Is glutathione an important neuroprotective effector molecule against amyloid beta toxicity?

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Abstract
Cellular thiols are critical moieties in signal transduction, regulation of gene expression, and ultimately are determinants of specific protein activity. Whilst protein bound thiols are the critical effector molecules, low molecular weight thiols, such as glutathione, play a central role in cytoprotection through (1) direct consumption of oxidants, (2) regeneration of protein thiols and (3) export of glutathione containing mixed disulphides.
The brain is particularly vulnerable to oxidative stress, as it consumes 20% of oxygen load, contains high concentrations of polyunsaturated fatty acids and iron in certain regions, and expresses low concentrations of enzymic antioxidants. There is substantial evidence for a role for oxidative stress in neurodegenerative disease, where excitotoxic, redox cycling and mitochondrial dysfunction have been postulated to contribute to the enhanced oxidative load. Others have suggested that loss of important trophic factors may underlie neurodegeneration. However, the two are not mutually exclusive; using cell based model systems, low molecular weight antioxidants have been shown to play an important neuroprotective role in vitro, where neurotrophic factors have been suggested to modulate glutathione levels.
Glutathione levels are regulated by substrate availability, synthetic enzyme and metabolic enzyme activity, and by the presence of other antioxidants, which according to the redox potential, consume or regenerate GSH from its oxidised partner.
Therefore we have investigated the hypothesis that amyloid beta neurotoxicity is mediated by reactive oxygen species, where trophic factor cytoprotection against oxidative stress is achieved through regulation of glutathione levels. Using PC12 cells as a model system, amyloid beta 25-35 caused a shift in DCF fluorescence after four hours in culture. This fluorescence shift was attenuated by both desferioxamine and NGF. After four hours, cellular glutathione levels were depleted by as much as 75%, however, 24 hours following oxidant exposure, glutathione concentration was restored to twice the concentration seen in controls. NGF prevented both the loss of viability seen after 24 hours amyloid beta treatment and also protected glutathione levels. NGF decreased the total cellular glutathione concentration but did not affect expression of GCS.
In conclusion, loss of glutathione precedes cell death in PC12 cells. However, at sublethal doses the surviving fraction respond to oxidative stress by increasing glutathione levels, where this is achieved, at least in part, at the gene level through upregulation of GCS. Whilst NGF does protect against oxidative toxicity, this is not achieved through upregulation of GCS or glutathione.

Introduction

Increasing age is the most reliable and robust risk factor for susceptibility to neurodegenerative disease (Bains & Shaw, 1997). Lovell *et al.,* (1995) showed support for the concept that the brain in Alzheimer's disease (AD) is under increased oxidative stress (OS) demonstrating lipid peroxidation changes in areas where degenerative changes occur. Further evidence suggesting that the pathogenesis of AD is as a result of oxidative damage includes elevated levels of iron in AD brains and a colocalised reduction in antioxidant status (Good *et al.,* 1996). Whilst the exact mechanisms underlying oxidative stress remain unclear, it has been proposed that the peptidergic fragment of amyloid beta (Aβ) that accumulates in AD may exert its toxicity through peroxide generation (Huang *et al.,* 1999).

Glutathione is arguably the most important AOX and free radical scavenger present in cells (Valencia *et al.*, 2001), where glutathione-associated metabolism is a major mechanism for cellular protection against agents that generate OS. Glutathione participates in detoxification at several different levels, and may scavenge reactive oxygen species (ROS), reduce peroxides, or be conjugated with electrophilic compounds. Thus, glutathione provides the cell with multiple defences not only against ROS but also against their toxic products. Most importantly, many of the glutathione-dependent proteins are inducible and therefore represent a means whereby cells can adapt to OS (Hayes & McLellan, 1999). The GSH redox status is critical for various biological events that include transcriptional activation of specific genes, modulation of redox-regulated signal transduction, regulation of cell proliferation, apoptosis, and inflammation (Rahman & MacNee, 2000). In addition, it has been shown previously that GSH levels decrease following addition of cytotoxic agents, and at the time of onset of apoptosis (van den Dobbelsteen *et al.,* 1996; Froissard & Duval, 1994).

The intracellular synthesis of GSH is mainly regulated by gamma glutamyl cysteinyl synthetase (γ-GCS) (Richman & Meister, 1975). Differences or alterations in the levels of protein can occur by a number of different mechanisms including alterations in the level of gene transcription and alterations in the stability or translatability of the resulting RNA. The expression of γ-GCS is sensitive to OS, where the existence of the OS-response element, AP-1, on the γ-GCSh (g-GCS heavy subunit) promoter has been clarified (Mulcahy *et al.,*1997).

Maintenance of a high intracellular (GSH)/(GSSG) ratio (>90%) minimises the accumulation of disulfides and provides a reducing environment within the cell. If there is a shift in the GSH/GSSG redox buffer, a variety of cellular signalling processes are influenced, such as activation and phosphorylation of stress kinases (JNK, p38, PI-3K) via sensitive cysteine-rich domains; activation of sphingomyelinase-ceramide pathway, and activation of the transcription factors AP-1 and NF-κB, eventually leading to increased gene transcription (Rahman & MacNee, 2000).

Froissard *et al.,* (1997) have reported that there is strong evidence to support the existence of a close relationship between the level of intracellular

glutathione and cell survival in PC12 cells, where mitochondrial GSH is critical for the maintenance of mitochondrial function and cellular viability (Seyfried *et al.,* (1999). However, whether a drop in GSH levels precede intracellular ROS production during apoptosis or vice versa is not certain. Hence any role of GSH depletion alone in triggering apoptosis is not clear (Hall, 1999).

Evidence is accumulating for an important role for glutathione in detoxification processes in the brain. Depletion of glutathione in newborn rats using buthionine sulfoximine (BSO) leads to mitochondrial damage in the brain (Jain et al , 1991). Furthermore, application of beta amyloid peptide, glutamate receptor agonists and BSO cause GSH depletion in cultured neurones and lead to induction of apoptosis. H_2O_2 has previously been suggested to mediate Aβ cytotoxicity based on antioxidant inhibition of toxicity and demonstration of lipid peroxidation in treated cells (Behl *et al.,* 1994b). The toxic effects of several other ROS generating compounds has been shown to be abrogated by neurotrophic factors such as BDNF, NGF, GDNF and bFGF (Cheng and Mattson, 1995; Chao and Lee). It has been postulated that this may be due to upregulation of the concentration of glutathione and/or the activities of antioxidant enzymes.

Therefore we have investigated the hypothesis that Aβ neurotoxicity is mediated by reactive oxygen species, where trophic factor cytoprotection against oxidative stress is achieved through regulation of glutathione levels at the gene level. Herein, we demonstrate that Aβ toxicity is associated with alterations in cellular redox status, and whilst NGF affords protection against toxicity, this is independent of GCS expression.

Methods

Maintenance of PC12 cell line

PC12 cells were routinely cultured in $75cm^2$ flasks in a water jacketed humidified 5% CO_2 incubator with maintenance media. (RPMI 1640 media with Glutamax I; 10% (v/v) HS, 5% (v/v) FBS, penicillin (0.5U/ml) and streptomycin (0.5mg/ml). Every 2-3 days, spent PC12 cell media was removed and replaced with fresh maintenance media, which had been pre-warmed to 37°C. Cells were passaged once a week, as described in section 2.2.1.3, when the cells were at a density of approximately 5×10^6 per ml. For experimental purposes, cells were seeded at 2×10^5 cells per ml and allowed to rest for 4 hours prior to treatment for times and doses indicated.

Determination of cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay

Cells (2×10^5 cells per ml, 100µl per well) were seeded into 96 well flat bottomed microtitre plates. Two hours prior to completion of the experiment, MTT solution (25µl of 5mg/ml in 0.01M PBS) was added to all wells including blanks. Plates were then incubated for a further 2 hours at 37°C, 5% CO_2. Lysis buffer (100µl of 20% w/v SDS, in DMF (50%), dH_2O(50%), pH 4.7 adjusted with 2.5% of 80% glacial acetic acid) was then added to each well

and the plates incubated for a further 16 hours at 37°C, 5% CO_2. The absorbance of the each well was then read at 570nm in a 96 well plate reader.

Dichlorofluorescein diacetate (DCFDA) staining

Cells (2 x 10^5 per ml, 2ml) were incubated with test agents in 35mm well plates. 30 minutes before the end of incubation, DCFDA solution (20µl of 7.5mM diluted stock in PBS) was added to the plates. After exactly 30 minutes cells were immediately harvested with a Gilson pipette and a rubber policeman and transferred to a flow cytometry tube. Samples were then immediately run through a Coulter EPICS flow cytometer with the intensity of light scatter and the FL1 fluorescence emitted between 505 and 535nm following excitation at 488nm (argon laser) was recorded for individual nucleoids. The median X was calculated for 10,000 nucleoid events.

Glutathione (GSH) Assay

Glutathione (reduced and oxidised) levels were determined using modified microtitre plate method (Punchard *et al.,* 1994). Actual glutathione levels were calculated by equating the changes in absorbance over time alongside standards of known GSH concentrations included on each plate. Glutathione (GSH), reduced standards were freshly prepared for each experiment from a stock (100mM in sterile distilled water). Cells (2x10^5) were then harvested and PBS (1ml) added to wash the pellet. Tubes were recentrifuged at 6600 x g for 1.5 minutes and the PBS was then removed, making sure to leave the pellet dry. SSA (3.33µl of 1% made up in distilled water) was then added to precipitate the protein and the tubes were immediately centrifuged at 13000 x g for 1.5 minutes. Stock buffer (96.6µl of 125mM sodium phosphate, 6.3mM disodium EDTA, pH 7.5 autoclaved) was then added to each tube. Standards and samples (25µl) were then aliquoted into a flat bottomed plates (96 well) in triplicate, the remaining aliquot was immediately frozen at -70°C for later use in the GSSG assay. Daily buffer (150µl of 0.3mg NADPH/ml stock buffer) and DTNB solution (50µl of 6mM DTNB in daily buffer) was then added to all wells. The plate was then loaded onto the carriage of a 96 well plate reader and glutathione reductase solution (25µl of 20U/ml) was then added as quickly as possible to initiate the reaction. The OD was then recorded every minute for 5 minutes at 410nm.

Glutathione (GSSG) Assay

Oxidised glutathione (GSSG) standards were freshly prepared for each experiment from an oxidised glutathione stock (1.5µM in sterile distilled water).

The remaining 25µl aliquot from the GSH assay was employed for this assay. To the frozen aliquot and standards (25µl), 2-vinylpyridine (2-VP), triethanolamine (0.5µl) were added. Samples and standards were then vortexed and centrifuged for 30 seconds at 13000 x g. Aliquots of standards and samples (10µl) were then plated out into a microtitre plate (96 well plate). Daily buffer (75µl of 0.3mg NADPH/ml stock buffer) and DTNB solution (25µl of 6mM DTNB in daily buffer) were added to the wells. The plate was then loaded onto the carriage of a 96 well plate reader and glutathione reductase solution (12.5µl of 20U/ml) was then added as quickly as possible to initiate the reaction. The OD was then recorded every minute for 5 minutes at 410nm.

Estimation of Protein Concentration

Protein concentrations were assessed according to a modified version of Sigma Procedure No. TPRO-562, based on the method of Smith *et al,* (1985). Protein standards of six concentrations were prepared from the protein standard solution (bovine serum albumin, 1mg/ml) diluted in coating buffer (15mM sodium carbonate, 35mM sodium hydrogen carbonate, pH 9.2). Each standard (10µl) was plated out in triplicate into a 96 well flat bottomed microtitre plate. Unknown samples (10µl) were also plated out in triplicate. Bicinchoninic acid solution (200µl) containing copper (II) sulphate pentahydrate 4% solution (50:1) was then added to all wells (samples and standards), the plate was then incubated at 37°C for 30 minutes. The absorbance of the plate was then measured at 570nm in a 96 well plate reader. Unknowns were calculated from a standard curve prepared using the software package GraphPad PRISM.

mRNA EXTRACTION

Cells ($2x10^5$) were harvested into a RT grade centrifuge tube (1.5ml). Cells were centrifuged for 1.5minutes at 13000 x g in an Eppendorf Centrifuge 5415D. Supernatants were removed and PBS (1ml) added, cells were centrifuged for another 1.5minutes at 13000 x g and the PBS removed. The extraction was carried out using the Dynal mRNA direct kit protocol. Pellets were resuspended into 200µl Dynal lysis/binding buffer (100mM Tris-HCl, pH 7.5, 500mM LiCl, 10mM EDTA, pH8.0, 1% LiDS and 5mM DTT) and aspirated twenty times to fully resuspend each pellet with fresh tips being used for each tube. The solution was then aspirated five times using 1ml sterile syringes and sterile 21 gauge needles to shear the DNA. This was then repeated using 1ml sterile syringes and sterile 25 gauge needles. Tubes were then centrifuged for 1 minute at 13000 x g in an Eppendorf Centrifuge 5415D. Resuspended Dynal Oligo $(dT)_{25}$ beads (30µl) were washed in Dynal lysis/binding buffer twice before being added to each tube of lysed cells. The tubes were then mixed on a Dynal sample mixer for 5 minutes at 22-25°C. Tubes were then magnetised on the Dynal MPC®-E magnet (Magnetic particle concentrator for microtubes of Eppendorf type (1.5ml)) and the colourless solution removed. Dynal wash buffer A (200µl of 10mM Tris-HCl, pH 7.5, 0.15M LiCl, 1mM EDTA, 0.1% LiDS) was then added to the pellet and the cells resuspended in it. The tubes were then remagnetised the colourless solution removed and Dynal wash buffer A added (200µl) and the cells resuspended. The process was then repeated using Dynal wash buffer B (200µl of 10mM Tris-HCl, pH 7.5, 0.15M LiCl, 1mM EDTA). After two washes with wash buffer B the cell pellet was resuspended in DEPC treated water (30µl of 0.1%). This was then transferred to a fresh tube (1.5ml), and master mix was added (39µl of 10mM DTT (7.5µl of 100mM), 1mM dNTPs (7.5µl dNTP mix), 25U RNAsin (1.8µl), 1U (3µl) RQ1 RNase-free DNase, 0.1% DEPC treated water (4.2µl) and Expand buffer (15µl)). Each tube was aspirated twice and incubated at 37°C for 60 minutes. DNase was heat inactivated at 70°C for 10 minutes in a preheated dry heating block. Samples were then centrifuged at 13000 x g for 1 minute in an Eppendorf centrifuge 5415D.

Reverse transcription of mRNA

Two RT grade PCR tubes were labelled for each sample, one positive and one negative. RNAsin 15U (1µl) was added to the negative tubes and 30U (2µl) to the positive tubes, mRNA extraction product (46µl) was added to the positive

tubes and mRNA extraction product (23µl) was added to the negative tubes. Expand RT 50U, (1µl) was then added to the positive tubes. For reverse transcription mRNA was incubated at 37°C for 1 hour in Expand RT buffer containing 10mM DTT, 1mM dNTPs, 25U RNAsin, 1U RQ1 RNase-free DNase. DNase was heat inactivated at 70°C for 10 minutes. The samples were then incubated at 42°C for 1 hour before being stored in the short term at 0 - 4°C, and at -20°C in the long term.

Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and GCS polymerase chain reaction (PCR)

The primers (50nmol of each) as stated in table 2.4 were synthesised by Gibco/BRL, Life Technologies, Paisley, Scotland. Each primer was diluted to 100 pmol/µl in sterile 1 x TE buffer (10mM Tris, 1mM EDTA, pH 8.0), and then further diluted tenfold in 1 x TE buffer and stored as 10µl aliquots at -20°C.

Primer Sequences

Primer	**Forward primer sequence (5' – 3')**	**Reverse primer sequence (5'-3')**	**Reference**
GAPDH	AGA ACA TCA TCC CTG CCT C	GCC AAA TTC GTT GTC ATA CC	Hall et al., 1998
GCS	CCT TCT GGC ACA GCA CGT TG	TAA GAC GGC ATC TCG CTC CT	El Mouatassium et al., 2000

PCR was performed in PCR buffer with sterile water, 200mM dNTPs and 10pmol of each primer. All reactions were covered in a drop of mineral oil and hot start conditions were used, full details of each PCR as stated in table 2.5. Reactions were initiated with 2.5U (0.5µl) Taq DNA Polymerase. All liquid handling was conducted using filter tips.

Optimal PCR Conditions

PRIMER	CONDITIONS	CYCLES
GAPDH	98°C 3 minutes + Taq 60°C 2 minutes 72°C 2 minutes ………………………………… 94°C 30 seconds 60°C 30 seconds 72°C 30 seconds ………………………………… 94°C 30 seconds 60°C 30 seconds 72°C 4 minutes	1 cycle 26 cycles 1 cycle
GCS	94°C 1 minute 94°C 45 seconds + Taq 56°C 1 minute 72°C 1 minute ………………………………… 94°C 45 seconds 56°C 1minute 72°C 1minute ………………………………… 72°C 10 minutes	1 cycle 31 cycles 1 cycle

Agarose gel electrophoresis of PCR products

Gel loading solution (2.5µl of 0.05% w/v bromophenol blue, 40% w/v sucrose, 0.1M EDTA, pH 8.0) was added to the PCR reaction products (10µl). Samples were loaded onto 1.5% agarose gels made up in 1 x TBE (89mM Tris, 89mM boric acid, 2.5mM EDTA, pH 8.0, 100ml Gel). Gels were electrophoresed at 80V for 2 hours in 350ml 1 x TBE. Post staining was achieved with ethidium bromide (10µl of a 10mg/ml solution) dissolved into 100ml of distilled water for 15 minutes in the dark at 22 - 25°C. Gels were visualised on an U.V. transilluminator table at 312nm. Expression levels were quantitated using the software package Phoretix 1D Advanced, Version 4.01 (Non Linear Dynamic Limited) and normalised to GAPDH. Gels were all loaded with a size ladder, usually a 123bp DNA ladder prepared according to manufacturer's instructions.

Results

It has been proposed that Aβ may exert its toxicity through peroxide generation. In order to confirm whether generation of ROS occurs in PC12 cells and whether these levels are modulated by exogenous agents, the effects of Aβ on intracellular peroxide levels were investigated in PC12 in the presence and absence of NGF. PC12 cells were incubated with NGF (5 and 10ng/ml) for 24 hours before addition of Aβ (25μM) and the fluorochrome DCFDA. Cells were harvested and immediately analysed through a flow cytometer. Exposure of PC12 cells to Aβ 25-35 (10μM) caused an increase in intracellular peroxide which was evident after 4 hours. Production of intracellular peroxide was inhibited by either desferioxamine or NGF (10ng/ml), where NGF had no effect on basal cellular peroxide in control cells (see Figure 1). Pre-treatment with 5ng/ml NGF prior to Aβ elicited a decrease in the median X value for DCF fluorescence to 0.300, indicative of a decrease in intracellular peroxide concentration (data not shown).

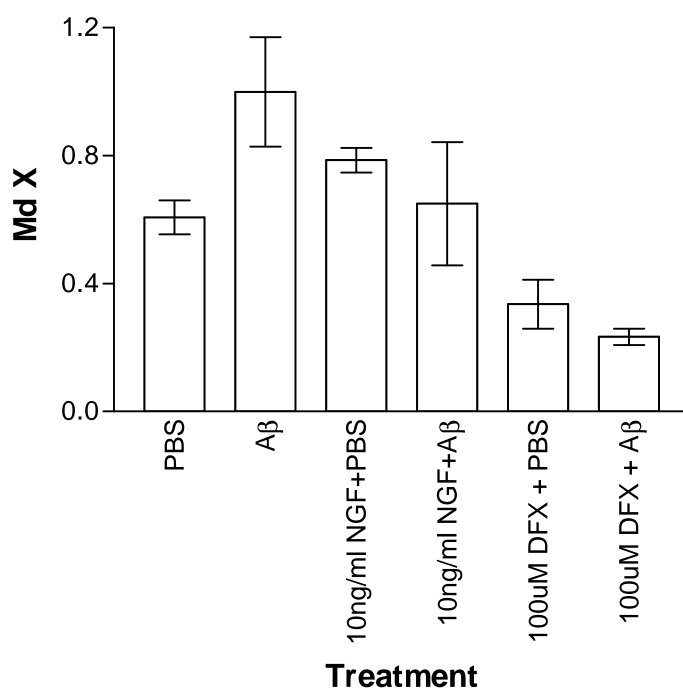


Figure 1. PC12 cells (2x10^5) were incubated for 4 hours at 37°C, 5%CO_2 before the addition of NGF (10ng/ml) or DFX (100uM) for 24 hours at 37°C, 5%CO_2. Aβ (25μM) was then added for 4 hours at 37°C, 5%CO_2. For the final 30 minutes of incubation, DCFDA (75μM) was added. Cells were then harvested and analysed by flow cytometry. Figure shows the average median X ± SEM.

In order to establish whether intracellular ROS production was associated with a reduction in cell viability, MTT reductive capacity was investigated after exposure to Aβ or H_2O_2. In the absence of NGF, Aβ (10μM) caused a 25% reduction in MTT reductive capacity after 24 hours treatment, and this effects was not enhanced by higher peptide concentrations. Much higher concentrations of hydrogen peroxide were required to elicit the same loss of

viability; after 24hours treatment with 150μM H_2O_2, MTT reductive capacity was reduced by 23% (Figure 2a and 2b).

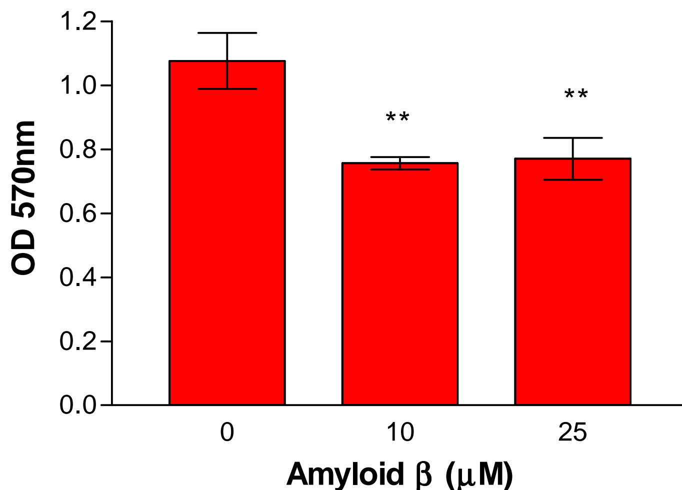


Figure 2a The effects of amyloid β (25-35) toxicity on PC12 cells. PC12 cells ($2x10^5$) were incubated with aged Aβ (10, and 25μM) for 24 hours at 37°C, 5% CO_2. Cells were then subject to the MTT assay. Graph shows the mean absorbance of formazan produced ± SEM for at least 3 replicates. ** represents P<0.01 (Dunnett's test).

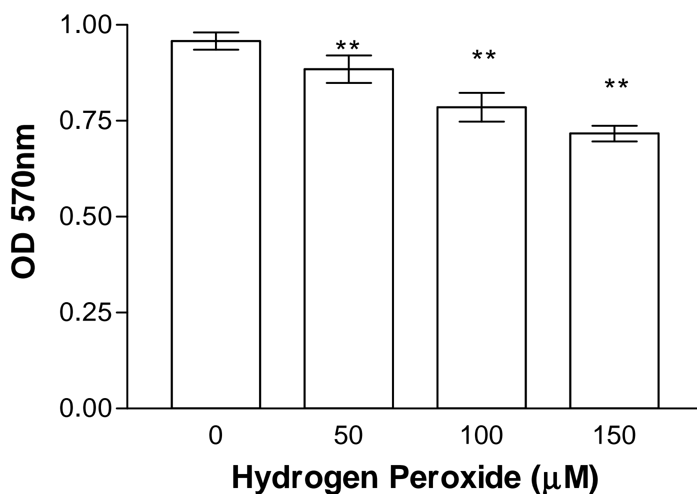


Figure 2b The effect of hydrogen peroxide on PC12 cell viability using the MTT assay. PC12 cells (2×10^5) were incubated for 4 hours at 37°C, 5% CO_2 before the addition of H_2O_2 (50, 100, and 150μM) for 24 hours at 37°C, 5% CO_2. Cells were then subject to the MTT assay. Data shown are the mean absorbance of formazan produced ± SEM of 6 replicates for each treatment. ** represents P values of <0.01 (Dunnett's test).

Previously, others have shown that NGF is an effective cytoprotectant against oxidative toxicity arising from glutamate or dopamine. However, this has not been shown for hydrogen peroxide or amyloid beta. Figure 3 demonstrates that NGF affords protection to PC12 cells against peroxide toxicity, when cells have been previously exposed to 5 or 10ng/ml NGF prior to oxidant challenge. Higher doses of NGF (50ng/ml) exerted a cytotoxic effect (data not shown).

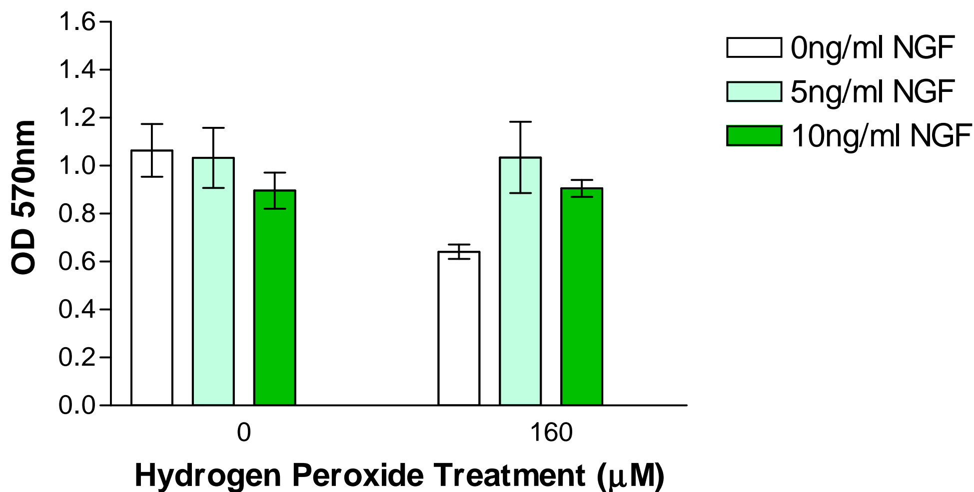


Figure 3 The effects of nerve growth factor on PC12 cell viability in the presence or absence of H_2O_2. PC12 cells (2 x 10^5) were incubated with NGF (5 and 10ng/ml) for 24 hours at 37°C, 5% CO_2. H_2O_2 (150µM) was then added for a further 24 hours at 37°C, 5% CO_2. Cells were then subject to the standard MTT assay. Graph shows the mean absorbance of formazan produced ± SEM for at least 14 replicates.

Glutathione participates in detoxification at several different levels, and can scavenge ROS and reduce peroxides. To determine whether intracellular protection is compromised by oxidative challenge, and to determine the kinetics of glutathione metabolism during 24hours of oxidative stress treatment elicited by either hydrogen peroxide or Aβ, total cellular glutathione was measured after 1, 4 and 24 hours. Figure 4 illustrates the rapid loss of intracellular GSH following addition of hydrogen peroxide to PC12 cells in culture, where levels reduced by 50% within one hour. In contrast, Aβ did not affect GSH levels until four hours after treatment. Nevertheless, Aβ treatments did cause a twofold increase in total intracellular glutathione in the surviving cell population at 24 hours ($p<0.05$), where peroxide induced a highly significant fourfold increase in GSH ($P<0.001$). Again after 24 hours peroxide treatment (160uM), there was a ten fold increase oxidised glutathione, but the concentration of GSSG only rose from 0.02% to 0.05% of total glutathione (data not shown).

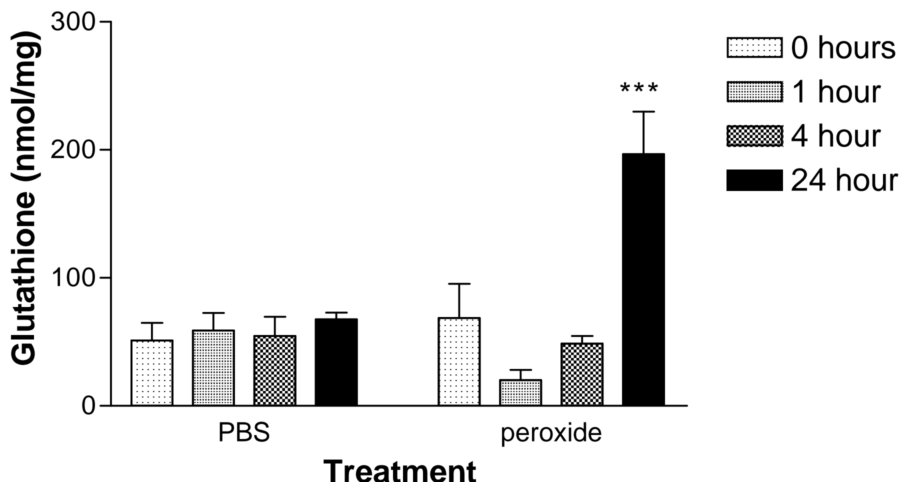


Figure 4. Kinetics of hydrogen peroxide effects on glutathione levels in PC12 cells. PC12 cells (2x105) were incubated with H_2O_2 (150 μM) for 0-24 hours at 37°C, 5%CO_2. Figure shows the mean glutathione value ± SEM of 3 replicates. * represents P<0.005 (Bon Feronni test)**

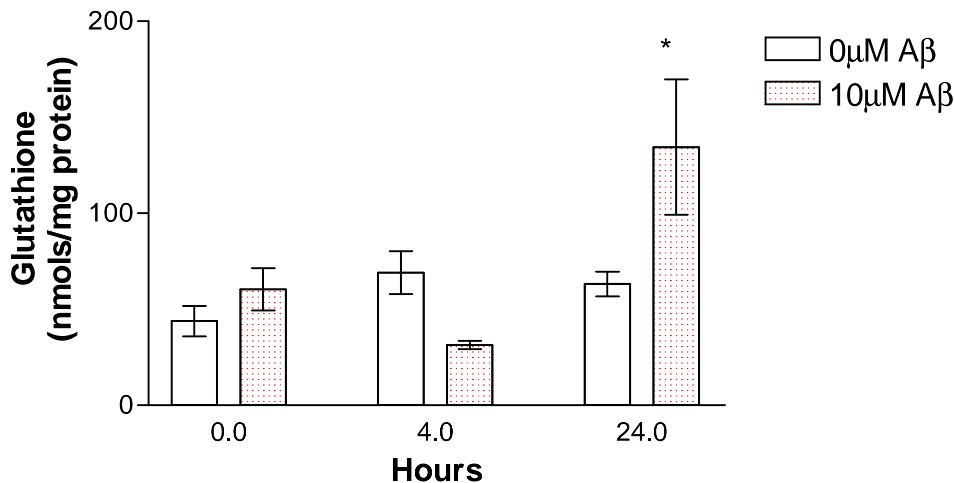
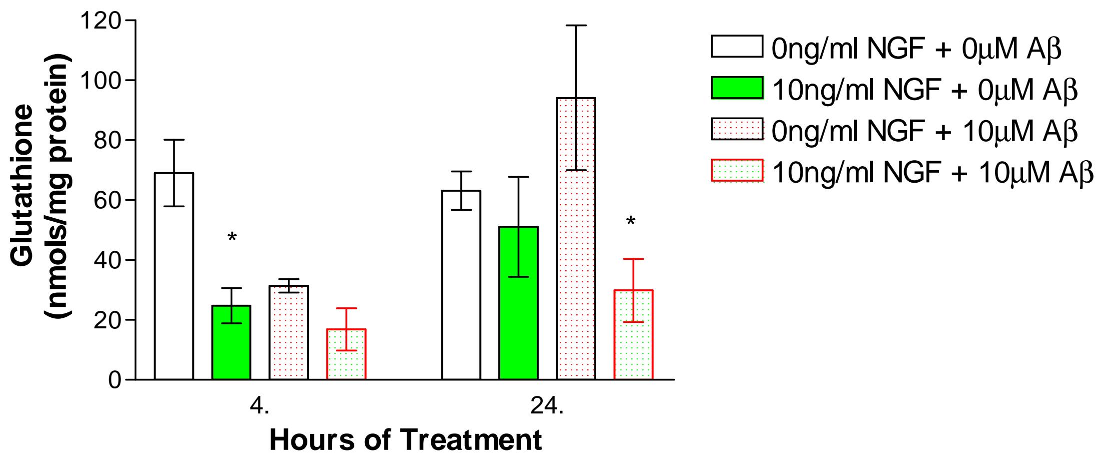


Figure 5. The effects of amyloid β peptide on glutathione levels in PC12 cells. PC12 cells ($2x10^5$) were incubated with $A\beta_{25-35}$ (10μM) for 0-24 hours at 37°C, 5% CO_2. Figure shows the mean glutathione value ± SEM of at least 4 replicates. * represents P<0.05 (Dunnett's test)

In order to determine whether part of the cytoprotective effects of NGF could be attributed to up-regulation of glutathione, the effects of NGF in the presence and absence of oxidant challenge on glutathione levels and on expression of GCS mRNA were examined. Figure 6 shows NGF to prevent the increase in GSH levels observed post- exposure to Aβ, where 10ng/ml NGF significantly reduced intracellular glutathione concentration after 4 and 24 hours respectively.

Figure 6a The effects of nerve growth factor on amyloid β peptide induced changes to glutathione levels in PC12 cells. PC12 cells ($2x10^5$) were incubated with NGF (10ng/ml) for 24 hours at 37°C, 5% CO_2 before incubation with $A\beta_{25-35}$ (10μM) for 0-24 hours at 37°C, 5% CO_2. Figure shows the mean glutathione value ± SEM of at least 3 replicates. * represents P<0.05 (Unpaired t-test)



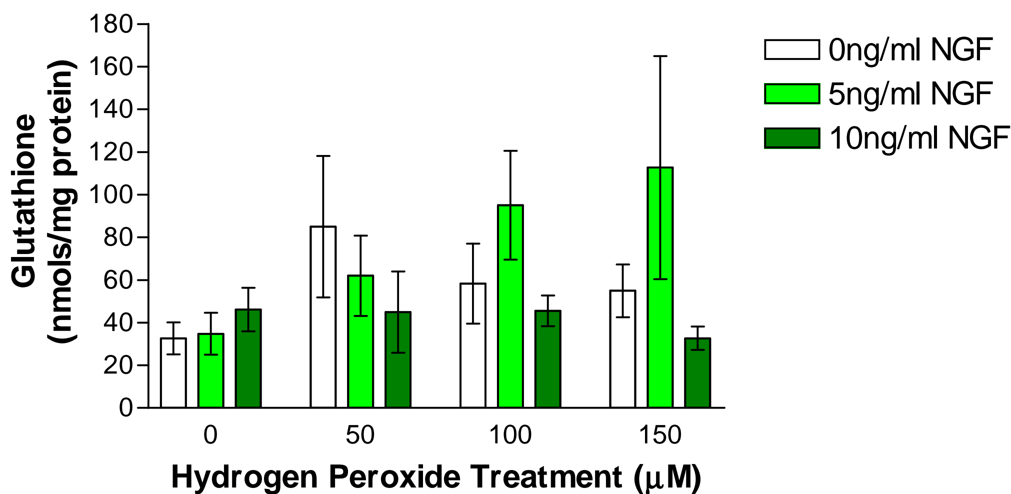
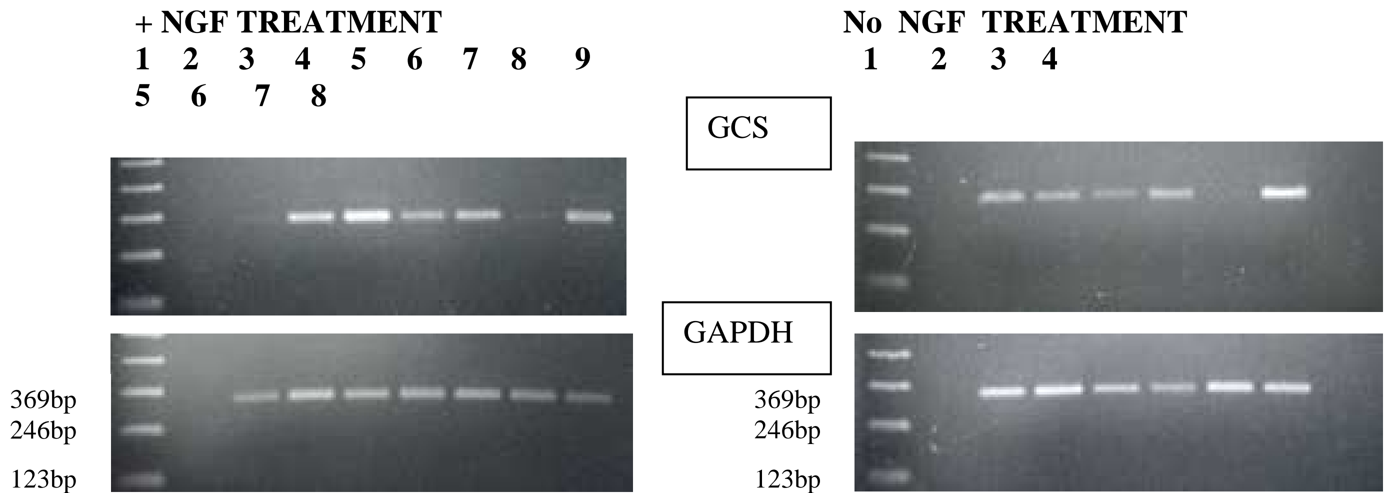


Figure 6b The effects of nerve growth factor on the action of hydrogen peroxide on glutathione levels in PC12 cells. PC12 cells ($2x10^5$) were incubated with NGF (5 and 10ng/ml) for 24 hours at 37°C, 5%CO_2 before addition of H_2O_2 (50, 100, 150µM) at 37°C, 5% CO for 24 hours$_2$. Figure shows the mean glutathione value ± SEM of 3 replicates.

Analysis of GCS mRNA expression in PC12 cells exposed to oxidative stress in the presence or absence of NGF is shown in figure 7. In the absence of peroxide challenge, NGF elicited an increase in GCS mRNA expression after two hours in PC12 cells, whereas mRNA levels were essentially unchanged in the absence of NGF. However, when PC12 cells were subject to an oxidative challenge for 4 hours after 24hours pre-incubation with vehicle or NGF, cells showed reduced expression of GCS mRNA irrespective of the presence or absence of NGF. Furthermore, 24hours after oxidative challenge in the presence in NGF, GCS mRNA expression was restored to that seen prior to oxidative challenge. In contrast, PC12 cells subject to oxidative challenge in the absence of NGF showed up-regulation of GCS expression 24 hours post-oxidative challenge, where image analysis using phoretix software demonstrated a twelve-fold increase in expression.

Figure 7. 1.5% Agarose gels of PCR products. PC12 cells ($2x10^5$) were incubated with NGF (10ng/ml) or PBS as control for 0-24 hours at 37°C, 5%CO_2. H_2O_2 (150μM) was then added for 4 and 24 hours at 37°C, 5%CO_2 and cells were harvested and mRNA extracted. Folowing reverse transcription specific PCRs were underatkaen for GAPDH and GCS.



Lane	Treatment	Lane	Treatment
1	123bp ladder	1	123 bp ladder
2	Blank	2	Blank
3	O hr NGF	3	1 hour PBS
4	1 hr NGF	4	2 hr PBS
5	2hr NGF	5	4 hr PBS
6	4 hr NGF	6	24 hr PBS
7	24 hr NGF	7	24hr PBS + 4 hr H_2O_2
8	24hr NGF+ 4hr H_2O_2	8	24 hr PBS + 24 hr H_2O_2
9	24hr NGF+24hr H_2O_2	9	

Discussion

The levels of intracellular peroxide can be quantitated using the nonfluorescent fluorochrome 2',7'-dichlorofluorescin diacetate (DCFDA) which is freely permeable to cells. It is de-esterified within cells by endogenous esterases to 2',7'-dichlorofluorescein (DCF). This becomes trapped within cells and accumulated DCF is able to interact with peroxides and hydroperoxides in the cell to form the fluorescent compound 2',7'-dichlorofluorescein (Behl *et al.,*1994a; Zhu *et al.,* 1994). There is an important caveat to the successful use of DCF as a peroxide sensor, and that it is also a suitable substrate for direct oxidation by xanthine oxidase, where inhibition by allopurinol can be used to determine any role of the latter.

DCF staining has previously been shown to increase with ROS production (Satoh *et al.,* 1997). Addition of H_2O_2 increases intracellular peroxide in PC12 cells (data not shown); this is in agreement with the work of other groups (Jang & Surh, 2001). Herein, we have shown that Aβ increases intracellular peroxides. The inclusion of the iron chelator DFX suggests this process is iron dependent, and suggests that Aβ cannot exert its toxicity without the availability of iron. These observations support the work of Monji *et al.,* (2002) who have demonstrated that Aβ (25-35)-associated free radical generation is iron dependent and strongly influenced by the aggregational state of the peptides.

Whilst NGF pre-treatment in unchallenged cells did not cause any significant lowering of intracellular peroxide levels in PC12 cells, it was effective in preventing the elevation in cytoplasmic peroxide observed following amyloid beta challenge. Previously trophic factors have been shown to protect cells against ROS toxicity in cultured neurones and PC12 cells, however these papers did not measure intracellular ROS directly (Chang and Mattson; 1995; Gong et al, 1999). In addition, these earlier studies have investigated oxidative stressors implicated in the pathogenesis of Parkinson's disease. Amyloid beta is implicated in cholinergic neurone loss in AD, and in our system, we have demonstrated the generation of ROS precedes cell death. Again, in our model, the toxic effects of amyloid beta were prevented by NGF.

It has been postulated that the cytoprotective effect of trophic factors may be dependent on the upregulation of glutathione and/or enzymes associated with ROS defence. Examination of the effects of ROS on cellular glutathione confirmed the observations of others, that GSH is rapidly consumed, but then recovers to offer enhanced protection against further insults. OS appears to be a key pleiotropic modulator of gene transcription acting through the generation of bioactive mediators (Morel & Barouki, 1999). The number of transcription factors whose activities are modulated by ROS is substantial, and include AP-1, p53, NF-κB, HSF, Sp-1 and GABP (Kehrer, 2000). In this respect, ROS appear to be actual modulators of gene transcription, independently of the degradation of biological macromolecules (Morel & Barouki, 1999). Therefore if ROS can induce an adaptive response, for example though induction of GCS, offering greater protection of the cell exposed to subsequent OS.

In this paper, we report that NGF (10ng/ml) did not cause any significant changes in GSH levels of unchallenged cells; this is in agreement with Kamata *et al.,* (1996) who showed NGF to protect cells from OS independently of GSH. However under H_2O_2 and Aβ challenge, increased levels of GSH were recorded versus control after 24 hours, with NGF (10ng/ml) preventing the elevation induced by oxidative stress. The data recorded with 5ng/ml NGF is consistent with the data previously published by Pan & Perez-Polo (1993) and Sampath *et al.,* (1994) who have both shown NGF to cause increases in GSH levels. GSSG levels were also assessed where H_2O_2 to caused increased GSSG concentration after challenge with 160uM peroxide. Nevertheless, it is evident from this data that much of the GSH is lost particularly at early time points, either through mixed disulphide formation with proteins or through efflux. The drop in GSH may therefore reflect an inhibition of synthesis, a deficit in the GSH salvage pathway, or an increased rate of efflux (Chandra *et al.,* 2000). In both scenarios, efficient removal of ROS and formation of GSSG is dependent on expression and activity of GPX, which in itself is redox sensitive, and the activity of the enzyme under oxidative stress. Specifically, the effect of NGF merits further investigation, since infusion of GDNF into the brain has been shown to increase the activity of GPx (Chao and Lee, 1999)

Yonezawa *et al.,* (1996) have shown that deprivation of one of the precursors of glutathione (cysteine) induces glutathione depletion and death in cultures of cells, but that rescue by free radical scavenger and a diffusible glial factor was not associated with a restoration of normal glutathione content. These authors thus suggest that the protecting agents act distal to glutathione. This is in agreement with Kamata *et al.,* (1996) who showed when cellular GSH was depleted by treatment with BSO, NGF still protected cells. These data largely support the hypothesis that NGF exerts its protection independently of GSH. NGF (10ng/ml) does not to cause any significant increases in glutathione levels, however, does protect against intracellular ROS and also cell death. To further clarify whether NGF exerts its protection via glutathione up regulation, studies on GCS expression were undertaken.

Cell cross-talk between glia and neurones appears critical to maintainence of neuronal viabililty. Glia conditioned medium has been shown to contain an unidentified diffusible factor which enhances resistance to oxidative stress by increasing transcription of GCS (Iwata-Ichikawa, 1999). In order to determine whether NGF can elicit a similar response in PC12 cells, we have evaluated GCS expression in PC12 cells, in relation to glyceraldehydes 3-phosphate expression (GAPDH). The glycolytic tetramer GAPDH/G3PDH is a multifunctional enzyme involved in cellular metabolism. It is an important glycolytic enzyme that catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, and it is the most widely used internal control gene (Suzuki *et al.,* 2000). Measurement of GAPDH transcript abundance was originally selected as a normalizer for other expressions because it encodes for a protein with a housekeeping function. As it is a glycolytic intermediate it is therefore expected to be present in all cells and exhibit minimal modulation. However GAPDH gene expression has been reported to increase during other non-glycolytic activities such as programmed neuronal cell death (Ishitani *et al.,* 1996).

During investigations into selective GCS gene expression, GAPDH expression remained constant. GCS expression was more variable in NGF treated cells compared to non-NGF treated cells. The only marked differences in non NGF treated cells were after 4 hours of peroxide challenge where expression was virtually nothing and after 24 hours of peroxide challenge where there was the greatest expression. In NGF treated cells GCS levels initially increased up to 2 hours after the NGF treatment then decreased. After 4 hours of peroxide challenge GCS levels were also greatly reduced showing the least expression, expression then increased after 24 hours of peroxide challenge. Tubulin is frequently used as a housekeeping gene for neuronal studies, however, transcription of this species is itself affected by the onset of apoptosis, rendering it a poor control gene.

One of the most variable factors appears to lie in the variation in GSH levels within resting cells. Indeed, GSH levels have also been shown to decrease in culture imposing the need for consistent experimental conditions (Reiners Jr *et al.,* 2000), including passage frequency and seeding density.

There is a poor correlation between mRNA and protein levels, as the presence of RNA in a cell is not necessarily indicative of peptide synthesis. Structurally authentic mRNAs may be poorly handled by the translational machinery in a cell. Even the efficient translation of a functional message may not result in biologically active peptide if post translational processes are absent, or if the peptide is rapidly degraded. The analysis of GCS gene expression must therefore extend to the description of the protein end-points of the expression pathway, included in this must be a consideration of protein function (Carter & Murphy, 1999).

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