

ROLE OF PHOSPHATIDYLINOSITOL 3-KINASE AND Akt IN THE INDUCTION
OF PROTEASOME EXPRESSION BY PROTEOLYSIS-INDUCING FACTOR
IN MURINE MYOTUBES

Stacey M. Wyke, Steven T. Russell, Helen L. Eley and Michael J. Tisdale*

Biomedical Sciences, Life and Health Sciences,
Aston University, Birmingham, B4 7ET, UK

Keywords: Proteasome expression; muscle; proteolysis-inducing factor;
phosphatidylinositol 3-kinase; Akt / PKB

*Correspondence: M.J. Tisdale, Life and Health Sciences, Aston University,
Birmingham, B4 7ET, UK; E-mail: m.j.tisdale@aston.ac.uk

Both proteolysis-inducing factor (PIF) and angiotensin II (Ang II) directly induce protein degradation in skeletal muscle through an increase in level of the ubiquitin-proteasome pathway, which involves activation of the nuclear transcription factor NF- κ B. The role of the serine / threonine kinase, Akt / PKB, in this process has been studied in murine myotubes expressing a dominant negative mutant of Akt (DNAkt), or a constitutively active Akt construct (Myr Akt). Both PIF and Ang II induced protein degradation in wild-type myotubes with a parabolic dose-response curve, while myotubes containing Myr Akt showed an enhanced response to both agents, and those containing DNAkt showed a reduced protein degradation. A similar effect was observed on the induction of chymotrypsin-like enzyme activity, as a measure of the 20S proteasome functional activity as well as expression of 20S proteasome α -subunits and the 19S subunit p42, as well as the loss of myosin. PIF induced phosphorylation of Akt at Ser⁴⁷³, and this was attenuated by the phosphoinositide 3-kinase (PI3-K) inhibitor LY294002, suggesting that it was due to activation of PI3-K. PIF induced an increase in inhibitor- κ B (I- κ B) phosphorylation, a reciprocal decrease in cellular levels of I- κ B, and an increased nuclear accumulation of NF- κ B, and these effects were also attenuated by LY294002. These results suggest that PIF induces activation of Akt through induction of PI3-K activity, and that this provides an alternative route for an increased nuclear binding of NF- κ B, through phosphorylation and subsequent degradation of I- κ B, resulting in an increased

protein degradation. Thus Akt may play a role in protein degradation in the presence of catabolic stimuli in addition to its role in protein synthesis in muscle.

INTRODUCTION

The ubiquitin-proteasome proteolytic pathway has been shown to play a major role in muscle wasting in starvation, sepsis, metabolic acidosis, diabetes, weightlessness, severe trauma, denervation and cancer cachexia [1]. This pathway can be activated by a number of factors, including proteolysis-inducing factor (PIF), a sulphated glycoprotein produced by cachexia-inducing tumours [2], cytokines such as tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) [3], angiotensin II (Ang II) [4] and glucocorticoids [5], while disuse atrophy results from an increase in oxidative stress [6]. Induction of protein degradation through the ubiquitin-proteasome pathway by PIF [7], TNF- α [8], Ang II [9] and reactive-oxygen species (ROS) [10] involves activation of the nuclear transcription factor NF- κ B. Activation of NF- κ B through muscle-specific transgenic expression of activated I κ B kinase β (IKK β) has been shown [11] to induce profound muscle wasting with an increase in mRNA levels for the C2 and C9 subunits of the proteasome, as well as for MuRF1, a muscle-specific E3 ligase, which is upregulated during atrophy, and loss of which provides partial protection of muscle loss after denervation [12]. Interestingly mRNA levels for another E3 ligase atrogin-1 / MAFbx, also implicated in muscle protein degradation [13], were normal, as were those for ubiquitin and the ubiquitin-conjugating enzyme E2_{14k}. Induction of atrogin-1 has been shown to be mediated by the Forkhead box O (Foxo) class of transcription factors [14].

Studies on the intracellular signal transduction pathways involved in activation of NF- κ B have mainly been performed using PIF and Ang II in murine

myotubes. Both PIF [15] and Ang II [9] have been shown to activate protein kinase C (PKC), and this was shown to be important in the induction of proteasome expression and NF- κ B activation, since myotubes transfected with a dominant-negative PKC α , showed no increase in nuclear binding of NF- κ B in response to PIF and no increase in proteasome expression [15]. In addition, calphostin C, a highly specific inhibitor of PKC attenuated phosphorylation and degradation of I κ B by both Ang II [9] and by 15-hydroxyeicosatetraenoic acid (15-HETE) [16] considered to be an essential signalling molecule in proteasome upregulation by PIF [17]. These effects of PKC are thought to be mediated by phosphorylation and activation of the upstream regulator kinase IKK β [18].

An alternative pathway for phosphorylation of IKK, which is utilized by both TNF- α [19] and platelet derived growth factor (PDGF) [20], involves the serine / threonine kinase Akt / PKB. Oxidative stress has also been shown to activate Akt, by increasing phosphorylation [21]. Activation of Akt by growth factor and cytokine treatment generally occurs via the phosphatidylinositide 3-kinase (PI3-K) pathway. However, IGF-1 induces hypertrophy by stimulating the PI3-K / Akt pathway [22], and constitutive activation of Akt induces rapid and significant skeletal muscle hypertrophy [23]. The hypertrophic effect is due to stimulation of translation, via regulation of glycogen synthelase kinase GSK and mTOR kinases [24]. Akt overexpression inhibits the Foxo transcription factors by increasing phosphorylation, inhibiting atrogen-1 expression [14].

In order to investigate the role, if any, of Akt in protein degradation we have transfected myotubes with plasmids expressing a dominant-negative

mutant of Akt, as well as a constitutively active Akt construct, and studied the effect on both protein degradation and proteasome activity in response to both PIF and Ang II. Using an inhibitor of PI3-K the effects have been associated with the state of activation of NF- κ B.

MATERIALS AND METHODS

Foetal calf serum (FCS), horse serum (HS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies (Paisley, UK). Mouse monoclonal antibody to I- κ B α was purchased from Biomol Research Laboratories Inc. (PA, USA) and to HA.11 from Cambridge Bioscience Ltd (Cambridge, UK). Mouse monoclonal antibodies to 20S proteasome α -subunits and p42 were from Affiniti Research Products (Exeter, UK). Mouse monoclonal antibody to myosin heavy chain was from Novocastra (Newcastle, UK). Rabbit polyclonal antisera to phosphorylated I- κ B α was from Merck Eurolab Ltd. (Leicestershire, UK) and polyclonal antisera to Akt that detects endogenous Akt1, Akt2 and Akt3 proteins, together with mouse monoclonal antibody to phospho-Akt (Ser 473) were from New England Biolabs (Herts, UK). Rabbit polyclonal antisera to mouse actin was from Sigma-Aldridge (Dorset, UK). Peroxidase-conjugated rabbit anti-mouse antibody and peroxidase-conjugated goat anti-rabbit antibody were purchased from Dako Ltd. (Cambridge, UK). Hybond A nitrocellulose membranes, L-[2, 6- 3 H] phenylalanine (sp. act. 2.07 TBq/mmol) and enhanced chemiluminescence (ECL) development kits were from Amersham Bioscience UK Ltd. (Bucks, UK). LY294002 was purchased from Calbiochem (through CN Biosciences, UK, Nottingham, UK). Lactacystin was purchased from Affiniti Research Products (Exeter, UK) and the chymotrypsin substrate succinyl-Leu-Leu-Val-7-amino-4-methylcoumarin (AMC) from Sigma-Aldridge (Dorset, UK). Expression vectors (pcDNA) encoding mouse Akt proteins fused in-frame to the hemagglutinin (HA) epitope [23] were kindly supplied by Dr.

Kenneth Walsh (Tufts University, School of Medicine, Boston, USA). Catch and Release V.2.0 reversible immunoprecipitation system was purchased from Upstate (Milton Keynes, Bucks, UK). This system was used to immunoprecipitate Akt from transfected myotubes using the HA.11 monoclonal antibody according to the manufacturer's instructions.

Cell culture The C₂C₁₂ myoblast cell line was grown in DMEM supplemented with 10% FCS plus 1% penicillin and streptomycin under an atmosphere of 5% CO₂ in air at 37°C. Cells were transfected with the plasmid DNA in OPTI-medium using Lipofect Amine (Gibco) at a ratio of 1:5 and incubated at 37°C for 18-48h prior to testing for transgene expression. Cells were passaged (1:10) in fresh growth medium for 24h and selected for growth in neomycin for 48-72h. Myotubes were formed by allowing confluent cultures of myoblasts to fuse in DMEM containing 2% HS over a 5-7 day period, with changes of medium every 2 days.

Purification of PIF PIF was purified from solid MAC16 tumours from mice with weight loss between 20 and 25%. The tumour homogenate was precipitated with ammonium sulphate (40% w/v), and the supernatant subjected to chromatography using a monoclonal antibody immobilized to a protein A matrix as described [25]. The immunogenic fractions were concentrated and used for studies without further purification, since the major contaminant was albumin [25].

Measurement of protein degradation The method for the measurement of protein degradation in murine myotubes has been described [2]. Briefly myotubes were prelabeled for 24h with L-[2, 6-³H] phenylalanine and were washed extensively prior to experimentation. Protein degradation was determined by the release of L-[2, 6-³H] phenylalanine into the medium after 24h incubation with various concentrations of PIF, Ang I or Ang II as depicted in the figure legends in the absence or presence of inhibitors added 2h before the agonists. Cold phenylalanine was added to prevent reincorporation of radioactivity into cells.

Measurement of proteasome 'chymotrypsin-like' activity 'Chymotrypsin-like' enzyme activity was determined fluorimetrically by the method of Orino et al [26] as previously described [7]. Myotubes were washed with ice-cold phosphate buffered saline (PBS) and sonicated in 20mM Tris HCl, pH 7.5, 2mM ATP, 5mM MgCl₂ and 1mM dithiothreitol at 4°C. The supernatant formed by centrifugation at 18,000g for 10min was used to measure 'chymotrypsin-like' enzyme activity by the release of aminomethyl coumarin (AMC) from the fluorogenic peptide succinyl-LLVY-AMC (0.1mM). Activity was measured in the presence and absence of the specific proteasome inhibitor lactacystin (10µM). Only lactacystin suppressible activity was considered to be proteasome specific.

Measurement of protein synthesis Myotubes were formed in 6-well multiwell dishes, and were supplemented with DMEM without HS and phenol red

18h prior to experimentation. PIF was added at the concentrations indicated followed by 2 μ l L-[2, 6-³H] phenylalanine (sp.act. 1.96TBq / mmol) in 8 μ l sterile PBS and the plates were incubated for 4h at 37°C under an atmosphere of 5% CO₂ in air. The reaction was arrested by washing three-times with 1ml ice-cold sterile PBS. Following removal of the PBS 1ml ice-cold 0.2M perchloric acid was added and the plates were kept at 4°C for 20min. The perchloric acid was substituted with 1ml 0.3M NaOH per well and incubation was continued for 30min at 4°C, followed by a further incubation at 37°C for a further 20min. The NaOH extract was removed and combined with a further 1ml wash of each well and 0.5ml 0.2M perchloric acid was added and left on ice for 20min. The extract was then centrifuged at 700g for 5min at 4°C and the protein-containing pellet was dissolved in 1ml of 0.3M NaOH and 0.5ml of the solution was counted for radioactivity after mixing with 8ml Ultima Gold XR scintillation fluid.

Western blot analysis Cytoplasmic proteins (5-15 μ g), obtained from the above assay, were also used for Western blotting I- κ B α for 20S proteasome α -subunits, p42, myosin, and phospho-I- κ B α , while the total cell extract (30 μ g protein) was used for Western blotting for Akt and phospho-Akt. 5 μ g of immunoprecipitated protein using HA.11 was used for immunoblotting for Akt. Proteins were resolved on 12% sodium dodecylsulphate: polyacrylamide gels and transferred to HybondTM nitrocellulose membrane. Membranes were blocked with 5% Marvel in PBS at 4°C overnight. The primary antibodies were used at a dilution of 1:1000, except for β -tubulin which was used at 1:40, and the

secondary antibodies were used at a dilution of 1:2000. Incubation was carried out for 2h at room temperature, and development was by ECL. Total cellular actin was used as a loading control. Blots were scanned by a densitometer to quantitate differences.

Electrophoresis mobility shift assay (EMSA) DNA binding proteins were extracted from myotubes by the method of Andrews and Faller [27], which utilizes hypotonic lysis followed by high salt extraction of nuclei. The EMSA binding assay was carried out using a Panomics EMSA 'gel shift' kit according to the manufacturer's instructions.

Statistical analysis Differences in means between groups was determined by one-way ANOVA followed by Tukey-Kramer Multiple Comparison Test. All experiments were repeated at least 3 times on separate occasions and the results shown are an average of the repeats.

RESULTS

Murine myotubes were transfected with plasmids encoding a dominant negative Akt (DNAkt) in which the two activating amino acid residues threonine 308 and serine 473 were changed to alanine [24], or a constitutively active Akt construct (Myr Akt), containing the c-src myristoylation sequence fused in-frame to the N-terminus of the HA-Akt (wild-type) coding sequence. Immunoprecipitation of cell lysates with anti-HA antibody, followed by Western blotting for Akt confirmed expression of both dominant negative and constitutively active Akt (Fig. 1A). When the myotubes were incubated with PIF protein degradation was increased with a maximal effect at 4.2nM as previously reported [15, 16] (Fig. 1B). Myotubes containing Myr Akt showed enhanced protein degradation over wild-type at all concentrations of PIF, while those containing DNAkt showed a reduced protein degradation at the maximum activating concentration of PIF. A similar result was obtained with both Ang I (Fig. 1C) and Ang II (Fig. 1D). The effect of Ang I was attenuated by co-treatment with the ACE inhibitor imidaprilat (50 μ M), suggesting that the stimulation of protein degradation was due to formation of Ang II. Both Ang I and Ang II induced protein degradation with a parabolic dose-response curve, similar to PIF, as described [4], with maximal effects as 0.05 and 0.5 μ M respectively. Protein degradation was significantly enhanced in myotubes expressing constitutively active Akt, while protein degradation was completely attenuated in myotubes expressing dominant negative Akt.

As anticipated myotubes expressing Myr Akt showed a significant increase in protein synthesis over wild-type cells (Fig. 2), while myotubes expressing DNAkt showed a significant reduction in protein synthesis. Myotubes exposed to PIF showed a reduction in protein synthesis with a maximal effect at 4.2nM (Fig. 2) as for the effect on protein degradation (Fig. 1B). However, there was no difference in the relative inhibition of protein synthesis in wild-type myotubes or in those expressing Myr Akt or DNAkt. This suggests that Akt is unable to reverse the depression in protein synthesis by PIF.

The effect on total protein degradation was mirrored by the effect on the 'chymotrypsin-like' enzyme activity, the predominant proteolytic activity of the proteasome. Thus proteolytic activity in the presence of PIF (Fig. 3A), Ang I (Fig. 3B) Ang II (Fig. 3C) was elevated in myotubes expressing Myr Akt over wild-type, while there was no increase in activity in myotubes expressing DN-Akt. In addition expression of both 20S proteasome α -subunits (Fig. 4A) and p42, an ATPase subunit of the 19S regulator (Fig. 4B) in the presence of Ang II showed a greater enhancement in myotubes expressing Myr-Akt over wild-type, while there was no increase in expression in myotubes expressing DN-Akt. Levels of myosin were also significantly reduced more in myotubes expressing Myr-Akt than in wild-type (Fig. 4C), while there was no decrease in myosin in the presence of Ang II, when the myotubes expressed DN-Akt. Thus instead of protecting myofibrillar proteins from degradation in the presence of catabolic stimuli, Akt appears to be essential for the degradative process and for induction of proteasome activity.

To investigate the mechanism of this effect myotubes were incubated with PIF and the effect on Akt phosphorylation at Ser⁴⁷³ was determined by immunoblotting. PIF increased Akt phosphorylation 2.5-fold within 30min of addition (Fig. 5A), with no effect on the total Akt in the cell (Fig. 5B). The maximal effect was seen at 4.2nM PIF, as with protein degradation (Fig.1B) and 'chymotrypsin-like' enzyme activity (Fig. 3A). The effect of PIF on Akt phosphorylation was completely inhibited by co-treatment with a 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), an inhibitor of PI3-K [28], suggesting that Akt activation occurs through a similar mechanism to growth factors and cytokines. The effect of PIF on the chymotrypsin-like enzyme activity was also attenuated by LY294002 (Fig. 6A) suggesting that PI3-K is also important in the induction of proteasome activity. LY294002 also attenuated the induction of proteasome expression by 15-HETE, considered to be a major signal transducer molecule in the induction of proteasome activity by PIF [17] (Fig. 6B). These results suggest that activation of PI3-K by PIF occurs downstream of the formation of 15-HETE.

To investigate the possibility that activated Akt was responsible for the phosphorylation and the consequent degradation of I- κ B α , the effect of LY294002 on this process was determined. PIF induced an increase in I- κ B α phosphorylation (Fig. 7A) and a reciprocal decrease in the cytosolic level of I- κ B α in myotubes (Fig. 7B), and this was completely attenuated by LY294002. These results suggest that activation of Akt by PIF results in the phosphorylation and subsequent degradation of I- κ B α . The decrease in cytosolic I- κ B α induced

by 15(S)-HETE was also attenuated by LY294002 (Fig. 7D). Degradation of I- κ B α should result in an increased nuclear accumulation of NF- κ B and the EMSA is shown in Fig. 8. Thus PIF induced an increased nuclear binding of NF- κ B, with a similar dose-response curve to that for the induction of the degradation of I- κ B α (Fig. 7B), and this effect was also completely attenuated by LY294002. Thus activation of PI3-K by PIF results in the activation of NF- κ B.

Activation of Akt inhibits the ability of tuberous sclerosis complex 2 (TSC2) to act as a Rheb-GTPase activating protein (GAP), allowing Rheb-GTP levels to rise causing activation of the mammalian target of rapamycin (mTOR). This has two primary downstream targets, the ribosomal S6 kinase (p70S6K) and the eukaryotic initiation factor 4E (e1F-4E) binding protein, phosphorylation of which leads to accelerated protein synthesis [29]. To determine whether this pathway was important in protein degradation myotubes were treated with the mTOR inhibitor rapamycin (25ng/ml), either before, or at various times after the addition of Ang II (Fig 9). Addition of rapamycin, either before, or up to 1h after addition of Ang II, completely attenuated the induction of protein degradation, whereas addition at later times had no effect. These results suggest that the PI3-K/Akt/mTOR pathway may be important for the synthesis of proteasome subunits, ubiquitin and ubiquitin ligase (E3) involved in the degradation of myofibrillar proteins.

DISCUSSION

The results of this study suggest that contrary to expectations, Akt is required for the induction of protein degradation through the ubiquitin-proteasome pathway by PIF and by Ang I and II. The mechanism of this effect appears to be through increased phosphorylation and subsequent degradation of I- κ B α , leading to increased nuclear accumulation of NF- κ B, which has been shown to act as a transcription factor for the induction of proteasome expression by both PIF [7] and Ang II [8]. Activation of Akt appears to occur through the PI3-K pathway, which is known to be activated by receptor and non-receptor tyrosine kinase [30]. This may be a potential mechanism by which PIF could activate PI3-K, since the tyrosine kinase inhibitors genistein and tryphostin 23 have been shown to attenuate PIF-induced proteasome expression [31]. Akt has previously only been considered to be involved in muscle hypertrophy and inhibition of Akt either by inhibition of PI3-K, or expression of a dominant negative Akt reduces the mean size of myotubes in culture [22]. Certainly in this study expression of DN-Akt significantly reduced protein synthesis compared with wild-type myotubes, whereas expression of constitutively active Akt slightly increased protein synthesis, although it did not protect against the fall in protein synthesis in the presence of PIF. Activation of Akt would be expected to inhibit atrogin-1 expression through phosphorylation and inactivation of Foxo transcription factors [14]. However, muscle atrophy can occur in the absence of an increased expression of this E3 ligase, since levels were normal in muscles of mice with activated IKK β , despite profound muscle wasting [11]. Instead

expression of MURF1 was increased, suggesting that it is not necessary to have increased expression of both E3 ligases to support an increased protein degradation.

Activation of Akt appears to be necessary not only for the activation of the transcription factor NF- κ B, leading to increased mRNA levels for proteasome subunits and MuRF1 [11], but also for the subsequent protein synthesis, possibly through the mTOR/p70S6K pathway. Rapamycin, a specific inhibitor of mTOR attenuated the increased protein degradation induced by Ang II when added before or up to 1h after the agonist suggesting a need for the mTOR pathway. Thus Akt may play a dual role in the activation of the ubiquitin-proteasome pathway.

Activation of Akt may provide an alternative pathway from PKC for the phosphorylation and degradation of I- κ B α and the nuclear accumulation of NF- κ B. Alternatively there may be some cross-talk between the pathways, since PKC-dependent Akt phosphorylation has been reported in JB6 [32] and MCF-7 cells [33], although in A549 and HEK293 cells PI-3K / Akt signalling is regulated by PKC in a negative manner [34]. Studies on interleukin-1 (IL-1)-induced cell proliferation have also suggested that the PLC-PKC cascade is required for the activation of Erk and Akt signalling [35]. Phosphorylation of other substrates in the muscle cell by PKC or Akt may influence the overall outcome of the stimulus. It is evident that even stimulation of the same pathway may lead to different outcomes. Thus PI3-K has been shown to activate IKK α , IKK β and NF- κ B-inducing kinase (NIK) by IGF II in the IGF-II dependent myoblast differentiation

process [36]. There may also be differences in the sequence of events. Thus LY294002 has been shown to specifically inhibit degradation of I- κ B α in RAW264.7 cells, stimulated with interferon- γ (IFN- γ), inhibiting the activation of NF- κ B [37]. Other results have suggested that NF- κ B is required for TNF- α -mediated Akt activation and that this lies upstream of the stimulation of Akt [38].

The results of the present study confirm the importance of NF- κ B activation in the stimulation of protein degradation in murine myotubes and provide evidence for an alternative role for Akt in this process. Akt may play a dual role in controlling muscle size, depending on the prevailing conditions.

Acknowledgments

This work has been supported by grants from Ark Therapeutics Ltd (S.T.R) and Novartis Medical Nutrition (H.L.E).

References

- [1] S.H. Lecker, V. Solomon, W.E. Mitch, A.L. Goldberg, Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *J. Nutr.* 129 (1999) 227S-237S.

- [2] M.J. Lorite, H.J. Smith, J.A. Arnold, A. Morris, M.G. Thompson, M.J. Tisdale, Activation of ATP-ubiquitin-dependent proteolysis in skeletal muscle in vivo and murine myoblasts in vitro by a proteolysis-inducing factor (PIF). *Br. J. Cancer* 85 (2001) 297-302.

- [3] M. Llovera, C. Garcia-Martinez, N. Agell, F.J. Lopez-Soriano, J.M. Argiles, TNF can directly induce the expression of ubiquitin-dependent proteolytic system in rat soleus muscle. *Biochem. Biophys. Res. Commun.* 230 (1997) 238-241.

- [4] P.M. Sanders, S.T. Russell, M.J. Tisdale, Angiotensin II directly induces muscle protein catabolism through the ubiquitin-proteasome proteolytic pathway and may play a role in cancer cachexia. *Br. J. Cancer* 93 (2005) 425-434.

- [5] S.S. Wing, A.L. Goldberg, Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. *Am. J. Physiol.* 264 (1993) E668-E676.

- [6] J.M. Lawler, W. Song, S.R. Demaree, Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle. *Free Rad. Biol. Med.* 35 (2003) 9-16.
- [7] S.M. Wyke, M.J. Tisdale, NF- κ B mediates proteolysis-inducing factor induced protein degradation and expression of the ubiquitin-proteasome system in skeletal muscle. *Br. J. Cancer* 92 (2005) 711-721.
- [8] Y-P. Li, M.B. Reid, NF- κ B mediates the protein loss induced by TNF- α in differentiated skeletal muscle myotubes. *Am. J. Physiol.* 279 (2000) R1165-R1170.
- [9] S.T. Russell, S.M. Wyke, M.J. Tisdale, Mechanism of induction of muscle protein degradation by angiotensin II. *Cell. Sig.* in press.
- [10] Y-P. Li, R.J. Schwartz, I.D. Waddell, B.R. Holloway, M.B. Reid, Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF- κ B activation in response to tumor necrosis factor α . *FASEB J* 12 (1998) 871-880.
- [11] D. Cai, J.D. Frantz, N.E. Tawa Jr., P.A. Melendez, B-C. Oh, H.G.W. Lidov, P-O. Hasselgren, W.R. Frontera, J. Lee, D.J. Glass, S.E. Shoelson, IKK β -

- NF- κ B activation causes severe muscle wasting in mice. *Cell* 119 (2004) 285-298.
- [12] S.C Bodine, E. Latres, S. Baumhueter, V.K-M. Lai, L. Nunez, B.A. Clarke, W.T. Poueymirou, F.J. Panaro, E. Na, K. Dharmarajan, Z-Q. Pan, D.M. Valenzuela, T.M. De Chiara, T.N. Stitt, G.D. Yancopoulos, D.J. Glass, Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294 (2001) 1704-1708.
- [13] M.D. Gomes, S.H. Lecker, R.T. Jagoe, A. Navon, A.L. Goldberg, Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc. Natl. Acad. Sci. (USA)* 98 (2001) 14440-14445.
- [14] M. Sandri, C. Sandri, A. Gilbert, C. Skurk, E. Calabria, A. Pichard, K. Walsh, S. Schiaffino, S.H. Lecker, A.L. Goldberg, Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* 117 (2004) 399-412.
- [15] H.J. Smith, S.M. Wyke, M.J. Tisdale, Role of protein kinase C and NF- κ B in proteolysis-inducing factor-induced proteasome expression in C₂C₁₂ myotubes. *Br. J. Cancer* 90 (2004) 1850-1857.

- [16] S.M. Wyke, J. Khal, M.J. Tisdale, Signalling pathways in the induction of proteasome expression by proteolysis-inducing factor in murine myotubes. *Cell. Signal.* 17 (2005) 67-75.
- [17] A.S. Whitehouse, J. Khal, M.J. Tisdale, Induction of protein catabolism in myotubes by 15(S)-hydroxyeicosatetraenoic acid through increased expression of the ubiquitin-proteasome pathway. *Br. J. Cancer* 89 (2003) 737-745.
- [18] A.C.O. Vertegaal, H.B. Kuiperji, S. Yamasoka, G. Courtois, A.J. van der Eb, A. Zantema, Protein kinase C- α is an upstream activator of the I κ B kinase complex in the TPA signal transduction pathway to NF- κ B in U20S cells. *Cell. Signal.* 12 (2000) 759-768.
- [19] M.E. Burrow, C.B. Weldon, L.I. Melnik, B.N. Duong, B.M. Collis-Burrow, B.S. Beckman, J.A. McLachlan, PI3-K / AKT regulation of NF- κ B signaling events in suppression of TNF-induced apoptosis. *Biochem. Biophys. Res. Commun.* 271 (2000) 342-345.
- [20] J.A. Romashkova, S.E. Makarov, NF- κ B is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 401 (1999) 86-90.

- [21] J.M. Taylor, P.J. Crack, J.A. Gould, U. Ali, P.J. Hertzog, R.C. Iannello, Akt phosphorylation and NF- κ B activation are counterregulated under conditions of oxidative stress. *Exp. Cell Res.* 300 (2004) 463-475.
- [22] C. Rommel, S.C. Bodine, B.A. Clarke, R. Rossman, L. Nunez, T.N. Stitt, G.D. Yancopoulos, D.J. Glass. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K / Akt / mTOR and PI(3)K / Akt / GSK3 pathways. *Nat. Cell Biol.* 3 (2001) 1009-1013.
- [23] K-M.V. Lai, M. Gonzalez, W.T. Poueymirou, W.O. Kline, E. Na, E. Zlotchenko, T.N. Stitt, A.N. Economides, G.D. Yancopoulos, D.J. Glass, Conditional activation of Akt in adult skeletal muscle induces rapid hypertrophy. *Mol. Cell. Biol.* 24 (2004) 9295-9304.
- [24] Y. Fujio, K. Walsh, Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J. Biol. Chem.* 274 (1999) 16349-16354.
- [25] P.T. Todorov, T.M. McDevitt, P. Cariuk, B. Coles, M. Deacon, M.J. Tisdale, Induction of muscle protein degradation and weight loss by a tumor product. *Cancer Res.* 56 (1996) 1256-1261.

- [26] E. Orino, K. Tanaka, T. Tamura, S. Sone, T. Ogura, A. Ichihara, ATP-dependent reversible association of proteasomes with multiple protein components to form 26S complexes that degrade ubiquitinated proteins in human HL-60 cells. *FEBS Lett.* 284 (1991) 206-210.
- [27] N.C. Andrews, D.V. Faller, A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* 19 (1991) 2499.
- [28] C. Vlahos, W. Matter, K. Hui, R. Brown, A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl) 8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269 (1994) 5241-5248.
- [29] D.J. Kwiatkowski, B.D. Manning, Tuberous sclerosis: a GAP at the crossroads of multiple signalling pathways. *Hum. Mol. Genet.* 14 (2005) R251-R258.
- [30] B. Vanhaesebroeck, S.J. Levers, K. Ahmadi, J. Timms, R. Katso, P.C. Driscoll, Synthesis and function of 3-phosphorylated inositol lipids. *Ann. Rev. Biochem.* 70 (2001) 535-602.

- [31] H.J. Smith, M.J. Tisdale, Signal transduction pathways involved in proteolysis-inducing factor induced proteasome expression in murine myotubes. *Br. J. cancer* 89 (2003) 1783-1788.
- [32] C. Huang, P.C. Schmid, W.Y. Ma, H.H. Schmid, Z. Dong. Phosphatidylinositol-3 kinase is necessary for 12-0-tetradecanoylphorbol-13-acetate-induced cell transformation and activated protein 1 activation. *J. Biol. Chem.* 272 (1997) 4187-4194.
- [33] M.E. Burow, C.B. Weldon, B.M. Collins-Burow, N. Ramsey, A. McKee, A. Kippel, J.A. McLachlan, C. Clejan, B.S. Beckman. Cross-talk between phosphatidylinositol-3 kinase and sphingomyelinase pathways as a mechanism for cell survival / death decisions. *J. Biol. Chem.* 275 (2000) 9628-9635.
- [34] H.C. Wen, W.C. Huang, A. Ali, J.R. Woodgett, W.W. Lin, Negative regulation of phosphatidylinositol 3-kinase and Akt signalling pathways by PKC. *Cell. Signal.* 15 (2002) 37-45.
- [35] A.R. Amin, Y. Ichigotani, M.L. Oo, M.H.U. Biswas, H. Yuan, P. Huang, N.N. Mon, M. Hamaguchi. The PLC-PKC cascade is required for IL-1 beta-dependent Erk and Akt activation: their role in proliferation. *Int. J. Oncol.* 23 (2003) 1727-1731.

- [36] J. Canico, P. Ruiz-Lozano, M. Carrasco, M. Palacin, K. Chien, A. Zorzano, P. Kaliman. Nuclear factor kappa B-inducing kinase and I kappa B kinase-alpha signal skeletal muscle cell differentiation. *J. Biol. Chem.* 276 (2001) 20228-20233
- [37] S.J. Park, S.C. Lee, S.H. Hong, H.M. Kim. Degradation of I κ B α in activated RAW264.7 cells is blocked by the phosphatidylinositol 3-kinase inhibitor LY294002. *Cell Biol Toxicol.* 18 (2002) 121-130.
- [38] F. Meng, L. Liu, P.C. Chin, S.R. D'Mello. Akt is a downstream target of NF- κ B. *J. Biol. Chem.* 277 (2002) 29674-29680.

Fig. 1 (A) Western blot of immunoprecipitated HA-tagged Akt in wild-type (lane 1), DNAkt (lane 2) and Myr Akt (lane 3) using rabbit polyclonal antisera to Akt. The lower band is the Ig light chain. (B) Effect of various concentrations of PIF (B), Ang I (C) or Ang II (D) on total protein degradation in murine myotubes containing wild-type Akt (Δ). Myr Akt (\blacklozenge) or DNAkt (\blacksquare). Differences from control are shown as a, $p < 0.05$, b, $p < 0.01$ or c, $p < 0.001$, while differences from wild-type are shown as d, $p < 0.05$, e, $p < 0.01$ or f, $p < 0.001$. All experiments were repeated at least 3-times.

Fig. 2 Effect of PIF on protein synthesis after 4h incubation in myotubes containing wild-type Akt (\blacksquare), Myr Akt (\blacklozenge) and DNAkt (\square). Differences from controls are indicated as a, $p < 0.05$, while differences from wild-type are shown as d, $p < 0.05$ or e, $p < 0.01$.

Fig. 3 The effect of PIF (A), Ang I (B) or Ang II (C) on the 20S proteasome chymotrypsin-like enzyme activity in wild-type Akt (Δ), Myr Akt (\blacklozenge) or DNAkt (\blacksquare) containing myotubes after 24h incubation. Differences from control are shown as a, $p < 0.05$ or c, $p < 0.001$, while differences from wild-type are indicated as e, $p < 0.01$ or f, $p < 0.001$.

Fig. 4 Expression of 20S proteasome α -subunits (A), p42 (B) and myosin (C) in myotubes containing wild-type Akt (\blacksquare), MyrAkt (\blacklozenge) and DNAkt (\square) in

response to Ang II. An actin loading control is shown in (D). A densitometric analysis representing the average from three blots is shown underneath. Differences from control are shown as c, $p < 0.001$, while differences from wild-type are shown as e, $p < 0.01$ or f, $p < 0.001$.

Fig. 5 (A) Western blots for the effect of PIF on phosphorylation of Akt and (B) for total cellular Akt. Myotubes were treated with for 30min in the absence or presence of 100 μ M LY294002 added 2h prior to PIF. The blots shown are representative of three separate experiments. The densitometric analysis is shown below the blot for the phospho Akt for myotubes in the absence (■) or presence (▣) of 100 μ M LY294002. Differences from control in the absence of PIF are shown as c, $p < 0.001$, while differences in the presence of LY294002 are indicated as f, $p < 0.001$. There was no difference in the level of total Akt in the cell for any of the treatments.

Fig. 6 (A) Effect of LY294002 (100 μ M) on the PIF-induced increase in 'chymotrypsin-like' enzyme activity in murine myotubes after 24h incubation. Myotubes were pre-incubated with LY294002 (■) or no-treatment (X) 2h prior to the addition of PIF. (B) Effect of LY294002 (100 μ M) on the 15(S)-HETE induced increase in 'chymotrypsin-like' enzyme activity in murine myotubes after 24h incubation. Myotubes were pre-incubated with LY294002 (■) or no-treatment (X) 2h prior to the addition of 15(S)-HETE. Differences from control are indicated as a,

p<0.05, b, p<0.01, or c, p<0.001, while differences in the presence of LY294002 are indicated as d, p<0.05 and f, p<0.001. Both experiments were repeated three times (n=9).

Fig. 7 Western blot for the effect of PIF on phospho I- κ B α (A), I- κ B α (B) and total cellular actin (C) determined 30min after addition of PIF. Myotubes were treated with 0 in the absence or presence of 100 μ M LY294002. The blots shown are representative of the three separate experiments. The densitometric analysis is shown below each blot for myotubes in the absence (■) or presence (▨) of LY294002. Differences from control are shown as a, p<0.05, b, p<0.01 or c, p<0.001, while differences in the presence of LY294002 are shown as d, p<0.05, e, p<0.01 or f, p<0.001. A Western blot for I- κ B α in myotubes 30min after treatment with 15(S)-HETE is shown in (D), while the actin loading control is shown in (E). Myotubes were treated for 30min with 0 15(S)-HETE in the absence or presence of 100 μ M LY294002. The densitometric analysis is the average of three replicate blots for myotubes in the absence (solid boxes) or presence (hatched boxes) of LY294002. Differences from control are indicated as c, p<0.001, while differences in the presence of LY294002 are shown as f, p<0.001.

Fig. 8 Effect of PIF on the nuclear translocation of NF- κ B in murine myotubes as determined by EMSA, in the absence (lanes 1-5) or presence (lanes 6-10)

of 100 μ M LY294002. Myotubes were treated for 30min with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 5 and 9) or 16.8nM PIF (lanes 5 and 10). Lane 11 is a positive control for NF- κ B and lane 12 is the positive control in the presence of a 100-fold excess of unlabelled NF- κ B probe. The densitometric analysis is an average of three replicate EMSA's. Differences from control are indicated as b, $p < 0.01$ and c, $p < 0.001$, while differences in the presence of LY294002 are shown as f, $p < 0.001$.

Fig. 9 Effect of Ang II on total protein degradation in murine myotubes in the absence (\blacklozenge) or presence of rapamycin (25ng/ml) added either before (Δ) (A) or at 30min(x), 1h(Δ), 2h(\blacktriangle) or 4h(\square) after Ang II (B). Differences from control are indicated as b, $p < 0.01$ and c, $p < 0.001$, while differences from Ang II alone are shown as f, $p < 0.001$.