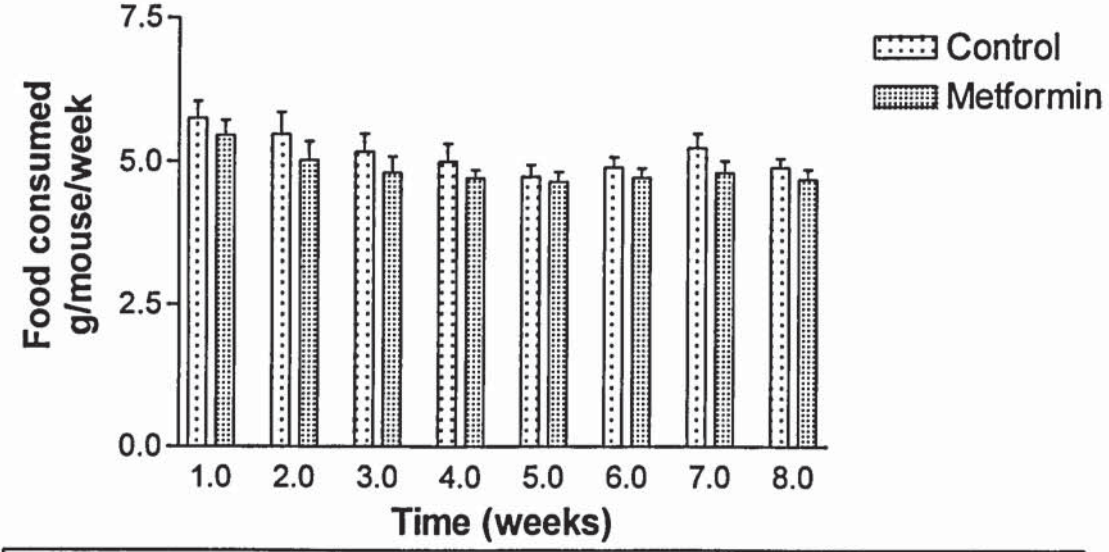


Figure 4-33: - The food consumed by each mouse was calculated every day. Then an average was taken for each day over the first, second, third week etc, this is average amount that is plotted on the graph. There is no difference in the amount of food consumed each day over the 8 week period by the control and metformin treated groups. ± SEM. (n=6)

Three sets on analysis were performed on weekly serum samples from tail blood; these include blood glucose, triglyceride and insulin concentrations.

Figure 4-34: - Blood glucose concentrations for per week over 8 weeks

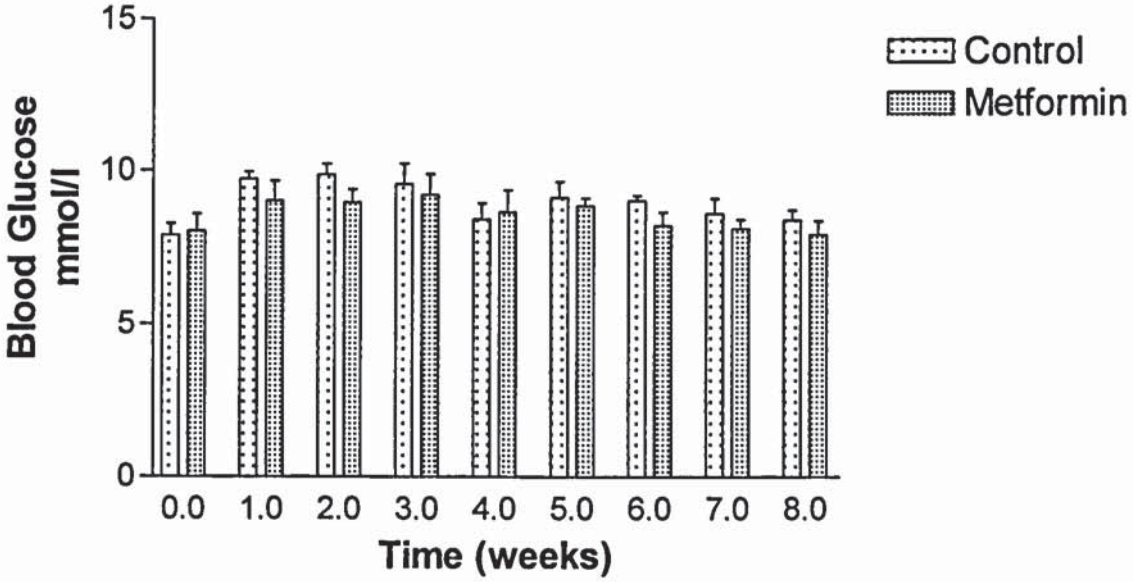


Figure 4-34: - Blood samples were taken from the tails of the lean male mice at the start of the study and then weekly thereafter. The blood glucose was taken using a blood glucose monitor and the average value of each group was plotted. There is no difference in the blood glucose concentrations between the control and metformin treated groups over this 8 week period. ± SEM. (n=6)

Figure 4-35: - Change in plasma triglyceride profile after 4 weeks in the presence and absence of metformin

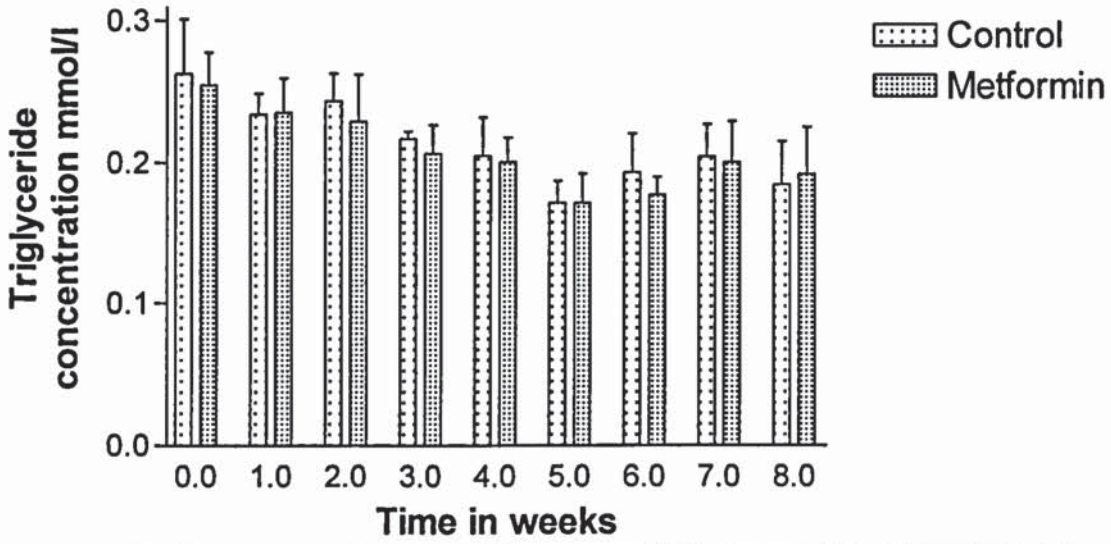


Figure 4-35: - Blood samples were taken from the tails of the lean male mice at the start of the study and then weekly thereafter. The blood triglyceride was determined using the INFINITY™ triglyceride reagent kit from Sigma and the average concentration of each group was plotted. There is no difference in the triglyceride concentrations between the control and metformin treated groups over this 8 week period. ± SEM. (n=6)

Figure 4-36: - Changes in insulin profile after 8 weeks treatment with metformin

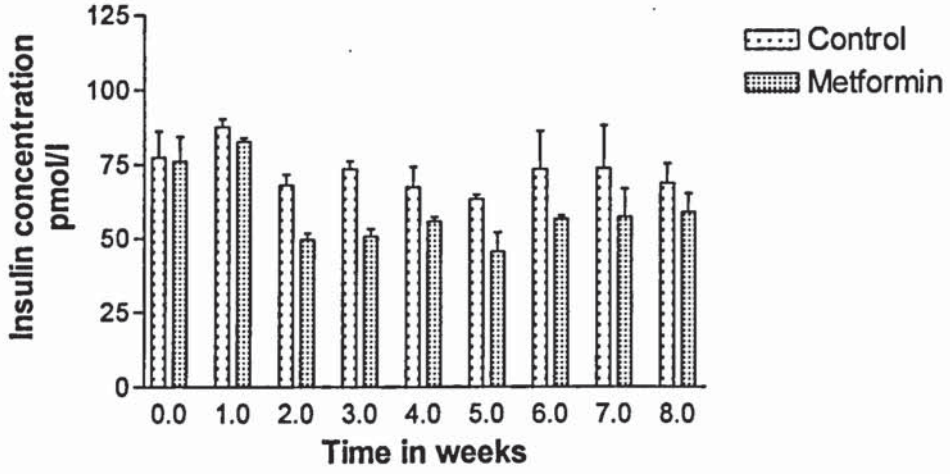


Figure 4-36: - Blood samples were taken from the tails of the lean male mice at the start of the study and then weekly thereafter. The blood triglyceride was determined using the ELISA insulin kit from Mercodia and the average concentration of each group was plotted. There is no difference in the insulin concentrations between the control and metformin treated groups over this 8 week period. The insulin values are non-fasting. (n=6)

Once again as in the previous studies there are no significant changes in body weight and food intake from that seen at the start of the study, and there are no changes in blood lipid, insulin or glucose serum concentrations over this time period.

4.7 Discussion

The results show that both the in vitro and in vivo studies showed an increase in contraction in the presence of metformin and noradrenaline. The effect of metformin increases relaxation in vitro but the effect is less marked during the in vivo studies. The increase in contraction and relaxation seen in vitro is consistent with other studies that have been performed. As these changes occur in vitro and regardless of whole animal treatment, the action of metformin must be independent of the internal in vivo environment and directly upon the vessel wall. There are several possible theories on how metformin is able to exert these effects; first it is appropriate to address the effect of increased contraction.

The maximal contraction effect in vitro is seen after 4 hours giving a maximal increase of 221% in contraction with 10^{-6}M. Treatment for 1 hour with metformin also increases contraction, but not to the same extent. Similar changes are seen over time with animals treated with metformin in vivo up to 8 weeks in the studies that were performed in our laboratory. The maximal effect is always seen at noradrenaline 10^{-6}M and the longer the treatment the greater the degree of contraction seen, though not to the same extent as the in vitro studies.

The tissue also responds with myogenic responses to stress, this is where the muscle contracts independently by itself without the aid of nerves or hormones. The smooth muscle in the vessel is able to increase its myogenic tone as a response to being stretched and therefore acts to resist the initial stretch on the vessel. In the same way a

decrease in stretching smooth muscle is accompanied by a reduction in the vessels tone (Sherwood 1993b). Therefore a possible explanation is that metformin may affect the ions involved in myogenic responses in smooth muscle and therefore may act via these ions to induce an increase in contraction when challenged with noradrenaline. There was no obvious increase in the stretching required to obtain the pre-tension of 1 gram in the tissue. As it was originally thought that metformin may induce relaxation causing the tissue to be required to be stretched to greater and greater length to generate the same tension, causing an optimal interaction of cross-bridging in the muscle and therefore allowing a greater contraction force to be produced. There was no obvious evidence for this from the traces. This suggests that metformin and other insulin sensitising drugs may be able to act directly with the vascular smooth muscle cells (VSMC) to cause vascular responses (Bhalla et al 1996). The effect on VSM will be further investigated by monitoring the effect of metformin on passive muscle tension, which will be addressed in detail in chapter 5 to see if metformin changes the muscle tension at rest. The full mechanisms of noradrenaline and acetylcholine on endothelium and VSM will be looked at in full detail in chapters 6 and 7 and therefore are not outlined here.

The effect of metformin on relaxation is not conclusive in this study, as after 1 and 4 hour in vitro treatment an increase in relaxation is clearly seen. In the longer-term studies there is either no increase in relaxation or an actual reduction in the relaxation seen in these tissues. Metformin is known to reduce blood pressure in various animal studies. For example Sterne 1969 showed that metformin reduces blood pressure in 30 minutes at doses of 50-100mg/kg of metformin. It has also been shown in other studies by Katakam et al 2000 that an increase in relaxation of insulin resistant animals treated with metformin and acetylcholine after 2 weeks. The explanation

given for this observation was an improvement in endothelium-dependent relaxation, caused by an increase in nitric oxide (NO), which will be investigated in detail in chapter 6, as maybe metformin is able to increase or alter NO production. These studies did document that NO-independent relaxation was still impaired. The mechanism by which metformin operates to enhance NO-induced relaxation is not fully understood. There is no direct effect on VSMC as metformin alone before acetylcholine did not induce any relaxation. Marfella et al 1996 suggested that metformin has this relaxation effect as in enhances agonist-induced NO production, as metformin treatment enhanced L-arginine-induced increases in blood flow in the forearm, but had no effect when given alone. Speculation suggests this may be due to enhanced intracellular calcium action, which chapter 7 will address by investigating calcium release in the presence and absence of metformin. At very high concentrations of metformin (>10mmol/l) the drug is able to directly induce relaxation and decrease intracellular calcium (Chen et al 1997). At the concentration used in the laboratory of 10^{-5}M metformin and seen during in vivo studies of metformin action is more likely to be to enhance agonist-induced NO mediated relaxation (Katakam et al 2000). Other possible explanations put forward by Bhalla et al 1996 suggest that metformin directly acts on VSMC and lowers agonist-stimulated intracellular calcium. Bhalla et al 1996 therefore suggested that in hypertensive type 2 diabetic patients the reduction in blood pressure may be due to an inhibition of agonist-stimulated intracellular calcium in VSMC.

On smaller vessels, which were unable to be studied accurately using our equipment, metformin has been shown to restore arteriolar vasomotion. The phenomenon of precapillary vasomotion consists of periodic phases of contraction and relaxation of these arterioles (Intaglietta 1988). It is the vasomotion that is critically lost in diabetic

patients, which has shown to be restored in studies with chronic metformin treatment in hamsters and bats (Bertuglia et al 1988 and Bouskela et al 1988).

Finally there were no significant changes in body weight, and food intake and water intake (water intake not illustrated here). Even though metformin is known to be an anorectic agent it only exerts these effects when the whole dose of 50-250mg/kg/day is given in one oral dose. Then it inhibits food intake for a period of 30-60 minutes in lean mice. However as this effect is short lived the mice compensate for this initial loss of food by eating slightly more after this period. Therefore over 24 hours there is no observed effect (Bailey & Flatt et al 1986). Large doses of metformin are required to maintain this anorectic effect as shown in clinical studies by Hermann 1979 in the dietary habits of type 2 diabetic patients. There was also no change in the circulating glucose and triglyceride concentrations during these studies. The in-vitro studies indicate that a therapeutic concentration of metformin acts directly on the aorta to increase maximum noradrenaline induced contraction. The effect is rapidly generated in-vitro, but it is only evident after in-vivo administration of the drug for 2 weeks in non-diabetic mice. The in vivo study also showed this effect of metformin was independent of significant changes in weight, triglyceride, glucose and insulin concentrations. Metformin has the ability to directly increase the contractility of aortic tissue in the presence of noradrenaline, additional and independent of its glycaemic control. So the effects of metformin are independent of its glucose and lipid and insulin lowering ability, which may take longer to be established.

The increase in both contraction and relaxation of the aorta may be how metformin is able to have an increase on diabetic patients survival, as patients treated with this drug are better equipped to cope with greater vascular challenges.

Chapter 5: - Length-tension of aorta, and the effect of metformin

5 Length-tension of aorta, and the effect of metformin

The aorta, being the main artery of the body has the ability to contract and to stretch, due to the high proportion of elastic fibres in the wall (Klabunde 2002b). There are several different ways that the elastic properties of arteries can be measured in vivo and in vitro. These techniques and measurements are detailed in this chapter; and some of these techniques are used to assess the effect of metformin. The loss of the elastic properties of the major arteries is part of the natural ageing process; again this is accelerated in diabetes and atherosclerosis.

5.1 Elastic properties of arteries

The majority of the elastic fibres in the artery lie in the central layer the tunica media along with collagen and smooth muscle fibres: the outer layer the tunica adventitia also contains some elastic fibres, see chapter 4.1.1 Figure 4-2: - The three layers of the artery wall. The elastic fibres are what allow the pulse to be detected in the body at key points, such as the radial and carotid pulse. As when the artery is compressed against a bone the artery is stretched by the force of the blood being ejected by the left ventricle of the heart (Watson 1998b). As it is the heart that produces the original pulsation the rate and rhythm of the beat can be judged from the pulse taken in the arteries.

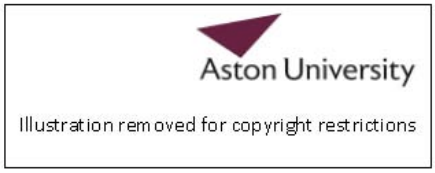
5.1.1 Vascular distensibility

Vascular distensibility is a vital component of circulatory function, as it is not only able to accommodate the stroke volume of the heart but it also averages out the pressure pulsations. It is this action that helps to provide a smooth and continuous supply of blood through out the network of tissues and smaller blood vessels. It is the combination of distensibility and resistance that mean there is virtually no pulsation by the time the blood reaches the capillaries. This progression of diminishing pulsations is known as the damping effect of the pressure pulse. The pulse pressure is determined from the systolic minus the diastolic pressure.

The resistance helps to dampen the pulse. As the blood at the front of the pulse wave moves forward it distends the next section of vessel, the greater the resistance of the vessel the more difficult it is to distend. Compliance also has a dampening effect, as the more compliant a vessel the greater the quantity of blood that travels at the wave front to cause a rise in pressure. Therefore dampening is directly proportional to the result of resistance times compliance (Guyton & Hall 1996c).

Vascular distensibility is measured and expressed as a fractional increase in volume for every millimetre of mercury rise in pressure. This can be expressed in the following calculation see Figure 5-1: - Units of vascular distensibility

Figure 5-1: - Units of vascular distensibility



Guyton A. C, Hall J. E (1996c). Textbook of medical physiology. 9th edition. Chapter 15. Vascular distensibility, and functions of the arterial and venous systems. Philadelphia, Pennsylvania. Published by W. B Saunders Company. 171.

5.1.2 Vascular compliance

Vascular compliance which can also be known as capacitance in some cases, is often a much more valuable measurement. It indicates the total quantity of blood stored in a section of vessel for each millimetre increase in mercury (Guyton & Hall 1996c). The compliance or capacitance of a vascular bed is determined by the equation below Figure 5-2: - Vascular compliance/ capacitance

Figure 5-2: - Vascular compliance/ capacitance

Guyton A. C, Hall J. E (1996c). Textbook of medical physiology. 9th edition. Chapter 15. Vascular distensibility, and functions of the arterial and venous systems. Philadelphia, Pennsylvania. Published by W. B Saunders Company. 171.

It is important to clearly distinguish between distensibility and compliance, as they are very different properties. For example a vessel may be very distensible but only have a small volume, which means it may be less compliant than a less distensible vessel with a much larger volume. This is because compliance can also be expressed as distensibility times volume. As veins are about 8 times more distensible than arteries and contain up to 3 times the volume, a vein is 24 times more compliant than

an artery (distensibility 8 x volume 3 = compliance 24). This is illustrated below Figure 5-1: - Units of vascular distensibility

Figure 5-3: - Compliance curves for arteries and veins

Klabunde R. E (2002c). Cardiovascular physiology web resources. Vascular compliance. http://www.oucom.ohiou.edu/cvphysiology/BP004.htm [Accessed 7/ 03/ 2002].

At low pressures it is clear to see in Figure 5.3 that venous compliance is greater than in arteries. The compliance of a vessel decreases with higher pressures and volumes; this is because the vessel becomes much stiffer under these conditions. Therefore at higher pressures and volumes venous and arterial compliance become similar. As a result of this phenomenon veins can be used as arterial grafts as they can withstand similar extremes of volume and pressure (Klabunde 2002c).

The velocity of the pressure pulse transmission through the normal healthy aorta is 3-5 m/sec; this is much faster in the smaller arteries where transmission is 15-35 m/sec. As a rule, the greater the compliance of the sections of the vessel, the slower the velocity of the blood. This explains why the blood travels much slower in the aorta compared to the smaller arteries, which are less distensible (Guyton & Hall 1996c).

The compliance of an individual vessel can also be altered by sympathetic stimulation of vascular smooth muscle. Sympathetic stimulation increases vascular tone, which reduces the compliance (Figure 5-4: - Arterial compliance curves), in the same way vascular relaxation increases vascular compliance. The contraction of the smooth muscles in the arteries not only reduces compliance but also reduces the arterial volume; as a result this increases the arterial pressure. Age and disease such as atherosclerosis, which cause structural changes, also reduce compliance; in the aorta this causes an increase in aortic pulse pressure.

Figure 5-4: - Arterial compliance curves

Klabunde R. E (2002c). Cardiovascular physiology web resources. Vascular compliance. http://www.oucom.ohiou.edu/cvphysiology/BP004.htm [Accessed 7/ 03/ 2002].

The more compliant the aorta, the smaller the increase in pressure during ventricle contraction. If the aorta was rigid or less compliant there is a much greater increase in pressure this is illustrated more clearly in Figure 5-5: - Changes in compliance

Figure 5-5: - Changes in compliance

Klabunde R. E (2002c). Cardiovascular physiology web resources. Arterial pulse pressure. http://www.oucom.ohiou.edu/cvphysiology/BP003.htm [Accessed 7/ 03/ 2002].

5.1.3 Delayed compliance

When the tissue is first stretched by an increase in blood flow, the pressure is initially higher than the steady state pressure that is later maintained. The fall that is seen in pressure while a constant volume is maintained is known as stress relaxation or delayed compliance (Klabunde 2002c). The vessel wall shows a delayed stretch and the pressure falls back towards normal over time (e.g. minutes/ hours). It is important to allow the relaxation to take place, as the compliance will be lower if the initial pressure is used, as the vessel appears much stiffer. Therefore compliance also contains a dynamic component, as it also depends on the rate of change of the volume. This allows the blood vessels to try to maintain a constant pressure irrespective of the length of the smooth muscle fibres (Guyton & Hall 1996d). This is due to the latch mechanism of the smooth muscle as it attempts to resist any change in length at first. Then the muscle fibres re-adjust as the myosin heads attach further along the actin filaments. As a result the length of the muscle changes but the tension

almost returns to normal, as there is a similar number of cross-bridges in the muscle generating the contractile force. This means that in nature an increase in volume such as that seen with a transfusion or the loss of volume as seen in haemorrhage can be dealt with by adapting according to the changes see Figure 5-6: - Delayed compliance

Figure 5-6: - Delayed compliance

Guyton A. C, Hall J. E (1996c). Textbook of medical physiology. 9th edition. Chapter 15. Vascular distensibility, and functions of the arterial and venous systems. Philadelphia, Pennsylvania. Published by W. B Saunders Company. 173.

5.2 Vessel stiffness

Arterial stiffness is a term used to describe the process of arterial stiffening and is used now in place of earlier terminology such as compliance, distensibility and elasticity. All of these factors decline during the ageing process and during metabolic disorders (Woodman and Watts 2003).

Larger mammals have larger aortas that obviously possess more elastin layers, in proportion to their wall size. Research by Wolinsky and Glagov (1967) in mice to pigs has revealed that the diameter and wall thickness increase in constant proportion,

but the thickness of the lamella remains constant. The lamella is a unit of the aortic medial structure. So the number of lamellar units increases directly proportional to the radius and wall thickness (Shadwick 1999).

Studies performed by Roach and Burton (1957) selectively digested the collagen or elastin fibres from human arteries. As seen in Figure 5-7: - Elastin and Collagen contribution to arteries. When the elastin fibres are removed by trypsin digestion, only the collagen fibres remain, this increases the tension generated in the vessel wall. In the same way when the collagen fibres were selectively digested using formic acid, leaving only the elastin behind, there is much less tension generated in the blood vessel wall.

Figure 5-7: - Elastin and Collagen contribution to arteries

Roach M, Burton A. C (1957). The reason for the shape of distensibility curves of arteries. *Can. J. Biochem. Physiol.* **35:** 181-190.

This illustrates that elastic force is dependent on contributions from both elastin and collagen. Roach and Burton (1957) also used this technique to illustrated hardening of human arteries, which normally occurs with age. They showed that there is an increase in collagen and the loss of initial low-compliance, the elastin dominated phase. This means that there is a greater collagen loading at lower circumferential strains.

5.2.1 Causes of vessel stiffness in diabetes

An increase in arterial stiffening has been demonstrated in both types of diabetes in several studies, two of the most recent being by Brooks et al 2001 and Aoun et al 2001. The most independent predictor of large artery stiffening is fasting glucose Salomaa et al 1995.

The incidence of type 2 diabetes increases with age, and age is also a major contributor to arterial stiffening. During ageing there are changes in the content of elastin and collagen fibres in the vessel walls. The elastin fibres become fractured and there is also an increase in collagen deposition, which contributes to an increase in arterial stiffness. The effect this has in the aorta is to cause a progressive increase in the diameter of the aortic arch, which decreases aortic distensibility. This has a large impact, as the aorta is responsible for the largest contribution to arterial buffering (Sterigopulos et al 2003).

Other contributing factors to arterial stiffening in type 2 diabetes include overall poor glycaemic control both fasting and postprandial (Wahlqvist 1988). There is also the formation of advanced glycation endproducts (AGEs), these cause structural changes

in the vessel wall such as crosslinkages and between proteins that contribute to arterial stiffening (Amar et al 2001). Other conditions such as dyslipidemia, hypertension and oxidative stress all may contribute to increasing arterial stiffness. Their contributions to macrovascular disease are dealt with in greater detail in chapter 4, section 4.2.2-4.2.3. The duration of diabetes and autonomic nerve dysfunction also play a significant role in the degree of arterial stiffness (Ahlgren et al 1999). Endothelial function may also be a significant factor in modifying the risk, which contributes to increased stiffness, as fewer vaso-relaxant substances such as nitric oxide are released from the endothelium (Wilkinson et al 2002).

It also seems that there may be a greater vascular risk for women with type 2 diabetes than men. As arterial stiffness has been shown to increase in both the abdominal aorta and common carotid artery in diabetic women compared to healthy controls of comparable age. The causes are still not fully understood but this study by Ahlgren et al 1995 showed that women have a greater cardio- and cerebro-vascular risk of associated diabetic complications. There was no significant correlation between identical studies carried out in men.

Another study by Suzuki et al 2001 showed that arterial stiffness was connected to a reduction in the arterial flow volume in the lower extremities of diabetic patients. This could explain why there is such an increased risk of lower limb amputation in diabetes. There is also genetic evidence that may point to a predisposed state of arterial stiffness and cardiovascular risk. Taniwaki et al 1999 has shown that ACE gene polymorphism is linked to impaired aortic and carotid distensibility in type 2 diabetic patients. It is the I allele of the ACE gene that is associated with stiffening of

the large arteries, although the causes of this increased stiffness are not yet fully understood.

One study by Duprez et al 2000 measuring elasticity indices in larger arteries, found that subjects with high-normal blood pressure showed a significant decrease in the large artery and small artery elasticity indices. This was accompanied by an increase in the intima media layer thickness in the carotid artery. They concluded that with further research this could be a valuable tool to screen patients for vascular damage and the impaired function of the endothelium. The use of intima media thickness measurement as a surrogate indicator of vascular compliance is becoming more widespread, but the interpretation of this measurement remains a topic of discussion.

Vascular compliance is reduced in both the large and small arteries of type 1 diabetic patients. These changes occur before any clinical complications become evident. This therefore confirms that vascular changes occur early in the disease and may increase the risk of a vascular event. Larger studies are needed to confirm this and to investigate the potential for pharmaceutical intervention to prevent this Romney and Lewanczuk 2001.

The altered characteristics of compliance for example may be an early marker of vascular damage (McVeigh 1996). Arterial stiffness is a powerful and independent risk factor for cardiovascular disease (Meaume et al 2001). Decreased arterial compliance can have a knock on effect and contribute to hypertension, left ventricle afterload, reduced myocardial perfusion and vasculopathy (Dart et al 2001). The majority of the risk factors predispose diabetic patients to an increased chance of a

cardiovascular event (~70% of type 2 diabetic patients have cardiovascular & cerebrovascular disease, Greiss and Herman et al 1995).

5.2.2 Measuring vessel stiffness

There are several methods that can be used on human subjects to measure arterial stiffness and these are all non-invasive methods. All of the methods operate on one of the main principles: -

1. Pulse wave velocity.
2. Pulse pressure or blood flow waveform analysis.
3. Distensibility from measurements of arterial pressure and diameter.

There are several devices on the market that will now make calculations or take values that are able to estimate vessel stiffness. The advantage to most of the methods is they are simpler and easier to use than in vitro equipment, which is also an invasive procedure, and they do not require any specialist training.

Augmentation index

The augmentation index is one of the measurements that can be taken. The pulse pressure occurs due to the forward projection of the blood being ejected from the left ventricle into the vessel, and the backward projection is due to the reflection of the wave (Woodman and Watts 2003). If arterial stiffening occurs the reflective wave increases, this enlarges systolic pressure as the two waves the forward and reflective wave become added together see figure 5.9 patient B. This acts to increase the central (aortic) waveform. The aortic or central augmentation index (AI) is measured by calculating the change in pressure between the shoulder of the aortic wave and the

peak of central systolic pressure wave (ΔP). The pulse pressure (PP) is also required for the equation below: -

Figure 5-8: - Augmentation index

Woodman R. J, Watts G. F (2003). Measurement and application of arterial stiffness in clinical research: focus on new methodologies and diabetes mellitus. *Med Sci Monit.* **9.** 5: RA85.

Factors such as endothelial dysfunction may affect the central pressure waveform. The AI and central waveform are good markers of arterial compliance and therefore whole body compliance.

Figure 5-9: - Augmentation index trace

The aortic pressure waveform is derived from the radial artery trace. The quantification of wave reflection, depends on arterial stiffness, this is done by identification of early and late systolic peaks. Trace A, shows a young normotensive subject. Trace B, shows a middle-aged hypertensive subject. (PP=pulse pressure)
Trace adapted from Woodman and Watts 2003. Med Sci Moit. 9. 5:RA81-89.

Pulse wave Velocity

Specialised software called the Sphygmocor™ analyses the distal and central pulse wave velocity. This measurement can be taken by a single operator. It uses the principle of an ECG-gated measurement. Even though the values were highly reproducible there was a 10.5% variation within-subjects (Woodman and Watts 2003). However the inter-subject variation is smaller compared to alternative methods of measuring arterial stiffness. As a result large test groups are required to show any degree of difference between separate trial groups.

Pulse contour analysis

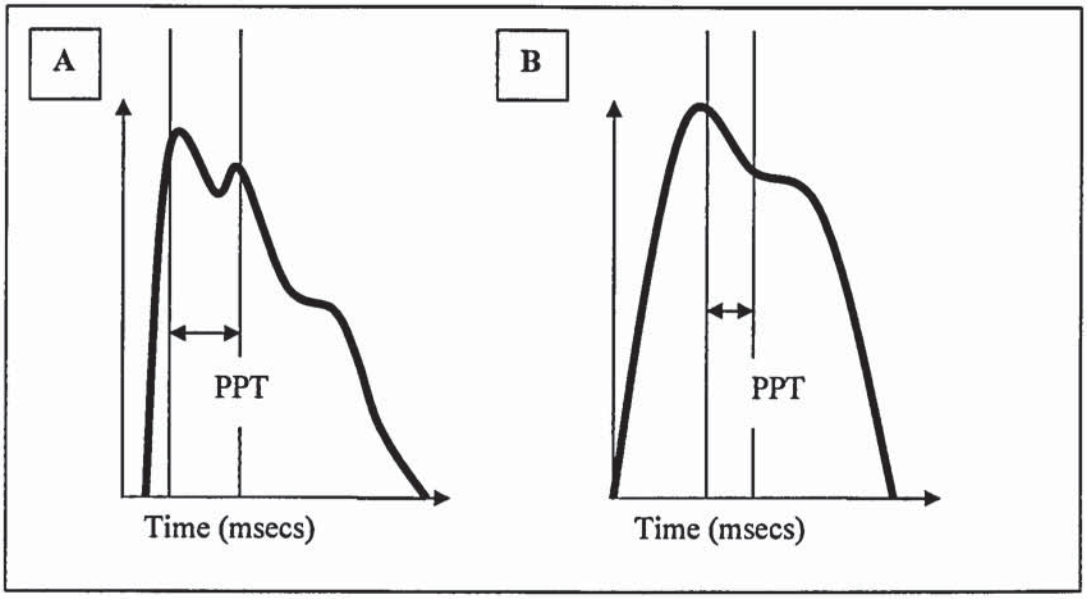
The CR200 (Hypertension diagnostics Inc/ Pulse Wave™ CR-2000 research cardiovascular profiling system, Minneapolis, USA) is a machine, which uses a pressure sensor to collect and amplify the waveform of the blood pressure. Analysis of the diastolic decay waveform can be used to determine the large vessel and small vessel compliance (Woodman and Watts 2003). The sensor is placed over the radial artery to take readings. The windkessel model equation is used to map the diastolic waveform; this is then matched to the cardiac output, capacitive and oscillatory compliance, systemic vascular resistance and impedance, distal and proximal blood pressure and blood inertia (Cohn 1995). Large artery compliance is derived from the gradient of the diastolic decay and small artery compliance is derived from the oscillatory component of diastolic decay. Small artery compliance was a better indicator of a cardiovascular event during a 7 years trial by Grey et al 2000, than that of larger arteries. Another study showed that there was a change in small artery compliance in type 2 diabetic patients compared to age-matched controls, but there was no difference in large artery compliance (McVeigh et al 1992). This is because

large arteries are more affected by changes in blood pressure and these values can vary greatly, while smaller arteries are more independent of this effect.

Digital volume pulse

The pulse trace system (Micro Medical™, Gillingham, Kent, United Kingdom) uses a finger photoplethsymography to generate a digital volume pulse (DVP). The waveform of the DVP is similar in characteristic and behaviour to the pulse pressure trace. Japanese researchers using the photoplethsymography discovered the second derivative closely correlates to the carotid augmentation index (Iketani et al 2000). The DVP consists of both a forward and a reflective travelling wave, which is similar to the pulse pressure waveform. The formation of a dichrotic notch occurs at the second peak in the waveform, which occurs during diastole. Software is able to analyse the DVP and project the second reflective wave. It is determined by measuring the time in milliseconds between the first peak and the dichrotic notch (see figure 5-10) of the reflected wave is divided by the height of the subject. This then obtains a figure called the stiffness index, which strongly correlates to the aortic wave velocity (Al Suwaidi et al 2000). The stiffness index can be used similarly to the central pulse wave velocity, which also correlates with the augmentation index (Woodman et al 2003). In the same way the reflective wave returns sooner in older and diabetic patients due to the increased stiffness, which overlaps with the forward travelling wave, causing the loss or reduction in the second peak.

Figure 5-10: - Digital pulse volume waveform



A, represents a young normal subject. B, represents a middle-aged diabetic subject. PPT= peak to peak time.

5.3 Passive tension

Passive tension tells us how easy or difficult it is to distend the muscle (Cothran 2003). The tension generated by elastic elements is called passive tension (Witteman 2003). The passive tension constantly exists even if the muscle is not active (Thompson 2002). When the muscle is at rest and the length is increased the tension will also become greater. On the passive curve as the preload is enlarged, if the resting length is above l_0 then the tissue begins to develop tension or force. Passive force is always just as active force directed from the point of the muscles attachment towards the centre of the muscle (Thompson 2002).

Different muscles obviously display different passive tension properties depending on the muscles designed structure and function. The slope of the length tension curve indicates the stiffness of the muscle. For example the steeper the curve the stiffer the muscle appears to be. Another tissue property is viscosity; any viscous material has a

slope that increases as the velocity of elongation increases. Elasticity is used as a model of muscular behaviour, and muscle behaviour is modelled on the same basis as springs (Thompson 2002).

5.4 Experimental detail and aims

The aortas were treated for comparable periods to the contraction and relaxation studies in chapter 4. These treatment times were 1 and 4 hours in vitro, 2, 4 and 8 weeks in vivo (250mg/kg/day) and all were treated in vitro with and without metformin 10^{-5}M. The lower aorta sections were used for these studies, as these sections were straighter and easier to thread the wire through, and to mount them on the equipment. The equipment and techniques used to measure passive tension are detailed in greater depth in section 2.4.1-2.4.2, chapter 2. The organ baths used during the experiment contained no metformin during the studies. Metformin was only present during the preincubation period in the in vitro studies, which took place in microfuge tube (1.5 ml) or metformin was present from the in vivo treatment. The aim of the study was to see if metformin altered smooth muscle tension in the aorta.

5.5 Results

The passive tension was measured at a range of time spans and models.

Figure 5-11: - Passive tension in thoracic aorta of lean mice incubated in the presence and absence of metformin for 1 hour

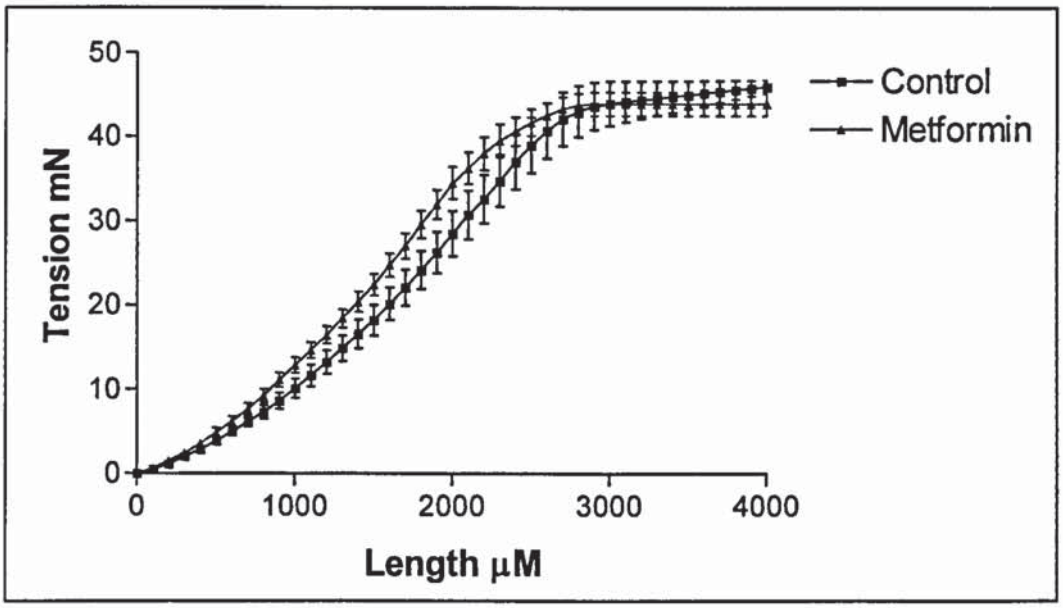


Figure 5-11: - The tissue was incubated again for 1 hour with and without the presence of metformin 10^{-5}M. The tissue was then passively stretched while at rest. The tissue treated with metformin showed a slight increase in tension, indicated by the slight shift of the curve to the left. The increase in the tissue tension seen with metformin 10^{-5}M incubation was not significant at this stage. ± SEM. (n=6), 10mN = 1g.

The tension was increased slightly as the curve shows a shift to the left after 1-hour treatment with metformin in vitro, but this was not statistically significant.

Figure 5-12: - Passive tension in thoracic aorta of lean mice incubated in the presence and absence of metformin for 4 hours

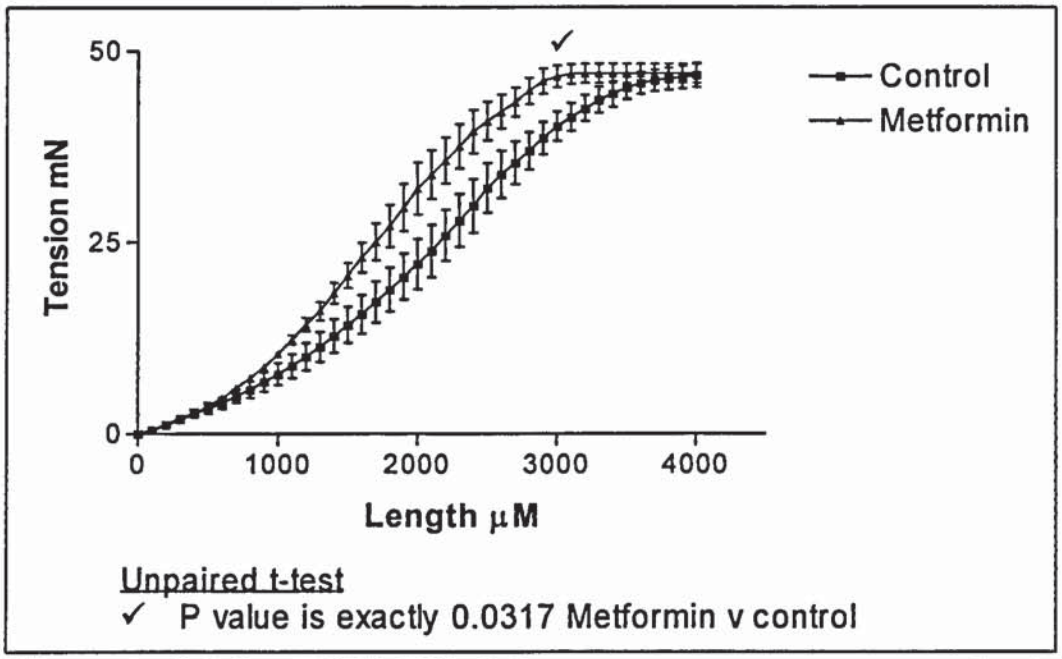


Figure 5-12: - The tissue was incubated again for 4 hours with and without the presence of metformin 10^{-5}M. The tissue was then passively stretched while at rest. The tissue treated with metformin showed a larger increase in tension, indicated by the shift of the curve to the left. The increase in the tissue tension seen with metformin 10^{-5}M incubation was only significant when the tissue was stretched to 3000μM in length. ± SEM. (n=6), 10mN = 1g.

The tension is greatly increased in the tissue treated with metformin for 4 hours in vitro and the curve shows a significant shift to the left.

Figure 5-13: - Passive tension of thoracic aorta of treated and untreated lean mice after 2 weeks

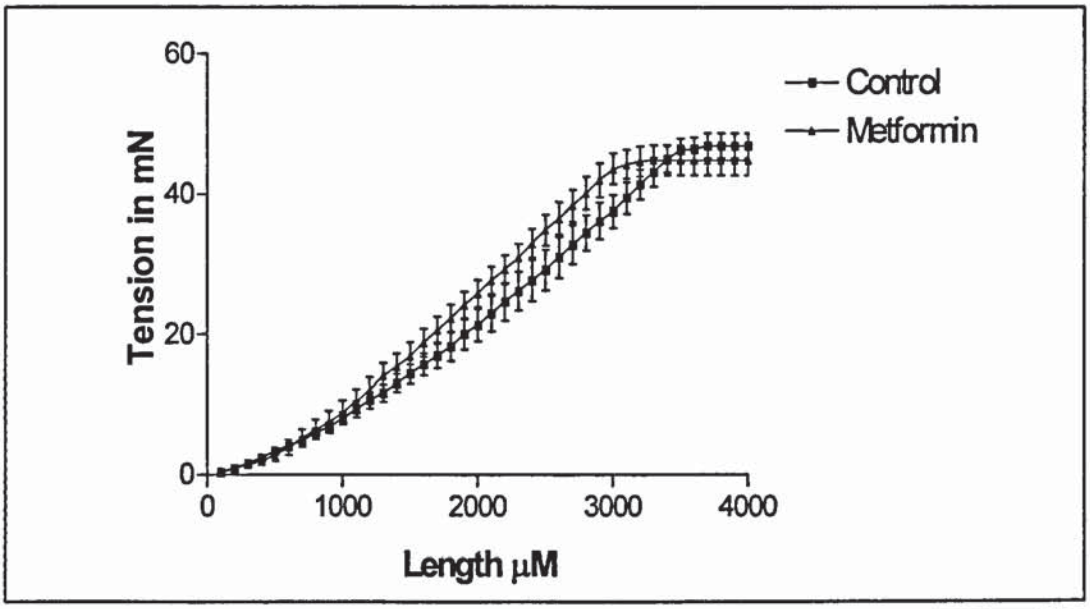


Figure 5-13: - Passive tension in thoracic aorta of treated and untreated lean male mice after 2 weeks.
The animals were treated with 250mg/kg/day equivalent to metformin10^{-5}M for 2 weeks. The aortic tissue was then removed and passively stretched while at rest. The tissue treated with metformin showed a slight increase in tension, indicated by the slight shift of the curve to the left. The increase in the tissue tension seen with metformin 10^{-5}M incubation was not significant at this stage. ± SEM. (n=6), 10mN = 1g.

There was no significant increase in the tension of the tissue treated with metformin, after 2 weeks.

Figure 5-14: - Passive tension of thoracic aorta of treated and untreated lean mice after 4 weeks

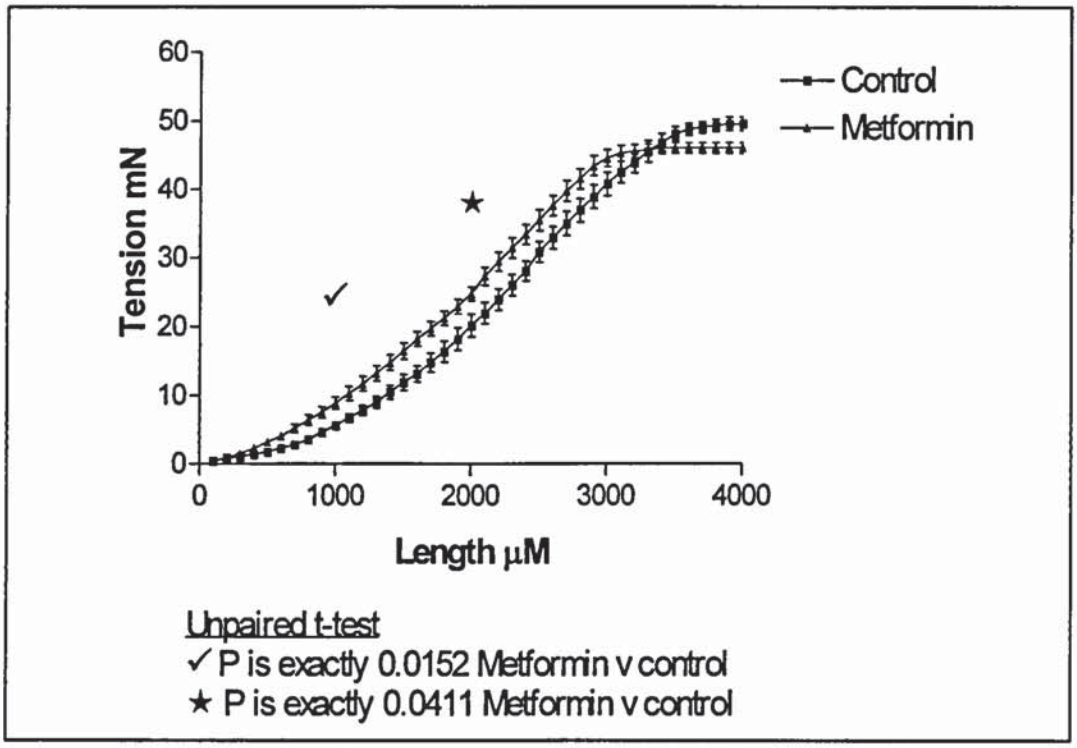


Figure 5-14: - The animals were treated with 250mg/kg/day equivalent to metformin10^{-5}M for 4 weeks. The aortic tissue was then removed and passively stretched while at rest. The tissue treated with metformin showed a slight increase in tension, indicated by the curve moving to the left. The increase in the tissue tension seen with metformin 10^{-5}M incubation was significant when the tissue was stretched to 1000μM-2000μM in length. ± SEM. (n=6), 10mN = 1g.

There was a significant increase in the tension of the tissue treated with metformin after 4 weeks at 1000 and 2000 μM.

Figure 5-15: - Passive tension of thoracic aorta of treated and untreated lean mice after 8 weeks

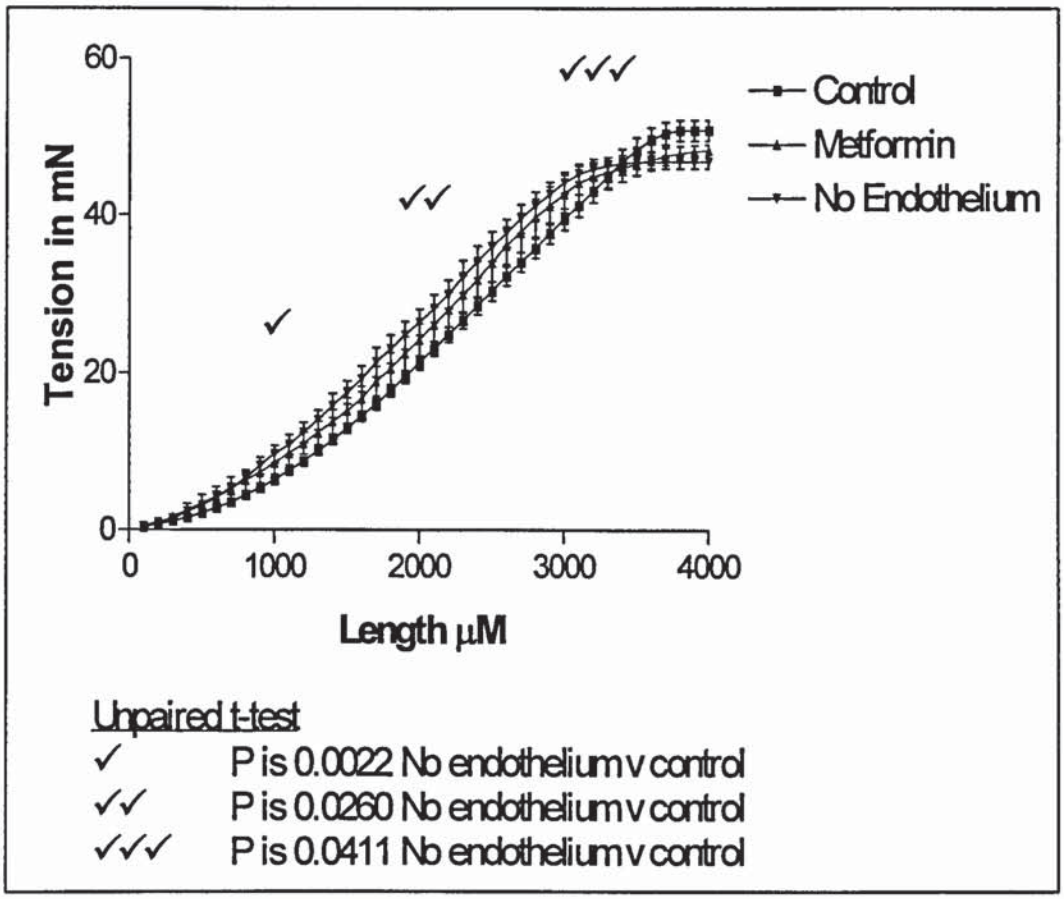


Figure 5-15: - The animals were treated with 250mg/kg/day equivalent to metformin10^{-5}M for 8 weeks. The aortic tissue was then removed and passively stretched while at rest. The tissue treated with metformin showed a slight increase in tension, indicated by the curve moving to the left. There was no increase in the tissue tension seen with metformin 10^{-5}M, but the results were significant with no endothelium present and was significant when the tissue was stretched to 1000μM-3000μM in length. ± SEM. (n=6), 10mN = 1g.

There was no significant increase in the tension of the tissue treated with metformin, after 8 weeks. The removal of the endothelium further increased the tension above that generated by metformin.

Figure 5-16: - Passive tension of thoracic aorta in normal lean mice with the endothelium removed and in obese mice

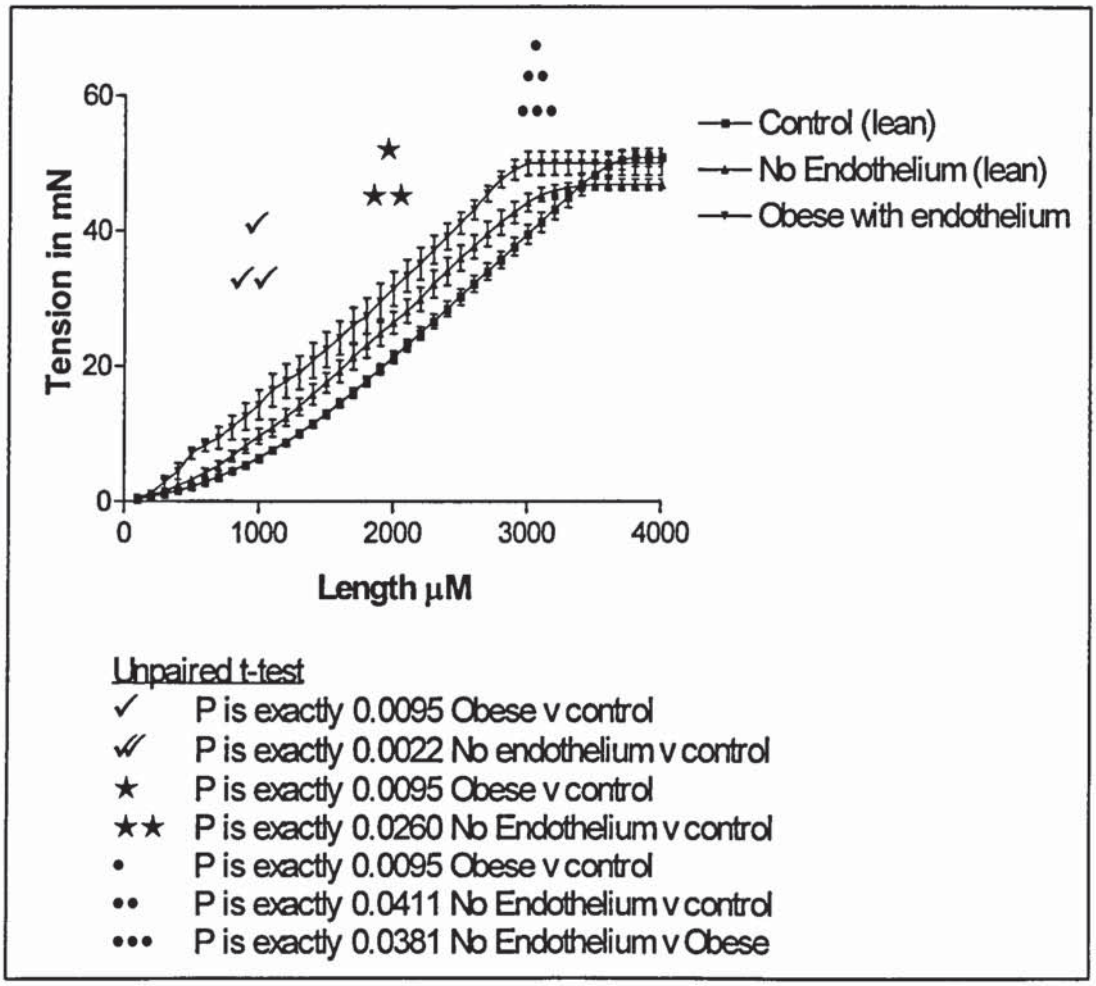


Figure 5-16: - The tissue was not incubated in this case the control was tissue taken from a lean male mice. For the second set of results lean male mice were again used but this time they also had the endothelium removed from the aorta. Finally a third group of mice were selected this time they were male obese mice. The lean mice with no endothelium showed an increase in tension shifting the curve to the right. The obese male mice showed an even greater increase in tension further deflecting the curve to the left. This indicates that there is additional damage to the obese tissue beyond the removal of the endothelium. ± SEM. (n=6), 10mN = 1g.

There was a greater increase in tension in the aorta of the obese mouse compared to the removal of the endothelium at comparable lengths.

Figure 5-17: - Passive tension of thoracic aorta of lean mice incubated for 4 Hour with papaverine

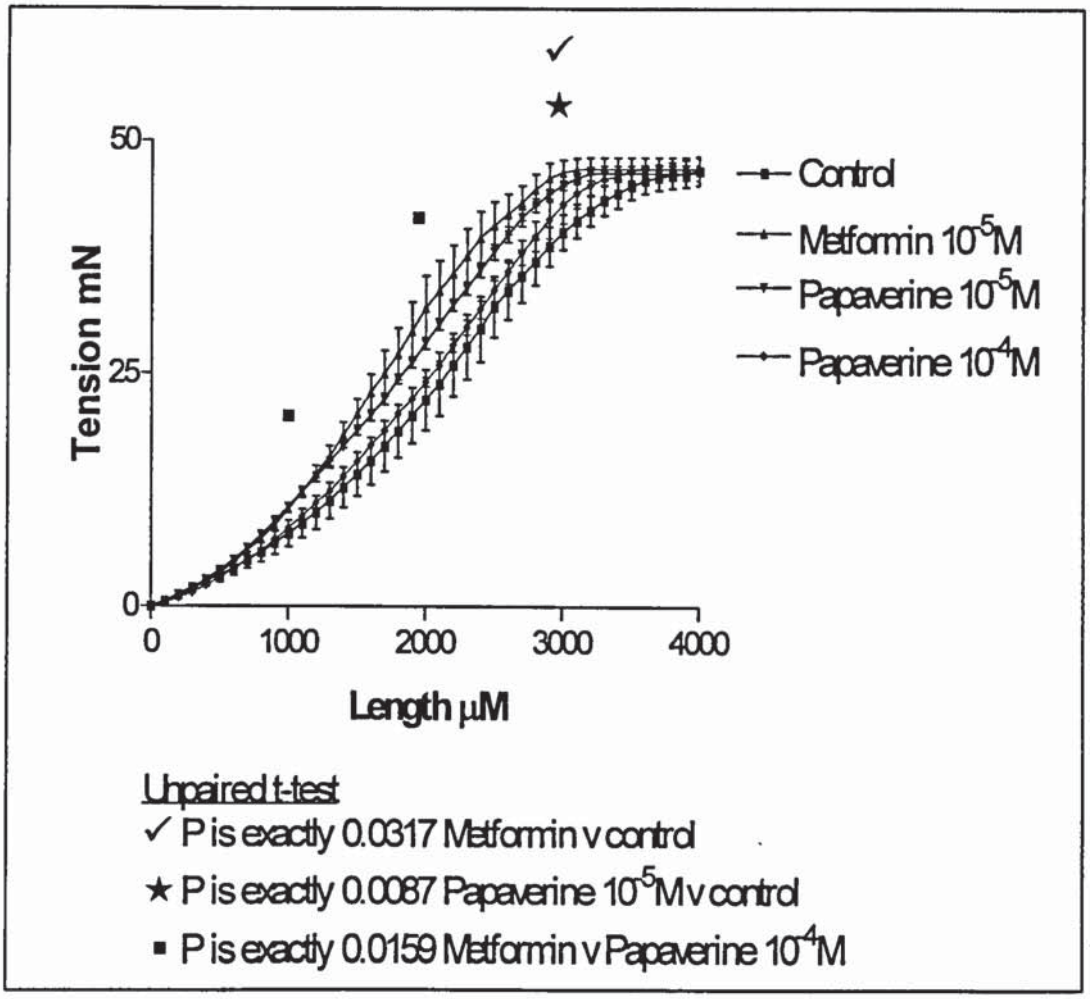


Figure 5-17: - The tissue was incubated again for 4 hours with metformin 10^{-5}M. The tissue was then passively stretched while at rest. The tissue treated with metformin showed a larger increase in tension, indicated by the shift of the curve to the left. The increase in the tissue tension seen with metformin 10^{-5}M incubation was only significant when the tissue was stretched to 3000µM in length. The papaverine was used on the metformin treated tissue in an attempt to act directly on the smooth muscle relaxing the tissue back to that of the control. Papaverine 10^{-4}M exposed to the tissue for 10 minutes had the greatest effect on the metfomin treated tissue relaxing it almost back to control tension. This shows that metformin acts directly on vascular smooth muscle in the aortic wall. ± SEM. (n=6), 10mN = 1g.

After 4 hours there was a significant shift of the length-tension curve to the left in the aorta treated with metformin, particularly significant at 3000µM. Papaverine was added for 10 minutes to the tissue after treatment with metformin; this should relax the smooth muscle tissue. There is little relaxation when papaverine is added as there is still a significant difference in tension between papaverine 10^{-5}M and the control at

3000μM. Then a greater concentration of papaverine was added and this increased the relaxation of the tissue. As a result there is no difference between papaverine 10^{-4}M compared to the control. There is however a significant difference between metformin 10^{-5}M and papaverine 10^{-4}M as a significant relaxation has been produced.

5.6 Discussion

The effect of metformin on the tension of the aorta can be seen in vitro after 1 hour, but its effects are only significant after 4 hours. Metformin appears to increase the tension in the aortic wall, as the length-tension curve is shifted to the left.

The in vivo studies showed that the effects of metformin to increase aortic tension were evident after 4 weeks. The effect of metformin was not as apparent after 8 weeks, which could indicate some adaptation of the tissue. The curve however was still shifted slightly to the left, even after 8 weeks. To ensure that the change in tension was due to the effects of metformin and not due to tissue damage, such as the removal of the endothelium, a control treated for a comparable time had the endothelium removed. This was achieved by dragging a wire around the lumen of the aorta to remove the endothelium. The results from the 8 weeks in vivo trial showed that a greater and significant tension was created in the tissue which had no endothelium. From this it was concluded that metformin had a direct effect upon the aortic tissue and did not involve damage to the endothelium.

Further studies were performed using lean and obese mice. The difference between the lean and obese mice was dramatic. The obese aortic curve was significantly shifted to the left, much more so than the effect of removing the endothelium. This showed that in obese mice there are structural changes associated with having diabetes and being overweight, which have a greater effect than just the removal of the endothelium. Studies carried out by Carter 2000 on lean mice showed that metformin triggered no structural change in the aorta by carrying out histology studies. This correlates with the findings in this study, as if metformin caused

structural changes the tension in the treated aorta would be more dramatic as seen in the obese mice.

The final study performed involved aortic sections being treated with metformin in vitro for 4 hours, also being exposed to the effects of a range of papaverine concentrations for 10 minutes. The higher concentration of papaverine 10^{-4}M, relaxed the metformin treated tissue back to the same tension as the control. This proved that metformin directly acts to increase smooth muscle tension. If metformin had affected other components of the aortic wall, papaverine would not have been able to reduce the tension.

Possible explanations for how metformin acts on vascular smooth muscle to increase tension are: -

Firstly, metformin may affect actin and myosin cross bridging in the smooth muscle. Secondly, metformin may effect calcium concentrations, which may increase muscle tension and increase contractility. The first of these will be dealt with in detail in this section. The second will be addressed in greater detail later in chapter 7.

Cross bridging is seen in smooth muscle just as in skeletal muscle but the arrangement of filaments is slightly different. The principle of cross bridging during muscle contraction is similar. The force generated by the muscle is proportional to the numbers of cross bridges that interact with in each sacromere (Berne & Levy 2000). The force generated by the muscle is lower if the muscle length is less than optimum, which is sometimes referred to as l_0. Any increases in tension can normally be associated in vivo with exercise or growth, however this can obviously not be the case

in vitro. Metformin therefore must act directly on the tissue; it cannot be due to changes in elasticity, which are normally measured using the passive tension curve, as additional collagen and elastin are not generated in vitro.

Figure 5-18: - Length-tension properties

Diagram A: - The passive curve behaves like a rubber band, due to the elasticity of the tissues collagen and elastin. The contracting muscle can generate greater force- the total force. The difference between total and passive force is the active force which represents the properties of the cross bridging
Diagram B: - Analysis of the sacromere shows that the force generated depends on the overlap of the filaments. L0 develops the most force as here the optimum number of filaments interact.

Taken from principles of physiology 3rd edition By Berne and Levy 2000.

It is therefore hypothesised that metformin must act to increase tension in the smooth muscle. This may be done by metformin increasing the cross bridging in the smooth muscle so the actin filaments are closer together, as a result for example of increased phosphorylation somewhere along the excitation coupling pathway, which initiates muscle contraction. There are several possible sites of action one potential site is the G-protein or CGMP dependent kinase. An unidentified substrate G1 has shown to be present in the plasma membrane cytoskeleton and is able to interact with smooth muscle cells, and able to effect relaxation (Baltensperger et al 1990). If metformin inhibits this action tension could be generated in the muscle. The connective tissue matrix can in some cases fulfil smooth muscle contraction (Shapovalov and Shuba 1994). This is less likely in this study as the effects of metformin were reversed by papaverine, which acts directly on the smooth muscle layer. Another possibility is metformin may inhibit the activation of spontaneously active phosphatases (Di Salvo et al 1983) found in the aorta, which is able to dephosphorylate phosphorylase.

Calcium may also be involved to some degree, as myosin binds to calcium via a G-protein coupled process. This therefore activates the myosin heads allowing them to cycle and cross bridge further along the actin filament, leading to a shortening of the muscle. Another possible reason is that metformin may alter ATP hydrolysis, which also causes a greater force to be generated in the muscle in a similar way. These explanations are only theories at this stage, and additional work and future studies are required to unravel the mechanism involving increased tension in metformin treated muscles.

Chapter 6: - Production of nitric oxide and the effect of insulin and metformin

6 Production of nitric oxide and the effect of insulin and metformin

Nitric oxide is one of the main vaso-relaxant chemicals in the circulatory system. It is produced by and acts upon the endothelial cell layer, and spreads to the underlying vascular smooth muscle to induce relaxation (Furchgott and Zawadski 1980). If there is damage or dysfunction of the endothelial cells then relaxation of the underlying vascular smooth muscle (VSM) is severely disrupted. Thus communication between endothelial cells and VSM is important, and nitric oxide is necessary for the full vaso relaxant effect to be seen on blood vessels.

This chapter addresses the issue of insulin action and how it may trigger nitric oxide release. In particular the experiments reported herein attempt to unravel, the pathway of insulin action controlling nitric oxide release. The effect of metformin on nitric oxide release will also be addressed in this chapter. It should be appreciated that only in vitro studies of nitric oxide production can be performed. This is due to the difficulty of identifying and tracing nitric oxide. It has a very short half-life in vitro of 1-2 seconds and in vivo of 0.1 seconds (Kelm and Schrader 1990) and is a highly reactive compound as it has an unpaired electron and is therefore metabolised within a few seconds in mammalian cells (Brady et al 1998). The methods available in our laboratory include direct measurement of nitric oxide using a fluorescent dye method

and indirect measurement by detecting nitrite and nitrate, which are nitric oxide metabolites.

6.1 Nitric oxide

6.1.1 Production and biosynthesis

Nitric oxide (NO) is formed from the amino acid L-arginine see Figure 6-1: - Synthesis of nitric oxide from L-arginine is controlled, by the enzyme nitric oxide synthase (NOS). L-arginine is hydroxylated to hydroxy-L-arginine, this is followed by an oxidation step forming NO and L-citrulline, this is shown below (Brady 1998).

Figure 6-1: - Synthesis of nitric oxide from L-arginine

Diagram taken from: Nitric oxide-The secret sympathy. Brady A. J. B (1998). Proceedings of the Royal college of physicians of Edinburgh. **28: 248.**

There are three isoforms of this enzyme, the one involved with vascular control is eNOS, endothelial nitric oxide synthase. The eNOS isoform and the neural isoform require calcium and calmodulin for their expression and activity in normal conditions. NO only has a short half life of a few seconds (Standl and Schnell 2000), but in this time it stimulates guanylate cyclase activity in the smooth muscle (Ignarro 1990). This causes an increase in cyclic-GMP triggering a decrease in intracellular calcium

and causing smooth muscle relaxation, this will be addressed in greater detail later. It has already been shown in rabbits that long-term inhibition of NO causes vascular disease. In severe diabetes the vasodilator action of insulin is impaired.

As early as 1977 Arnold et al suggested that endothelial relaxant capable of stimulating cGMP might be nitric oxide. For many years nitric oxide was known as endothelial derived relaxation factor (EDRF), but it was confirmed independently by other groups Ignarro et al 1986, and Palmer et al 1987.

6.1.2 Physiological actions of nitric oxide

Nitric oxide is capable of being an inter and an intra cellular messenger, it is capable of this as it is a highly diffusible, non-polar colourless gas with low solubility (Wilhelm et al 1977). In concentrations less than 2μM it stimulates guanylate cyclase as briefly discussed earlier, which can triggers relaxation of the blood vessels. This will be addressed later in section 6.2.2: - The chemical and cellular process involving NO induced relaxation. The stimulation for NO release comes in several forms. It can be released due to shear stress of blood flow on the endothelial luminal surface (Korenaga et al 1994). Or, NO release can be ligand mediated (Klabunde 2000d). Others suggest that oxygen sensors are located on the endothelium, which trigger NO release in hypoxic conditions (Bassenger and Busse 1988). In the micro-circulation there is a tonic release of nitric oxide (NO). This suggests that NO is involved in the maintenance of normal muscle tone (Brady 1998).

NO also inhibits vasoconstrictor influences such as angiotensin II and sympathetic vasoconstriction (Klabunde 2000d). NO is also capable of the inhibition of platelet adhesion to the vascular endothelium (VE) making it antithrombotic. There is also inhibition of leukocyte adhesion to VE making NO anti-inflammatory. NO also has an antiproliferative action particularly on smooth muscle hyperplasia, which may follow from vascular injury. NO inhibits these effects and cGMP inhibitors, suggesting cGMP might be responsible for NO effects on proliferation. It is however not proof that the effect is induced by G-protein activation alone (Moncada and Higgs 1993). It is mainly endogenous substances that inhibit cell growth during the S phase, as exogenous NO inhibits DNA synthesis but exogenous cGMP has no effect (Sarkar et al 1994). cGMP dependent and independent pathways mediate the anti-proliferative effects of NO. It is currently believed that the action is fundamentally via the cGMP-independent pathway. NO also inhibits the migration of VSM needed for neointima formation (Sarkar et al 1996). This migration is also considered to involve cGMP-independent pathways because exogenous cGMP only partially inhibits the action (Kanagy et al 1996).

At higher concentrations of 10μM or more NO can have a cytotoxic effect that can triggers cell death. It can interact with oxygen radicals and generates the production of highly reactive peroxynitrite (Ritter 2001), which is highly toxic but capable of relaxing VSM either directly or indirectly by secondary messenger pathways to increase cGMP.

Table 6-1: - Functions of endothelium-derived NO

Table taken from:- Bayraktutan (2002). Free radicals, diabetes and endothelial dysfunction. *Diabetes, Obesity and Metabolism.* 4: 225.

6.1.3 Breakdown of nitric oxide

NO reacts with the iron in the haem group of haemoglobin (Hb) and forms dinitrosyl complexes (Doyle and Hoekstra 1981). The combination of NO with haemoglobin is the principal pathway of endogenous NO breakdown and its ultimate fate, (Mateo et al 2000). NO has a diffusivity 1.4 times greater than oxygen. Therefore it rapidly diffuses into the vascular compartments where it is deactivated by Hb before it has a chance to react with any cell components (Meuleman 1994). NO can interact with Hb in various ways and under varying conditions. The main pathways through which NO interacts with Hb are illustrated in Figure 6-2: - Reactions of nitric oxide with haemoglobin, are explained below.

Figure 6-2: - Reactions of nitric oxide with haemoglobin

Diagrams taken from: - Mateo A. O, De Artinano M. A. A (2000). Nitric oxide reactivity and mechanisms involved in its biological effects. *Pharmaceutical research* 42. 5: 421-427.

In normal arterial blood the concentration of oxygen is normally high and it exists attached to Hb, forming oxyhaemoglobin (HbO_2). This form reacts with NO to produce nitrate and methaemoglobin (metHb), which takes place in reaction 1 illustrated in the figure above. This reaction must take place a little slower than generally assumed in order for NO to effect biological targets (Gross and Lane 1999).

In anaerobic situations NO can combine with deoxyhaemoglobin (deoxyHb) which then forms nitrosilated HB (HbNO): this is the second reaction illustrated in Figure 6-2: - Reactions of nitric oxide with haemoglobin. The rate that HbNO dissociates is very slow and the affinity of NO for deoxyHb is 1500 times greater than that of carbon monoxide. In venous blood a mixture of HbNO and metHb is formed: the ratios depend upon the initial concentrations of both forms of Hb.

The third and final reaction in this series takes place in plasma where NO becomes oxidised to form nitrite: in this state NO can remain stable for hours. In the blood however the nitrite becomes further oxidised to form nitrate when it reacts with HbO_2 (Kelm et al 1992).

The end product concentration of nitrites is low and that of nitrates is about 100 times higher (Green et al 1982). The concentrations of nitrate, metHb and HbNO vary according to the degree of oxygen saturation of the blood (Wennmalm et al 1992). More recent evidence from Han et al 2002 suggests that nitric oxide may be involved with three successive reactions with the same haem molecule, converting oxyhaemoglobin to haemoglobin (Fe II) combined with nitric oxide.

6.2 Vascular smooth muscle relaxation

There are two distinct methods of relaxation. The first involves the G-protein coupled pathways of either Gs stimulation or Gi inhibition. These can effect adenylyl cyclase (AC) cGMP can act to lower/ inhibit cAMP phosphodiesterase (PDE) which reduces the breakdown of cAMP. In vascular smooth muscle (VSM) the increase in cAMP causes relaxation by inhibiting myosin light chain kinase (MLCK). The fall in MLC phosphorylation decreases the interaction between the actin and myosin filaments. So any drug or hormone that increases cAMP causes vasodilatation (Klabunde 2002e). The second involves the pathway detailed in section 6.2.2.

6.2.1 Physical process of relaxation

Myosin light chains are 20-KD regulatory subunits that are found on the myosin heads. The cGMP reduces calcium influx and phosphorylation of myosin light chain kinase (MLCK), which leads to a decrease in the phosphorylation of MLC. This decreases the smooth muscle tension causing vasodilatation (Klabunde 2002d).

6.2.2 The Chemical and cellular process involving nitric oxide induced relaxation

CGMP dependent relaxation

The main compound involved in relaxation is guanylyl cyclase (GC). It is able to combine with NO, as GC contains a haem group, forming a dinitrosyl complex as described earlier. When the two molecules combine a histidine molecule is released. This stimulates the conversion of guanosine 5'-triphosphate (GTP) into guanosine-3'-5' monophosphate (cGMP). So histidine forms the basis of the enzyme activity of GC, due to the conformational change of the enzyme (Traylor and Sharma 1992).

The cGMP that has been formed is able to act as a secondary messenger, capable of activating cGMP-dependent kinases, aiding various proteins to become phosphorylated and inducing vasorelaxation (Walter 1989). When the cGMP concentration increases and cGMP dependent protein kinase is stimulated and activated there is a fall in intracellular calcium (Ca^{2+}). There are several different pathways through which intracellular Ca^{2+} is reduced and these are described here. The first involves the phosphorylation of phospholambane, which activates Ca^{2+} ATPase in the sacroplasmic reticulum (Cornwell et al 1991). The stimulation of Na^{+}/ Ca^{2+} exchange also involves the extrusion of Ca^{2+} from inside the cell (Furukama et al 1991). Ca^{2+} L-type channels can also be inhibited (Mery et al 1991). The entry of Ca^{2+} is therefore activated when cGMP concentrations are low, which causes contraction. When cGMP concentrations are high as in relaxation, Ca^{2+} is removed or entry inhibited to have the desired effect (Milbourne et al 1995).

CGMP plays many other important roles and is able to inhibit Ca^{2+} release from intracellular Ca^{2+} stores (Meisherei et al 1986). It is also able to stimulate the formation of inositol triphosphate (IP_3) (Hirata et al 1990). It is also important to appreciate the role of cGMP beyond protein kinase activation. Other roles of cGMP include being able to directly bind to (K^{+}) potassium channels to activate them. All of these factors act to bring about relaxation, including cGMP inhibition of cyclic 3'-5' adenosine monophosphate (cAMP) phosphodiesterase, which increases cAMP leading to relaxation, briefly discussed earlier. The above information is shown diagrammatically in Figure 6.4 Mechanism of NO (EDRF) action. These mechanisms take place in the vascular smooth muscle.

cGMP independent relaxation

In vascular smooth muscle NO is able to activate the enzyme ADP-ribosyltransfoerases (ADPRT) (Brune et al 1989). Ribosylation of intracellular protein and protein kinase involves the transfer of the ADP-ribose group from nicotine adenine dinulcleotide (NAD^+) to an amino acid residue in the protein.

This mechanism may be involved in arterial tone control. As an antagonist of ADPRT 1,4-naphthoquinone can reverse NO effects on some contractile responses in isolated arteries (Kanagy et al 1996). Any fall in NO acts to elevate vascular tone. An acute fall in NO causes a vasoconstriction response as a consequence of reduced cGMP (therefore increasing Ca^{2+}). If NO concentrations continue to decrease over time less ribosylation of G-protein also leads to an increased sensitivity to any vasoconstrictor agents (Kanagy et al 1996).

In VSM cGMP dependent actions can cause the addition of nitrosyl groups to thiol residues (s-nitrosylation). This process can occur to receptor proteins and G-proteins. s-nitrosylation can also activate Ca^{2+} dependent K^+ channels by acting on their proteins in a similar manner. s-nitrosylation is also the method that activates endogenous ADPRT which causes ADP-ribosylation as described earlier. The cGMP independent pathways are summarised in Figure 6.3

Figure 6-3: - cGMP independent pathways in Vascular smooth muscle

The g-protein is inhibited by the addition of ADP-ribose. All cell modifications involve covalent linkages, which are difficult to reverse these bonds therefore remain even after NO has been deactivated. All of the above mechanisms act to bring about relaxation.
Diagram adapted from Mateo and De Antinano et al (2000).

6.2.3 Role of acetylcholine in muscle relaxation

Acetylcholine binds to receptors on the endothelial cell surface. The activated receptors communicate with the inhibitory G-protein (Gi), which in turn activates inositol (1, 4, 5) trisphosphate (IP_3) (Ritter 2001). IP_3 then triggers the release of calcium from the endoplasmic reticulum (ER). The increase in calcium activates the calcium dependent protein calmodulin (Cam), which then goes on to activate the enzyme nitric oxide synthase (NOS). L-arginine is the amino acid that is the physiological substrate for the enzyme NOS. L-arginine is transported into the endothelial cells by a cation transporter (Y^+). The NO that is generated in the endothelium diffuses into the underlying VSM, where the cGMP dependent and cGMP independent pathways are activated as previously described in section 6.2.2

Figure 6-4: - Mechanism of NO (EDRF) action

This diagram was complied from 3 separate sources: - 1. Ritter and Chowienczyk (2001), 2. Hartell (2001), 3. Mateo and De Artinano (2000).

At concentrations above 0.1μM (10^{-6}M) acetylcholine triggers graded contraction as it reacts on muscarinic receptors (Furchgott 1955 & Furchgott and Bhadrakam 1953). This is why all of the experiments undertaken use 10^{-6}M acetylcholine as the maximum administred concentration.

6.2.4 Role of insulin in relaxation

Insulin also stimulates NO production in vascular endothelial cells via a shared signalling pathway which triggers the production of eNOS the enzyme which produces NO. The pathway is summarised in Figure 6-5: - Insulin signalling pathways related to production of nitric oxide. This could explain why insulin resistance causes impaired vasodilation.

Figure 6-5: - Insulin signalling pathways related to production of nitric oxide

"Insulin signalling pathways related to production of nitric oxide" is taken from Montagnani M and Quon M. J (2000) Diabetes, Obesity and Metabolism. Vol.2 p288

This pathway is fundamentally different from the activation of NO by G-protein coupled cholinergic receptors on the endothelium. As insulin is able to stimulate nitric oxide release from vascular endothelium, it may explain how insulin resistance contributes to vascular complications associated with diabetes (Vincent et al 2003).

6.3 The endothelium

Over 1.2 trillion endothelial cells line the blood vessels; in total they cover an area of approximately 400m^2 and weigh about 1.5kg (Rehman 2001). The endothelium is a selectively permeable barrier to macromolecules; it provides a non-adhesive and non thrombogenic surface that helps to maintain the fluidity of the blood (Rehman 2001). As well as a barrier and transporter, the endothelium is able to act as a paracrine and endocrine organ. It secretes a number of biologically active chemicals that regulate a wide range of functions. For instance some of these mediators regulate immune responses, coagulation and vascular tone. The endothelium is also able to influence the underlying VSM as well as monocytes, macrophages and fibroblasts.

It is the endothelial cells that are the main mediators of vasodilatation in arteries (Furchgott at el 1984). Below some of the key roles and organ specific cells that endothelial derived mediators act on are listed.

- Vasodilators produce NO, prostaglandins and natriuretic peptides
- Vasocostrictors are also released from endothelium: they include thromboxane A2, endothelin (ET), prostaglandin H2 and components of the rennin-angiotensin system.
- Both pro- and anti- thrombotic factors are produced here such as platelet activating factor (PAF) and von willebrand factor (vWF).
- Fibrinolytic activators and inhibitors such as tissue plasminogen activator (tPA) and also plasminogen activator inhibitor-1 (PAI-1).
- Arachidonate metabolites also known as prostanoids.
- Adhesion molecules such as leukocyte adhesion molecules, intracellular adhesion molecular-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1).

- It also produces multiple cytokines that do jobs for example involving growth factors, pro and anti inflammatory mediators, tumour necrosis factors (TNF), chemokins and steroids.

So the endothelium can act as an inhibitory regulator of vascular contraction. The characteristics of the endothelium can alter due to local and systemic changes such as trauma, hyperglycaemia and hyperlipidemia. Endothelial dysfunction occurs when normal organ function can no longer be sustained (Bayraktutan 2002).

6.3.1 Endothelial function in vasodilatation

As noted in section 6.3 various mediators are released from or act on the endothelium. In this section the factors, which are able to cause vasodilatation by binding to or being released from endothelium are addressed, as this is the main focus of this chapter.

Nitric oxide

Nitric oxide (NO) is a gas and a free radical produced by the enzyme endothelial Nitric oxide synthase (eNOS). It is formed through a series of reactions involving the precursor L-arginine. NO induces relaxation of the blood vessels, this was first discovered over 25 years ago (Ignarro 2002). It is thought to do this by activating guanylate cyclase, which triggers the production of the secondary messenger; cyclic guanidine monophosphate (cGMP) (Dawson & Dawson, 1997). cGMP then goes on in sequence to stimulate cGMP-dependent protein kinases, which dephosphorylates the myosin chains in the muscle, causing relaxation. The phosphorylation of eNOS by Akt/ protein kinase B is a potential calcium-dependent regulatory mechanism of

eNOS activation. Nitric oxide also plays a key role in the regulation of blood pressure and vascular remodelling as well as in angiogenesis (Dimmeler et al. 1999)

Another factor that activates NO generation is the effects of shear stress. This has been shown to be blocked by wortmannin, a PI3 kinase inhibitor, indicating that the NO mechanism is stimulated along the PI3 kinase pathway. eNOS activation by shear stress is thought to regulate enzyme activity in a calcium-independent fashion and is observed in the absence of extracellular calcium. Small amounts of intracellular free calcium are essential though for eNOS activity (Dimmeler et al. 1999).

There are various stimuli that act to induce the release of NO from the endothelium these include mechanical stretching, stress, hypoxia, acetylcholine and vasopressin, , endothelin, bradykinin, histamine, adenine nucleotides, thrombin and 5'-hydroxy tryptamine (Rehman 2001). Nitric oxide is a paracrine hormone capable of acting in its immediate environment, as it is inactivated by circulating haemoglobin from the blood.

Endothelins

The endothelin (ET) family of peptides (approximately 21 amino acids) there are three types ET-1, ET-2, ET-3 all of which are coupled to G-protein receptors. ET-1 causes vasoconstriction described by Hopfner (1999). There are two types of receptors; - endothelin receptor type A (ETA) and endothelin receptor type B (ETB). ETA when stimulated by ET-1 causes vasoconstriction (Halcox 2001), this receptor has a selective affinity for this isoform. Receptor ETB is able to bind to all isoforms with an equal affinity. ETB releases endothelium-derived nitric oxide (NO) (Goraca

2002) and other chemicals, which trigger vasodilation. In some vascular regions ETB receptors may cause vasoconstriction instead and ETB receptors are important in clearing ETs, enabling endothelial cells to remove ETs from the circulation. Long-term exposure to insulin was shown to cause an increase in the ETA receptors, which are found on vascular smooth muscle, this enhances intracellular calcium over long periods. Calcium triggers vasoconstriction, narrowing blood vessels, but very high concentrations of calcium are toxic, triggering cell death. The opposite occurs in short-term incubation periods. Since insulin suppresses any effect that ET-1 has on the vascular muscle, it is possible that insulin causes decreased ET-1 activity, which may prevent the pathophysiology of ischaemic disease. ET-1 plays a very important part in helping to regulate both vascular tone and growth. It could also play a key role in the development of diabetic complications (Migdalis et al 2002). Studies in diabetic animals show that chemicals that block the ET system may be beneficial, as they could reduce vascular complications normally associated with diabetes.

Nitric oxide is very important for the maintenance of homeostasis in the vasculature, particularly under chronic conditions such as insulin resistance, obesity and dyslipidemia. The defects in the endothelium and smooth muscle cells are potentially caused by hyperlipidemia and hyperinsulinaemia.

Natriuretic peptide (NP)

There are 3 distinctly different gene products that make up the NP family. These are atrial natiuretic peptide (ANP), brain natiuretic peptide (BNP) and finally type C natiuretic peptide (CNP). The main one of interest is CNP that is produced by the vascular endothelium and macrophages (Komatsu et al 1992).

Full details of CNPs biological action are not completely known. CNP administered intravenously can reduce blood pressure, cardiac output, urine volume and sodium excretion (Stingo et al 1992). CNP is similar to NO as it stimulates the production of cGMP and it inhibits cell proliferation and synthesis of DNA in VSM. CNP also improves the intimal thickening that occurs after vascular injury (Furuya et al 1993). CNP can affect ET-1 and has been shown to inhibit its release in porcine endothelial cells (Kohno et al 1992). In this way it is suggested that CNP is able to counteract vaso-constriction as a result of suppressing ET production. Prostaglandin I_2 (PGI_2) has a very similar effect (Yokokawa et al 1991). Finally CNP can up-regulate ETB receptor mRNA expression in VSM cultures (Eguchi et al 1994).

Prostacyclins

Prostacyclins are released as a result of physical or chemical damage to the cell membrane. Other mediators that trigger prostacyclin release include bradykinin, thrombin, serotonin and platelet-derived growth factor (PDGF), adenine nucleotide and interleukin-1 (Gryglewski et al 1988). Prostaglandins act in a paracrine fashion on the abluminal surface of VSM causing relaxation it also prevents platelets clumping at the site of the endothelium. These actions of vasodilatation and anti-

platelet actions are induced via the cAMP pathway in VSM and platelets (Rehman 2001).

Aspirin is able to prevent prostacyclin formation but does not affect normal blood pressure, suggesting prostacyclins play no role in normal blood pressure control. Vascular tissue produces less prostacyclins as the tissue ages and in diseased states such as diabetes and atherosclerosis. Prostacyclins are also capable of increasing the activity of other enzymes such as those, which metabolise cholesterol esters in VSM. This suppresses cholesterol ester accumulation by macrophages and prevents growth factors from being released, which thicken the vascular wall (Willis et al 1986). This indicates a link between prostacyclin synthesis and thrombotic and atherosclerotic episodes in the vascular wall.

Kinins

Vascular kinins induce vasodilatation through the release of several potent vasodilatation factors such as NO and prostacyclin. Angiotensin-converting enzyme (ACE) inhibitors prevent kinin degradation and are able to prolong their natural effects, lowering blood pressure causing cardioprotection and the regression of cardiovascular hypertrophy and make insulin sensitivity greater. The main vasodilators are overviewed in this section, but the endothelium has other molecules such as tPA and PAI-1 and leukocyte adhesion molecules, which have an effect, these are addressed in greater detail in the review by (Rehman 2001).

6.3.2 Endothelial dysfunction

Endothelial dysfunction is associated with reduced NO generation and action (Beckman et al 2002). There is a diminished basal production of NO in diabetes (Pieper 1998). There is a natural decline in endothelial function, which increases with age. This occurs above 40 years of age in men, and above 55 years in women (Hsueh et al 1997). The loss of nitrogen oxide function is caused mainly by free radicals, connected to increased vascular constriction, increased adhesion of both leucocytes and platelets and platelet aggregation. Damage to the endothelium can cause vasoconstriction to dominate and lead to conditions such as hypertension (Scäfers 2003). There also appears to be an increase in the proliferation of vascular smooth muscle, which contributes to acceleration of atherogensis. This naturally occurs in ageing due to the involvement of free radicals in the ageing process. However in diabetic patients free radical production is accelerated, causing an earlier onset of complications associated with endothelial dysfunction (Higashi and Yoshizumi 2003).

Increased oxidative stress has been demonstrated in type 2 diabetes, and this could be a possible explanation for further damage to endothelium-dependent relaxation (Chowienczyk et al 2000). It is thought that reactive oxygen species are able to inactivate endothelium-derived NO, by directly inhibiting NOS and reducing the signals sent by guanylate cyclase. There is also the potential down regulation of the intracellular enzyme superoxide dismutase (SOD), which dismutes the superoxide anion (O_2-) to the less severe reactive oxygen species (ROS) hydrogen peroxide (H_2O_2), which could account for a decrease in NO as O_2- concentrations are elevated, further adding to endothelial dysfunction (Ulker et al 2001). O_2- is one of the main reactive oxygen species ROS as it is directly able to cause VSM contraction

(Gryglewsk et al 1986). When H_2O_2 has been produced it can be degraded by H_2O_2 scavengers, such as catalase and glutathione peroxidase (GPx). Under high glucose concentrations GPx activity is decreased, this leads H_2O_2 causing cellular damage adding to endothelial dysfunction (Kashiwagi et al 1994).

By introducing oxygen free radical scavengers there is an improvement in endothelium-dependent relaxation. This has been shown both in vivo and in vitro, as superoxide dismutase is not reduced and improves NO release. The glycation of proteins is thought to be one of the main sources of free radicals in diabetes, which helps to generate reactive oxides. The superoxide anions are created in glycated proteins almost 50 fold more than in non-glycated proteins. This supports the theory that glycohaemoglobin plays a significant role in the endothelial dysfunction that is associated with diabetes as it later becomes modified by glycation and auto-oxidative glycosylation (Vlassara et al 1994).

The highly reactive compound peroxynitrite ($ONOO^-$) as described earlier, formed from NO combining with O_2-, depletes important plasma antioxidants like cysteine and glutathione, which increases the oxidative stress on the endothelium leading to additional damage (Van De Vliet et al 1994).

The endothelium also has receptors for AGEs, which can be internalised into sub-endothelial space (Wautier et al 1994). AGEs impair endothelial dependent relaxation by glycosylation or modifying LDLs that go on to inactivate and disrupt NO formation (Bucala et al 1993).

In diabetes, hyperglycaemia is the most likely causal factor of the increased oxidative stress on the endothelium (Bayraktutan 2002). These factors are mediated through various mechanisms, which include alterations to the cellular redox state by changes to NADH/NAD+ ratios. Changes in protein tyrosine kinase regulation, dysregulation of protein kinase C and accumulation of sorbitol (Bayktutan 2002), these factors are dealt with in greater detail in this review. Only the hyperactivity of the sorbitol pathway will be addressed at length here, as it is believed to contribute to increased oxidative stress. Hyperglycaemia increases intracellular NADH/NAD+ and reduces NADPH/NADP+ ratios due to hyperactivity of the sorbitol (polyol) pathway. This creates a cytosolic redox imbalance as NADH/NAD+ increase mimics tissue hypoxia giving the state the name "hyperglycaemic pseudohypoxia" (Williamson et al 1993). Aldose reductase is the first and key rate- limiting enzyme in the pathway, and it catalyses the reaction of NADPH-dependent reduction of glucose to sorbitol.

Table 6-2: - Potential causes of increased oxidative stress in diabetes mellitus

Table taken from:- Bayraktutan (2002). Free radicals, diabetes and endothelial dysfunction. *Diabetes, Obesity and Metabolism.* **4: 225.**

Sorbitol is then catalysed to fructose by the enzyme sorbitol dehydrogenase (Kador et al 1995). Any accumulation of sorbitol increases the intracellular osmolarlity, which causes the polyol pathway changes. Aldose reductase inhibitor can provide a preventative role in diabetic complications such as nephropathy and neuropathy. New evidence though suggests closer links between myo-inositol depletion, glycation and increased oxidative stress in diabetes are summarised in Table 6.2.

Obesity often present in type 2 diabetes can also lead to endothelial dysfunction (Williams et al 2002). This is probably through obesity-induced insulin-resistance. The exact relationship between endothelial dysfunction and obesity related insulin resistance is unclear (Tounian et al 2001). The endothelial dysfunction in diabetes mellitus is a result of decreased NO synthesis and increased degradation (Chaudhuri 2002), which has been outlined.

6.3.3 Reversal of endothelial dysfunction

This will only be basically outlined here further detail can be found in (Bakyratutan 2001). Insulin therapy for 6 months decreases glucose, free fatty acids and triglycerides (TG), which normalise endothelial function. By lowering TG and increasing high-density lipoproteins (HDL) using 3 months of ciprofibrate therapy, endothelium-dependent vasodilatation was increased (Evans et al 2000).

ACE inhibitors also cause improvements as discussed earlier in section 6.3.1. Acute 1-week treatment with antioxidant vitamin E showed improvement of endothelial cell function (Chowienczyk et al 2000), but this was lost after 2 months treatment (Gazis et al 1999).

6.4 Experimental detail and aims

The following sets of experiments were performed to investigate the release of nitric oxide, and if metformin is able to influence NO. 8.1. The ability for insulin to trigger NO release was determined using the dye DAF-2DA as outlined in chapter 2 section 2. The experiments carried out in this section are outlined below. All of these studies used sections of aorta with endothelium and VSM intact and prepared as described in chapter 2, section

- The dye method was also used to establish if metformin had any effect on insulin and acetylcholine release of NO. The aortic sections were incubated for 1 and 4 hours with metformin.
- The second set of experiments used inhibitors of the glucose pathway to establish if this pathway is also responsible for NO production. Aortic sections of tissue were used here, but only limited exposure of each inhibitor was allowed to prevent tissue decay.
- The last set of experiments used 2 dyes DAF-2DA to detect NO and FURA-2AM to detect Ca^{2+} this used sophisticated equipment and the expertise of Dr Nick Hartell, to determine if insulin release of NO is calcium dependent or independent. Thin slices of rat aortic tissue were used in this section, prepared for imaging as outlined in chapter 2, section 2.8.1.

6.5 Results

Studies were performed on aortic sections incubated for 1 and 4 hours with metformin. The samples were then incubated with the nitric oxide sensitive dye DAF2DA prior to the study. Then the tissue was subjected to 5 minutes exposure to insulin or acetylcholine. The effects of using insulin after a 1 hour incubation are shown in figure 6-6, and with insulin in the presence of metformin figure 6.7.

Figure 6-6: - The effect of a 1 hour pre-incubation and the effect of insulin on NO release

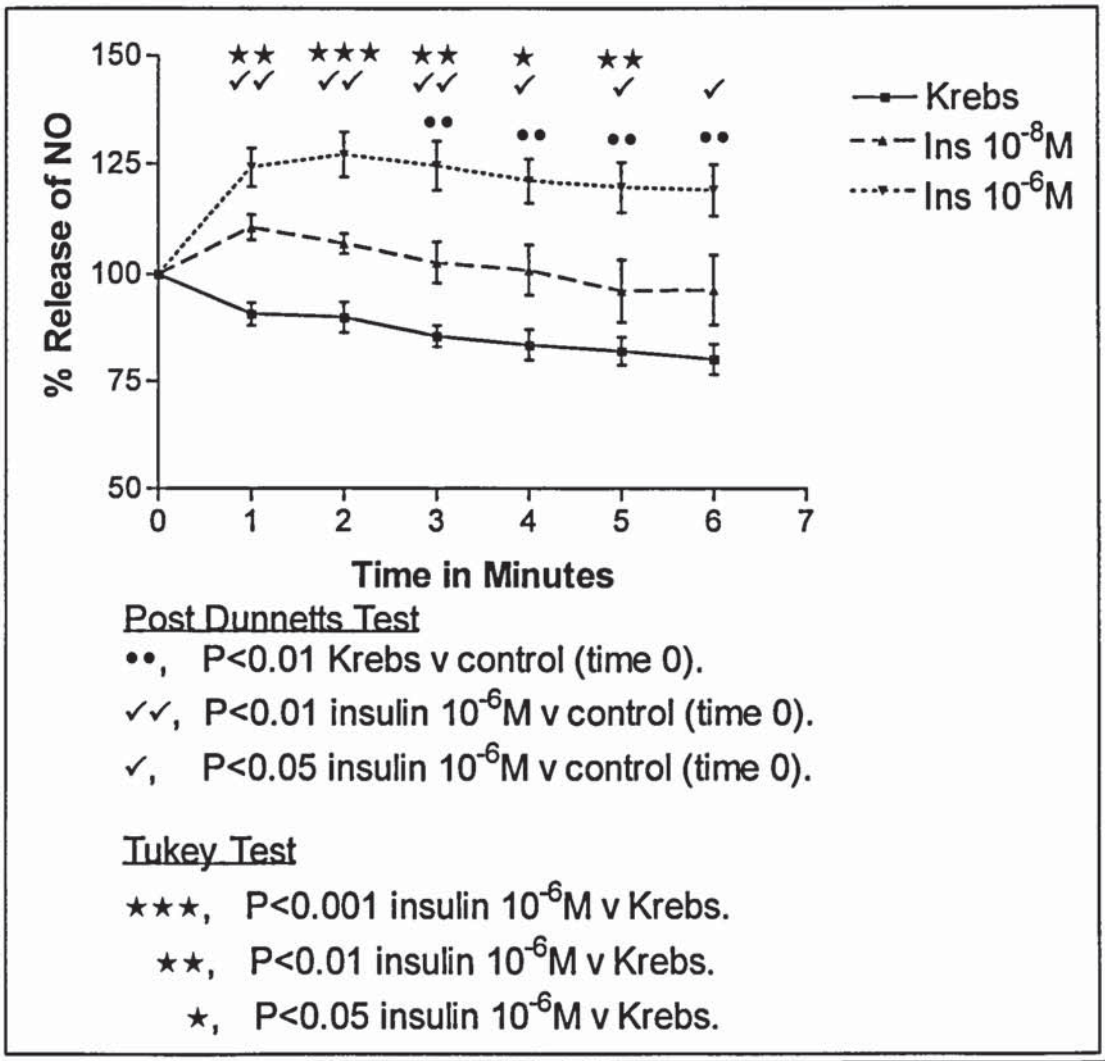


Figure 6-6: - Insulin triggers the release of nitric oxide compared to the administration of Krebs as a control. The release of NO by insulin also appears to be dose dependent. The greater the insulin concentration, the greater the release of NO. The maximum release of nitric oxide is triggered by insulin 10^{-6}M after 2 minutes. ± SEM. (n=6)

Insulin definitely triggers the formation and release of NO. At 10^{-6}M insulin this was significant NO release compared to a control of adding Krebs to vascular tissue. The maximal effect of insulin was seen after 2 minutes. The response of insulin at 10^{-8}M is not significant, but 10^{-6}M causes a greater release of nitric oxide (NO), suggesting that insulin causes a dose-dependent release of NO. The greater the concentration of insulin the bigger the release of NO.

Below in figure 6.7 a similar experiment was performed in the presence of metformin 10^{-2}M. Metformin was used at a larger concentration as no significant changes were detected at 10^{-5}M. In this case higher metformin concentrations were used to try and produce an increase in NO that could be detected by this system.

Figure 6-7: - The effect of a 1 hour pre-incubation with metformin, and the effect of insulin on NO release

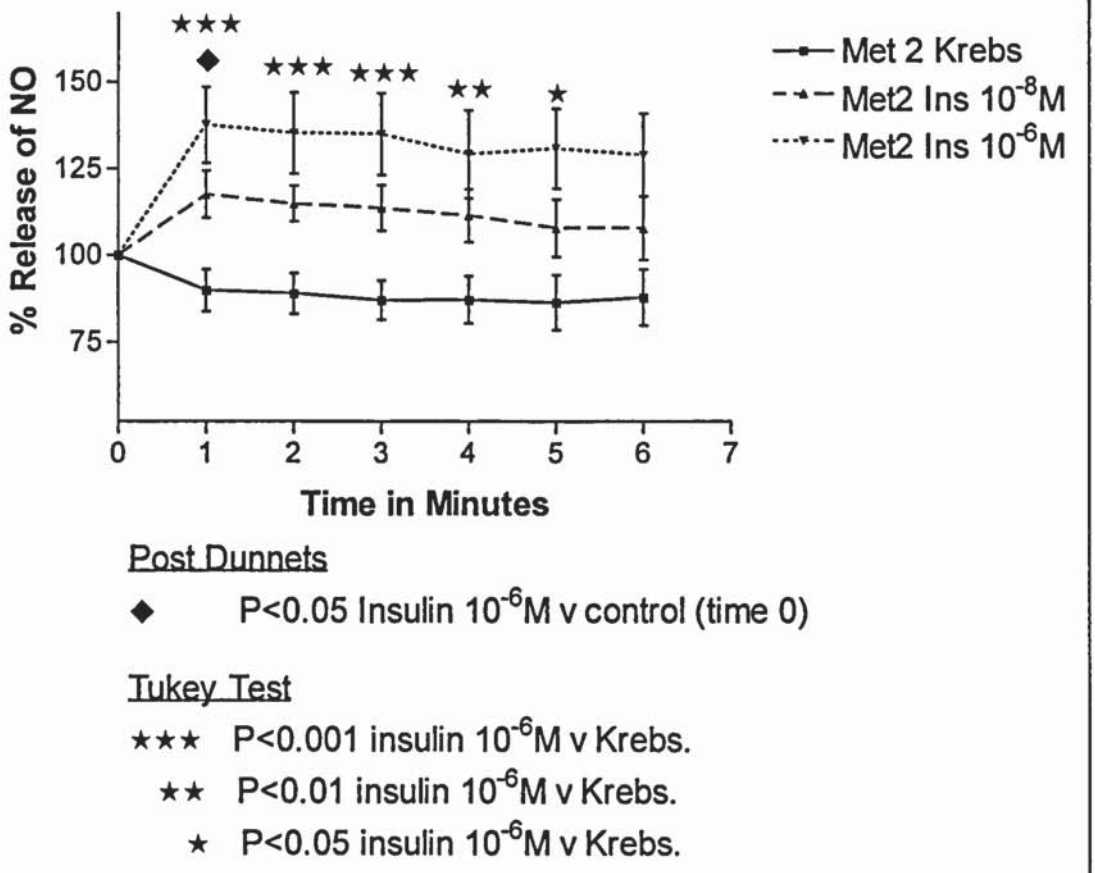


Figure 6-7: - Insulin triggers the release of nitric oxide compared to the administration of Krebs as a control. The release of NO by insulin also appears to be dose dependent. Again the greater the insulin concentration, the greater the release of NO. Metformin 10^{-5}M however does not appear to cause much additional increase in nitric oxide. ± SEM. (n=6)

Again insulin triggers NO release, but metformin triggers little additional increase in the production of NO. This suggests that metformin may not increase vaso-relaxation by causing a significant increase in NO, production from the insulin pathway. This may point to another mechanism being responsible for increasing aortic relaxation in the presence of metformin

The second set of experiments in this series used the same principles as the previous 2 experiments, but acetylcholine was used instead of insulin to trigger NO release. The results in the presence of acetylcholine are shown below.

Figure 6-8: - The effect of a 1 hour pre-incubation and the effect of acetylcholine on NO release

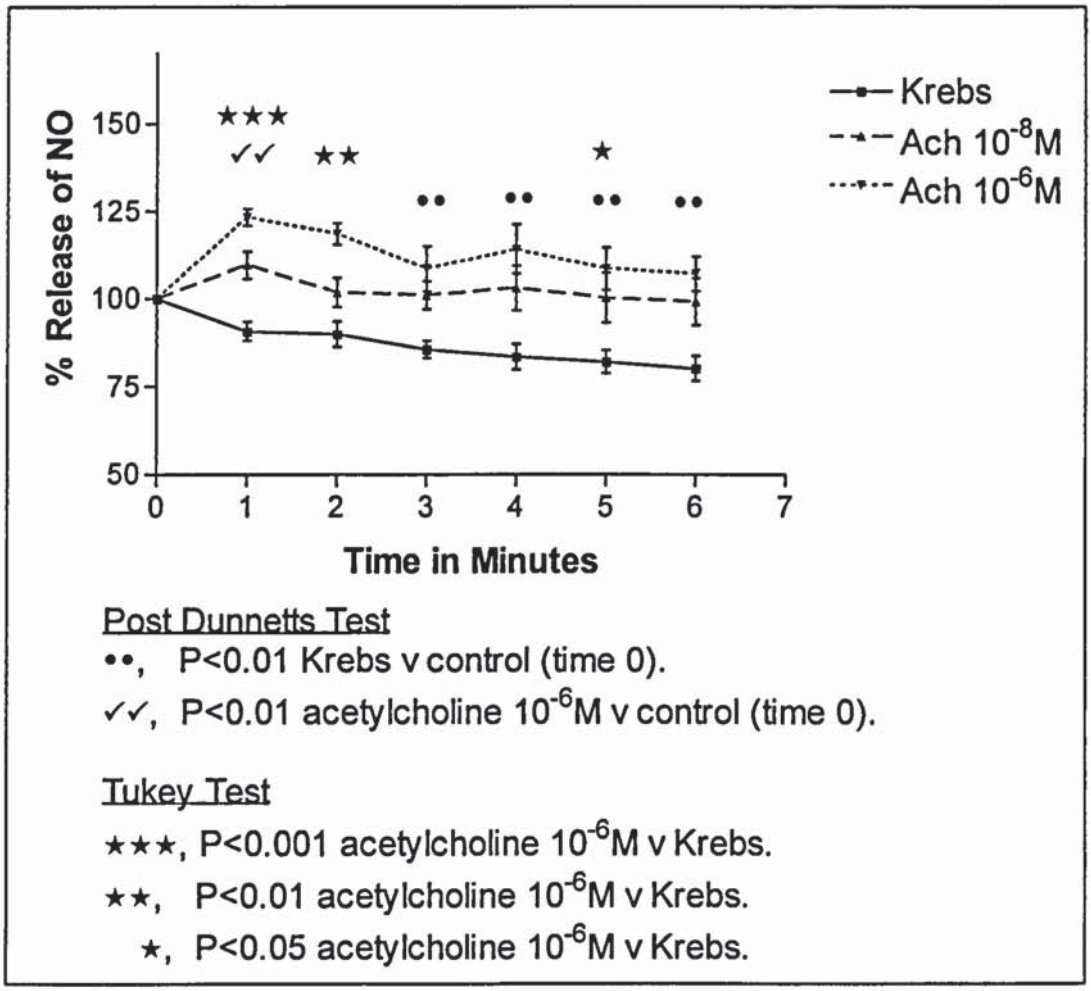


Figure 6-8: - Acetylcholine triggers the release of nitric oxide compared to the administration of Krebs as a control. The release of NO by acetylcholine appears to be dose dependent. The greater the acetylcholine concentration, the greater the release of NO. The maximum release of nitric oxide is triggered by acetylcholine 10^{-6}M. ± SEM. (n=6)

Acetylcholine is known to trigger the release of NO, but unlike insulin the release of NO is sustained for longer, still having a significant effect on NO release after 5 minutes.

Figure 6-9: - The effect of a 1 hour pre-incubation with metformin, and the effect of acetylcholine on NO release

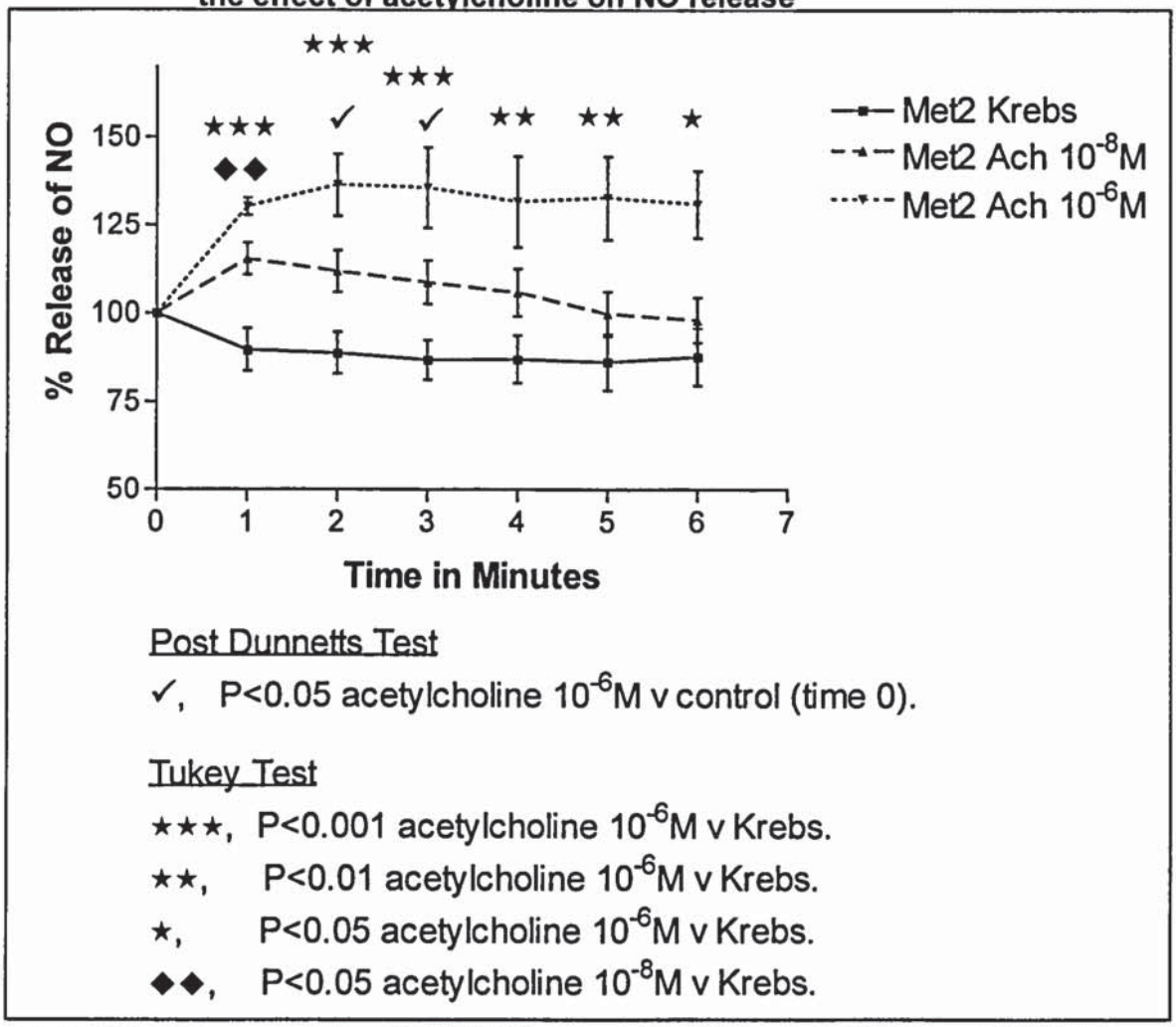


Figure 6-9: - Insulin triggers the release of nitric oxide compared to the administration of Krebs as a control. The release of NO by insulin also appears to be dose dependent. Again the greater the insulin concentration, the greater the release of NO. Metformin 10^{-5}M however does not appear to cause much additional increase in nitric oxide. ± SEM. (n=6)

Acetylcholine still triggers NO release from the endothelium and vascular smooth muscle. There is little additional release of NO in the presence of metformin, again suggesting that enhanced relaxation may not be stimulated by increased NO concentrations.

Similar experiments on insulin and acetylcholine were carried out with a 4 hour incubation with metformin; the concentrations of NO detected were smaller, but similar in pattern. The results are not shown here, as the fall in NO is believed to be due to tissue degeneration and preparation conditions after this time (4 hours).

An additional set of experiments was performed in aortic sections to compare the nitric oxide produced after 1 minute. The tissue was stimulation with either acetylcholine or insulin, after the 1 hour control incubation. Metformin was also added as seen earlier for a 1 hour incubation prior to acetylcholine and insulin being applied. The results are shown below.

Figure 6-10: - The effect of a 1 hour pre-incubation with metformin, and the effect of Insulin and acetylcholine after 1 minute on NO release

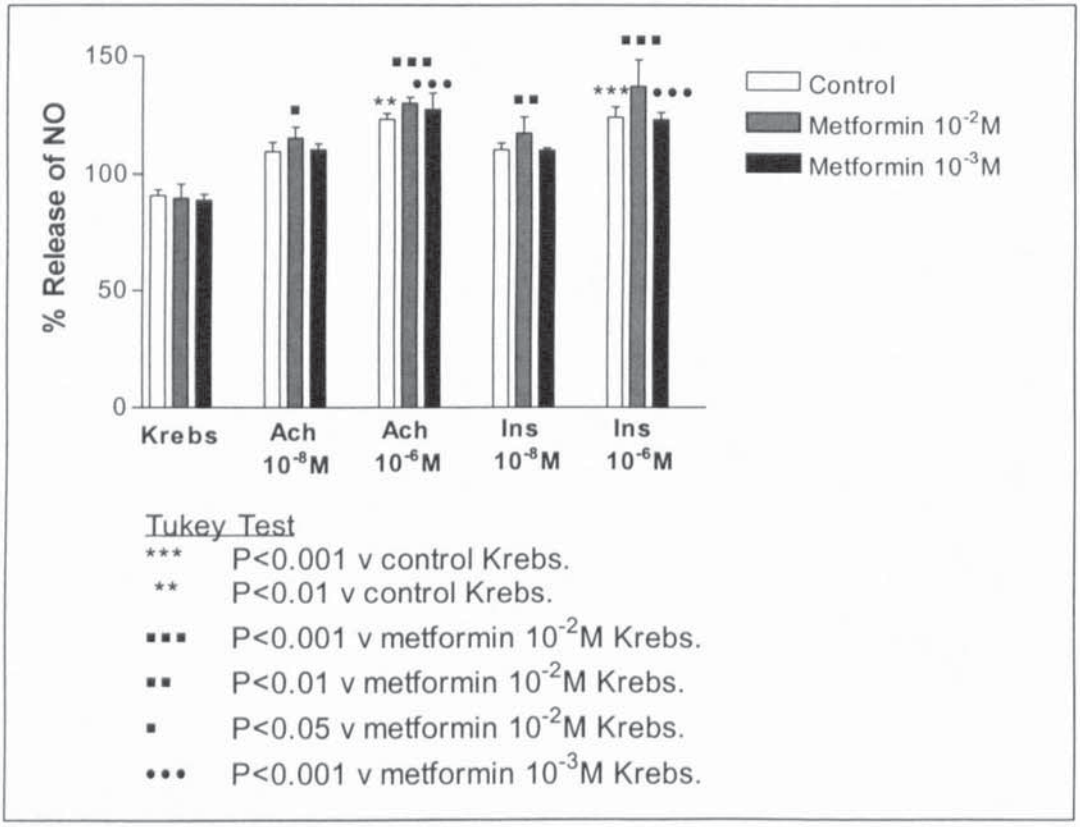


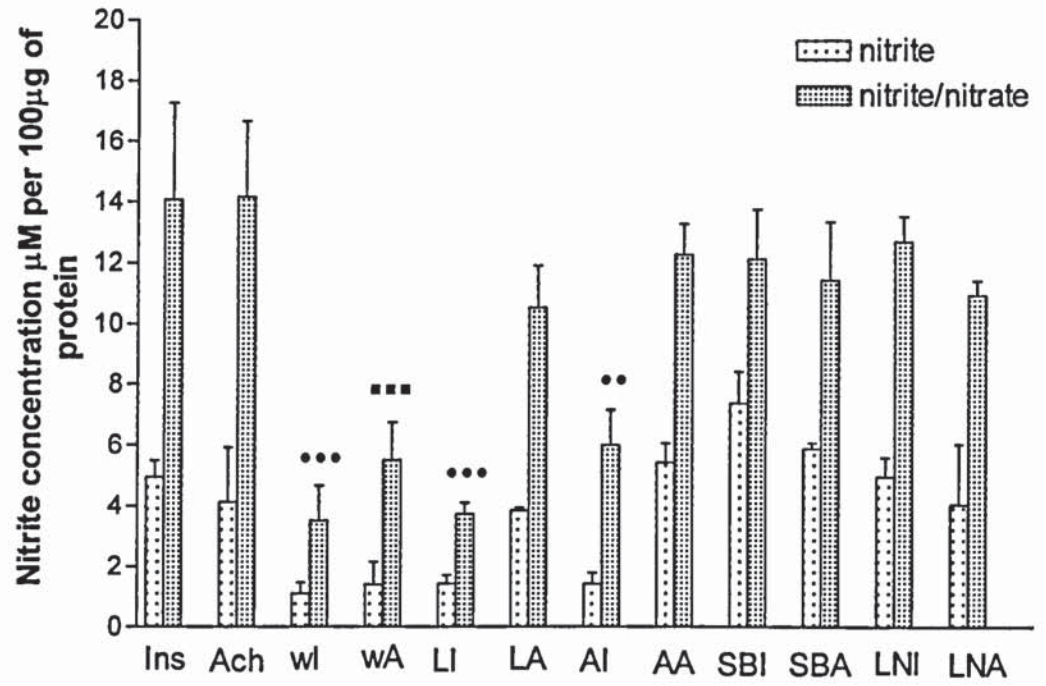
Figure 6-10: - The results after 1 minute are only significant when compared to the Krebs control. Metformin 10^{-5}M does not cause any additional increase in nitric oxide release induced by either insulin or acetylcholine. ± SEM. (n=6)

These results show that insulin causes a comparable release of NO to acetylcholine at 1 minute. The results also show that metformin has no significant effect on NO release after a 1 hour incubation in either insulin or acetylcholine induced NO release.

Due to the limitation of the methodology and tissue degeneration it is not possible to test these effects at longer time periods as tissue showed degradation and reduced release of NO, because of this at 4 hours. In vivo studies are difficult to ensure an adequate number of treatments with tissue in a similar physiological state. As it is difficult to reduce variation in the results and to accurately dosage the animals with out direct oral administration, which can cause stress to animal models. Stress also effects endothelial function whether it is physical stress or oxidatively induced stress.

The final experiment shown in figures 6-11 again used aortic slices, which were incubated for 30 minutes with the inhibitors of the insulin and NO pathways. The inhibitors used and their abbreviations are shown in the key to Figure 6.11. After the incubation they were exposed to either acetylcholine or insulin for 10 minutes. Then the nitrite and total nitrite content (nitrite/ nitrate) of the samples were determined using the DAN reaction as detailed in chapter 2, section 2.5.3. The Griess reagent reaction was originally used, but was not sensitive enough to accurately detect the amounts of nitrite and nitrate produced.

Figure 6-11: - Production of nitrite and nitrate stimulated after 10 minutes exposure to insulin or acetylcholine after 30 minute incubation with various inhibitors.



Key

First letter of samples 3-8, and first 2 letters of samples 9-12 indicate the inhibitors used shown on the key below.

W = Wortmanin, IC50 concentration 5.0nM
L= LY294002, IC50 concentration 1.4 µM
A = Akt inhibitor, IC50 concentration 5.0 µM
S = SB202190 IC50 concentration 16nM
LN = L-NAME IC50 concentration 500nM

Additional letters indicate whether insulin or acetylcholine was used.

I or Ins = Insulin 10^{-6}M
A or Ach = Acetylcholine 10^{-6}M

Figure 6-11: - The first letter in the key below indicates the inhibitor the tissue was exposed to for 30 minutes and whether insulin 10^{-6}M or acetylcholine 10^{-6}M was administered for the 10 following 10 minute period. This set of experiments was performed to establish if insulin triggers nitric oxide produced by a similar pathway to glucose uptake. The greatest degree of inhibition was seen in tissue treated with Wortmannin and LY294002 in the presence of insulin 10^{-6}M. Acetylcholine stimulated tissues showed limited inhibition of nitric oxide release in the presence of the inhibitors, as it does not induce NO release via the glucose uptake pathway. ± SEM. (n=6)

Wortmanin and LY294002 both act on the enzyme phosphatidylinositol 3-kinase (PI3 kinase) and inhibit its action, but at different sites of the enzyme complex (p85 and p110). Normally phosphatidyl 4, 5 bisphosphate is converted to phosphatidylinositol 3, 4, 5 triphosphate, in the insulin signalling pathway by the enzyme (PI3 kinase). LY294002 is considered the better inhibitor of the 2. As wortmanin can be toxic to cells and induce cell death. This can clearly be seen in Figure 6-11 as wortmanin induces a great fall in NO in both insulin and acetylcholine treated samples. This suggests the fall here is due to cell death. This cannot be tested visually on vascular slices, as it is difficult to distinguish endothelium and VSM under the light microscope. LY294002 causes a significant fall in insulin treated samples, which act via PI3 in the insulin-signalling pathway. Acetylcholine also shows a slight fall, but acetylcholine is able to trigger cGMP independent pathways that trigger NO release.

The most conclusive result comes with the Akt inhibitor as this part of the pathway action only occurs in insulin. Here a significant fall in NO stimulated by insulin and Akt inhibitor takes place as the pathway of glucose uptake is blocked. Blockage is not total due of the IC50 being used and the restricted time period of 30 minutes incubation to prevent tissue degradation, which naturally occurs over time. Little fall in acetylcholine and Akt inhibitor is seen, therefore only Akt/ protein kinase B is involved solely in insulin stimulation of NO and not that of acetylcholine. SB202190 causes the inhibition of the Map-kinase pathway and appears to have no significant effect on either insulin or acetylcholine stimulated NO release. Finally L-NAME a NOS inhibitor does not cause a significant fall in NO, this is surprising but may be due to the IC50 not being high enough to block NOS, and/ or the period of incubation only being 30 minutes (to ensure the tissue was still viable), a limitation of this study.

The experiments illustrated in figures 6-12 to 6-18 were the experiments performed in collaboration with Dr Nick Hartell. The experiments are documented in detail in chapter 2 in sections 2.8.2 - 2.8.5 using an epifluorescent microscope to take real time images of nitric oxide and calcium production when stimulated by acetylcholine and insulin.

Figure 6-12: - Rate of release of nitric oxide and calcium from the endothelium and vascular smooth muscle of aortic slices stimulated by acetylcholine

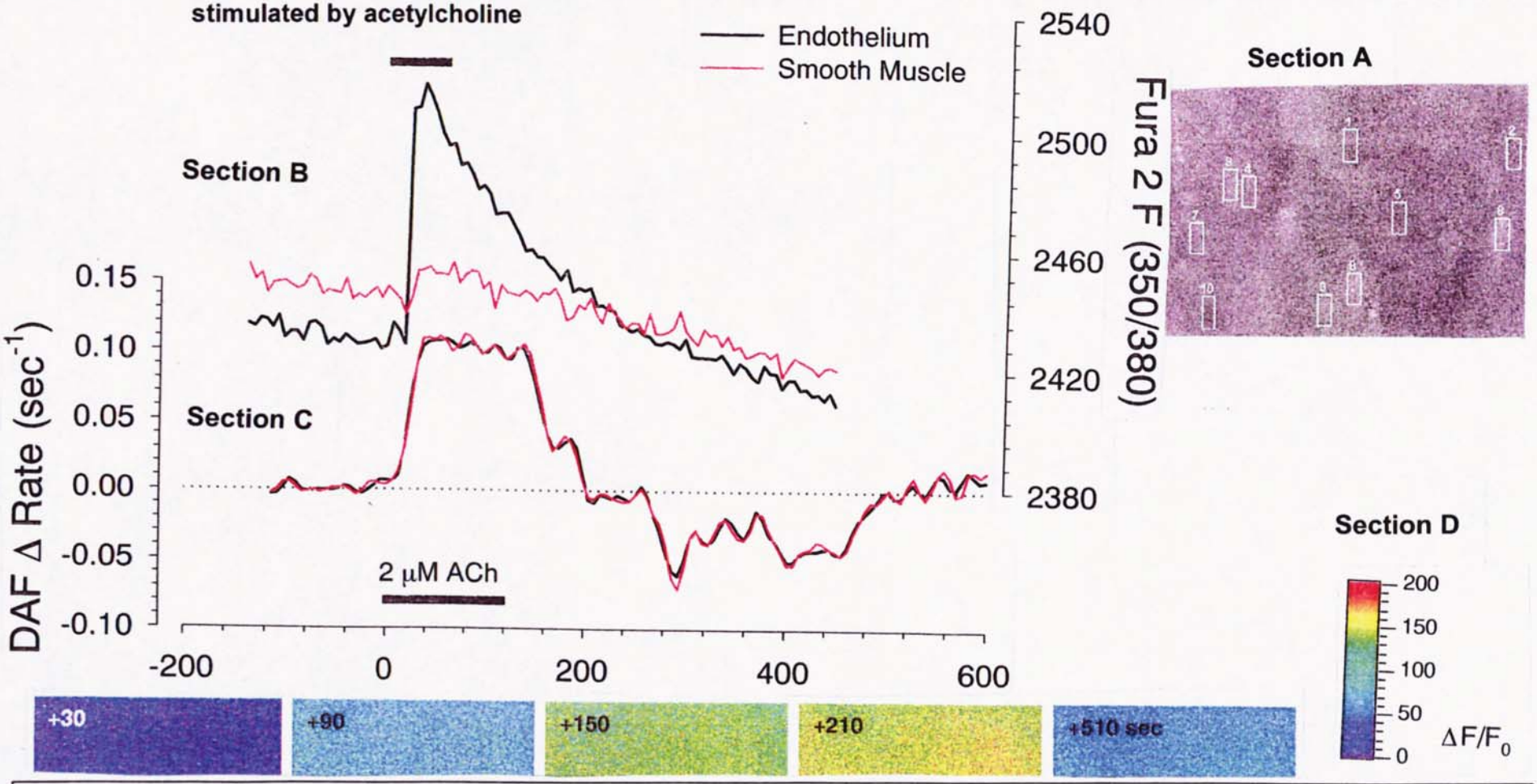


Figure 6-12: - The aortic slice is loaded with both FURA-2AM which images changes in calcium and DAF-2DA, which measures changes in nitric oxide. **Section A**, represents the positions on the aortic slice selected for imaging.**Section B**, shows the release of calcium. This is represented by the uppermost black and red traces. Showing a greater amount of calcium is released from the endothelial cell layer on the administration of.. **Section C**, shows the rate of release of nitric oxide, this is indicated by the lower black and red traces. The rate of release of nitric oxide is greatest during the acetylcholine infusion. Nitric oxide is released from the endothelium and spreads to the underlying smooth muscle. **Section D**, indicates the intensity of the release of nitric oxide monitored by the DAF-2DA dye. The warmer the colour (red, orange, yellow) the greater the NO release.

Figure 6-13: - Total release of nitric oxide and calcium from the endothelium and vascular smooth muscle of aortic slices stimulated by acetylcholine

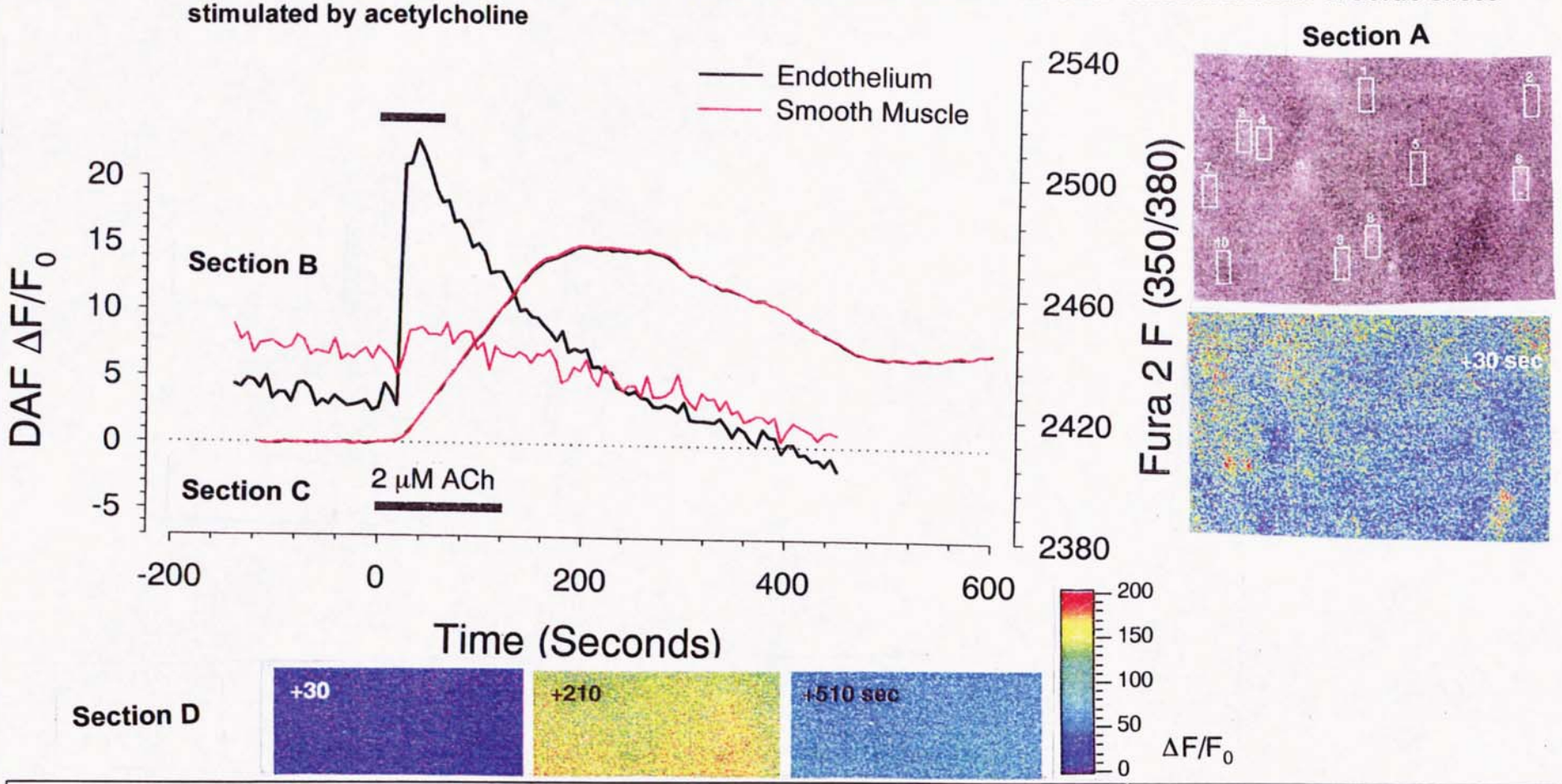


Figure 6-13: - The aortic slice is loaded with both FURA-2AM which images changes in calcium and DAF-2DA, which measures changes in nitric oxide. **Section A**, represents the positions on the aortic slice selected for imaging. **Section B**, shows the release of calcium. This is represented by the uppermost black and red traces taken from the far left. Showing a greater amount of calcium is released from the endothelial cell layer on the administration of acteylcholine. **Section C**, shows the total release of nitric oxide, this is indicated by the lower black and red traces, taken from the far left. The total release of nitric oxide increases over time, due to the accumulation of NO in the medium. **Section D**, indicates the intensity of the release of nitric oxide monitored by the DAF-2DA dye. The warmer the colour (red, orange, yellow) the greater the NO release.

Figure 6-14: - Total release of nitric oxide and calcium from the endothelium and vascular smooth muscle of aortic slices stimulated by insulin.

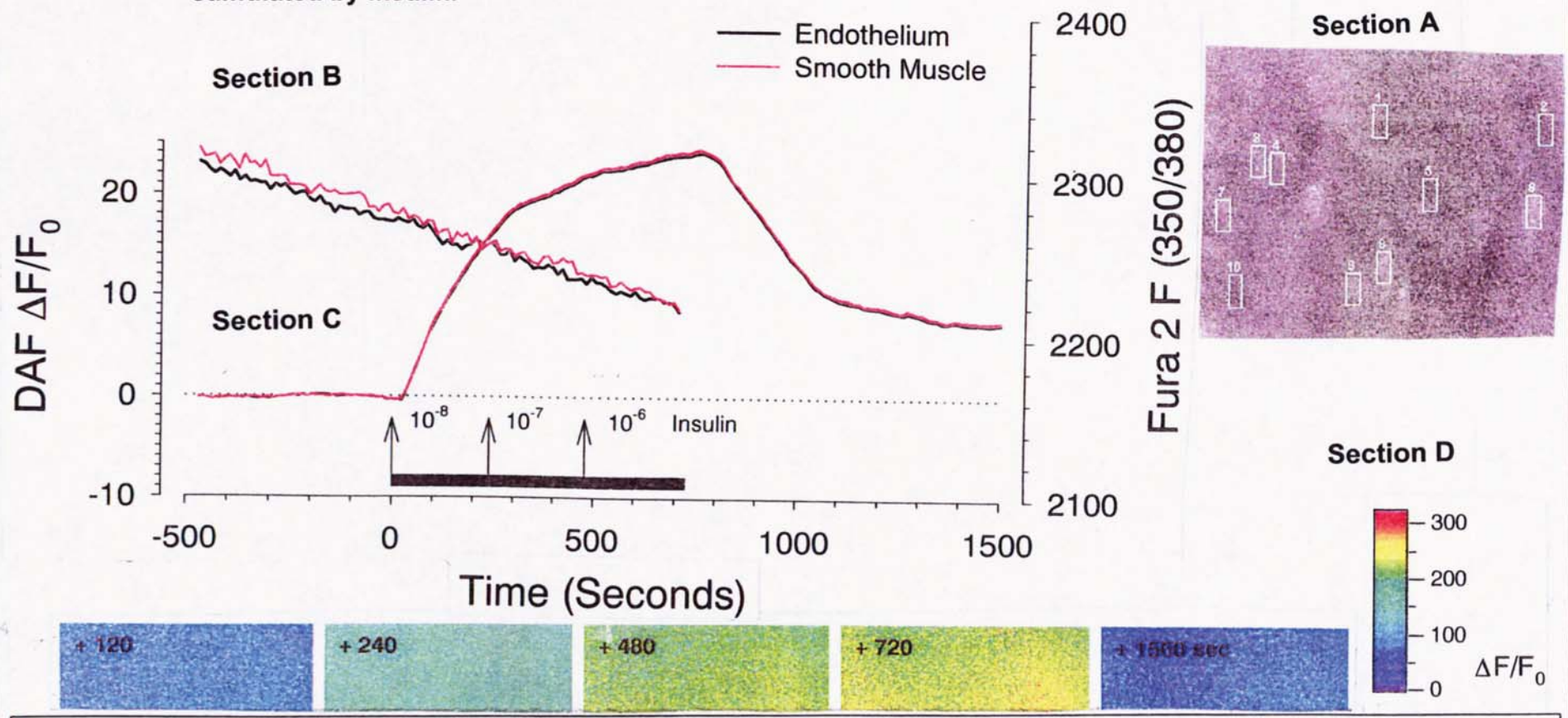


Figure 6-14: - The aortic slice is loaded with both FURA-2AM which images changes in calcium and DAF-2DA, which measures changes in nitric oxide. **Section A**, represents the positions on the aortic slice selected for imaging. **Section B**, shows the release of calcium. This is represented by the sloping black and red traces taken from the far left. Showing a fall in calcium concentration in both the endothelium and smooth muscle on the administration of insulin. **Section C**, shows the total release of nitric oxide, this is indicated by the lower black and red traces, taken from the far left. The total release of nitric oxide increases over time with increasing insulin concentrations, due to the accumulation of NO in the medium. **Section D**, indicates the intensity of the release of nitric oxide monitored by the DAF-2DA dye. The warmer the colour (red, orange, yellow) the greater the NO release.

Figure 6-15: - Total amount of nitric oxide produced with acetylcholine and increasing concentrations of insulin.

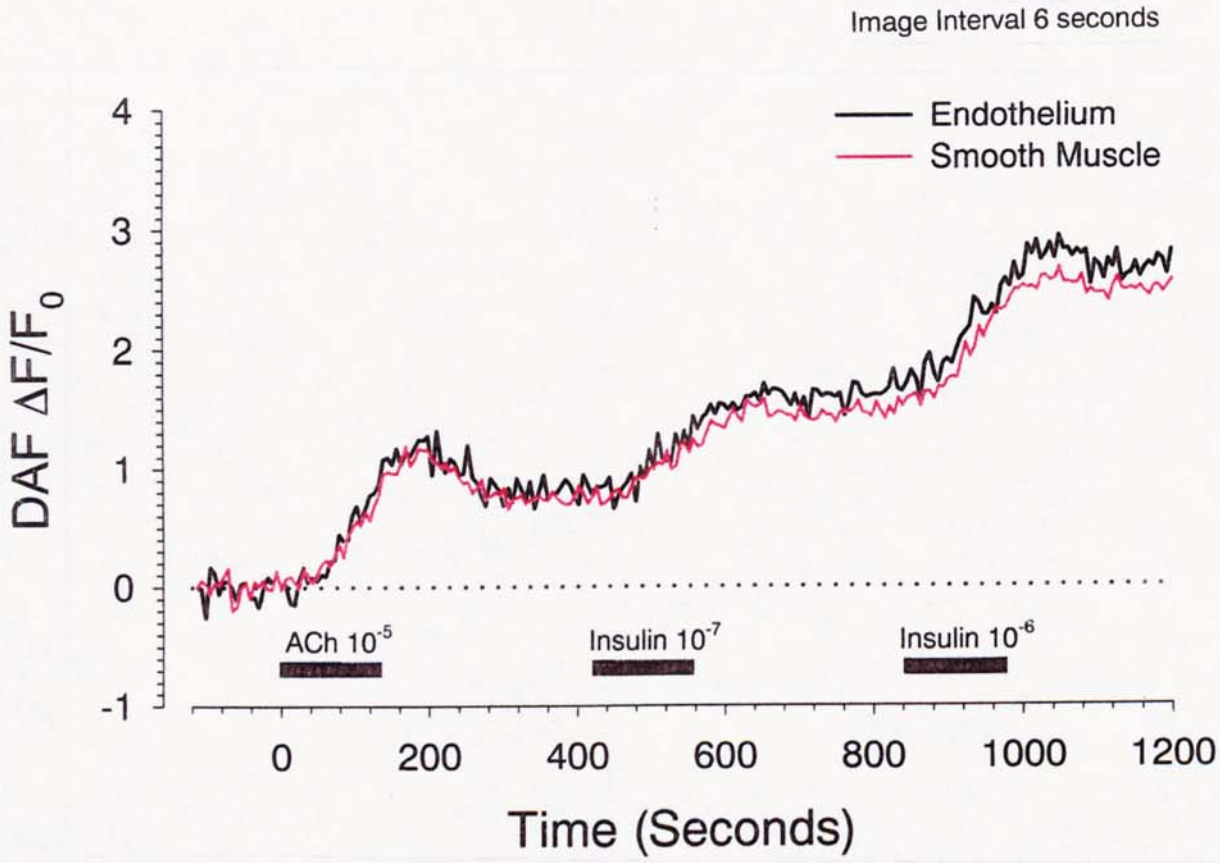


Figure 6-15: - This indicated the total amount of nitric oxide produced over time. Acetylcholine 10^{-5}M was used to assess the viability of the endothelial cells and induced the release of nitric oxide. Then insulin was added and nitric oxide increased in a dosage dependent manner with increasing insulin concentrations 10^{-7}-10^{-6}M.

Figure 6-16: - Rate of release of nitric oxide produced with acetylcholine and increasing concentrations of insulin.

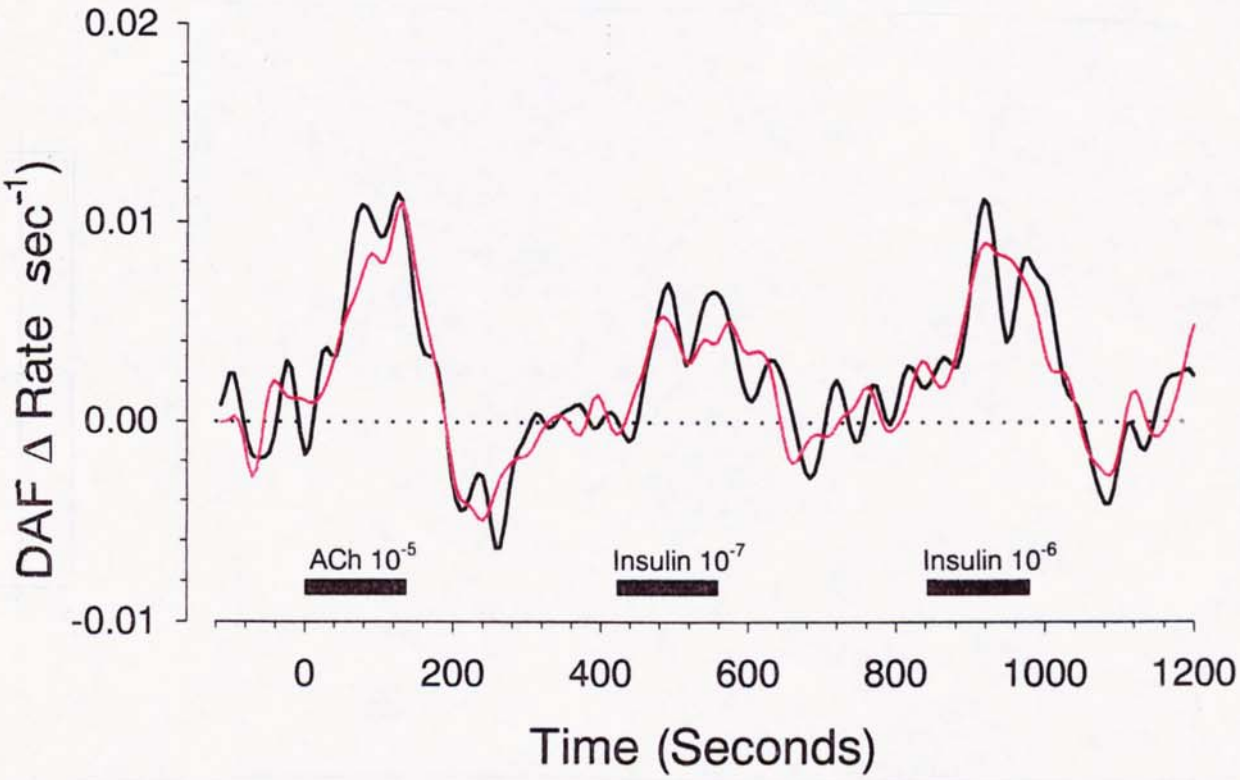


Figure 6-16: - A similar experiment was carried out but this time to determine the rate of release of nitric oxide. Here it can be seen that acetylcholine 10^{-5}M induces a large and rapid release of nitric oxide. Insulin 10^{-7}M induced a slower and reduced release of nitric oxide. Insulin 10^{-6}M induced a greater response than insulin 10^{-7}M and was comparable in size to the response triggered by acetylcholine10^{-5}M.

Figure 6-17: - Total amount of nitric oxide produced with similar concentrations of insulin.

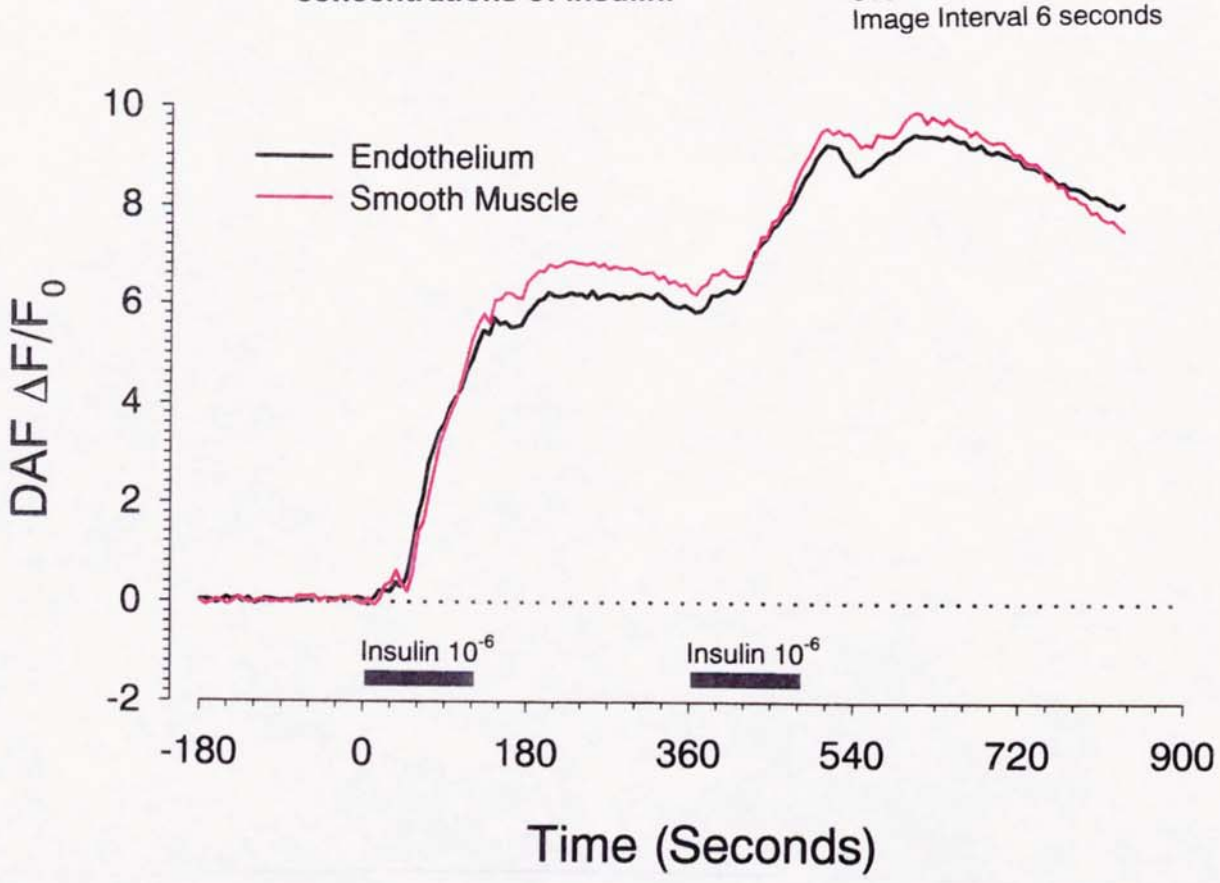


Figure 6-17: -The addition of the same concentration of insulin after a period of 6 minutes gives a further increase in the Nitric oxide. The greatest nitric oxide production was in the vascular smooth muscle and the peak first initiated from the endothelial cell layer at time 0.

Figure 6-18: - Rate of nitric oxide produced with similar concentrations of insulin.

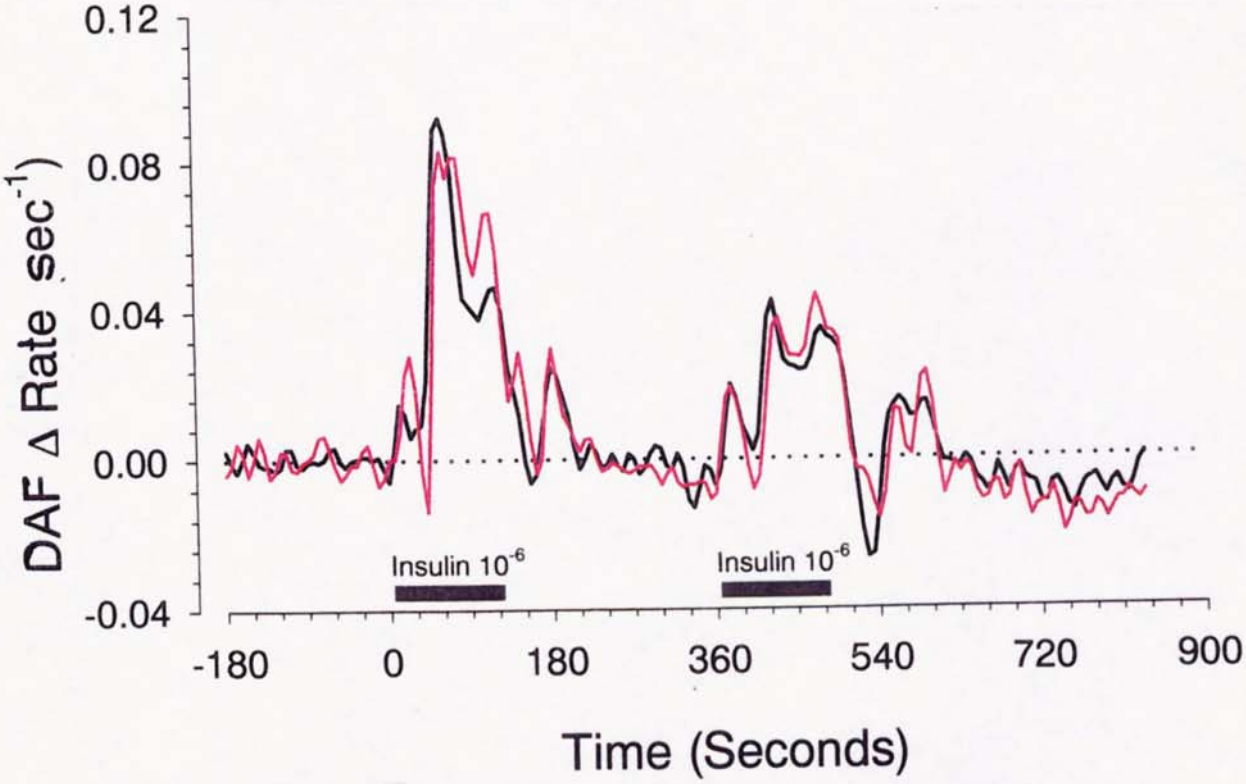


Figure 6-18: - Insulin 10^{-6}M gave a large response on its first addition, causing a large concentration of nitric oxide to be released. On its second addition a lower concentration was observed. This suggests that there is either a period of recovery required to allow further nitric oxide release, or the tissue is already releasing a maximum amount of nitric oxide.

6.6 Discussion

The tissue was always checked with acetylcholine to ensure the endothelium had remained intact. In order to ensure the viability of the tissue the incubation period was kept to a minimum to allow maximum detection of NO. Figures 6.6 and 6.7 show clearly that insulin is able to release NO and from figure 6.10 it can be seen that NO release by insulin is comparable to that produced by acetylcholine. The maximal effect of insulin was seen after approximately 2 minutes and this is consistent with other studies (Zeng et al 1996 and Zeng et al 2000). In studies carried out, but not illustrated in this study, even though insulin releases NO it was not found to physically relax pre-contracted aortic section. This may be due to insulin also stimulating endothelin-1, which causes contraction.

This suggests that insulin is likely to be a physiologically important vasodilator and may play a role in the tonic stimulation of endothelial NO production, maintaining the health and integrity of the endothelium (Cleland et al 2000). From these studies it was also concluded that metformin created no significant increase in NO production.

Graph 6.11 shows that insulin stimulates the PI3K signalling part of the eNOS pathway, as inhibition is caused by LY294002. Glucose disposal is also triggered via this pathway, so if dysfunction of this pathway occurs caused by insulin resistance it would create a blunted vasodilatation effect of insulin in endothelial cells. So PI3K is involved in glucose uptake and in NO production in endothelial cells (Hsueh et al 1997).

The conclusive evidence that insulin stimulates NO production via the shared pathway occurs with the Akt inhibitor. As in insulin treatment the Akt inhibitor significantly reduces NO production, but not in the case of acetylcholine. As here insulin and acetylcholine stimulation of NO have separate pathways. It has been shown that Akt directly phosphorylates eNOS at Ser1177 (Dimmeler et al 1999 and Fulton et al 1999). The inhibitor SB202190 does not effect insulin or acetylcholine production of NO. Insulin signalling in response to glucose follows the Map-kinase pathway, which promotes insulin-mediated growth of VSM (Xi et al 1997). This is not involved in acute insulin-induced production of NO. The effects of insulin-stimulated growth are not known and need to be studied long-term, which was not possible in this study.

Figure 6.12 represents the rate of NO release, which is measured by DAF Δ Rate (sec-1). This system allows temporal information to be collected, so cause and effect can be determined very quickly. Blute et al 2000, has clearly shown with DAF-2DA fluorescence dye that the greater the slope of NO-evoked fluorescence, the greater the concentration of NO released. This shows that the initial rate of NO release is highest between 0-120 seconds, the period in which 2μM of acetylcholine (Ach) was administered. The release of NO appeared in the case of Ach to be dependent on an increase in intracellular Ca^{2+}, which FURA -2AM measures. As the FURA -2AM dye shows a spike in the Ca^{2+} released from the endothelial cells, which corresponded to NO release, both the endothelial and smooth muscle layers of the aorta closely mirrored the quantity of NO released. Evidence suggests acetylcholine stimulates a two-fold increase in cGMP during relaxation of the aorta (Pfister and Campbell 1992), and NO can additionally stimulate MaxiK calcium channels during pig aorta relaxation (Tanaka et al 2000). Recent information indicates that NO synthesis is

calcium dependent, requiring the involvement of calmodulin dependent protein kinase II (Schneider et al 2003).

Figure 6.13 also represents the same experiment but a different form of NO release by DAF is presented. In this case the initial fluorescence F_0 is compared to the change in fluorescence over time ΔF. Even though the rate of NO release changes the total amount of NO in the medium is cumulative. The most rapid rise in NO corresponds to the 0-120 seconds high release rate of NO. The graph then plateaus indicating no additional NO is being added to the surroundings, before NO levels later decrease due to NO clearance or inactivation.

Insulin was administered as shown in Figure 6.14 to the aortic slice. Again a cumulative effect of NO is observed. However the period of administration between the different concentrations of insulin should have been increased, as it is difficult to interpret from the graph whether there is additional NO release with higher doses of insulin, or whether maximum release was triggered by the initial 10^{-8} M concentration. However what can be clearly seen is that the increase in NO release does not appear to be dependent on intracellular Ca^{2+}, as in fact the Ca^{2+} levels fell as indicated by the decreased fluorescence of the FURA-2AM dye. Another possibility is that insulin may require lower concentrations of Ca^{2+} to initiate NO release via the insulin-stimulated pathway, or cause a Ca^{2+} efflux from the tissue. As shown earlier in Figure 6.4 and 6.5 Ca^{2+} is not involved in insulin production of NO and Ca^{2+} entry is blocked and its extrusion promoted in VSM, which could be the reason behind Ca^{2+} fall. It has been suggested that insulin stimulation of eNOS via a Ca^{2+} independent pathway involving tyrosine kinases is plausible (Montagnani and Quorn 2000).

However in Figure 6.15 a more stepwise accumulation of NO was observed on the addition of Ach 10^{-5} M. Then an increasing amount of NO was present when insulin at 10^{-7} M and 10^{-6} M was added respectively. The rate of release of NO is shown in Figure 6.16. Ach 10^{-5}M induced the highest concentration of NO. While insulin 10^{-7}M gave a reduced NO release, but insulin 10^{-6}M NO release is comparable to acetylcholine, what was seen was a dose-response release in the concentration of NO when insulin concentrations were increased. From this particular graph it can also be seen that the NO release first appears in the endothelial cell layer. This is indicated by the black line on the trace as a spike in the increase in NO concentration.

Graph 6.17 shows that addition of the same concentration of insulin after a period of 6 minutes gives a further increase in the NO. The greatest NO production was in the vascular smooth muscle (VSM) and the peak first initiated from the endothelial cell layer at time 0.To understand more clearly what is happening here Figure 6.18 gives more detailed information. Insulin 10^{-6}M gave a large response on its first addition, causing a large concentration of NO to be released. On its second addition a lower concentration was observed. This suggests that there is either a period of recovery required to allow further NO release, or the tissue is already releasing a maximum amount of NO. NO production may be limited by the enzyme eNOS. However this additional NO release is still able to raise the total NO content in the tissue as seen in Figure 6.17

Many thanks to Dr N. Hartell for providing access to the apparatus and the use of his expertise in computing and graphics analysis as well as the interpretation of these data.

There has recently been some speculation on the reliability of results obtained using these fluorescent dyes in particular DAF-2DA as a consistently and linearly reproducible index of NO. It is speculated Briollet et al (2001), that the change in calcium concentration within the medium may account in part for the change in fluorescence. If this is the case it must be a minor interference because the results in Figure 6.14 show that the rate of NO release was very different to the Ca^{2+} activity. The second problem associated with this dye is that it is photosensitive, so it becomes more reactive and sensitive over time. This has been eliminated from the experiments by ensuring that baseline readings of the background fluorescence have been taken and that they are constant readings over time, showing that any additional rises must be due to changes in the tissue.

In conclusion to this chapter insulin triggers significant NO release for short periods of 2 minutes maximum. This indicates insulin can have no long-term effects on the release of NO, and acetylcholine action is predominant. Metformin was shown to have no significant effect on NO release by either insulin or acetylcholine treated vascular tissue. This suggests that the additional relaxation seen earlier with acetylcholine and metformin treated aortic ring sections in chapter 4, may be due to longer periods of acetylcholine exposure, or it poses the question is metformin inducing NO independent relaxation via an alternative pathway or action on ion channels. Insulin stimulates NO release via an insulin-signalling pathway similar to glucose uptake. This was confirmed using specific inhibitors that trigger inhibition of steps in the glucose signalling pathway and this also inhibits NO release. Finally confirmation of the dose dependent release of NO, induced by increasing concentrations of insulin. Insulin also triggers NO release independent of Ca^{2+} due to

the insulin-signalling pathway in the endothelium, working via the glucose uptake pathway, which does not involve Ca^{2+}. Ca^{2+} concentration in fact falls as VSM involves the removal of Ca^{2+} and inhibition of Ca^{2+} entry into the cell during VSM relaxation. This may be because metformin can interact with the membrane (Wilcock et al 1991) and may be able to activates the calcium extrusion pump to remove calcium, and prevent its re-entry.

Alternative actions of metformin other than through NO are postulated in further discussion in chapter 8, the most likely is through ion channel stimulation.

Chapter 7: - The effect of metformin on calcium in vascular smooth muscle

7 Effect of metformin on NA-induced calcium release in vascular smooth muscle

7.1 Vascular smooth muscle

Vascular smooth muscle is the main layer that mediates vasoconstriction and relaxation to bring about changes in blood vessel diameter. The previous chapter focused on relaxation of VSM via the endothelium. This chapter aims to observe the effects of noradrenaline on the VSM, and to see if these effects are changed after being treated with metformin. As seen in chapter 4, metformin is able to increase VSM contraction in the presence of noradrenaline; here the study attempts to find if the reason for this increase is a change in calcium handling in the VSM. Calcium plays a very important role during both vaso-contraction and relaxation; this could potentially explain metformin's effects on the vasculature.

7.1.1 Physical process of contraction

Vasoconstriction involves calcium binding to a regulatory calcium dependent protein called calmodulin. It is then that the calcium-calmodulin complex activates the myosin light chain kinase (MLCK). This enzyme MLCK has the capacity to phosphorylate the myosin light chains (MLC) in the VSM. The presence of ATP is crucial for this stage to occur. The MLC is a 20 kD regulatory protein present on the myosin heads. Once MLC is phosphorylated by MLCK the cross bridge interaction between the actin and myosin filaments can occur (Klabunde 2002d).

The contraction of VSM is very different from other smooth muscle. VSM can cause sustained, slow and tonic contractions. VSM contains both actin and myosin components, but it contains no troponin which is a regulatory protein. VSM filaments are not arranged in distinct bands as skeletal or cardiac muscle, but they are organised in a fashion to maintain tonic contractions, which reduce the size of the lumen (Klabunde 2002d). The typical arrangement seen in VSM is illustrated in Figure 7.1. The actin fibres are attached to structures known as dense bodies, which may also be attached at the cell membrane (Guyton & Hall 1996c).

Figure 7-1: - Physical structure of smooth muscle

Guyton A. C, Hall J. E (1996c). Textbook of medical physiology. 9th edition. Chapter 8. Contraction and excitation of smooth muscle. Philadelphia, Pennsylvania. Published by W. B Saunders Company. 96.

Under the electron microscope, 15 times more actin filaments are observed than myosin filaments. The dense bodies serve the same function as the Z lines in skeletal muscle. The characteristic of smooth muscle is it shows a greater degree of shortening, almost 2/3 of its length. This is the main feature that allows it to change the diameter of blood vessels very quickly (Guyton & Hall 1996c).

There are two separate cyclic interactions that occur during interaction of the actin and myosin filaments that generate muscle contraction (Tawada and Sekimoto 1991). One first is a non-productive cycle, during this stage myosin heads rapidly attach to and detach from actin. The other involves a productive interaction cycle, where the myosin heads split the ATP and generate force.

7.1.2 Chemical process of contraction

Contraction of VSM can be stimulated by electrical, mechanical and chemical stimuli; even passive stretching can generate myogenic contractions of VSM (Klabunde 2002d). The main mechanism of vasoconstriction in the blood vessels is through the α1-adrenoceptors (Garcia-Sainz et al 1999) that catecholamines control vascular tone. In this section the chemical compounds able to trigger VSM contraction will be briefly overviewed.

Adrenaline and noradrenaline

Both adrenaline and noradrenaline are very powerful vasoconstrictors; they are tonically active and extra amounts are released during sympathetic neurotransmission in periods of exercise or stress (Koshimizu et al 2003). They both act via α1-adreneoceptor (Garcia-Sainz et al 1999). Sympathetic nerves also innervate the adrenal medulla to release these hormones into the circulation (Guyton and Hall 1996e). The exact mechanism, by which noradrenaline triggers vasoconstriction is addressed in greater detail in section 7.1.3.

Angiotensin II

As little as 1μg is able to generate an increase in blood pressure of 50mm Hg or more in man. It mainly acts upon the small arterioles; it depresses blood flow in regions where it is released. Its main action is to act on all of the arterioles simultaneously, increasing total peripheral resistance, and as a result increasing arterial pressure (Guyton and Hall 1996e). Angiotensin II has a direct vasoconstrictor effect and causes arterial wall growth via the AT1 receptor (Weber 2002). Its main effects are on the peripheral blood vessel systems such as the splanchnic, renal and cutaneous systems, with less effect on the brain and skeletal muscle systems. There are 2 Angiotensin II receptors, AT1 and AT2. It is the AT1 receptor that is predominant in adults. They are found in the smooth muscle and mediate the effects of angiotensin II by activating phospholipase C, inhibition adenylate cyclase, opening calcium channels and tyrosine kinase activation (Ardaillou 1999).

Vasopressin

Vasopressin is a peptide hormone also known as antidiuretic hormone; its main actions are normally on the kidney. It is formed principally in the posterior pituitary gland (Rang et al 1996). Vasopressin can have a profound effect on the circulatory system, during severe haemorrhage circulating concentrations of vasopressin rise and increase arterial blood pressure, bringing the pressure back to near normal. In VSM vasopressin acts on the V1-receptor, which requires higher concentrations to become activated, it mobilises intracellular calcium by the phosphatidylinositol pathway (Zingg 1996). The actual receptor subtype present in vascular smooth muscle is the V1aR, which consists of 418 amino acids with a molecular weight of 46745 (Kimura 1997). Vasopressin is a potent vasoconstrictor (Guyton and Hall 1996e).

Endothelin

In VSM endothelin-1 (ET-1), binds to a G-protein which activates phospholipase C. This hydrolyses phosphatidylinositol-4,5-biphoshphate (PIP_2) into 2 separate products of diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP_3) (Van Renterghem et al 1988). Upstream of IP_3 tyrosine kinases also participate in the contractile response of the α-adrenoceptor (Mateo et al 1997), IP_3 acts to mobilise calcium from intracellular stores. IP_3 may become further phosphorylated to inositol tetrakis phosphate (IP_4) (Marsden et al 1989) making it easier for calcium to enter VSM via calcium channels. DAG also acts on protein kinase C (PKC), which generates a maintained contraction in VSM. This is summarised in Figure 7.2.

Figure 7-2: - Mechanism of endothelin-1 action

Mateo and De Artinano (1997). Highlights on endothelins: A review. *Pharmacological research.* 36. 5. 344.

7.1.3 Contraction stimulated by noradrenaline

Noradrenaline (NA) acts on separate receptor pathways to generate a contractile response in different regions of the aorta. In the upper abdominal aorta NA acts upon α1D-adrenoceptor, while in the lower abdominal aorta it acts upon α1A-adrenoceptor. (Yamamoto and Koike 2001). The studies carried out in chapter 4 involved the upper aorta in vasoconstriction, while the passive tension studies in chapter 5 involve the lower aorta. The actions of the α-adrenreceptor will be focused on here, as this is the main pathway stimulated by NA. It is important though to also appreciate that there are other pathways that are also involved in vasoconstriction, and these will be briefly addressed here.

Figure 7-3: - Signal transduction used by adrenergic receptors

Diagram adapted from Figure 8.9. Chapter 8 of Wingard L. B, Brody T. M, Larner J, Schwartz A (1991). Human pharmacology, molecular to clinical. p88.

Pathway A is a α2-adrenoceptor coupled to the Gi protein; this is an inhibitory G-protein, which inhibits the catalytic activity of adenylate cyclase (AC). This leads to a reduction in cAMP concentrations and reduces the activation of cAMP-dependent protein kinases (Wingard et al 1991).

In pathway B the α2-adrenoceptor also stimulates the exchange of sodium (Na+) and hydrogen (H+) system. This causes an influx of Na+ and an efflux of H+, this occurs for example in human platelets (Wingard et al 1991). This causes cell alkalisation and therefore an increase in pH, which leads to elevated intracellular calcium. This in turn activates the membrane bound enzyme phospholipase A2 (PLA2). Arachidonic acid is

released and enzymatically converted to thromboxane A2, this produces platelet aggregation.

Pathway C is the main α2-adrenoceptor mediated pathway that is involved in signalling in the blood vessels (Wingard et al 1991). The activation is again via Gi, which leads to the activation of membrane calcium channels. This triggers an influx of extracellular calcium, which contributes to VSM contraction.

Finally pathway D, which activates α1-adrenoceptors by noradrenaline itself, which acts in a similar manner to the ET-1 receptor described in section 7.1.2. Noradrenaline activates the Gp regulatory protein, which stimulates the membrane, bound enzyme phospholipase C (PLC), which leads to intracellular Ca^{2+} mobilisation (Petitcolin 2001). This cleaves the substrate phosphatidylinositol bisphosphate (PIP2) to produce 2 new components diacylglycerol (DAG) and inositol trisphosphate (IP3). IP3 triggers the phasic release of calcium from intracellular stores such as the endoplasmic reticulum. DAG activates protein kinase C by sensitising it to calcium, which leads to further phosphorylation of intracellular enzymes/ proteins containing the amino acids serine or threonine (Lullman et al 2000). This leads to PKC-mediated contractile responses (Abebe and Agrawal 1995). There is also a tonic component of contraction that requires Ca^{2+} entry in the presence of the agonist causing Ca^{2+} influx (Berridge 1993). A second NA induced pathway acts via Gi and involves calcium amplification (Petitcolin 2001).

7.2 Calcium pathways and types of calcium channels

In VSM the mechanisms of calcium (Ca^{2+}) release that trigger contraction may vary not only with stimuli, but also may vary between vascular beds (Lagaud et al 1999). Calcium entry from extracellular spaces plays an important role in smooth muscle (SM) activation (Khoyi et al 1999). In the rat aorta there are two types of calcium channel present, these are voltge-dependent calcium channels, which allow calcium influx, and receptor operated calcium channels (Huang and HO 1996).

During active force development VSMs respond to stretch. Stretch induces the release of Ca^{2+} from intracellular stores in a sigmoidal pattern, as well as Ca^{2+} influx across the plasma membrane (Davis et al 1992). The increase in calcium was due to the potential activation of stretch-activated cation channel. There are many different voltage gated calcium channel subtypes, these are not addressed here but are discussed by Hering et al 2000.

There is the influx of calcium across the plasma membrane via voltage-operated channels and the capacitative entry of Ca^{2+} activated by depletion of its stores (Fasolate et al 1994). The capacitative Ca^{2+} entry pathway is believed to be activated by tyrosine kinase (Hollenberg 1994). When there was a depletion of internal Ca^{2+} stores, which respond to NA, there was an observed increase in resting tone when Ca^{2+} was re-added, even in the absence of agonist. There are 2 Ca^{2+} entry pathways in rat aorta that rely on the depletion of the internal Ca^{2+} compartment. The first is the classic capacitative Ca^{2+} entry pathway and is promoted by the depletion of the internal pool sensitive to NA. The second is dependent on the depletion of the α1-

adrenoceptor-sensitive Ca^{2+} pool (Noguera et al 1998). Calcium entry can also be via receptor operated Ca^{2+} channels

Some evidence suggests that NA mediated contraction that operates via the tyrosine kinase pathway can mediate contraction without altering intracellular calcium and is a MLC independent pathway in rat aorta (Fang et al 2002). In guinea-pig aorta it has been demonstrated that tyrosine phosphorylation is not caused by Ca^{2+} entry through a transplasmalemmal route, but by increased Ca^{2+} sensitivity of the intracellular contraction pathway (Masui and Wakabayashi 2000). Evidence for 2 independent Ca^{2+} pathways was postulated as far back as 1981 (Meisheri et al 1981).

There are also secondary messenger operated channels such as the arachidonic acid sensitive channel illustrated in Figure 7.3.There is also another ion channel family described as transient receptor protein (TRP) which is divided into 3 distinct groups, canonical TRPC family, vanilloid (TRPV) and melastatin (TRPM), TRP channels control slow cellular processes such as VSM contractility and cell proliferation (Berridge et al 2003).

7.2.1 Calcium contribution to contraction

The α1-adrenoceptors stimulated by NA cause the release of Ca^{2+} from the sarcoplasmic reticulum (SR), which has been shown to contribute about 20% to the final contractile response (Koch et a 1990). Other pathways therefore must contribute towards 80% of contraction, some of which may be calcium independent. Depleted Ca^{2+} store can be replenished and this will be addressed in detail in section 7.2.3.

7.2.2 Internal calcium stores

The main intracellular store of calcium is the sarcoplasmic reticulum (SR) in VSM, the channels here are controlled by the redox-status of the cell (Himpens and Missiaen 1993). This store of Ca^{2+} can be released by IP3 as described earlier and in diagram 7.3. In other non-muscular cells the main intracellular Ca^{2+} stores are in the endoplasmic reticulum. Once these stores have been emptied they need to be refilled for further or additional activation at a later point.

7.2.3 Replenishing calcium stores

Intracellular signalling causes the release of calcium from intracellular stores and changes in the permeability of the cell membrane as previously described. The increase in intracellular calcium is contributed to by various pathways; such as release of calcium from intracellular SR stores by IP3, fluxes from the membrane surface and voltage –sensitive Ca^{2+} channels and receptor-operated Ca^{2+} channels (Blatter 1995). Once these calcium stores have been used they begin to refill, but it is not fully understood how this takes place. There are various mechanisms proposed, one possibility is the when IP3 sensitive Ca^{2+} stores are depleted there is a rapid refilling of stores due to an influx of Ca^{2+} across the membrane. This is the capacitive model first put forward by Putney 1986, and later confirmed by others (Somlyo et al 1985 and Van Breemen 1989).

What has been difficult to confirm is the connection between the plasma membrane and Ca^{2+} stores. This has remained very controversial with a lot of speculation on how it works. One potential possibility is that Ca^{2+} directly enters SR (Casteels et al 1981). Another option is Ca^{2+} may enter via the restricted region of the cytoplasmic region,

which is very narrow and lies between the cell membrane and Ca^{2+} store (Putney 1986). Here it may enter calcium stores by the use of the SR Ca^{2+} pump. In both of these theories Ca^{2+} stores refill by bypassing the bulk of the cytoplasm. In contrast to these ideas it has been put forward that Ca^{2+} does enter the bulk of the cytoplasm before being taken up into Ca^{2+} stores (Takemura 1989). As well as triggering Ca^{2+} release IP3 also promotes Ca^{2+} re-entry and refilling of Ca^{2+} stores. Evidence suggests that Ca^{2+} stores are refilled under physiological conditions by a direct pathway between the store and the extracellular medium (Casteels and Droogmans 1981). In the case of noradrenaline, papaverine inhibits the caffeine sensitive Ca^{2+} stores (Noguera and D'Ocon 1993).

More recent studies have suggested that depleted Ca^{2+} stores stimulate entry via the release of small intracellular messengers called Ca^{2+} influx factor (Randriamampita and Tsien 1993). Other mechanisms may involve phosphates and a diffusible intracellular messenger (Parekh et al 1993). All of these pathways have potential for restoring calcium stores, and the mechanism may vary according to cell type. This means that a number of these mechanisms may be able to operate simultaneously in the same tissue, or system.

7.3 Experimental details and aims

The present study used both VSM sections of aorta incubated for 1 and 4 hours in the presence and absence of metformin. A7r5 cells were also used, which were grown to confluence and then incubated in the presence and absence of metformin for 24 hours, and used in time period studies of 0, 10 and 20 minutes. The process of growing VSM is detailed in chapter 2; section 2.6.1-2.6.5 and additional details on the VSM cell line

appear in chapter 3. The intracellular calcium concentration was then determined before and after being stimulated with noradrenaline (NA), see section 2.8.2. The aim of this study is to investigate if metformin alters basal calcium and calcium release triggered by noradrenaline during contraction.

7.4 Results

The aortic tissue was cut into flat sections and incubated for 30 minutes with the calcium sensitive dye FURA/2AM. This calcium sensitive dye allows intracellular calcium concentrations to be determined as described in section 2.8.2. Sections of aortic tissue were incubated with metformin for 1, 4 hours.

Figure 7-4: - Calcium concentration induced by noradrenaline in the presence and absence of metformin after a 1-hour incubation

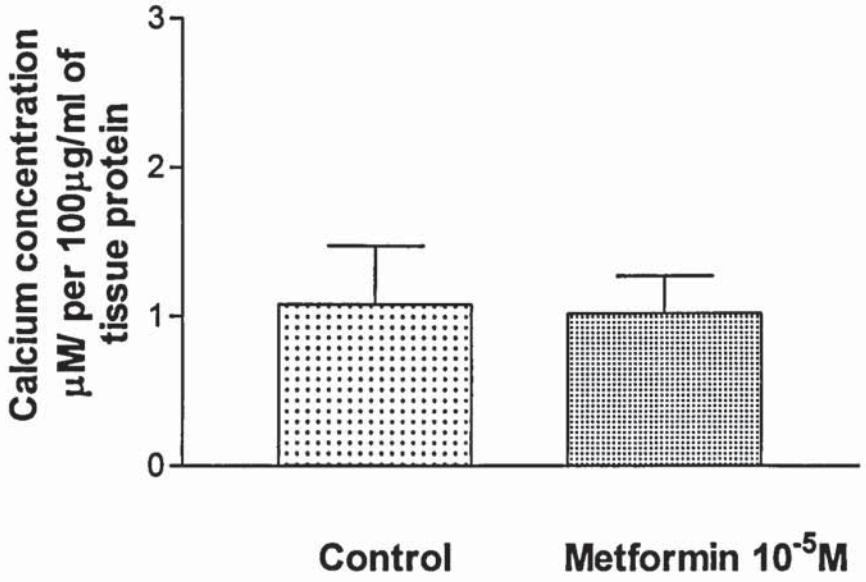


Figure 7-4: - Aortic tissue samples were incubated with or without metformin 10^{-5}M for 1 hour. The tissue was then exposed to noradrenaline 10^{-6}M for 10 minutes. The increase in calcium released was determined by subtracting the calcium values before and after noradrenaline was added. There was no significant difference between control and metformin treated samples after a 1 hour incubation. ± SEM. (n=6)

There is no statistical difference between calcium concentrations, where after a 1-hour incubation with and without metformin, which is then stimulated by noradrenaline. Stimulation with noradrenaline produced similar calcium concentrations in the control and metformin treated samples.

Figure 7-5: - Calcium concentration induced by noradrenaline in the presence and absence of metformin after a 4-hour incubation

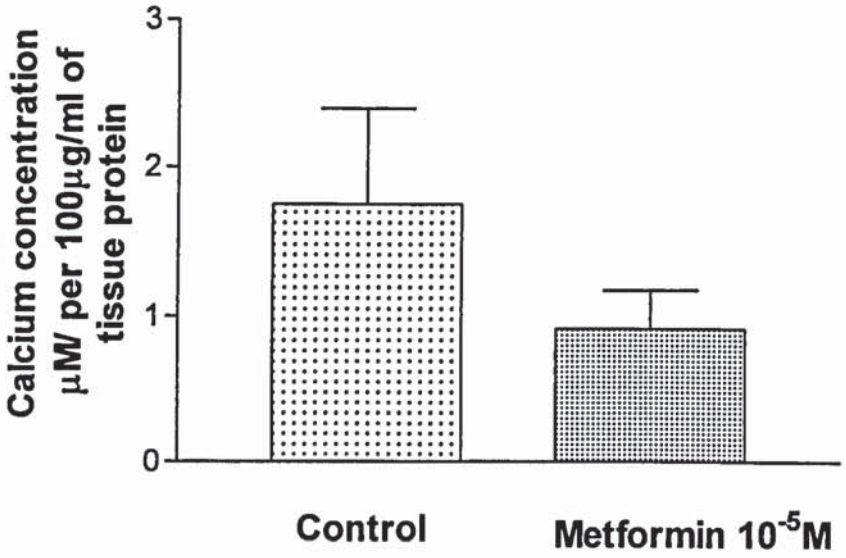


Figure 7-5: - Aortic tissue samples were incubated with or without metformin 10^{-5}M for 4 hour. The tissue was then exposed to noradrenaline 10^{-6}M for 10 minutes. The increase in calcium released was determined by subtracting the calcium values before and after noradrenaline was added. The control showed a greater increase in calcium than the metformin treated tissues. The results however were not quite significant between the control and metformin 10^{-5}M treated samples after a 4 hour incubation. ± SEM. (n=6)

The calcium concentration in aortic sections was decreased after exposure to metformin 10^{-5}M after a 4-hour incubation. It can be seen that noradrenaline did not stimulate as much calcium to be released in the metformin treated tissue, as the control. The results are not quite statistically significant (P>0.05).

Longer-term exposure to metformin was achieved using A7r5 muscle cells. These cells were exposed to metformin treatment for 24 hours, to determine if there is a different effect of metformin beyond the 4-hour limit that the aortic sections can test. As aortic sections begin to decay beyond the 4-hour barrier the cellular model is ideal to overcome this limitation. However, metformin can affect cell growth, although no significant inhibition is seen after 24 hours, cells were grown to confluence before their exposure to metformin. This allowed an optimum cell number to be maintained before treatment with metformin 10^{-5}M.

Figure 7-6: - Contraction induced in A7r5 cells incubated with and without metformin for 24 hours after 5 minutes of noradrenaline administration 10^{-5}M

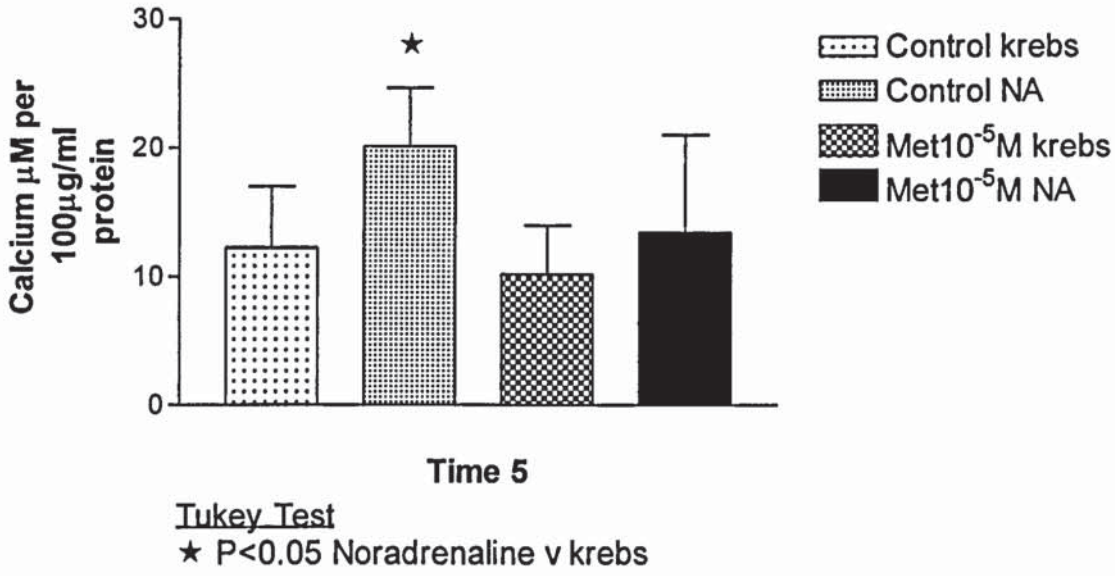


Figure 7-6: - Confluent A7r5 cells were incubated with and without metformin 10^{-5}M for 24 hours. After this period they were exposed to noradrenaline 10^{-5}M for 2 minutes. Krebs was also added to the cells to check that the release of calcium was not stress induced by the administration of the drug. The control samples showed a significant increase in calcium release induced by noradrenaline compared to the krebs solution. This showed that little calcium was released due to the stress of administration of the drug to the tissue. There was no significant increase in the metformin treated tissue. ± SEM. (n=6).

In the control tissue there is an immediate and statistically significant increase in calcium released from A7r5 cells, was seen after stimulation with noradrenaline. Metformin treated tissue shows a less dramatic increase in calcium, which is not significant.

Figure 7-7: - Contraction induced in A7r5 cells incubated with and without metformin for 24 hours after 10 minutes of noradrenaline administration 10^{-5}M

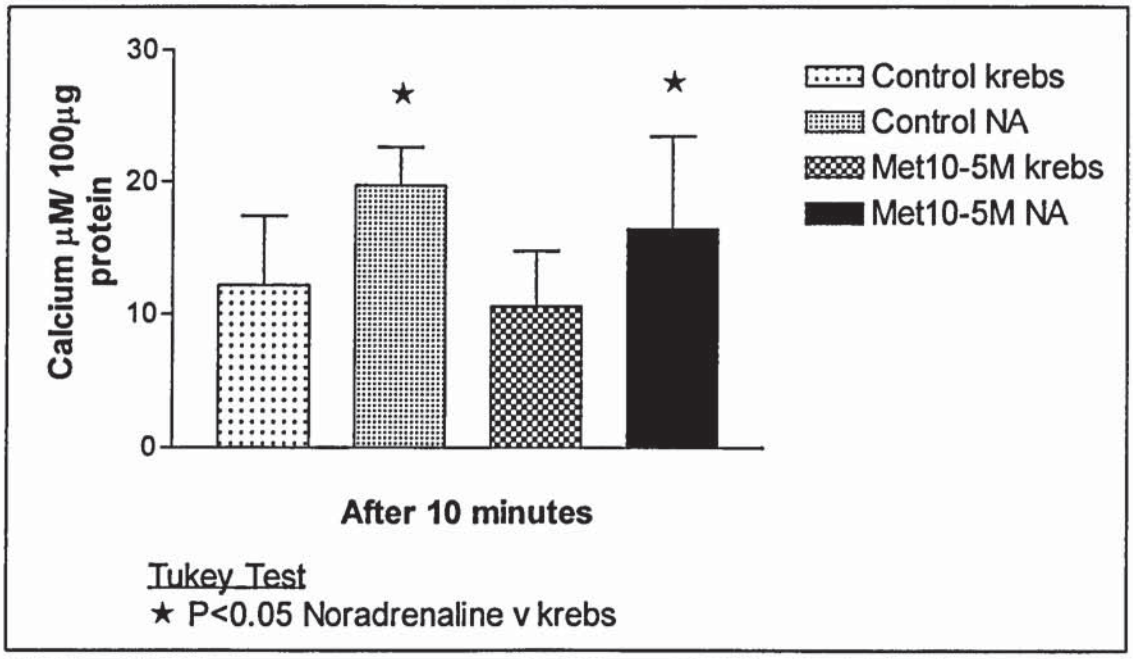


Figure 7-7: - Confluent A7r5 cells were incubated with and without metformin 10^{-5}M for 24 hours. After this period they were exposed to noradrenaline 10^{-5}M for 10 minutes. Krebs was also added to the cells to check that the release of calcium was not stress induced by the administration of the drug. Both the control and metformin 10^{-5}M treated samples showed a significant increase in calcium release induced by noradrenaline compared to the krebs solution. This showed that little calcium was released due to the stress of administration of the drug to the tissue. ± SEM. (n=6).

In the A7r5 cells there is a statistically significant increase of $P<0.05$ in calcium after 10 minutes of stimulation with noradrenaline, in both the control and metformin treated VSM samples. This shows that noradrenaline activates a rise in intracellular calcium, in order for contraction to take place. The overall concentration of calcium however is still lower in the metformin treated tissue.

Figure 7-8: - Contraction induced in A7r5 cells incubated with and without metformin for 24 hours after 20 minutes of noradrenaline administration 10^{-5}M

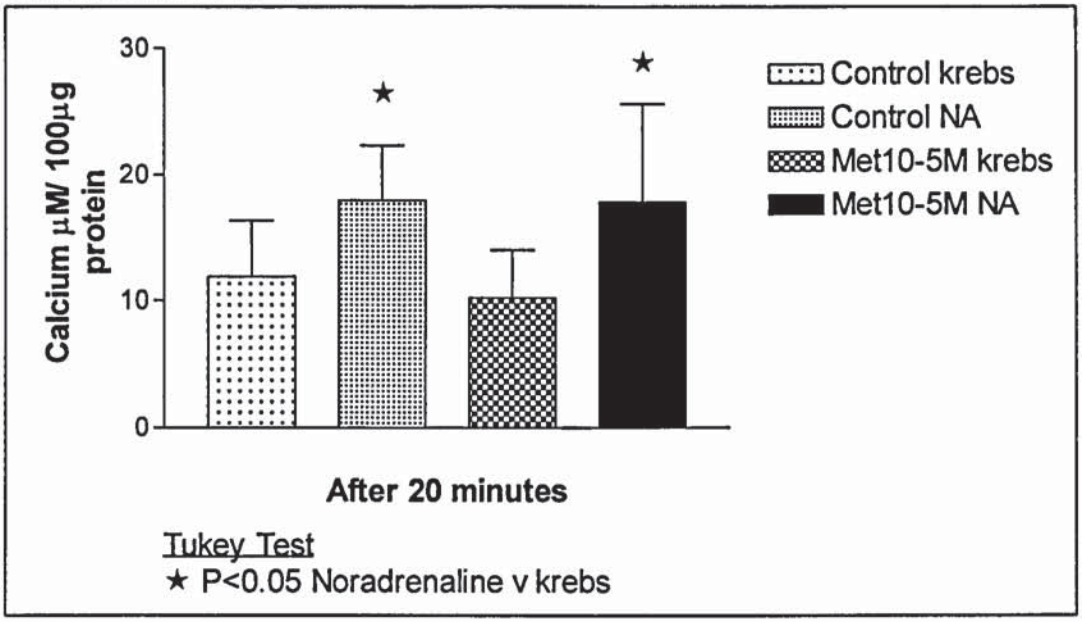


Figure 7-8: - Confluent A7r5 cells were incubated with and without metformin 10^{-5}M for 24 hours. After this period they were exposed to noradrenaline 10^{-5}M for 20 minutes. Krebs was also added to the cells to check that the release of calcium was not stress induced by the administration of the drug. Both the control and metformin 10^{-5}M treated samples showed a significant increase in calcium release induced by noradrenaline compared to the krebs solution. This showed that little calcium was released due to the stress of administration of the drug to the tissue. ± SEM. (n=6).

In the both samples there is a statistically significant increase after 20 minutes in calcium release induced by NA in both the control and metformin treated A7r5 cells. The increase in calcium is slightly greater in the metformin treated VSM, but this can be seen more clearly later in figure 7.8. Figure 7.8 illustrates the difference in calcium after various time periods of exposure to noradrenaline compared to the control (basal) calcium. Therefore it is clearer in this figure to see the changes induced by noradrenaline and metformin.

Figure 7-9: - Increase in calcium over time

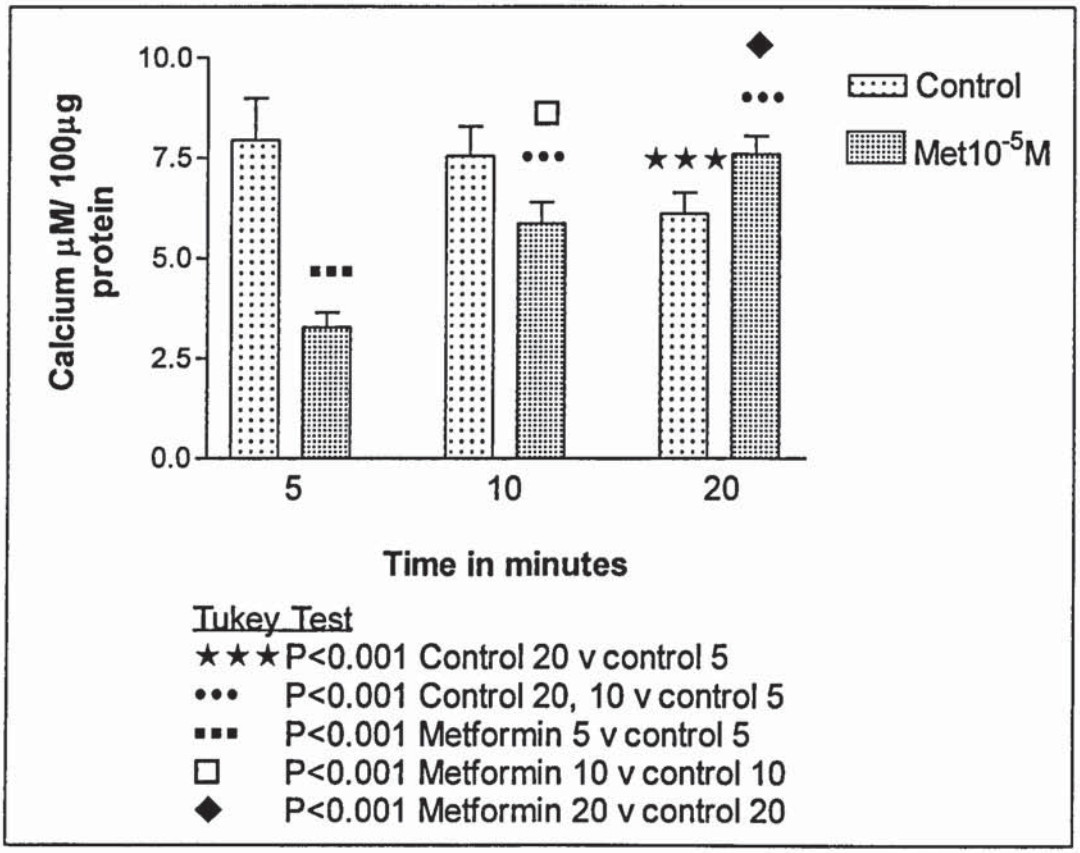


Figure 7-9: - Confluent A7r5 cells were incubated with and without metformin 10^{-5}M for 24 hours. After this period they were exposed to noradrenaline 10^{-5}M for 2, 10 and 20 minutes. On this graph the increase of calcium is plotted which is calculated by the noradrenalin induced calcium release minus that released by krebs which is stress induced, the results were then plotted over time. It can be seen that in the control samples the amount of calcium release triggered falls slowly over the 20 minute period. The opposite happens with metformin there is a gradual increase in calcium concentrations over the same 20 minute period. ± SEM. (n=6).

This graph illustrates more clearly that the release of calcium induced by NA is generated immediately and sustained for 10 minutes, before there is a decrease in calcium concentrations. Metformin on the other hand produced a slow, but consistent increase in calcium concentrations over the 20 minutes period. This may suggest metformin activates slow acting calcium channels to generate this increase over time.

7.5 Discussion

There was an obvious increase in calcium induced by noradrenaline, but there was little statistical difference in Ca^{2+}concentrations after 1 and 4 hours exposure in aortic tissue sections. The metformin treated tissues though appear to have a lower concentration of calcium compared to the control tissue treated with Krebs alone. Sharma and Bhalla 1995 observed a significant decrease in intracellular Ca^{2+} in metformin treated VSM cultured from rat thoracic aorta, however their studies were for longer periods varying from 1 to 24 hours. These studies Sharma and Bhalla 1995 were carried out on diabetic patients, and metformin was hypothesised to directly inhibit agonist stimulated intracellular calcium in VSM, by inhibiting the platelet derived growth factor (PDGF) and angiotensin II-induced calcium release.

Over time noradrenaline causes an immediate and sustained increase in control A7r5 cells see Figure 7.8 and metformin-induced increase in calcium is slower with a consistent calcium increase. This may be due to the fact that metformin can increase noradrenaline action over time. Metformin has been shown to increase circulating NA with and without intact postganglionic sympathetic nerve action (Peuler et al 2001).

In diabetic patients there is an increase in vasoconstriction and the response of these patients to intra-arterial noradrenaline is greater, but the concentration of noradrenaline did not differ from normal healthy controls. The arterial α-adrenergic tone was also greater. The increase in vasoconstriction was therefore attributed to an inappropriate increase in α-adrenergic receptors relative to the level of systemic sympathetic nervous system activity (Hogikyan et al 1999). The enhanced contractile response noted in diabetic patients was not associated with an increase in intracellular

Ca^{2+}, but instead may be due to an increase in Ca^{2+} sensitivity of the contractile proteins (Chow et al 2001), which could be what occurs in the presence of metformin.

Metformin activates AMP-activated protein kinase (AMPK) in hepatocytes, and AMPK could be a potential site of metformin action and could explain the pleiotropic effects of metformin (Zhou et al 2001). Other potential mechanisms recently uncovered show that in the liver cell membrane is activated by a Cl- /HCO3- exchanger, which depends on HCO3- formation. Any activation of this pathway disturbs the intra-extracellular balance of Cl- ions. This then enhanced Cl- efflux via the Cl- channels (Lutz et al 2001). It is possible that metformin could be associated with this pathway, altering Cl- handling which effects contraction and relaxation of VSM.

Metformin may also be able to effect basal intracellular calcium, as metformin is able to insert itself into the membrane (Wilcock et al 1991). In this way metformin may be able to hold the membrane in a constantly depolarised state, which depletes the free intracellular calcium. When noradrenaline is administered metformin is able to attenuate its action, and increase intracellular calcium release. The most likely pathway for metformin being able to exert this effect is on the G-proteins or other membrane bound messengers. Noradrenaline acts via the α1D-adrenoceptor in the upper aorta (Yamamoto and Koike 2001), which is where these sections were taken from for the contraction studies. The α1D-adrenoceptor subtype is only effectively coupled to the accumulation of inositol phosphates (Richardson 2003). Therefore it is most likely metformin is able to influence this signalling pathway and cause an increase in inositol, which is able to release calcium from intracellular stores. This is

the most feasible pathway for metformins actions on calcium, which may explain the increase in aortic contraction.

Chapter 8: - General discussion

8 General Discussion

8.1 Rationale behind the study

The basis for this study was founded on the observations made by the United Kingdom Prospective Diabetes Study which demonstrated that metformin did not only lower blood glucose (UKPDS 33, Turner R. C et al 1998a) and blood pressure (UKPDS 38, Turner R. C et al 1998b), but also had a significant impact in reducing both micro- and macrovascular complications associated with type 2 diabetic patients (Levy 2001). In particular metformin reduced the incidence and severity of macrovascular disease independently of its blood glucose lowering effect. This meant that treatment with metformin increased survival of type 2 diabetic patients, compared to other antidiabetic drugs, due mainly to a reduction in macrovascular complications (UKPDS 34, Turner R. C et al 1998c). Despite this crucial phenomenon induced by metformin little is known about its effects on the vascular system, which previously had been overlooked. Although there had been several reports and anecdotal accounts in the literature that metformin could improve aspects of both microvascular and macrovascular function (Wiernsperger 2000), this area had not previously received detailed study. Research at Aston by Carter 2000 on aortic contraction and relaxation in long-term metformin treated mice, highlighted that metformin could affect vascular tissue, and provided a clear rationale for more detailed investigation.

The overall aims and objectives of this study were to determine the effect of metformin on macrovascular function and to assess the mechanisms involved. This included studies on the time period for metformin to affect the vascular tissue, and the possible mechanisms that enhance contraction and relaxation, and the role of the endothelium and vascular smooth muscle in mediating this influence of metformin. The previous chapters of this thesis have attempted to investigate and address these issues.

8.2 Summary of key results

The initial focus of the present studies was to establish permanent and reliable models of vascular function to test the effects of metformin. The A7r5 vascular smooth muscle (VSM) cell line was characterised in chapter 3, and used in subsequent studies that only required the long-term investigation of the VSM such as those involving calcium studies in chapter 6. The A7r5 cells were also used for studies on metformin effects on VSM growth. It was found that at high concentrations of metformin (10^{-2}M) VSM growth was significantly inhibited after 4 days. This confirmed and extended similar studies by Bűnting et al 1986. The underlying mechanism by which metformin exerts this inhibition of VSM growth is not however understood, and only preliminary investigation of the mechanisms was achieved in this research programme. Though speculation by Stith et al 1998 suggests that inhibition of cell division by metformin may be due to the inhibition of tyrosine kinases associated with cell proliferation.

In chapter 4 the in vitro and in vivo studies involving metformin treatment examined the contractile and relaxation responses of isolated aorta to noradrenaline and acetylcholine. In vitro the greatest contractile and relaxation response were seen after 4 hours in metformin treated aortic rings, showing that metformin could directly affect the VSM during contraction and relaxation via the endothelium. This type of in vitro incubation with metformin had not been previously performed in haemodynamic studies on blood vessels, particularly with noradrenaline. Carter 2000 noted the increase in contraction in vitro in mouse aorta, but clinical studies had generally reported little or no effect on blood pressure (Bailey 1992). However in vivo studies performed by Muntzel et al 1997 in spontaneous hypertensive rats (SHRs) observed that metformin elevated blood pressure (BP) under autonomic blockade. An elevation in BP may be caused in vivo by non-neural mechanisms such as a direct release of renin with consequent rise in angiotensin II, antidiuretic hormone (ADH), or by the direct vasoconstrictor actions of metformin on VSM. Each of these mechanisms could theoretically account for an affect of metformin in vivo; although there is no evidence that metformin affects either renin, angiotensin II or ADH. From our studies in vitro it is evident that metformin can directly affect VSM in a relatively small time period. There is little documentation to indicate the mechanism for metformin inducing increased vascular contraction. This has remained relatively overlooked until now, but recent and as yet unpublished data by Bouskela suggest that a topical metformin can act directly in hamster cheek pouches. In this model metformin can depolarise cell membranes by 5mV, creating a tendency towards metformin favouring vasoconstriction (Wiernsperger and Bouskela 2003). While this could explain increased vasomotion in capillary beds induced by the effects of metformin on

arterioles and afferent capillary vessels, it does not explain the apparent protection of metformin against macrovascular diseases.

There is much greater published information available to support enhanced vasorelaxation with metformin than vasoconstriction. The relaxation studies from this investigation showed that metformin increased relaxation of mouse aorta significantly after 4 hours in vitro, but in vivo there was a different story with metformin not showing any significant effect to enhance relaxation. There may be several reasons for this observation; one of the first is that many of these studies have used chronic metformin treatment, which cannot be directly compared to that used by a typical type 2 diabetic patients. In the studies performed in this thesis, there has been an attempt to ensure that metformin concentrations have been used that represents those found circulating at therapeutic concentrations in the plasma, which may take longer to have a significant effect on relaxation than the time periods covered during in vitro studies. Some of the studies that have shown increased relaxation of blood vessels using metformin are discussed here. Mather et al 2001 demonstrated that type 2 diabetic patients treatment with 500mg twice daily of metformin significantly improved with acetylcholine-stimulated blood flow compared to control patients receiving a placebo. This Illustrates that metformin is able to improve blood flow in insulin resistant animals and improve endothelial function. In studies performed by Sitori et al 1984, metformin was administered at 850mg for 6 months to diabetic and non-diabetic patients with peripheral atherosclerosis. In these studies a 40 % increase in blood flow was seen during plethysmography, conducted after a standardised ischaemia. It was also shown that the effect of metformin was reversible after patients were switched to placebo treatment. Further studies by Verma et al 2000, showed that metformin

administered at 500mg/kg by oral gavage for 6 weeks restored the vascular actions of insulin and improved acetylcholine-stimulated vasorelaxation in the aortas of male Sprague Dawley rats. It was hypothesised that this effect could be explained if metformin exerts acute arterial vasorelaxation by stimulating K+ efflux (Peuler et al 1999). Studies by Katakam et al 2000, treated mice in vivo with 300mg/kg metformin and in vitro with 100μmol/l of metformin. They observed that at high concentrations metformin (>10mmol/l) could directly effect VSM and induce relaxation and decreased intracellular calcium (Chen et al 1997), while at clinical concentrations metformin appeared to enhance agonist-induced NO mediated relaxation. It was Marfella et al 1997 who demonstrated that metformin treatment accompanied by L-arginine (the natural precursor of NO), amplified a reduction in blood pressure (BP) after 8 weeks of metformin treatment (850mg), without affecting type 2 diabetic patients heart rate or plasma catecholamines.

The findings from chapter 4 that metformin could directly affect VSM were further confirmed in chapter 5. In this section the passive tension of mouse aortic sections was measured, so the tissue was at rest and not treated with any other agonist. Metformin treatment alone was compared to control aortic sections not incubated with metformin. The study showed that metformin could increase tension in the VSM even at rest. This confirms that metformin can directly act on VSM even at relatively low concentrations (10^{-5} M), which may be as Bouskela suggests by depolarising the cell membrane (Wiernsperger and Bouskela 2003). This may be due to the documented fact that metformin has a net positive charge at physiological pH, and therefore may be able to carry out ionic interactions with the cell membrane (Wilcock et al 1991). The increase in tension was also shown to be reversible in the studies from this thesis

by treatment with papaverine, which is able to act directly on VSM to induce relaxation, confirming the action of metformin must be on VSM.

In order to confirm if metformin could affect agonist-induced relaxation, by enhancing NO release, chapter 6 investigated the effect of metformin on NO release in aortic sections of lean mice stimulated by acetylcholine. From these studies at 1 hour it was shown that metformin did not significantly enhance NO production, and a similar effect was observed but with a smaller NO response after 4 hours. The reduction in NO after 4 hours was probably due to tissue degeneration and unfortunately was a limitation of the experiment. This however, poses the question that if enhanced relaxation is not due to an increase in NO, what could be inducing the relaxation. If metformin can favour contraction via noradrenaline, by acting directly on VSM, maybe relaxation can also be mediated directly on VSM. Marfella et al 1997 only showed a fall in BP when accompanied by both metformin and L-arginine. So metformin alone may not have the same effect on NO, especially at lower concentrations. This was obviously the case in this thesis, as metformin alone showed no change in NO despite the use of thorough and high sensitivity protocols and assay methods, it was only on the administration of acetylcholine that metformin enhanced vasorelaxation.

The important observation that insulin triggers NO release via a shared pathway with the glucose uptake pathway was also shown in chapter 6. The key observation was that this release of NO was independent of calcium activation unlike the traditional NO activation pathway. Soon after these studies were conducted, similar observations were reported elsewhere (Storey et al 2001). This calcium independent pathway is

activated by insulin phosphorylating Akt (Storey et al 2001), which then goes on to phosphorylate eNOS at the serine residue 1179 (Montagnani et al 2001).

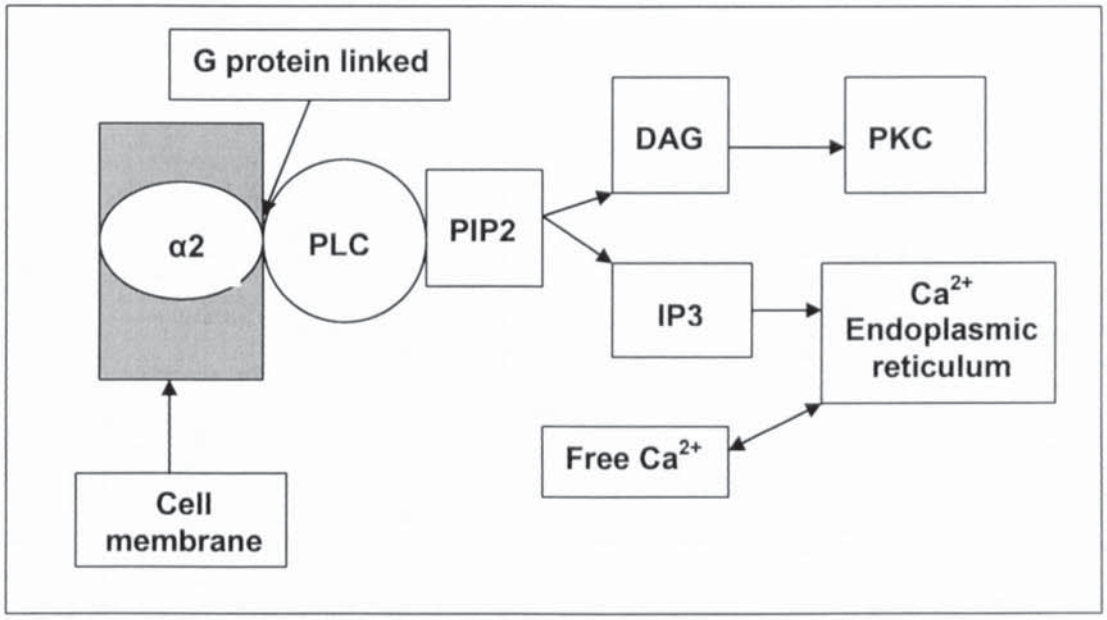
As metformin clearly did not cause relaxation by NO, but was able to increase relaxation, as well as enhance contraction, the next obvious line of investigation was to carry out studies on calcium handling in the vascular muscle cells. Chapter 7 mainly concentrated on the effect of metformin on calcium in relation to contraction. Metformin appeared in the studies carried out in this thesis to have slightly, but not significantly lowered noradrenaline-induced calcium concentrations after 24 hours of treatment of A7r5 cells with metformin, and also after 1 and 4 hours of pre-incubation with metformin. There is some contradictory evidence in the published literature about what metformin does to calcium concentrations at rest. Dominguez et al 1996 showed 24-metformin treatment of VSMC did not alter basal intracellular calcium. While Sharma and Bhalla 1995 showed 1μg/ml metformin significantly increased ($P<0.05$) resting intracellular calcium and slightly but not significantly reduced intracellular calcium at 2μg/ml of metformin. This may suggest that metformin inhibits the influx of extracellular calcium when the cell is at rest (Ren et al 1999). During contraction there was no significant difference between the metformin and control increase of intracellular free calcium triggered by noradrenaline. This observation was shared by Dominguez et al 1996, who demonstrated no rise in calcium in metformin-treated VSM cells during their stimulation with arginine vasopressin. This was observed in a different situation by Chow et al 2001 in which diabetic patients appeared to have a sustained contractile response to noradrenaline, which was not associated with any increase in intracellular calcium. This suggests that

metformin may enhance the sensitivity of calcium of contractile proteins, but does not directly alter Ca^{2+} concentration.

8.3 Hypothesis of metformin actions

Metformin treatment conclusively increases contraction in all cases when it is induced by noradrenaline. The aortic rings tested in the contractile studies used the upper section of the aorta, which predominantly contains α1D-adrenoceptors (and α2-adrenoceptors), which are linked to phospholipase C and release intracellular calcium (Rang et al 1996) see Figure 8.1. Noradrenaline acts at the α1D-adrenoceptors and also at the α2-adrenoceptors. It is possible that metformin could interact with the receptor itself or G-protein mediators of the activated receptor, as metformin can bind to cell membranes because of its ionic charge at physiological pH (Wilcock et al 1991). This pathway of activation or enhancement would then trigger calcium release from the sacroplasmic reticulum (SR) in the VSM. The contraction observed in the presence of metformin was greater but slower to develop, than that seen in the control aortic section. This may be because the calcium is released from VSM SR stores. When these stores are depleted they need to be replenished, which occurs by calcium entering the bulk cytoplasmic compartment before being pumped into the sacroplasmic reticulum (SR) (Blatter 1995). This could explain the greater but slower increase in contraction in response to noradrenaline. As the SR stores are being replenished this slows the increase in calcium influx. As calcium influx concentrations also appear to be unchanged this may further suggest that metformin may additionally enhance the sensitivity of contractile proteins to calcium.

Figure 8-1:- Adrenoceptor pathway



Noradrenaline activates the Gq regulatory protein, which stimulates the membrane bound enzyme phospholipase C (PLC), which leads to the activation of intracellular Ca^{2+} from organelles such as ER. This cleaves the substrate phosphatidylinositol bisphosphate (PIP2) to produce two new components diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP3). IP3 triggers the phasic release of calcium from intracellular stores such as the endoplasmic reticulum. DAG activates protein kinase C (Idris, Gray and Donnelly 2001) by sensitising it to calcium, which leads to further phosphorylation of intracellular enzymes and other proteins containing the amino acids serine or threonine. This then leads to PKC-mediated contractile responses. There is also a tonic component of contraction that requires Ca^{2+} entry in the presence of the agonist causing Ca^{2+} influx.

Relaxation may be affected by changes on the G protein or protein kinases in the VSM muscle that triggers K+ efflux and are induced by acetylcholine via the endothelium see Figure 8.2. This is not a favoured explanation for the action of metformin, which did not affect NO production in this study (Wiernsperger and

Bouskela 2003). Many different ionic channels may be involved with vasomotion, but recent information in hepatocytes suggests metformin might affect chloride channels (Lutz 2001). Metformin could also effect Cl- ions, which may enhance relaxation; the effect of metformin may in fact be inhibited by chloride channel blockers. This information as yet is unpublished in detail but documented by Wiernsperger and Bouskela 2003. This may explain arteriolar vasomotion, which could be mediated by a cGMP-dependent linked chloride current as documented by Matchkov 2001.

Figure 8-2: - nitric oxide pathway

This diagram was complied from 3 separate sources: - 1. Ritter and Chowienczyk (2001), 2. Hartell (2001), 3. Mateo and De Artinano.

Unfortunately there was not the time in the limits of this study to validate the possible role of chloride channels in metformin action, but the next line of future inquiry should definitely involve monitoring cGMP activity and the movement of Cl- ions in VSM, in relation to metformin treatment.

8.4 Conclusions

Metformin is able to directly affect the vascular system via its effects on contraction and relaxation mechanisms. It is also able to increases passive vascular muscle tension. These effects are shown to be direct as the effects in vitro occur in 1 and 4 hours, which is not long enough to affect the structure of the aortic vessel. The in vivo response appeared to take longer to become evident. The maximal effect of metformin on vascular contraction occurred after 8 weeks. The effect on relaxation is not significantly enhanced in the presence of metformin. The reason for this is probably due to vascular adaptation, and the fact that in vivo the metformin concentration is decreased as the drug is excreted, therefore taking longer for the tissue to be exposed to the beneficial effects of metformin. The effect of increased contraction was not elicited through an increase in calcium, as the calcium concentration was actually reduced in the presence of metformin. Moreover, enhanced relaxation was also shown not to be mediated through any effects of metformin on nitric oxide production.

From the evidence the effects of metformins on the vascular system, a potential future line of investigation could investigate whether metformin is able to affect G-protein pathways, which induce both contraction and relaxation, as well as cell growth. This could be the potential mechanism by which metformin is able to exert its beneficial vasomotive effects, which enhance vascular function and contribute to increased

survival in type 2 diabetic patients treated with this drug. Thus, if the vascular system is able to cope with greater extremes in vasoconstriction and relaxation, it is more able to cope with the changes in blood vessel dynamics and to resist forces of friction and turbulence against the vascular wall (Wiernsperger 2000).

8.5 Future use of metformin

As metformin has been shown to have beneficial vascular effects at low circulating plasma concentrations such as 10^{-5}M, it may be beneficial for all diabetic patients to take a low dose of this drug long-term, to reduce the micro and macrovascular complications that impede diabetic patients quality and quantity of life. Metformin is mainly targeted for use in obese patients, but limited research has been performed on non-obese patients (for example Clarke and Campbell 1977, Hermann et al 1991 carried out non-obese studies) to establish if metformin has universal properties in both types of patient, these studies suggest it might.

There is also the potential to widen this treatment to other patients at risk, notably those with features of the metabolic syndrome and those vascular problems with chronic endpoints such as stroke or myocardial infarction. Metformin has been shown not just to be an anti-hyperglycaemic compound, but its effects go beyond its initial glucose, lipid and insulin lowering properties. Metformin could potentially be a preventative treatment for serious vascular endpoints in other diseases and not just restricted to use in type 2 diabetes. This view is shared by Libby 2003 who suggests that metformin could provide cardiovascular protection in type 2 patients and many other conditions of vascular risk. Metformin is also considered to be a unique compound with a broad range of mechanisms, which improve both the endocrine and

cardiovascular systems (Standl 2003). Indeed some of its metabolic and vasoprotective actions are independent of insulin and the diabetic state. This is very much the age of evidence based medicine as highlighted by Standl 2003, and metformin already has evidence that it can improve clinical outcomes in type 2 diabetes.

This thesis has provided additional information to show that metformin acts independently of its antihyperglycaemic effect to exert potentially advantageous effects on the vasculature. The research programme described herein has established a direct effect of metformin on the vascular smooth muscle, which may involve activation of ion channels, which are G-protein linked.

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Appendices

Appendix 1: - Animal models

The ob/ob mouse as shown in the diagram below: - shows an obese ob/ob mouse on the left with a normal lean (+/+) mouse.

The ob/ob mouse lacks the hormone leptin, an adipocyte peptide that acts in the brain to induce satiety: hence ob/ob mice over eat. If these mice are treated with exogenous leptin, their metabolism is normalised and their weight is reduced along with the food intake (Bray and Ryan, 1997). Leptin was first identified by a mutation (premature stop codon) in the Ob gene in the ob/ob mice (Zhang et al, 1994). The obese mice as well as being insensitive to leptin, are prone to develop a form of type 2 diabetes.

Both of the above mice were born on the 2/12/02 and are approximately 6 months/ 24 weeks old in the figure above. The obese mouse on the left weighs 91.3g and the lean mouse weighs 34.4g. Making the obese mouse approximately 2.5 times larger, even though they are the same age. The dimensions of the obese mouse are: - 19cm in length and 6 cm wide. While the lean mouse is 18cm in length and 3cm wide. Both mice are of similar length, but the obese mouse is double the width of the lean.

Appendix 2

Physiological salt solution

Used to bath tissue during compliance studies of aortic tissue.

Adapted from: -

Palmer A. M, Thomas C. R, Gopaul N, Dhhia S, Änggård E. E, Poston L, Tribe R. M (1998) Dietary antioxidant supplementation reduces lipid peroxidation but impairs vascular function in small mesenteric arteries of the streptozotocin – diabetic rat. *Diabetologia*. Vol. 41, pp 148-156.

Compound	Molecular Weight (g)	g/ litre	Required Molarity (mM)
NaCl	58.44	6.925	118.50
KCl	74.56	0.354	4.75
$MgSO_4 7H_2O$	246.48	0.288	1.17
KH_2PO_4	136.09	0.163	1.20
$NaHCO_3$	84.01	2.100	25.00
D-Glucose	180.16	2.090	11.60
*1$CaCl_2$	-	-	-

The following compounds were added prior to use along with $CaCl_2$.

Compound	per/ liter
EDTA 10^{-2}	1ml
Ascorbic acid 10^{-1}	0.5ml
*2Propranolol	0.1ml

The solution was pre-gassed with 5% CO_2: 95% O_2 for about 20 minutes. The pH of the buffer was then adjusted to pH 7.4, using NaOH prior to use.

*1 2.5ml of 1M solution of $CaCl_2$ was added per litre. It was added in the solution to ensure adequate calcium content of the solution. It was added just prior to use, to avoid $CaCl_2$ precipitation.

*2 The propranolol has an equal blocking action on the effects of noradrenaline at both the β_1 and β_2 receptor preventing relaxation, (Rang et al 1996) and ascorbic acid and EDTA prevent the oxidization of noradrenaline and binds calcium respectively.

Appendix 3a

Schematic diagram of isometric tension method

Figure 2.1. The organ bath was attached to a transducer. The surgical stainless-steel (ss) wire hooks were arranged to measure the passive force-internal circumference relationship: *g*, was the length of the vessel segment cut by a double-bladed scalpel; *f*, separation of wires measured by the micrometer; F, isometric force measured by the transducer. Reproduced from He, G. –W, Angus J. A, & Rosenfeldt, F. L (1998). This was based on the original system by Mulvany and Halpern (1977) for small vessels under isometric tension on a wire

Appendix 3b

Transducer, Pioden Controls Ltd, [Dynamometer, UF1, Ranges 0-25g]

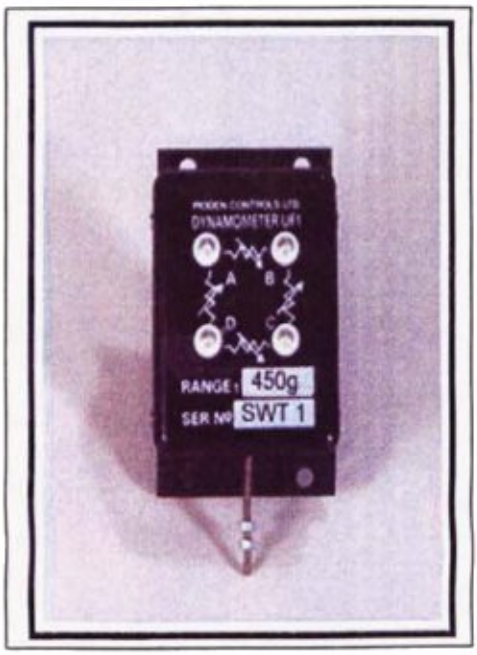


Figure 2.2. The transducer utilizes an unbonded strain gauge, which is protected from overload. It is widely specified for industrial and research applications where measurement of low forces combined with high sensitivity and robustness are required. The UF1 can be linked to both digital displays and chart recorders.

Model UF1 Specifications.

Output sensitivity: - 4mV/V FRO
Accuracy: - Better than 0.5%
Maximum overload: - 2.5Kg on all ranges
Operating temperature range: - -40°C to +80°C
Supply voltage: - 10 Volts max
Weight: - 45g
Information obtained from: - http:/www.pioden.com/uf1.htm

Appendix 4: - Metformin administered in vitro

The concentration of metformin administrated to mice in their drinking water is normally at the concentration of 250mg/kg/day. However in this case the concentration of interest is the circulating concentration of metformin when this dose is administrated. This was extrapolated from the information provided by Lord et al (1983), who carried out a study on the acute time dependent effect of metformin administered at an oral dose of 60mg/kg/day.

60mg/kg/day metformins circulating concentration after 24 hours = 0.41 µg/ml

250mg/kg/day metformin circulating concentration after 24 hours = ?

$$\frac{250\text{mg/kg/day}}{60\text{mg/kg/day}} = 4.167$$ conversion factor to convert 60mg/kg/day to 250mg/Kg/day

0.41µg/ml circulating metformin after 24 hours in 60mg/kg/day

0.41µg/ml circulating metformin x 4.167 = 1.70847 µg/ml

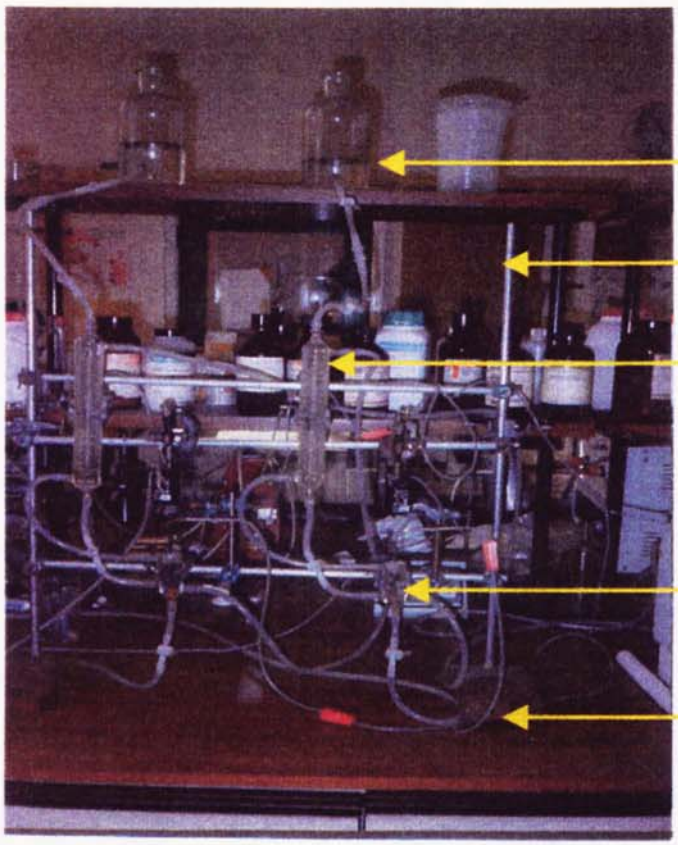
Therefore for a dose of 250mg/kg/day the circulating plasma concentration after 24 hours is approximately 1.71 µg/ml.

This is equivalent to 10^{-5}M metformin concentration.

Hence the above information was extrapolated from known published data by Lord et al (1983) for circulating metformin concentrations in 60mg/kg/day treated mice to our 250mg/kg/day metformin dose. Unpublished data from the Aston laboratory confirms the plasma metformin concentration of approximately 1.7 µg/ml (studies by C. J Bailey).

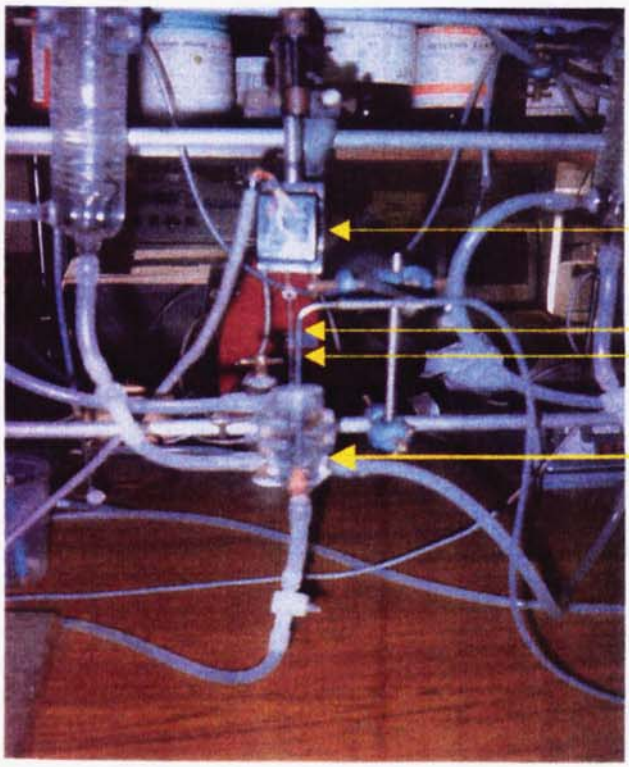
Appendix 5: - Mulvany Halpern equipment

Below is the apparatus of the Mulvany Halpern Myograph equipment used to perform the compliance studies, as described in the materials and methods.



Key of Components of the Mulvany Halpern Myograph System.

- Reservoir of physiological salt solution. See Appendix II.
- Clamping scaffold.
- Heated outer water jacket. (Keeps the water at 37 °C, which in turn keeps the krebs under constant physiological conditions.)
- Incubation chamber Volume used 10ml.
- Tubing to gas and maintain tissue viability.



Close up of the Mulvany Halpern Myograph incubation chamber.

- Transducer, converts movement of tissue into an electrical signal.
- Two wires one attached to each side of the tissue sample.
- Incubation chamber, contains 10ml of physiological saline solution into which metformin, noradrenaline and acetylcholine are injected.

Appendix 6: - Metformin administered in vivo

Metformin was administered to mice at a dosage of 250mg/kg/day. In order to ensure that the correct dosage was received the average daily intake of water by lean Ob/Ob (= +/+) was determined. The average daily intake was approximately 5 ml as noted previously by Bailey et al (1986). The calculation to determine the amount of metformin added to the drinking water is shown below.

Lean mice drink approximately 5 ml a day
Lean mice weight ~ 40g

In order to determine the amount of metformin added to the drinking water: -

$$\frac{\text{Metformin (mg) x body weight (g)}}{\text{kg}}$$

$$= \frac{250 \times 40}{1000}$$

= 10mg/ 5ml
= 2000mg/ 1L
= 2g/ 1L

Appendix 7a

The INFINITY triglyceride reagent contents

ATP	2.5 mmol/l
Mg^{2+}	2.5 mmol/l
4-Aminoantipyrine	0.8 mmol/l
3,5 DHBS	1 mmol/l
Peroxidase (Horseradish)	>5400U/l
GK (Micobial)	>560 U/l
GPO (Micobial)	>6000U/l
Lipoprotein Lipase (Micobial)	>31000U/l
Buffer (pH 7.0 at 25°C)	53 mmol/l
Sodium azide	0.05% w/v

(abbreviations given on next page)

Appendix 7b

Background and Principle of Test

Triglycerides + H_2O —Lipase→ Glycerol + Free Fatty Acids

Glycerol + ATP —GK→ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 —GPO→ DAP + H_2O_2

H_2O_2 + 4AAP + 3,5DHBS —POD→ Quinoneimine dye + $2H_2O$

A Trinder (1969) type colour reaction was catalysed by peroxidase. The absorbance of this dye was proportional to the concentration of triglycerides present in the sample.

Key

GK = glycerol kinase

GPO = glycerol phosphate oxidase

DAP = dihydroxyacetone phosphate

POD = peroxidase

Appendix 8: - Triglyceride calculation

The calculation below was used to determine the triglyceride content of the plasma samples. The glycerol standard used was made to the concentration 195mg/dl (Fisons G/0600, Batch 68).

$$\text{Triglycerides} = \frac{\text{Absorbance of Unknown}}{\text{Absorbance of Calibrator}} \times \text{Calibrator value}$$

Example:

Absorbance of Calibrator = 0.50

Absorbance of Unknown = 0.30

Value of Calibrator = 195 mg/dl (2.2 mmol/l)

$$\text{Triglycerides, mg/dl} = \frac{0.30}{0.50} \times 195 = 117 \text{ mg/dl}$$

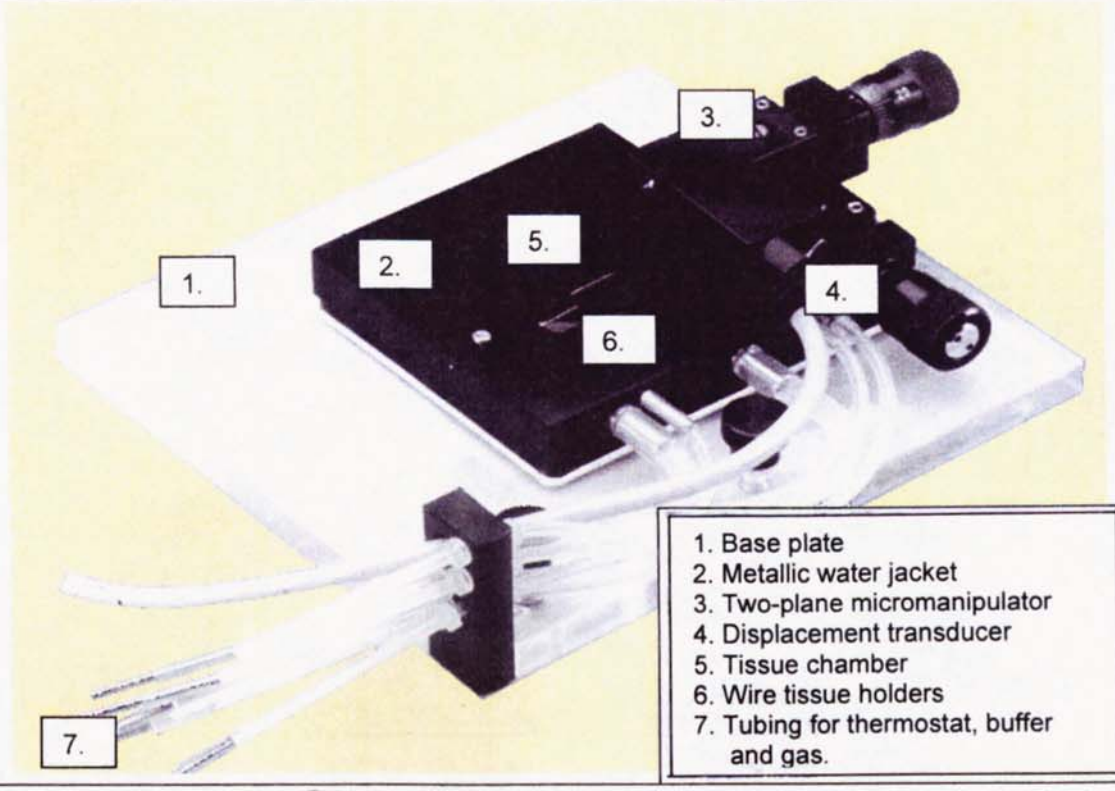
$$\text{Triglycerides, mmol/l} = \frac{0.30}{0.50} \times 2.2 = 1.32 \text{ mmol/l}$$

To directly convert mg/dl to mmol/l the unit conversion is: - mg/dl x 0.0113 = mmol/l. The above conversion is specifically for the Sigma diagnostics kit "INFINITY™ TRIGLYCERIDES REAGENT". It measures triglyceride values in the range from below 0.1 mmol/l up to 800mg/dl (9 mmol/l) and all the studies in the present study were within this range.

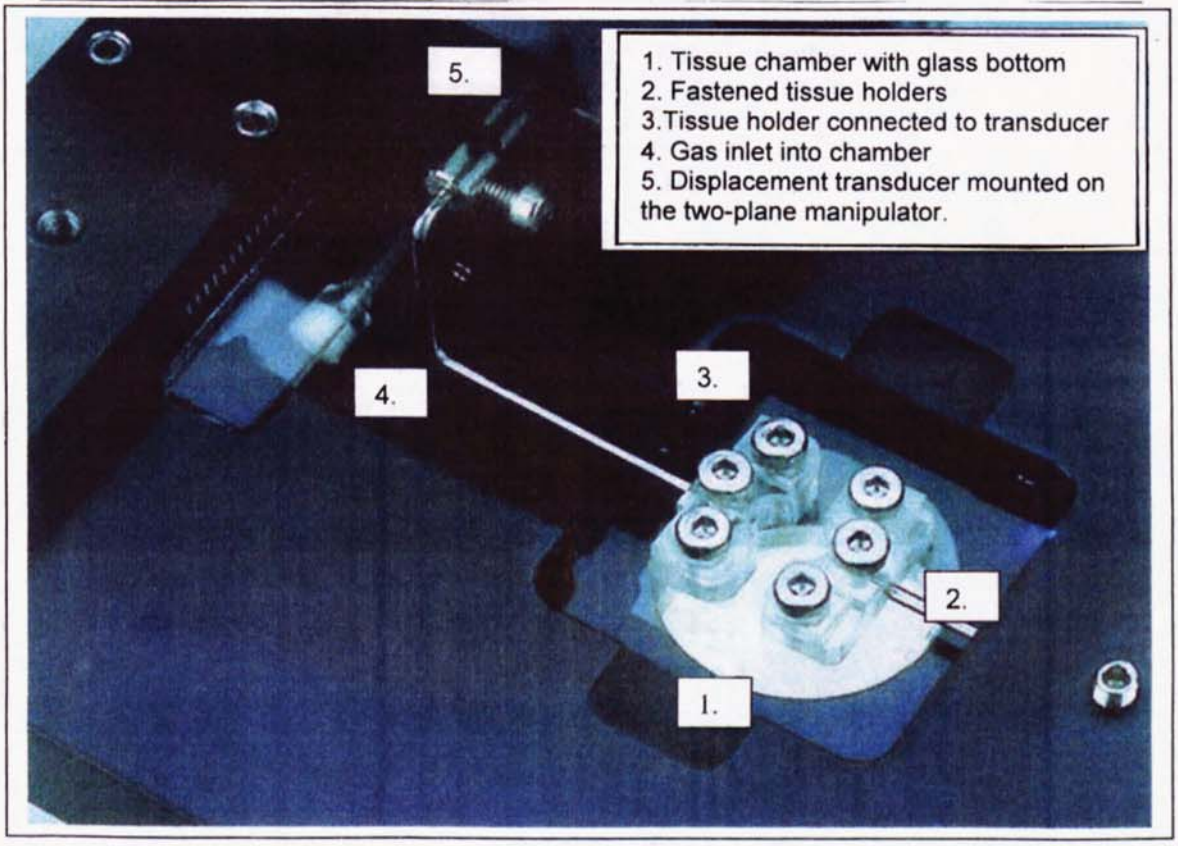
Appendix 9

Small Vessel Myograph System:- model MYO-01

A. Components of the tissue bath



B. Components of the chamber



C. Attaching tissue to MYO-01 small vessel myograph

Applying the tissue ring onto the holders.

A. Cut 2 pieces of tungsten wire 4cm in length, fix one end by tightening the screw on both tissue holders.
B. Drive one wire through the lumen of the vessel.
C. Attach the other end of the wire by tightening the other screw.
D. Drive the other wire through the lumen of the tissue ring. Attach the wire using the second screw. Then attach the tissue holders into the organ bath, and the transducer.

(Taken from: -Small vessel myograph system. User manual for the bath, Experimetria Ltd.)

Appendix 10: - Nitrite/ nitrate determination

Appendix 10a: - Assay Buffer

A 0.1M phosphate buffer was made using: -
81ml $Na_2HPO_4.12H_2O$ (Mr= 358.14) – ml NaH_2PO_4 (Mr=156.01) at pH 7.4

81.0 ml of 0.2M Na_2HPO_4 + 19.0ml of NaH_2PO_4 re needed,
make up to 200ml with distilled water

From stock 0.1M solution make a 14mM solution.

0.1M ÷0.014M = 7.1 fold dilutions
1ml of 0.1Mstock phosphate buffer in 6.1ml water = total volume 7.1ml
4ml in 24.2ml = 28.4ml

Appendix 10b:- The Assay mix

The following solutions were prepared in 25ml of assay buffer (See above)
KEEP REFRIDGERATED IN FOIL OR COLOURED BOTTLE
SHELF LIFE 2 WEEKS

Nitrate Reductase
Stock solution of 200U/ Litre
Equivalent to 5U/ 25ml
Dissolve 1 5U bottle into 25ml of 14mM Sodium Phosphate buffer.

Glucose-6-phosphate Dehydrogenase (100 units/ bottles)
Stock solution 400U/ Litre
Equivalent to 100U/ 250ml
10U/ 25ml of14mM Sodium Phosphate buffer.

Each bottles of Glucose-6-phosphate Dehydrogenase contains 100 Units. Add 1ml of distilled water into each bottle. This is then frozen down in 100µl aliquots.

1 x 100µl aliquots contain 10 units this should be dissolved into the same 25mls as the above.

Glucose-6-phosphate 2.5mM

1M = 304.1g in 1 litre
1mM = 0.3041g in 1 litre
1mM = 0.0003041g in 1ml
2.5mM = 0.0076025g in 1ml
2.5mM = 0.01900625g in 25ml of 14mM Sodium Phosphate buffer.
Dissolve the above into the same 25mls as the above 2 solutions.

Appendix :- The NADPH preparation

MAKE UP FRESH EACH TIME

It is important that this solution is made up fresh each time it is required

1M = 833.4g in 1 litre
1mM = 0.8334g in 1 litre
1mM = 0.008334g in 10ml
1 µM = 0.000008334g in 10ml
10 µM= 0.00008334g in 10ml
10 µM= 0.08334mg in 10ml of 14mM Sodium Phosphate Buffer

This amount was too small to measure, so a grain or two of the powder was added to the 10ml solution.

Appendix 10c:-

Sulfanilamide Solution NED Solution

1% sulphanilamide in 5% phosphoric acid.

Phosphoric acid 85% concentrated

Take 85% concentrated phosphoric acid dilute (85/5 =17 fold dilution)

e.g 1ml of acid in 16ml of water

3ml 48ml

Discard 1ml giving 50ml final volume

Sulfanilamide 1% solution

1.0g in 100ml

0.5g in 50ml store in fridge in foil container

NED Solution

An equal quantity of NED solution and sulfanilamide solution was required as they were used in equal amounts.

0.1% N-1-(napthyl) ethyl-endediamine dihydrochloride (NED) in water

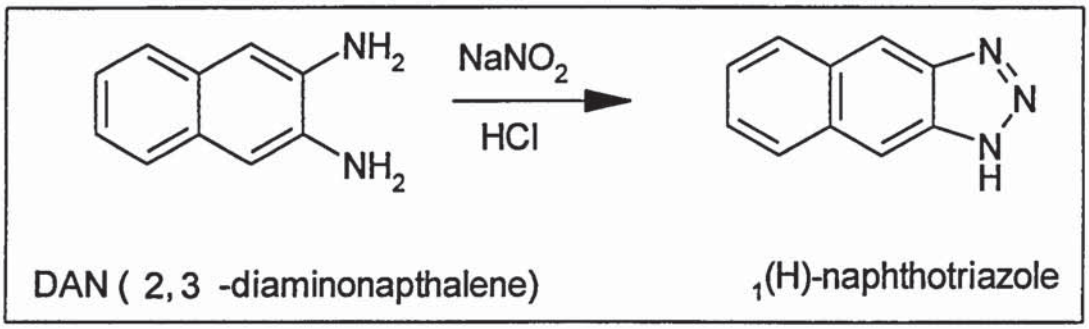
0.10g in 100ml

0.01g in 10ml

0.05g in 50ml store in fridge in foil container

Appendix 11: - DAN

Misko et al (1993) have produced a rapid sensitive assay, which allows the quantification of nitrite/ nitrate. The reaction involves nitrite combining with 2,3-diaminonaphthalene to form a fluorescent product known as 1-(H)-naphthatriazole. This reaction is shown in detail below.

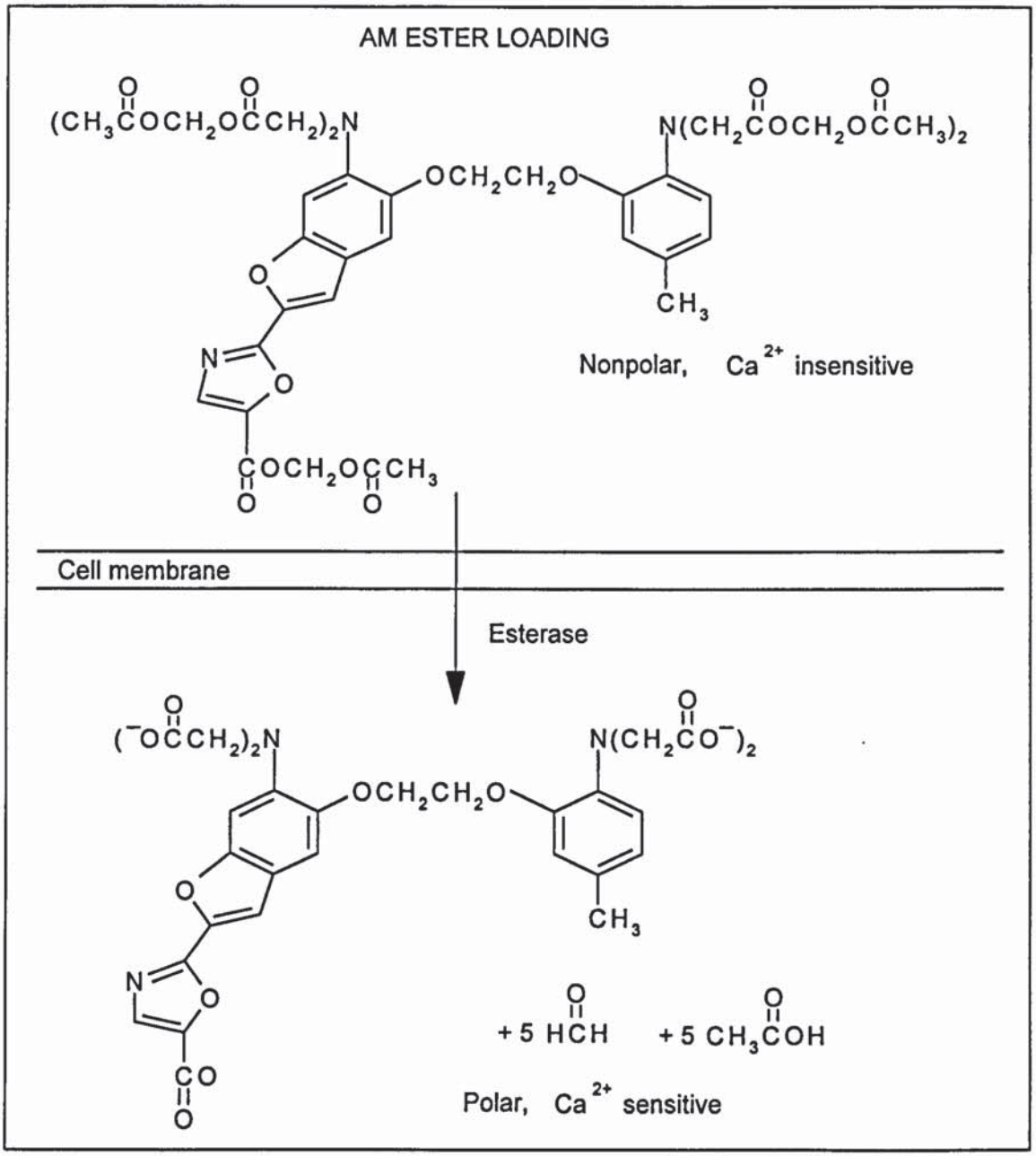


DAN (2,3 -diaminonapthalene) $_1$(H)-naphthotriazole

The assay can detect nitrite concentrations as low as 10nM, making it 50-100 times more sensitive than the Griess assay.

Appendix 12

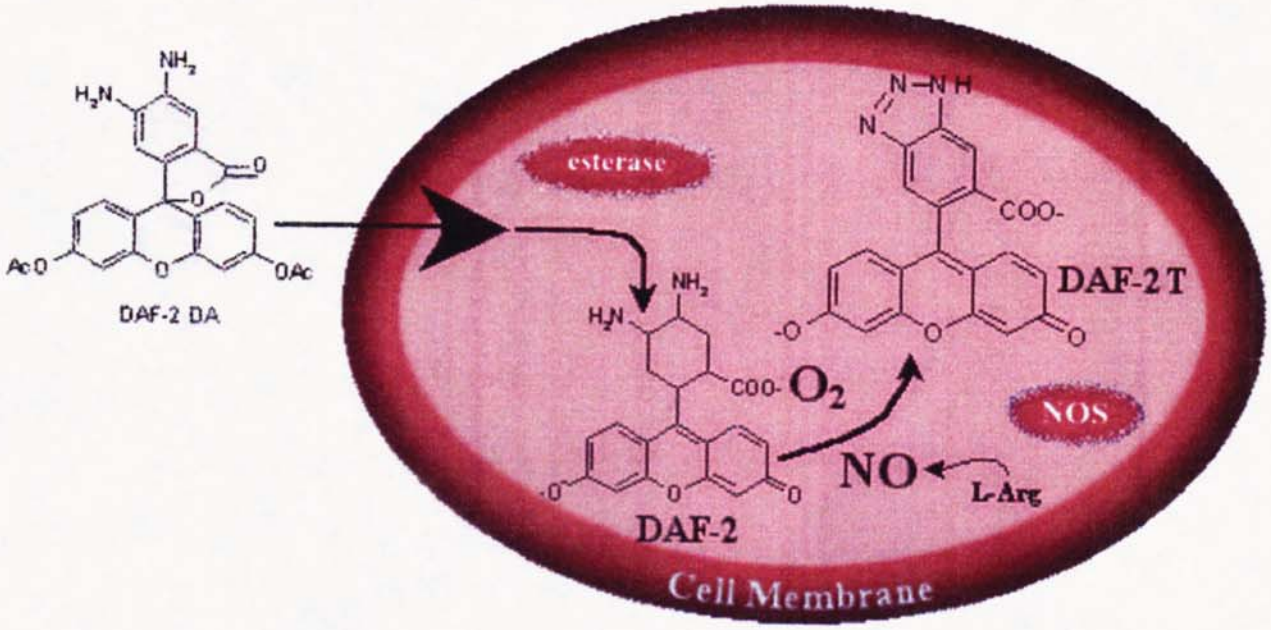
FURA/2AM cleavage in the cell



Cells were loaded using membrane-permeant acetoxymethyl (AM) ester derivatives of fluorescent indicators, in this case fura-2. Note the generation of potentially toxic by-products (formaldehyde and acetic acid). Diagram from section 20.1- Introduction to Ca^{2+} measurements with fluorescent indicators. (Accessed 21/06/02: - http:/www.probes.com/handbook/print/2001.html

Appendix 13

An example of DAF-2DA cleavage in the cell



DAF-2DA is hydrolysed by cytosolic esterase, which releases DAF-2 a relatively non-fluorescent compound, which does not leak into the surrounding medium. In the prescence of Nitric oxide and oxygen a fluorescent DAF-2 triazole product is formed.

Appendix 14

Krebs-Ringer buffer solution

Adapted from: -

Krebs, H. A. (1950). Body size tissue Respiration. Biochimica et Biophysica Acta. Vol. 4, pp 249-269.

Compound	Molecular Weight (g)	g/ liter	Required Molarity (mM)
NaCl	58.44	6.920	118.00
KCl	74.56	0.354	5.00
$NaHCO_3$	84.01	2.100	25.00
$MgSO_47H_2O$	246.48	0.290	1.18
KH2PO4	136.09	0.324	1.17

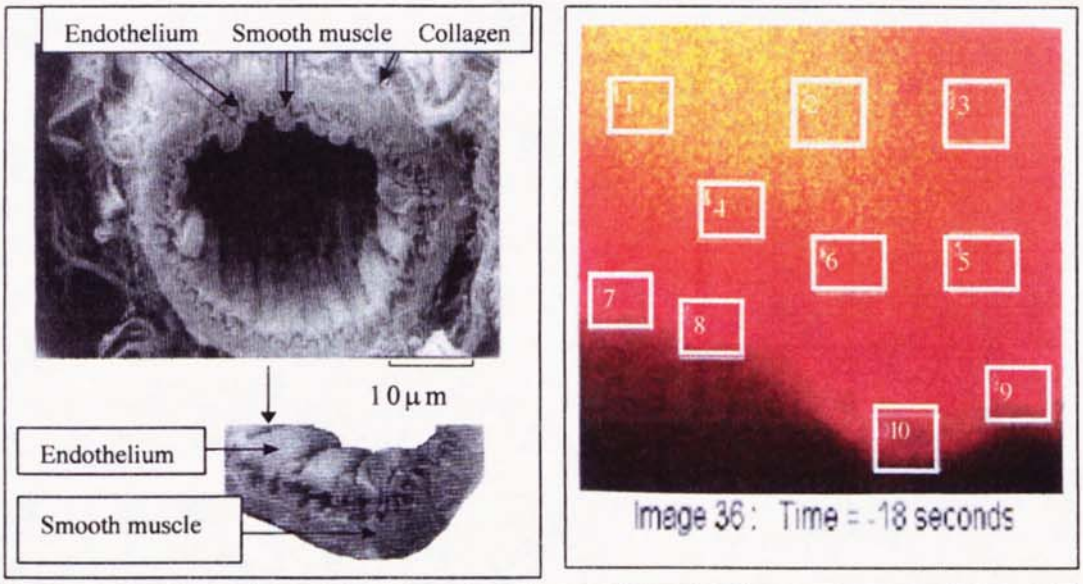
This solution was pre-gassed using 5% CO_2: 95% O_2 for approximately 20 minutes.

$CaCl_2$ was added from a pre-prepared stock solution of 1.27M. This was added as 1µl per ml of buffer solution.

This solution was used in the imaging studies as none of the ingredients are shown to interfere with the system

Appendix 15a

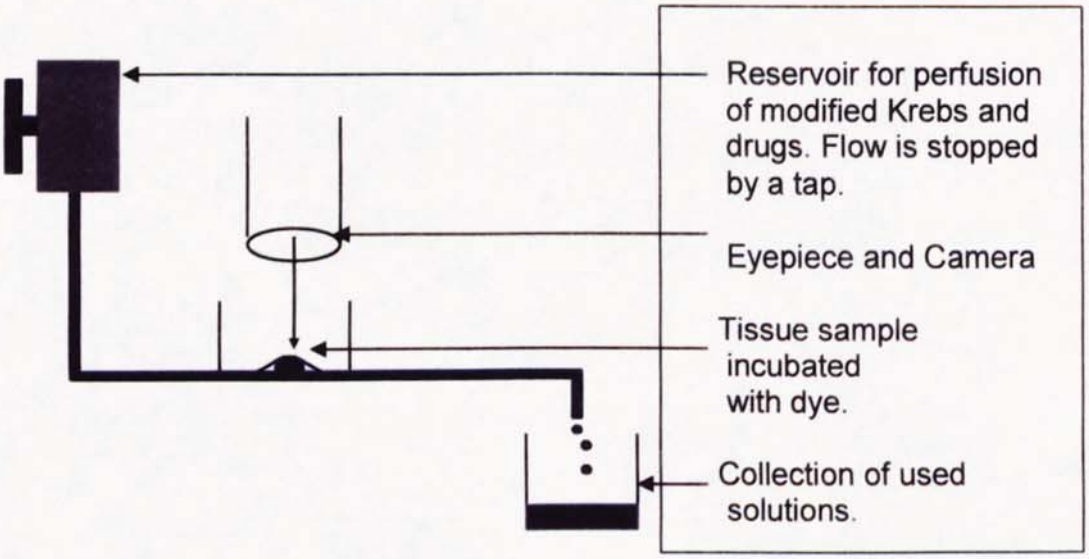
Specific selection of regions on aortic tissue



Slices of aorta are produced as shown on the left above. The image is then focused on under the fluorescent microscope as shown on the right and regions of interest are selected. The changes in fluorescence in each region are detected and this information is used to produce a trace of the changes in fluorescence over time in each region. Boxes 1-6 are regions selected in the smooth muscle, while regions 7-10 are the endothelium.

Appendix 15b

A schematic diagram of the perfusion system



Appendix 16

Chemical Lots used and their suppliers

The chemicals used are listed below in alphabetical order, and if more than one lot or batch has been used these appear in date order: -

Acetylcholine (Sigma), Lot 35H0784

Akt inhibitor (Calbiochem), Lot B47190

BCA, Bicinchonic acid (Pierce), Lot CL52976

Calcium (liquid form), Lot 70237369

DAN, 2,3 Diaminonaphthalene (Avocado, 97%), Lot F4223A

DAF-2DA , 4, 5-Diaminofluorescein Diacetate, (Calbiochem), Lot B37791

FURA/2AM, 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N, N, N', N'-tetraacetic acid, pentaacetoxymethyl Ester (Calbiochem), Lot B45863

Glucose-6-phospate (Sigma), Lot 40k7014

Glucose-6-phospate dehydrogenase (Sigma), Lot 31k7692

Insulin (Sigma), Lot 79H0566

L-NAME, NG-Nitro-L-argininemethylester, Hydrochloride (Sigma), Lot 51k1351

LY294002 (Calbiochem), Lot 121K46081

Metformin, 1,1 Dimethylbiguinide hydrochloride (Sigma), Lot 77H1491, Lot 71K2125

NADPH (Sigma), Lot 80K7019

NED, N-1-napthyl(etyl)enediamine dihydrochloride (Sigma), Lot 120K0048

Nitrate reductase (Sigma), Lot 31K1458, Lot 12K1665, Lot 062K1189

Noradrenaline, bitartrate salt (Sigma), Lot –108F0758

Papaverine (Sigma), Lot 91K1114

DL Propranolol, (Sigma), Lot 33F-0495, Lot 97H0311.

SB (Calbiochem), Lot B37330

Sodium nitrate, (Sigma), Lot

Sodium nitrite, (Sigma), Lot 123HO257.

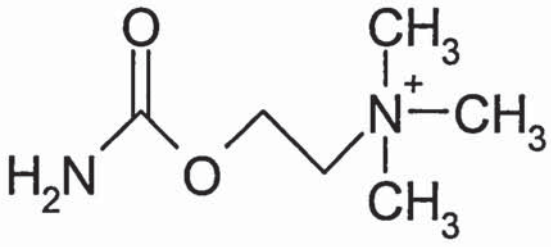
Sulfanilamide (Sigma), Lot 51K1263

Wortmannin (Sigma), Lot 51K4033

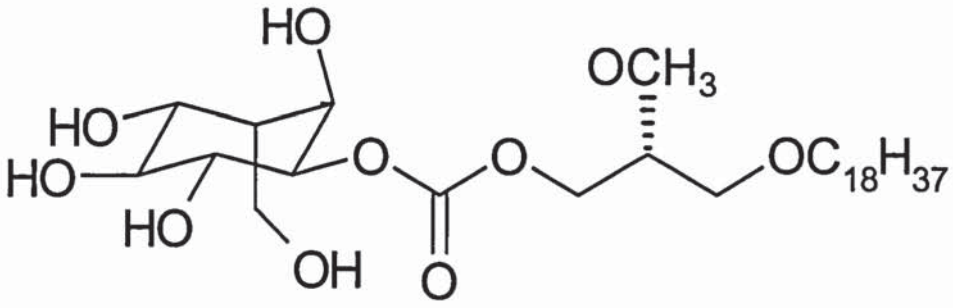
Appendix 17:- Chemical Structures

The chemical structures of the drugs, dyes, inhibitors and reagents used appear below in alphabetical order.

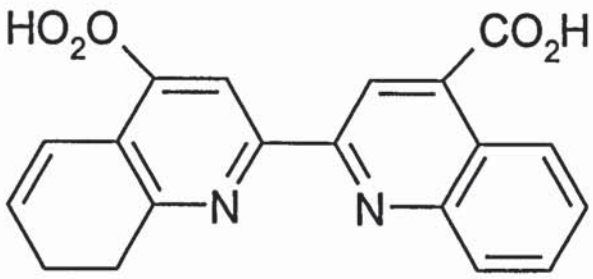
Acetylcholine



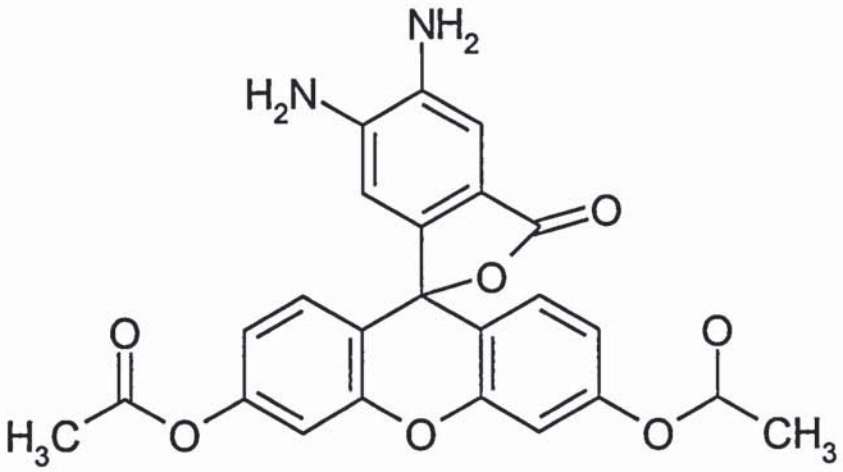
Akt inhibitor



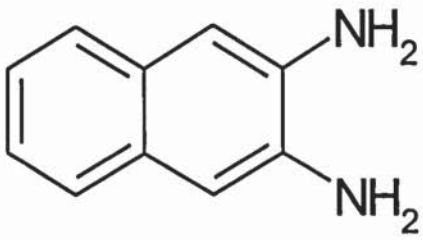
BCA: - Bicinchonic Acid



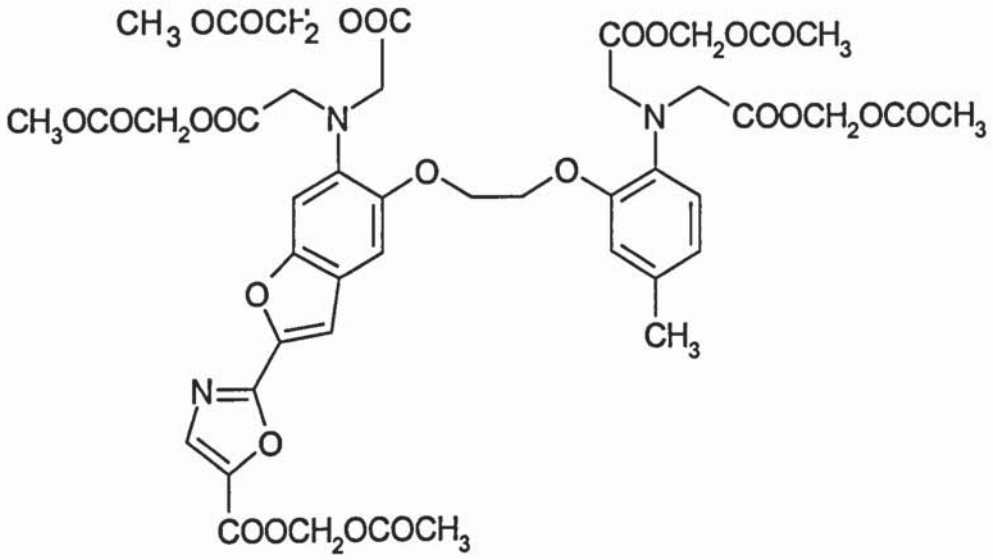
DAF-2DA: - 4,5-Diaminofluorescein Diacetate



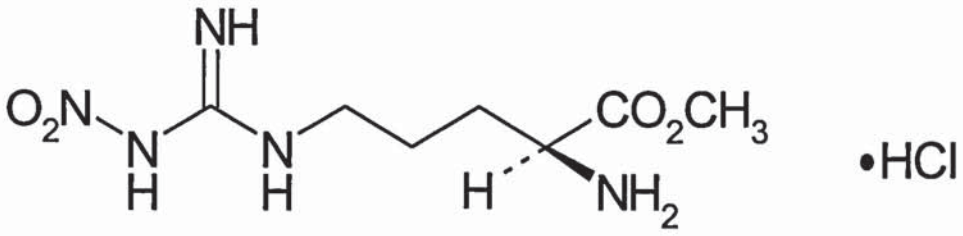
DAN (2,3 diaminonapthalene)



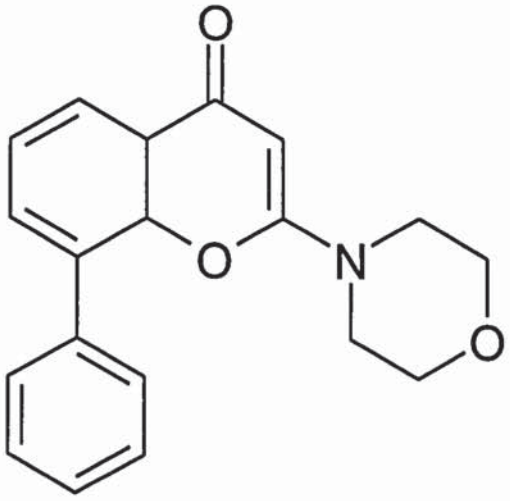
FURA-2AM: - 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic Acid, Pentaacetoxymethyl Ester



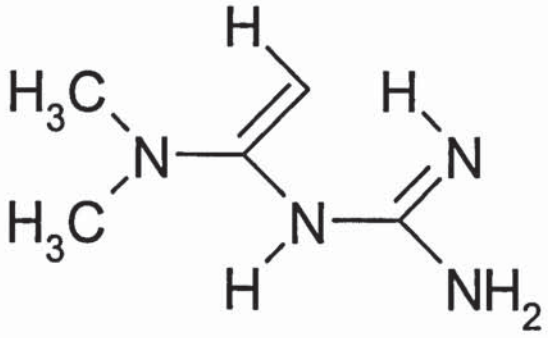
L-NAME: - NG-Nitro-L-arginine Methyl Ester, Hydrochloride



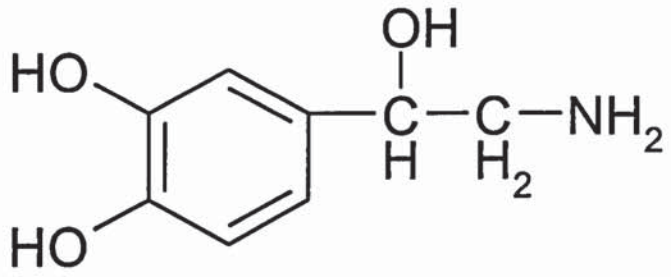
LY294002



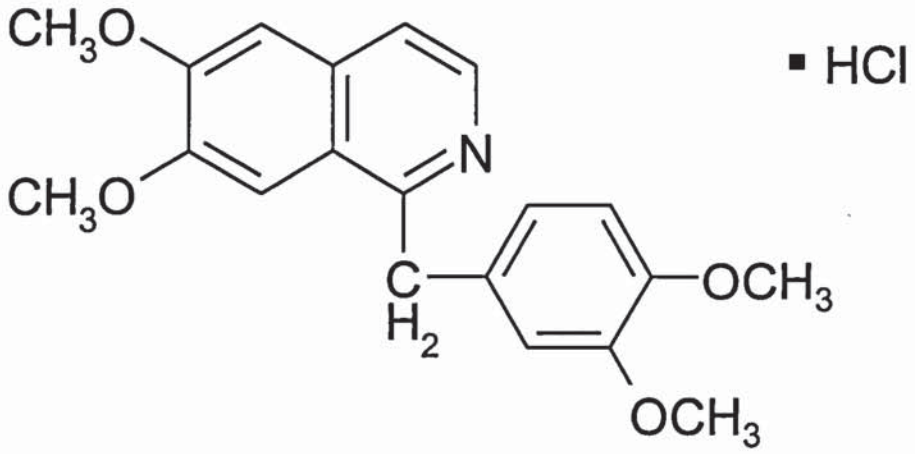
Metformin: - 1,1-Dimethylbiguanide Hydrochloride



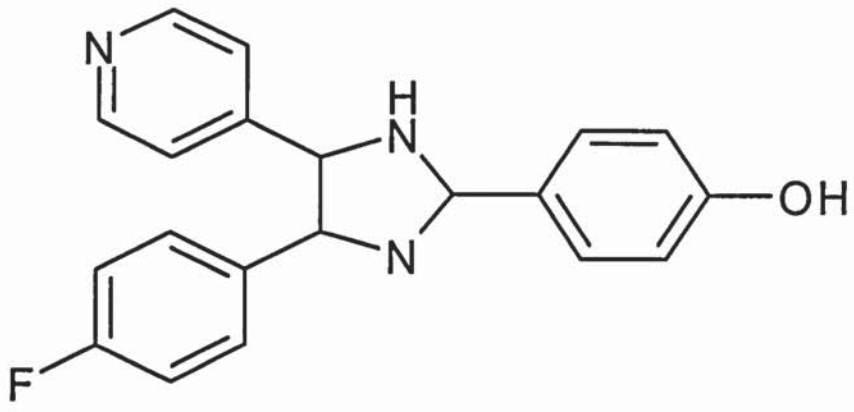
Noradrenaline



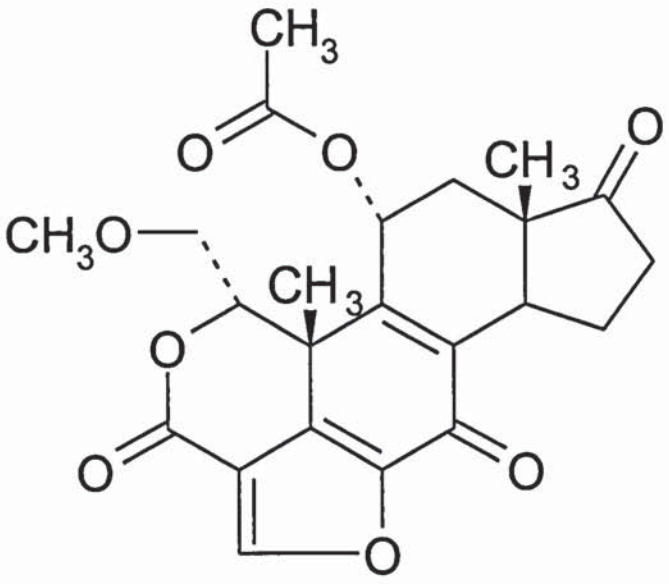
Papaverine



SB 202190



Wortmannin



Appendix 18: - Abbreviations

AC: -	Adenylate cyclase
ACE: -	Abgiotensin converting enzyme
ACH: -	Acetylcholine
ADPRT: -	ADP-ribosltransferases
AI : -	Augmentation index
AMI : -	Acute myocardial infarction
AMP kinase:-	Adenyl monophosphate kinase
AGE: -	Advanced glycation endproducts
ATP: -	Adenosine triphosphate
BDA: -	British Diabetic Association
BMI: -	Body mass index
BP: -	Blood pressure
CAM: -	Calmodulin
CAMP: -	Cyclic adenosine 3', 5' monophosphate
CEE: -	Chicken embryo extract
CGMP: -	Cyclic guanosine 3',5' monophosphate
CPK : -	Creatinine phosphokinase
GTP: -	Guanosine 5' tri-phosphate
CHD: -	Coronary heart disease
CK: -	Creatine kinase
CRP: -	C-reactive protein
CVD: -	Cardiovascular disease
DAG: -	Diacylglycerol
DCCT: -	Diabetic control and Complications Trial
DMEM : -	Dulbecos modified Eagle's medium
DVP: -	Digital volume pulse
ED: -	Endothelial dysfunction
EDRF: -	Endothelial derived relaxation factor
ELISA: -	Enzyme linked immunosorbant assay
ENOS: -	Endothelial nitric oxide synthase
ER: -	Endoplasmic reticulum
ET: -	Endothelin

FFA : -	Free fatty acids
GC: -	Guanylyl cyclase
GPX: -	Glutathione peroxidase
GSH : -	Glutathione reductase
Hb: -	Haemoglobin
HbO2: -	oxyhaemoglobin
HCO3: -	Hydrogen carbonate
HbNO: -	nitrosilated haemoglobin
HDL: -	High density lipoprotein
HMG: -	3-hydroxy-3-methylglutaryl-CoA
HOMA : -	Homeostasis model assessment
ICAM-1: -	Intracellular adhesion molecule-1
IGF-1: -	Insulin-like growth factors
IP3: -	Inositol 1, 4, 5 trisphosphate
IP4: -	Inositol tetrakis phosphate
LDL: -	Low density lipoproteins
MAP: -	Mitogen activated protein
MCP-1: -	Monocyte chemo attractant protein-1
ME: -	Methylglyoxal
MetHb: -	Methaemoglobin
MK: -	Myokinase
MI: -	Myocardial infarction
MRFIT : -	Multiple risk intervention trial
NA: -	Noradrenaline
NIDDM: -	Non insulin dependent diabetes mellitus
NO: -	Nitric oxide
NOS: -	Nitric oxide synthase
PAF: -	Platelet activating factor
PAI-1: -	Plasminogen activator inhibitor-1
PAS : -	Periodic acid-Schiff
PDGF: -	Platelet derived growth factor
PIP2: -	Phosphatidylinositol 4, 5 bisphosphate
PIP3: -	Phosphatidylinositol 3, 4, 5 trisphosphate
PI3-kinase : -	Phosphatidylinositol 3 kinase

PKC: -	Protein kinase C
PLA2: -	Phospholipase A2
PLC: -	Phospholipase C
PP: -	Pulse pressure
SHR: -	Spontaneous hypertensive rats
SOD: -	Superoxide dismutase
SR: -	Sacroplasmic reticulum
T2DM: -	Type 2 diabetes mellitus
TG: -	Triglyceride
TNF: -	Tumour necrosis factor
ROS: -	Reactive oxygen species
TNF: -	Tumour necrosis factor
TRP: -	Transient receptor protein
TRPM: -	Transient receptor protein melastatin
TRPV: -	Transient receptor protein vanilloid
SM: -	Smooth muscle
SMMHC: -	Smooth muscle myosin heavy chain
UKPDS: -	United Kingdom prospective diabetes study
VCAM: -	Vascular cell adhesion molecule-1
VE: -	Vascular endothelium
VLDL: -	Very low density lipoprotein
VSM: -	Vascular smooth muscle
VSMC: -	Vascular smooth muscle cells
VWF: -	Von Willebrand factor
3DG: -	3-deoxyglucosone

Temporal co-ordination of insulin-stimulated release of nitric oxide from vascular endothelium

Authors:- H. E Archer, Dr N. Hartell, Dr C. J Bailey.

Department of Pharmaceutical and Biological Sciences, Aston University, Birmingham, B4 7ET.

Background

Insulin is known to trigger the release of nitric oxide (NO) from the arterial wall, mediating a vasodilator effect (Hsueh and Law 1999.)

NO is a very unstable free radical gas with a half-life of only a few seconds (Rehman 2001.) NO is inactivated mainly by materials such as haemoglobin, oxygen and superoxide mutase, and is degraded to nitrite (NO_2^-) and nitrate (NO_3^-) (Vallance 2001).

Aims

To visualise the spatial release and distribution of nitric oxide and calcium in real time, to determine whether insulin stimulated NO release is calcium (Ca^{2+}) dependent.

The effect of insulin was compared to acetylcholine which is known to release NO in a calcium-dependent manner.

The fluorescent dyes DAF-2DA andFURA-2AM were used to visualise NO and Ca^{2+} (respectively) via a continuous recording camera.

Methods

An isolated thoracic aorta preparation was opened longitudinally and placed endothelial side up onto a nitrocellulose membrane.

Thin slices (approx 200μM) were cut with a Mickleway chopper. and secured in a superfusion chamber by 2 tracks of silicon grease.

The tissue is then incubated with the dyes for 30 minutes to allow the dye to penetrate the cells. Extracellular dye is then removed by washing with a modified calcium free Krebs buffer.

Regions of special interest within and between cells were selected by confocal microscopy in the endothelial region and the underlying vascular smooth muscle (VSM) of the aortic slice.

The DAF-2DA dye was excited at λ 495nm, and the emission at 515nm. While the FURA-2AM dye is excited at 350nm it is divided by the decrease in fluorescence seen at 380nm. It is the ratio between these two wavelengths that is directly proportional to calcium.

Images were taken at 6 second intervals. A steady baseline was established before any of the drugs were administered.

Results

Directly comparative results were obtained from the same piece of tissue.

Figure 1 shows that Acetylcholine at 2μM increased NO, which was immediately accompanied by an increase in intracellular Ca^{2+}.

Insulin concentrations of 10^{-8}, 10^{-7},10^{-6} M also produced a rapid increase in NO as shown in Figure 2, with no accompanying increase in intracellular calcium. The calcium baseline declined with time, suggesting that subsequent actions of insulin are Ca^{2+} independent.

Figure 1:- The release of nitric oxide and calcium triggered by acetylcholine.

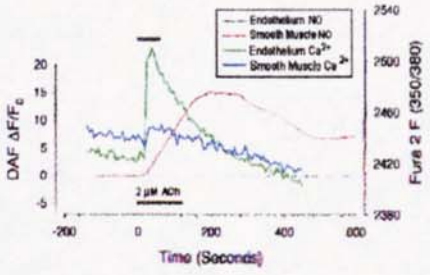
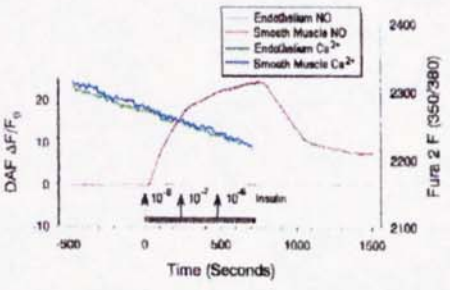


Figure 2:- The release of nitric oxide and calcium triggered by insulin.



Conclusion

This study indicated that insulin acts on the endothelium to release NO via a Ca^{2+} independent pathway. This contrasts with acetylcholine which acts via a calcium dependent pathway.

NO release via the Akt/PKB (protein kinase B) pathway was recently shown by Dimmeler et al (1999). Part of this pathway contributes to the postreceptor insulin signalling mechanism.

It is suggeste that the PI3 (phoshatidylinositol-3-kinase) and Akt pathway played an important role in th activation of eNOS (endothelial Nitric oxide synthase.) Therefore the same pathway through which insulin activates glucose transport in skeletal muscle cells may trigger NO release from endothelial cells.

Summary

Unlike acetylcholine insulin triggers NO release independently of Ca^{2+}. Therefore insulin resistance in endothelial cells may contribute to the increased risk of hypertension (Weston 2001) and atherosclerosis (Wheatcroft 2001) in diabetic patients.

References

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. (1999) Activation of nitric oxide in endothelial cells by Akt-dependent phosphorylation.*Nature*. Vol.399,pp 601-605.

Hsueh WA, Law RE. (1999). Insulin signalling in the arterial wall. *American journal of Cardiology*.84 (1A) :21J-24J.

Rehman HU (2001). Vascular endothelium as an endocrine organ. *Proceedings of the Royal College of Physicians Edinburgh*. Vol.31, 149-154.

Weston C. (2001) NO hiding place: nitric oxide in cardiovascular disease. *British journal of Cardiology*. Vol.8, Issue 10, 544-548.

Wheatcroft SB, Williams IL, Kearney MT. (2001) Insulin resistance, the endothelium and atherosclerosis. *British journal of Cardiology*. Vol 8. Issue 9, pp

Vallace P. (2001) Nitric oxide *Biologist* Vol. 48, 153-158.

Effect of metformin on aortic contractility.

H. E Archer, H. C. S Howlett, N. F Wiernsperger, C. J Bailey.

Department of Life and Sciences, Aston University, Birmingham, B4 7ET.

Background

The UKPDS in 1998[1] showed that initial antidiabetic drug treatment of overweight type 2 diabetic patients with metformin, reduced chronic macrovascular complications independently of glycaemic control. This study aims to investigate the effects and time period required for metformin to induce these effects on the vasculature.

Increased contraction and relaxation of tissue in the presence of metformin has been previously observed in patients with peripheral vascular disease by Sitori et al 1984[2], and Katakam et al 2000[3] demonstrates metformin enhances nitric oxide (NO) mediated relaxation in insulin resistant rats.

Aims

The aim of this study was to determine the time period of exposure to a therapeutic concentration of metformin required to produce an increase in maximum aortic contractility in response to noradrenaline.

Methods

Elastic thoracic aortic ring sections were obtained from male lean mice. Excess connective tissue was removed before the tissue was mounted onto tensioned wire tissue holders, attached to a strain gauge, and maintained in oxygenated physiological salt solution (PBS) as instructed by Palmer et al 1998[4] at 37°C.

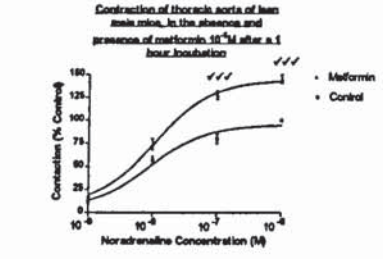
The aortic section was tensioned at 1g (Rossoni et al 2002[5]). An initial adaptation period of ~ 45 minutes was given to equilibrate the tissue. This simulates the tension exerted by circulating blood.

For in vitro studies tissue was exposed to 1.71µg/ml of metformin (Lord 1983[6]), this is approximately 10^{-5}M, which is equivalent to the therapeutic circulating concentration of metformin seen in human subjects with 1-3g/day and mice treated with-250mg/kg/day.

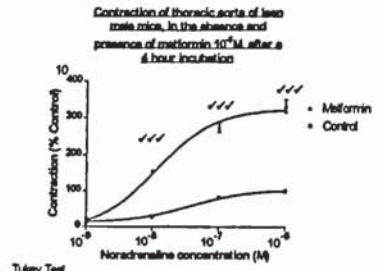
The aortic sections were exposed for 1 and 4 hours respectively, before being subjected to a range of noradrenaline concentrations (i.e 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M.)

Ex vivo studies were conducted using mice treated with metformin at a dose of 250mg/kg/day in their drinking water for 2, 4 and 8 weeks respectively, before aortic sections were removed for contractility studies. The weight, total triglyceride profile, glucose and insulin concentrations of these animals were also investigated.

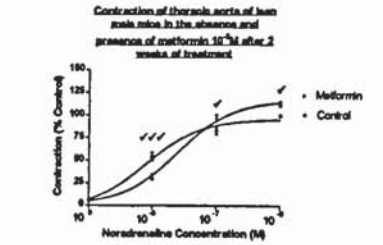
Results



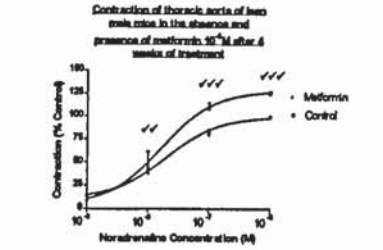
Tukey Test
✓✓✓ P<0.001 Metformin v Control



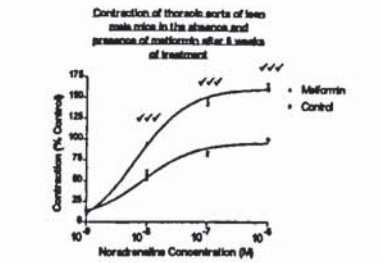
Tukey Test
✓✓✓ P<0.001 Metformin v Control



Tukey Test
✓ P<0.05 Metformin v Control
✓✓✓ P<0.001 Metformin v Control



Tukey Test
✓✓ P<0.01 Metformin v Control
✓✓✓ P<0.001 Metformin v Control



Tukey Test
✓✓✓ P<0.001 Metformin v Control

Comments

In vitro studies showed a 1-hour incubation with 10^{-5}M metformin, increased aortic contraction by a maximum of 49% ($p<0.001$) compared to control at 10^{-6}M noradrenaline. This was further enhanced after a 4-hour incubation giving an increase of 221% ($p<0.001$) compared to the control.

After treatment in vivo, the effects on noradrenaline induced contraction took much longer to be displayed. There was no effect after 24 hours of treatment with metformin (250mg/kg/day)

The first response was displayed at 2 weeks when there was an increase in maximum noradrenaline induced contraction by 21% ($p<0.05$). At 4 and 8 weeks the effect was further enhanced giving rises of 29% ($p<0.001$) and 65% ($p<0.001$) respectively.

Conclusion

The in vitro studies indicate that a therapeutic concentration of metformin acts directly on the aorta to increase maximum noradrenaline induced contraction. The effect is rapidly generated in vitro, but it is only evident after in vivo administration of the drug for 2 weeks in non-diabetic mice. The in vivo studies also showed this effect of metformin was independent of significant changes in weight, and plasma concentrations of triglyceride, glucose and insulin.

Summary

Metformin has the ability to directly increase the contractility of aortic tissue in the presence of noradrenaline, additional and independent of its glycaemic control, total triglyceride concentration and circulating insulin concentration.

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Direct effect of Metformin on Vascular Contractility.

Authors:- H. E Archer[1], H. C. S Howlett[2], N. F Wiernsperger[2], C. J Bailey[1]

[1]Life and Health Sciences, Aston University, Birmingham, UK,

[2]Merck-Sante, Lyon, France.

Background

The biguanide metformin reduced macrovascular complications by 32% compared to conventional diet over 10 years, when used as initial antidiabetic therapy in overweight type 2 diabetic patients during the United Kingdom Prospective Study (UKPDS 34)[1]. Metformin also reduced diabetes-related endpoints by 39%, and diabetes-related death by 42%. These effects were greater than achieved by intensive use of insulin and sulphonylureas, although glycaemic control was similar with all therapies. This suggests metformin may have a cardiovascular protective action beyond its antihyperglycaemic properties (Kirpichnikov 2002)[2].

Therefore metformin may have independent actions on the vascular system. Studies have shown the action of metformin on contraction and relaxation of blood vessels in the presence of metformin by Sitori et al 1984[3] and Katakam et al 2000[4] respectively.

Aims

This study examines the acute effects of metformin on vascular contractility and passive tension:-The time periods studied were 1 and 4 hour incubation periods with metformin invitro. Maximum contractility of mouse aorta in vitro was measured in response to noradrenaline, followed by acetylcholine induced relaxation after maximal contraction tissue. Passive tension generated by the aortic vessel wall was also measured.

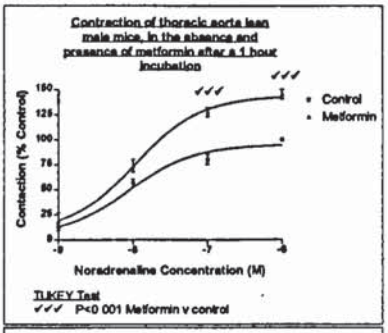
Methods

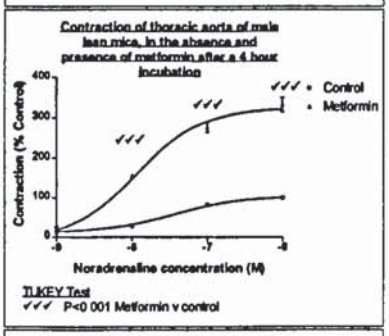
Thoracic aortic ring sections from lean male mice, were mounted onto tensioned wire tissue holders, attached to a strain gauge, and maintained in oxygenated physiological salt solution (PSS) as described by Palmer et al 1998[5] at 37°C. The upper aortic section was tensioned at 1g: this was equivalent to 9.8mN, the optimal tension of the tissue (Rossini et al 2002)[6]. An initial adaptation period of ~ 45 minutes was given to equilibrate the tissue. This mimics the tension exerted by circulating blood in the mouse.

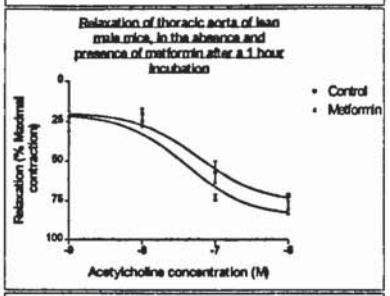
Aortic tissue was exposed in vitro to 1.71µg/ml of metformin, approximately 10^{-5}M (Lord et al 1983)[7], which is equivalent to the plasma concentration of metformin observed in humans treated with 1-3g/day and mice treated with 250mg/kg/day. The aortic sections were exposed to metformin for 1 and 4 hours and then contracted with noradrenaline (i.e 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M), to produce a cumulative concentration-response curve (Sheykzade et al 2000)[8]. At maximum contraction the effect of acetylcholine was assessed on relaxation, similar concentrations are also used (i.e 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M.)

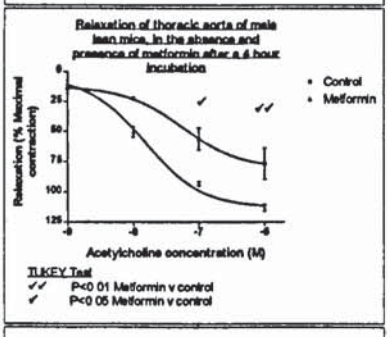
Lower thoracic aorta regions were used to determine passive tension of the aortic tissue. A Small Vessel Myograph (MYO-01) system was used to study passive tension. The tissue is stretched transversely after exposure to the presence and absence of metformin 10^{-5}M for 1 and 4 hours at 37°C in aspirated physiological buffer. The tension of the tissue is then determined.

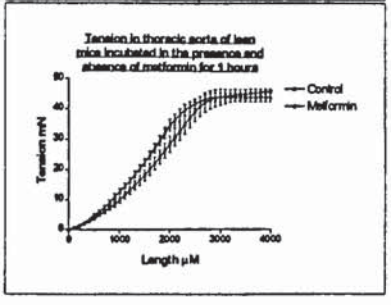
Results

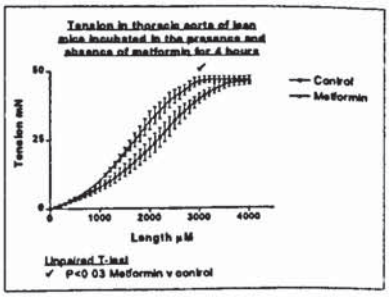












Comments

In vitro a 1 hour exposure of the upper section of aorta to metformin showed an increased noradrenaline-induced aortic contraction by a maximum of 49% ($p<0.001$) compared to control. This effect was further enhanced after a 4-hour incubation with metformin, giving an increase of 221% ($p<0.001$) compared to the control..

After incubation with metformin for 1 hour the effect of acetylcholine was not significant, only caused an additional 8.45% relaxation. At 4 hours acetylcholine, increased the relaxation response by 36.85% with 10^{-6}M acetylcholine ($P<0.01$) compared to the control.

The passive tension was measured by transverse stretching of the lower aortic section. After 1 hour there was ~2-3 % increase in tension in the tissue treated with metformin, this was not statistically significant. After a 4 hour study a larger passive tension was seen in the metformin treated tissue ~6-10 % increase, ($P < 0.03$ v control at 3000mm).

Conclusion

The in-vitro studies indicate that a therapeutic concentration of metformin (10^{-5}M) can increase maximum noradrenaline (10^{-6}M) stimulated contraction via vascular smooth muscle, after 1 hour by 49% and by 221% after 4 hours. Acetylcholine-stimulated relaxation via endothelium also increased after 4 hours by 36.85%. Metformin also increases passive tension generated by the aortic vessel wall after 4 hours.

Therefore metformin can act directly in the vascular wall to alter vascular contractility via effects on both vascular smooth muscle and endothelium.

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