SKELETAL MUSCLE ATROPHY: A LINK BETWEEN DEPRESSION OF PROTEIN SYNTHESIS AND INCREASE IN DEGRADATION

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Running title: Mechanism of induction of skeletal muscle atrophy
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Both proteolysis-inducing factor (PIF) and angiotensin II have been shown to produce a depression in protein synthesis in murine myotubes concomitant with an increased phosphorylation of eukaryotic initiation factor 2 (eIF2α). Both PIF and angiotensin II were shown to induce autophosphorylation of the RNA-dependent protein kinase (PKR), and an inhibitor of this enzyme completely attenuated the depression in protein synthesis, and prevented the induction of eIF2α phosphorylation. The PKR inhibitor also completely attenuated the increase in protein degradation induced by PIF and angiotensin II, and prevented the increase in proteasome expression and activity. To confirm these results myotubes were transfected with plasmids that express either wild-type PKR, or a catalytically inactive PKR variant, PKRΔ6. Myotubes expressing PKRΔ6 showed no increase in eIF2α phosphorylation in response to PIF or angiotensin II, no depression in protein synthesis and no increase in protein degradation or increase in proteasome expression. Induction of the ubiquitin-proteasome pathway by PIF and angiotensin II has been linked to activation of the transcription factor nuclear factor-κB (NF-κB). Inhibition of PKR prevented nuclear migration of NF-κB in response to both PIF and angiotensin II, by preventing degradation of the inhibitor protein I-κB. Phosphorylation of PKR and eIF2α was also significantly increased in the gastrocnemius muscle of weight losing mice bearing the MAC16 tumor, suggesting that a similar process may be operative in cancer cachexia. These results provide a link between the depression of protein synthesis in skeletal muscle and the increase in proteasome expression.

Muscle atrophy is a characteristic feature of cancer cachexia, and the degree of muscle loss has been correlated with the reduced survival rates of cancer patients (1). Muscle mass is determined by both the rates of protein synthesis and degradation. Emery et al. (2) have suggested that muscle mass in cancer cachexia is regulated primarily by alterations in the protein synthetic rate, while changes in protein degradation are largely secondary. However, both a reduced rate of protein synthesis and an increased rate of degradation were observed in muscle biopsies from newly diagnosed cancer patients with weight loss (3).

Changes in muscle protein synthesis and degradation rate in cancer cachexia probably arise from the presence of tumor-derived factors, such as proteolysis-inducing factor (PIF), or increased production of endogenous factors, such as cytokines or angiotensin II (Ang II). Using murine myotubes as a surrogate model of skeletal muscle both PIF and Ang II have been shown to inhibit protein synthesis (4, 5) and increase protein degradation (4, 6). Both agents stimulate protein degradation through an increased expression and activity of the ubiquitin-proteasome proteolytic pathway (7, 8), by a similar mechanism, involving activation of the nuclear transcription factor NF-κB (8, 9). For both PIF (7) and Ang II (8) protein degradation is inhibited by specific proteasome inhibitors, while mutations in IκBα, which prevent activation of NF-κB and the ubiquitin proteasome proteolytic pathway (9), also inhibit protein degradation by PIF, confirming a causal relationship. Much less is known about how these agents affect protein synthesis, although, with PIF at least, this has been suggested to arise from an effect on translation efficiency (4).

There are two points of control on initiation of protein translation, binding of the...
initiator methionyl-tRNA (met-tRNA) to the 40S ribosomal subunit, and binding of mRNA to the 43S ribosomal subunit. This study will concentrate on the first mechanism. Binding of met-tRNA to the ribosomal subunit occurs as a tertiary complex with eukaryotic initiation factor 2 (eIF2) and GTP (10). eIF2 is released in its GDP-bound state and to return to the GTP form the GDP is exchanged for GTP in a reaction catalysed by the guanine exchange factor eIF-2B (11). This recycling of GTP can be inhibited by phosphorylation of eIF2 on its α-subunit, sequestering eIF-2B with GDP, so that it is not available to catalyze nucleotide exchange on non-phosphorylated eIF2 (12). Mammalian cells possess four different eIF2α kinases, double-stranded RNA-dependent kinase (PKR), haeme-regulated inhibitor kinase (HRI), PERK and the yeast GCN2 (13). Each of these kinases respond to distinct stress conditions that affect transcription and protein synthesis. eIF2α phosphorylation has been shown to be central to activation of NF-κB (14), while PKR has been shown to activate I-κB kinase (IKK), leading to degradation of the inhibitors I-κBα and I-κBβ and the concomitant release of NF-κB (15). Thus eIF2α phosphorylation might provide a link between the depression of protein synthesis and the increase in protein degradation observed in skeletal muscle of cachectic subjects.

This study examines the mechanism for the depression of protein synthesis in skeletal muscle by both PIF and Ang II using murine myotubes as a surrogate model. It confirms the importance of eIF2α phosphorylation in the depression of protein synthesis by both agents and provides a mechanism for activation of the ubiquitin-proteasome proteolytic pathway and increased muscle protein degradation, through activation of NF-κB by a PKR-dependent process.

**EXPERIMENTAL PROCEDURES**

**Materials** - Fetal calf serum (FCS), horse serum (HS) and Dulbecco’s modified Eagle’s medium (DMEM) and Escherichia coli DH5α cells were purchased from Life Technologies (Paisley, United Kingdom). L-[2,6-3H] Phenylalanine (sp.act. 2.07TBq/mmol), hybond A nitrocellulose membranes and enhanced chemiluminescence (ECL) development kits were from Amersham Biosciences Ltd (Bucks, United Kingdom). Mouse monoclonal antibodies to 20S proteasome α-subunits and p42 were from Affiniti Research Products (Exeter, United Kingdom). Rabbit polyclonal antibody to total eIF2α was purchased from New England Biolabs (UK) (Herts, United Kingdom). Lipofectamine, rabbit monoclonal antibody to phospho-eIF2α (Ser 51), to phospho-PKR (Thr 446) and to total PKR (C-term) were purchased from Insight Biotechnology Ltd (London, United Kingdom). Mouse monoclonal antibody to myosin heavy chain was from Novocastra (Newcastle, United Kingdom) and to I-κBα was from Biornol Research Laboratories (PA, USA). Anti-PIF monoclonal antibody was produced as described (16). Rabbit polyclonal antisera to phosphorylated I-κBα and Phosphosafe™ Extraction Reagent were from Merck Eurolab Ltd (Leicestershire, United Kingdom), as was the PKR inhibitor and the eIF2α inhibitor, Salubrinal. Rabbit polyclonal antisera to mouse β-actin, angiotensin II, IGF1 the chymotrypsin substrate succinyl-Leu-Leu-Val-Tamino-4-methylcoumarin (AMC) and insulin were from Sigma Aldridge (Dorset, United Kingdom). Peroxidase-conjugated rabbit antibody to total PKR was produced using WIZARD Magnesil™ purification kit (Promega, Southampton, UK) according to the manufacturer’s instructions. The authenticity of the plasmids was confirmed by nucleotide sequence analysis (Birmingham University, UK).
Primers for PCR analysis were purchased from MWG Biotech (Ebersberg, Germany).

**Transformation of bacteria**—E-coli DH5α were transformed with pcDNA3 plasmids containing wild-type PKR and the catalytically inactive variant PKRA6, as well as empty vector using heat shock and selected with ampicillin (50µg/ml). PCR analysis was employed to confirm that the plasmids contained an insert of the correct size, and the authenticity of the inserts was confirmed by sequence analysis. Plasmid DNA was extracted from positive colonies grown overnight in LB media containing ampicillin (50µg/ml).

**Cell Culture and transfection**—C2C12 murine myoblasts were maintained in DMEM supplemented with 10% FCS, glutamine and 1% penicillin-streptomycin under an atmosphere of 10% CO2 in air at 37°C. When the myoblasts reached confluence they were allowed to fuse to form myotubes by replacing the propagation medium with DMEM containing 2% HS, with medium changes every 2 days. Differentiation was complete within 5-7 days and cells were used experimentally within 4 days of differentiation. Myotubes were maintained in unsupplemented DMEM without phenol red for 18h prior to experimentation. For transfection myoblasts were seeded at a density such that they reached 50-60% confluence the next day. Plasmid DNA (8µg) in 0.5ml reduced serum medium was mixed with 20µl of lipofectamine in 0.5ml medium and the DNA and lipofectamine were combined, mixed gently and incubated at room temperature for 20 min. The complex was added to myotubes in a 20cm2 flask containing 5ml media without antibiotics, and the cells were incubated for 24h before dilution and incubation for a further 24h in media without antibiotics. Transfected cells were selected for growth in neomycin (50µg/ml) for 48-72h. Myotubes were formed by allowing confluent cultures of myoblasts to fuse as above.

**Purification of PIF**—PIF was purified from solid MAC16 tumors excised from mice with a weight loss between 20 and 25% as previously described (16). Tumors were homogenised in 10mM Tris-HCl, pH 8.0, containing 0.5mM phenylmethylsulfonyl fluoride, 0.5mM EGTA and 1mM dithiothreitol at a concentration of 5mg/g tumor. Protein was precipitated by the addition of 40% (w/v) ammonium sulphate and the supernatant was subjected to affinity chromatography using anti-PIF monoclonal antibody (16) coupled to protein A on an Affi-Gel column. The immunogenic fractions were concentrated and used without further purification. The endotoxin content of the preparation was below the level of detection.

**Measurement of protein degradation**—Protein degradation in myotubes was determined as previously described (18) using six-well multiwell dishes. Cells were labeled by the addition of 74kBq of L-[2,6-3H] phenylalanine to each well and incorporation was maintained for 24h. The medium was removed and the tubes extensively washed followed by a 2h preincubation in DMEM without phenol red for 2h. For the experiment indicated cells were incubated with PIF or Ang II for 24h in the absence or presence of the PKR inhibitor added 2h prior to the agents, and in the presence of 2mM ‘cold’ phenylalanine to prevent reincorporation of radioactivity. The extent of protein degradation was determined from the radioactivity released into the medium, and was calculated as a fraction of the total radioactivity incorporated into the myotubes.

**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 2µl (370kBq) L-[2,6-3H] phenylalanine (sp.act. 196TBq/mmol) in 8µl sterile PBS and the plates were incubated for 4h at 37°C under an atmosphere of 10% CO2 in air. The reaction was arrested by washing three-times with 1ml ice-cold sterile PBS. Following removal of PBS 1ml 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 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. To measure the intracellular
amino acid pool the perchloric acid extract was neutralized with 0.2M potassium hydroxide and the insoluble potassium perchlorate removed by centrifugation (4500g, 10min). The radioactivity of the supernatant was determined as above.

Measurement of RNA - RNA was estimated in the perchloric acid supernatant in the protein synthesis experiment, by centrifugation of the samples at 700g for 5min at 4°C, and by measuring the absorbance at 232nm and 260nm. The following equation uses the modified formula of Ashford and Pain (19) and corrects for any absorbance due to the presence of aromatic amino acids.

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\text{RNA (µg/ml)} = \left[ A_{260} \times 32.9 \right] - \left[ A_{232} \times 6.11 \right]
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Measurement of proteasome activity - The 20S proteasome functional activity was determined by measuring the ‘chymotrypsin-like’ enzyme activity the predominant proteolytic activity of the β subunits by the method of Orino et al (20). Activity was determined as described (18) by the release of aminomethyl coumarin (AMC) from the fluorogenic peptide succinyl-LLVY-AMC. Activity was determined in the absence and presence of the specific proteasome inhibitor lactacystin (10µM). Only lactacystin-suppressible activity was considered to be proteasome specific. Activity was normalized to the protein content of the cell extract determined using a standard colorimetric protein assay (Sigma).

Western blot analysis - Samples of cytosolic protein (5-10µg) were resolved on 10% sodium dodecylsulfate polyacrylamide gels (SDS / PAGE) (6% for eIF2α) and transferred to 0.45µm nitrocellulose membranes, which had been blocked with 5% Marvel in Tris-buffered saline, pH 7.5, at 4°C overnight. The primary antibodies were used at a dilution of 1:1000 except for eIF2α (1:500), actin (1:200), and myosin (1:100), and the secondary antibodies were also used at a dilution of 1:1000. Incubation was for 1h at room temperature (actin, p42) or overnight, and development was by ECL. Blots were scanned by a densitometer to quantitate differences.

Electrophoretic mobility shift assay (EMSA) - DNA-binding proteins were extracted from myotubes as described (21) using 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. Assays were conducted using a biotin labelled double-stranded oligonucleotide having a consensus recognition sequence for NF-κB (5’AGTTGAGGGGACCTTT CCCAGGC-3’). Unlabelled probe was added to negative controls, while a positive probe was also supplied by the manufacturer. Protein-DNA complexes were separated using nondenaturing PAGE.

Statistical analysis - Results are presented as mean ± SEM for at least three replicate experiments. Differences in means between groups were determined by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. P-values less than 0.05 were considered significant.

RESULTS

PIF induced a time and concentration-dependent decrease in protein synthesis in murine myotubes, and the maximum effect was observed after 4h incubation and at a concentration of 4.2nM (Fig. 1), as previously observed with murine myoblasts (4). The depression of protein synthesis by PIF (30%) was similar to that previously reported for tumor necrosis factor-α (TNF-α) in soleus muscle (22%) (22) and for interleukin-1 (IL-1) in gastrocnemius (30%) and soleus muscles (25%) (23). The effect of PIF was specific since it was attenuated by co-incubation with anti-PIF monoclonal antibody (10µg/ml) (Fig 1). The concentrations of PIF causing significant inhibition of protein synthesis (2.1 to 10.5nM) were the same as those previously shown to stimulate protein degradation (24) and the dose-response curve showed a similar parabolic relationship. We have previously shown (5) that Ang II also inhibits protein synthesis in myotubes at similar concentrations to those inducing protein degradation (6). There was no effect of PIF on the uptake of labeled phenylalanine into myotubes (data not shown), confirming that the effect was on protein synthesis, rather than depletion of the amino acid pool in the cell. There was also no significant effect of PIF on the total cellular RNA, even up to 24h incubation (data not shown), suggesting that the depression in protein synthesis resulted from an effect on translational efficiency, as previously reported (4).
Suppression of protein synthesis through phosphorylation of the α subunit of the translation initiating factor eIF2 is known to occur in response to many forms of cellular stress (25). Since PIF can be considered as a stress factor in cancer cachexia, the effect on eIF2α phosphorylation was determined. The results presented in Fig. 1B show that PIF, at the concentration (4.2nM) producing the maximal depression in protein synthesis in myotubes (Fig. 1A), induced an increase in the phosphorylated form of eIF2α, without an effect on the total eIF2α. Phosphorylation of eIF2α increased with time of exposure, and was significant within 2h of addition of PIF. Moreover, the dose-response curve for the induction of eIF2α phosphorylation (Fig. 1C) was the same as for the effect on protein synthesis (Fig. 1A). The effect was specific, since it was completely attenuated by co-treatment with anti-PIF monoclonal antibody. Mammalian cells possess at least four different eIF2α kinases (13). Since it is not known which eIF2α kinase is activated in response to PIF a commercially available PKR inhibitor 8-[1-(1H-imidazol-4-yl)meth-(Z)-ylidene]-6,8-dihydro-thiazol[5,4-e]indol-7-one (PKRI) (26), was selected for further study. The data in Fig. 2A show the inhibitor to attenuate the reduction in protein synthesis induced by PIF at concentrations between 100 and 210nM, but not at a concentration of 300nM. The reason why the higher concentration did not have an effect is not known. Moreover, at a concentration of 210nM the inhibitor was effective at reversing inhibition of protein synthesis at all concentrations of PIF (Fig. 2B), and this was associated with complete inhibition of the increase in eIF2α phosphorylation (Fig. 2C). In controls (Fig. 1, 2 and 3) there was a mild expression of phosphorylated eIF2α, which possibly arose due to the effects of serum deprivation during the experimental treatments, as previously suggested for cortical neurons under the influence of Aβ peptide (27). This is confirmed by treating controls with the PKR inhibitor (Fig. 2D), where levels of phospho eIF2α were reduced below basal values. The PKR inhibitor was also effective in attenuating the depression in protein synthesis induced by Ang II (Fig. 3A). Ang II also induced an increase in eIF2α phosphorylation (Fig. 3B), with a similar dose response curve to the effect on protein synthesis. Phosphorylation of eIF2α by Ang II was also attenuated by the PKR inhibitor (Fig. 3B).

These results suggest that PKR may be involved in the phosphorylation of eIF2α in response to PIF and Ang II. Upon activation, PKR undergoes phosphorylation at multiple sites, although Thr-446 and Thr-451 phosphorylation have been shown to be critical for the kinase activity of human PKR (28). Using an antibody which only detects PKR phosphorylated on Thr-446, Western blotting showed an increase in phosphorylation within 30 min of treating myotubes with PIF (Fig 4A) or within 2h of treatment with Ang II (Fig 4B), which was maintained over the 4h time course of the experiment. Activation of PKR precedes, but overlaps the time course for phosphorylation of eIF2α in response to PIF (Fig. 1B). These results confirm activation of PKR in response to PIF and Ang II, and suggest that it is responsible for the phosphorylation of eIF2α. Phosphorylation of PKR was also significantly increased in gastrocnemius muscle of weight losing mice bearing a cachexia-inducing tumor, when the weight loss exceeded 16% (Fig 4C). Activation of PKR was accompanied by a corresponding increase in eIF2α phosphorylation (Fig 4D). These results suggest that the same events are occurring in the skeletal muscle of cachectic mice, and may be responsible for the depression in protein synthesis.

In order to determine whether PKR is responsible for phosphorylation of eIF2α, and the subsequent depression of protein synthesis by PIF and Ang II, murine myoblasts were transfected with plasmids that express wild-type, or catalytically inactive dominant-negative PKR (PKRA6) proteins and allowed to differentiate into myotubes. As an additional control myoblasts were also transfected with the non-recombinant empty vector pcDNA3(-). PKRA6 has previously been shown to be devoid of kinase activity towards eIF2α (29). When incubated with PIF both pcDNA3(-) and wild-type cells showed phosphorylation of PKR (Fig 5A), while myotubes containing the mutant PKRA6 showed no response. The time course for pcDNA3(-) and wild-type PKR transfected cells was the same as with non-transfected cells (Fig 4A). Myotubes containing
PKRΔ6 also showed no phosphorylation of eIF2α in response to PIF (Fig 5B). In addition, while pcDNA3(-) and wild-type cells showed an equal or greater depression of protein synthesis than non-transfected cells in response to PIF (Fig. 5C) or Ang II (Fig. 5D), there was no effect on PKRΔ6 cells. These results confirm that activation of PKR by PIF and Ang II is responsible for the phosphorylation of eIF2α and the subsequent depression of protein synthesis.

Phosphorylation of eIF2α (14) and activation of PKR (15) has been linked to the induction of NF-κB transcriptional activity, which in turn is required for the induction of protein degradation by both PIF (7) and Ang II (8), through an increased expression and activity of the ubiquitin-proteasome proteolytic pathway. This suggests a link between the inhibition of protein synthesis and the increase in protein degradation in skeletal muscle in cancer cachexia. To confirm this, the effect of the PKR inhibitor on protein degradation was determined (Fig. 6). Using the same concentration as used to abrogate the depression in protein synthesis, the PKR inhibitor completely attenuated the increase in protein degradation induced by both PIF (Fig. 6A) and Ang II (Fig. 6B). Furthermore, while myotubes containing pcDNA3(-) and wild-type PKR showed the same increase in protein degradation as non-transfected cells in response to PIF (Fig. 6C) and Ang II (Fig. 6D), there was no increase in protein degradation in myotubes containing PKRΔ6. These results provide a link between the depression in protein synthesis induced both by PIF and Ang II and the increase in protein degradation.

Since induction of muscle protein degradation is mediated through an increased activity and expression of the ubiquitin-proteasome pathway the effect of activation of PKR was determined (Fig. 7). In the presence of PIF ‘chymotrypsin-like’ enzyme activity in pcDNA3(-) and wild-type PKR containing myotubes were elevated to the same extent as non-transfected cells, while in myotubes containing PKRΔ6 there was no increase in ‘chymotrypsin-like’ enzyme activity (Fig. 7A). A similar result was obtained with the PKR inhibitor (supplementary material Fig. 1A). Expression of 20S proteasome α-subunits and p42, an ATPase subunit of the 19S regulator, that promotes ATP-dependent association of the 20S proteasome with the 19S regulator to form the 26S proteasome, determined by Western blotting, were increased in the presence of PIF, at the same concentrations as those found to increase total protein degradation (Fig. 6A). However, this increase in expression was not seen in the presence of the PKR inhibitor (supplementary Fig. 1B and C). Similarly levels of the myofibrillar protein myosin were found to decrease in the presence of PIF, but this decrease was completely attenuated by the PKR inhibitor (supplementary Fig. 1D). In addition, although upregulation of 20S proteasome α subunits and p42 and a decrease in myosin expression were seen in myotubes containing pcDNA3(-) and wild-type PKR, they were not seen in myotubes containing PKRΔ6 (supplementary Fig. 2). These results confirm that the inhibition of PKR attenuates the increased protein degradation in myotubes in the presence of PIF by downregulating the increased expression and activity of the ubiquitin-proteasome proteolytic pathway.

Induction of the ubiquitin-proteasome pathway in response to both PIF (9) and Ang II (8) requires activation of the nuclear transcription factor NF-κB. Release of NF-κB from the inactive complex with I-κB in the cytosol requires phosphorylation of I-κB, leading to degradation of the I-κB, and movement of the free NF-κB into the nucleus. Both PIF (supplementary Fig. 3A) and Ang II (supplementary Fig. 3B) cause a 3-to-4 fold increase in phosphorylation of I-κBα with a maximal effect at those concentrations having the largest effect on protein degradation, and this was not seen in the presence of the PKR inhibitor. Phosphorylation of I-κBα was accompanied by a decreased cytosolic concentration of I-κBα (supplementary Fig. 3C and D), and increased nuclear accumulation of NF-κB (supplementary Fig. 3E and F). Both effects were blocked by the PKR inhibitor. Furthermore, degradation of I-κBα and nuclear accumulation of NF-κB in response to PIF (Fig. 7B and D) and Ang II (Fig. 7C and E) was seen in myotubes transfected with pcDNA3(-) and wild-type PKR, but not in those containing the mutant PKRΔ6. These results suggest that phosphorylation of PKR results in increased protein degradation through an increased phosphorylation and degradation of I-κBα,
probably through activation of the upstream kinase IKK.

**DISCUSSION**

Atrophy of skeletal muscle is generally considered to result from hypoanabolism combined with an increase in protein catabolism. This study investigates the mechanism by which two factors PIF and Ang II, which are linked to cancer cachexia (6, 7), inhibit protein synthesis in murine myotubes, and how in turn this may be related to an increased protein degradation through the ubiquitin-proteasome proteolytic pathway. This is the first report to link a depression of protein synthesis with an increase in protein degradation in skeletal muscle. Both PIF and Ang II (5) decrease protein synthesis by 40%, and the concentrations of both agents that are maximally effective in the depression of protein synthesis are the same as those that are maximally effective in the induction of protein degradation. The effect of PIF on protein synthesis is suggested to result from an effect on translational efficiency, since total RNA levels were not affected by concentrations of PIF maximally depressing protein synthesis. Since about 85% of the total RNA is ribosomal, this indicates that inhibition of protein synthesis is not caused by a decrease in the number of ribosomes.

The effect of both PIF and Ang II on translation appears to arise from an increased phosphorylation of eIF2α, which in turn would inhibit binding of initiator met-tRNA to the 40S ribosomal subunit. Since eIF2B is present in cells at a lower molar concentration than eIF2α, phosphorylation of a small fraction of eIF2α can lead to a severe block in protein synthesis. Inhibition of protein synthesis in rat liver by vasopressin is caused by a reduction in eIF-2B activity, due to an increase in phosphorylation of eIF-2α (30), while the inhibition of protein synthesis in apoptosis by tumor necrosis factor-α (TNF-α) is also associated with increased phosphorylation of eIF2α (31). Phosphorylation of eIF2α by PIF and Ang II seems to occur through activation of PKR, since a PKR inhibitor (26) attenuated the inhibitory effect of both agents on protein synthesis. To confirm the role of PKR in phosphorylation of eIF2α, and the subsequent depression in protein synthesis, myoblasts were transfected with plasmids that overexpress heterologous wild-type PKR, or a dominant-negative PKR variant, which is unable to autophosphorylate or activate substrate proteins (PKRΔ6) (17). Neither PIF nor Ang II induced activation of PKR or phosphorylation of eIF2α in the PKR variant, and there was no depression in protein synthesis, confirming that activation of PKR was responsible for both events. In gastrocnemius muscle of weight losing mice bearing the MAC16 tumor there is evidence for a progressive increase in phosphorylation of both PKR and eIF2α at weight losses greater than 12%, suggesting that a similar mechanism may be responsible for the depression of protein synthesis in this cachexia model (32). In this model there was continuous activation of PKR and phosphorylation of eIF2α, which increased with increasing weight loss, suggesting a constant stimulation, probably due to continuous tumor release of PIF or Ang II.

PKR is normally activated by viral ds RNA, although other polyanionic agents such as heparin have also been shown to cause activation in vitro (33). Treatment of cells with interferon-γ also results in phosphorylation and activation of PKR (34), even in the absence of ds RNA. The mechanism of activation by PIF and Ang II, is not known, but may be through PACT, a cellular protein activator of PKR (35), although PIF is also a polyanionic molecule similar to heparin, and may cause direct activation. Activation of PKR by TNF-α in MCF-7 breast cancer cells is responsible for the induction of apoptosis and the activation of caspase-8 (31). PIF has also been shown to stimulate apoptosis and activate caspase-8 in murine myotubes (36), although the mechanism by which it induces this effect has not been elucidated. It is possible that activation of PKR by PIF may lead to apoptosis. PKR has been shown to play a dual role in cell survival and death (37). PKR has been shown to activate a survival pathway, mediated by NF-κB to delay apoptosis, and cell death is also induced by PKR through the phosphorylation of eIF2α. Thus at least part of the signalling pathways involved in muscle atrophy may also be involved in regulating muscle cell survival and death.

Activation of PKR may provide the link between the inhibition of protein synthesis and
induction of muscle protein degradation, leading to muscle atrophy in response to diverse cellular stress in a range of conditions in addition to cancer cachexia, including HIV-AIDS, sepsis, burns and weightlessness. The PKR inhibitor employed in the current study was capable of attenuating not only the depression of protein synthesis induced by PIF and Ang II, but also the increase in protein degradation. That this effect was due to activation of PKR was confirmed using myotubes containing the mutant plasmid PKRΔ6, which showed no increase in protein degradation in response to PIF or Ang II, in contrast with myotubes containing wild-type PKR. The effect appears to be due to antagonism to the induction of the ubiquitin-proteasome proteolytic pathway through inhibition of nuclear accumulation of NF-κB, which is required by both PIF (7) and Ang II (8) for increased proteasome expression and activity. An increased nuclear binding of NF-κB has also been shown to mediate muscle protein loss by TNF-α (38), and reactive oxygen-species (39), which are thought to be responsible for muscle atrophy associated with disuse and weightlessness (40). Both PKR (15) and GCN2 (14) have been linked to activation of NF-κB. Activation of NF-κB by PKR occurs by a mechanism independent of its eIF2α kinase activity. PKR activates IκB kinase (IKK) leading to degradation of IκBα, and the concomitant release of NF-κB. Activation of NF-κB by PKR is known to physically interact with IKK (15) through its catalytic domain, since mutants lacking the dsRNA-binding domain still form a complex with IKK (41). PKR is thought to stimulate the autophosphorylation of IKKβ by protein-protein interactions, and not by direct phosphorylation (42). However, phosphorylation of eIF2α is required for activation of NF-κB in response to either endoplasmic reticulum stress or amino acid starvation (14). The results of the current study confirm that activation of PKR is essential for activation of NF-κB, since myotubes containing the mutant plasmid PKRΔ6 show no enhancement in phosphorylation of IκBα in response to either PIF or Ang II, no degradation of IκBα, and no increase in nuclear binding of NF-κB. However, since such mutants also show no increase in eIF2α phosphorylation it does not rule this out as a contributing factor.

Previous studies have shown a role for protein kinase C (PKC) in the induction of proteasome expression by both PIF (43) and Ang II (8). A key step in protein degradation by both PIF and Ang II is the transient formation of reactive oxygen species (ROS), possibly through activation of NADPH oxidase by arachidonate and PKC (44). Formation of ROS leads to upregulation of the ubiquitin-proteasome pathway through activation of NF-κB. Preliminary data (unpublished) suggest that PKR may be involved in arachidonate release and the subsequent activation of PKC. Thus both PKR and PKC are likely to be on a common pathway leading to activation of NF-κB, and protein degradation.

Inhibitors of NF-κB activation such as eicosapentaenoic acid (EPA) (18) and resveratrol (45) have been shown to attenuate muscle protein degradation in cachexia, and lead to stabilization of the atrophy process. However, for muscle mass to increase it is also necessary to stimulate protein synthesis, and agents which attenuate NF-κB activation, such as EPA, have no effect on protein synthesis (46). It is possible to stimulate the depressed protein synthesis in the muscle of cachectic mice by the administration of the amino acids leucine, arginine and methionine (46), and, when combined with EPA, this produces a doubling of the ratio of protein synthesis to protein degradation over that of EPA alone. The results of the current study suggest that a PKR inhibitor alone may be as effective as this combination by targeting the critical molecular step in the atrophy process.

This study provides the first evidence of a direct relationship between the depression of protein synthesis in skeletal muscle by PIF and Ang II, through activation of PKR, and eIF2α phosphorylation, and the enhanced degradation of the myofibrillar protein myosin, through activation of NF-κB resulting in an increased expression and activity of the ubiquitin-proteasome proteolytic pathway. This suggests that agents which target PKR may be effective in the treatment of muscle atrophy in cancer cachexia, or other wasting conditions.
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FOOTNOTES

1 This work has been supported by a grant from Novartis Medical Nutrition.

2 The abbreviations used are: PIF, proteolysis-inducing factor; Ang II, angiotensin II; IGF1, insulin-like growth factor 1; eIF2α, eukaryotic initiation factor 2; PKR, RNA-dependent protein kinase; NF-κB, nuclear factor-κB; IKK, I-κB kinase; I-κB, inhibitor protein-κB.

FIGURE LEGENDS

Fig. 1 Effect of PIF on protein synthesis and relationship to phosphorylation of eIF2α. (A) Effect of different concentrations of PIF on protein synthesis in the absence (■) or presence (□) of anti-PIF monoclonal antibody (10µg/ml). Anti-PIF antibody was added 2h prior to PIF and protein synthesis was quantitated after incubation with PIF for 4h. (B) Time course for the effect of PIF (4.2nM) on phosphorylation of eIF2α. A densitometric analysis representing the average of 3 blots is shown underneath. Phosphorylation of eIF2α was measured by immunoblot analysis using rabbit monoclonal antibody specific to eIF2α phosphorylated at serine 51. Levels of total eIF2α were determined by using an antibody that recognises both the phosphorylated and non-phosphorylated forms of this translation initiation factor. (C) Effect of PIF concentration on the phosphorylation of eIF2α after 4h incubation in the absence or presence of anti-PIF antibody (10µg/ml). A densitometric analysis of phospho eIF2α representing the average of three separate blots is shown underneath. 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 anti-PIF antibody are indicated as d, p<0.05 or f, p<0.001.

Fig.2 Effect of inhibition of PKR on protein synthesis in murine myotubes in the presence of PIF. (A) Effect of different concentrations of a PKR inhibitor (PKRI) on protein synthesis in myotubes in the presence of PIF (4.2nM). The inhibitor was added 2h prior to PIF. (B) Dose-response for protein synthesis in myotubes exposed to different concentrations of PIF in the absence (■) or presence (□) of the PKR inhibitor (210nM). (C) Western blot of eIF2α phosphorylation in myotubes in the presence of various concentrations of PIF with (□) or without (■) the PKR inhibitor (210nM). (D) Western blot showing the effect of the PKR inhibitor (210nM) on phospho eIF2α expression in control cultures. A densitometric analysis of three separate immunoblots is shown underneath. Differences from control are indicated as b, p<0.01 or c, p<0.001, while differences from PIF alone are indicated as e, p<0.01 and f, p<0.001.
Fig. 3 Effect of inhibition of PKR on protein synthesis in murine myotubes in the presence of Ang II. (A) Dose-response for Ang II in the absence (◼) or presence (□) of the PKR inhibitor (PKRI) (210nM). (B) Western blot of eIF2α phosphorylation in the presence of various concentrations of Ang II, with (□) or without (◼) the PKR inhibitor (210nM). The densitometric analysis represents the average of three separate immunoblots. Differences from control are indicated as a, p<0.05, or b, p<0.01 while differences from Ang II alone is shown as d, p<0.05.

Fig. 4 Effect of PIF and Ang II on activation of PKR. Western blots of phospho PKR in murine myotubes with time after treatment with PIF (4.2nM) (A) or Ang II (0.5µM) (B) and in gastrocnemius muscle of mice bearing the MAC16 tumor and different extents of weight loss (C). The degree of phosphorylation of eIF2α in gastrocnemius muscle of the same mice is shown in (D). The method for transplanting the tumor has been described (40). The densitometric analysis represents the average of three separate immunoblots and is the ratio of phospho PKR to total PKR. Differences from time zero are indicated as b, p<0.01 or c, p<0.001.

Fig. 5 Effect of mutation of PKR on activation by PIF and Ang II. Western blots of phospho PKR (A) and eIF2α (B) with time after treatment with PIF (4.2nM) in myotubes transfected with pcDNA3(-) (◼), wild-type PKR (◼) and PKRΔ6 (□). The effect of PIF (4.2nM) and Ang II (0.5µM) on protein synthesis in myotubes transfected with pcDNA3(-), wild-type PKR and PKRΔ6 in comparison with non-transfected myotubes (◼) is shown in C and D respectively. Differences from control are shown as a, p<0.05, b, p<0.01 or c, p<0.001, while differences of PKRΔ6 from non-transfected cells, pcDNA3(-) and wild-type PKR are shown respectively as e, h and k, p<0.001 or f, i and l, p<0.001.

Fig. 6 Effect of inhibition of PKR on protein degradation in murine myotubes in the presence of PIF (A) or Ang II (B). Myotubes were treated with various concentrations of PIF or Ang II, either alone (◼), or in the presence of the PKR inhibitor (PKRI) (210nM) (□), added 2h prior to the catabolic stimuli. Protein degradation was measured after a further 24h incubation, as described in methods. 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 the PKR inhibitor are indicated as e, p<0.01 or f, p<0.001. Protein degradation in response to PIF (C) and Ang II (D) in non-transfected myotubes (◼) and in myotubes transfected with pcDNA3(-) (◼), wild-type PKR (◼) and PKRΔ6 (□). Differences from non-transfected myotubes are shown as d, p<0.05; e, p<0.01 or f, p<0.001, differences from pcDNA3(-) as g, p<0.05, h, p<0.01 or i, p<0.001 and differences from wild-type PKR as j, p<0.05, k<p<0.01 or l, p<0.001.

Fig. 7 Activation of PKR and induction of the ubiquitin-proteasome pathway through activation of NF-κB. (A) Effect of PIF on chymotrypsin-like enzyme activity in non-transfected myotubes (◼) and in myotubes transfected with pcDNA3(-) (◼), wild-type PKR (◼) and PKRΔ6 (□). Differences from control are indicated as a, p<0.05, b, p<0.01, or c, p<0.001. Differences from non-transfected myotubes are shown as e, p<0.01 or f, p<0.001, differences from pcDNA3(-) as h, p<0.01 or i, p<0.001 and differences from wild-type PKR as l, p<0.001. Cellular levels of IκBα (B and C), determined by Western blotting and nuclear accumulation of NF-κB, determined by EMSA (D and E) after treatment with PIF (4.2nM) (B and D) or Ang II (0.5µM) (C and E). The first four lanes are the untreated controls while the last four measurements were made after 30min. In the EMSA the lines marked +ve ctl is a positive control for NF-κB (HeLa nuclear extract supplied by the manufacturer of the kit) while the lane marked –ve ctl contains a positive control for NF-κB together with a 100-fold excess of unlabelled NF-κB probe. Myotubes were either not transfected (C2C12), or were transfected with empty plasmid (pcDNA3) or with wild-type PKR or the mutant PKR (A6). A densitometric analysis representing three separate blots is shown underneath. Differences from control are indicated as either b, p<0.01 or c, p<0.001.
Figure 1

A

![Graph showing protein synthesis (% control)]

PIF (nM) | 0 | 2.1 | 4.2 | 10.5 | 16.8
---|---|---|---|---|---
Anti-PIF antibody | - | + | - | + | - | +

B

| PIF (4.2nM) Time (h) | 0 | 0.5 | 1 | 2 | 4 |
|-----------------------|---|---|---|---|---|
| Arbitrary units (ph/tot) | 0.6 ± 0.1 | 0.86 ± 0.13 | 1.2 ± 0.4 | 2 ± 0.35^a | 2.4 ± 0.49^b |

C

| PIF (nM) | 0 | 2.1 | 2.1 | 4.2 | 4.2 | 10.5 | 10.5 | 16.8 | 16.8 |
|----------|---|---|---|---|---|---|---|---|---|
| Anti-PIF ab | - | - | + | - | + | - | + | - | + |
| Arbitrary units (ph/tot) | 0.2 | 0.47 | 0.04 | 0.5 | 0.01 | 0.1 | 0 | 0.1 | 0 |

^a, ^b, ^c, ^d, ^e, ^f, ^g, ^h, ^i, ^j, ^k, ^l, ^m, ^n, ^o, ^p, ^q, ^r, ^s, ^t, ^u, ^v, ^w, ^x, ^y, ^z, ^AA, ^AB, ^AC, ^AD, ^AE, ^AF, ^AG, ^AH, ^AI, ^AJ, ^AK, ^AL, ^AM, ^AN, ^AO, ^AP, ^AQ, ^AR, ^AS, ^AT, ^AU, ^AV, ^AW, ^AX, ^AY, ^AZ, ^BA, ^BB, ^BC, ^BD, ^BE, ^BF, ^BG, ^BH, ^BI, ^BJ, ^BK, ^BL, ^BM, ^BN, ^BO, ^BP, ^BQ, ^BR, ^BS, ^BT, ^BU, ^BV, ^BW, ^BX, ^BY, ^BZ,
Figure 2

A

B

120
100
80
60
40
20
0

Protein synthesis (% control)

PIF (4.2nM) - + - + - + - + - +
PKR I (100nM) - - + + - - - -
PKR I (210nM) - - - - + + - -
PKR I (310nM) - - - - - - + +

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Protein synthesis (% control)

PIF (nM) 0 2.1 4.2 10.5 16.8
PKR I (210nM) - + - + - + - +
Figure 2

| PIF (nM) | PKR I (210nM) | Arbitrary units (ph/tot) |
|----------|---------------|--------------------------|
| 0        | -             | ±0.1                     |
| 2.1      | -             | ±0.02                    |
| 2.1      | +             | ±0.07                    |
| 4.2      | +             | ±0.05b                   |
| 4.2      | -             | ±0.02b                   |
| 10.5     | +             | ±0.02b                   |
| 10.5     | -             | ±0.02b                   |
| 16.8     | +             | ±0.02b                   |
| 16.8     | -             | ±0.03                    |

Figure 3

| Ang II (µM) | PKR I (210nM) | Protein synthesis (% control) |
|-------------|---------------|------------------------------|
| 0           | -             | d                            |
| 0.1         | +             | b                            |
| 0.5         | -             | d                            |
| 1           | +             | b                            |
| 2.5         | -             | d                            |
Figure 3

![Phospho eIF2α and Total eIF2α](image)

**Ang II (µM)**
- 0
- 0.1
- 0.1
- 0.5
- 0.5
- 1.0
- 1.0
- 2.5
- 2.5

**PKR I (210nM)**
- -
- -
- +
- -
- +
- -
- +
- -
- +

**Arbitrary units (ph/tot)**
- 0.4 ± 0.1
- 0.6 ± 0.1
- 0.3 ± 0.03
- 1.1 ± 0.03
- 0.5 ± 0.1d
- 0.6 ± 0.04
- 0.3 ± 0.02d
- 0.5 ± 0.1
- 0.3 ± 0.03

Figure 4

**A**

![Phospho PKR and Total PKR](image)

**PIF (4.2nM)**
- 0
- 0.5
- 1
- 2
- 4

**Arbitrary units (ph/tot)**
- 1.1 ± 0.08
- 1.7 ± 0.1
- 1.8 ± 0.15
- 1.9 ± 0.16
- 2 ± 0.02

**B**

![Phospho PKR and Total PKR](image)

**Ang II (0.5µM)**
- 0
- 0.5
- 1
- 2
- 4

**Arbitrary units (ph/tot)**
- 0.2 ± 0.04
- 0.4 ± 0.05
- 0.6 ± 0.09
- 0.8 ± 0.1
- 1 ± 0.08
Figure 4

**Figure 4**

**C**

Phospho PKR

Total PKR

| Weight loss (%) | 0 | 12 | 16 | 18 | 20 | 22 | 25 |
|-----------------|---|----|----|----|----|----|----|
| Arbitrary units (ph/tot) | 0.06 | 0.3 | 0.9 | 1.0 | 1.0 | 1.0 | 1.3 |

**D**

Phospho eIF2α

Total eIF2α

| Weight loss (%) | 0 | 12 | 16 | 18 | 20 | 22 | 25 |
|-----------------|---|----|----|----|----|----|----|
| Arbitrary units (ph/tot) | 0.2 | 0.6 | 0.7 | 0.9 | 0.9 | 1.0 | 1.0 |

Figure 5

**A**

pcDNA3  Wild type  PKRΔ6

Phospho PKR

Total PKR

| Time (h) | 0 | 0.5 | 1 | 2 | 4 | 0 | 0.5 | 1 | 2 | 4 | 0 | 0.5 | 1 | 2 | 4 |
|----------|---|-----|---|---|---|---|-----|---|---|---|---|-----|---|---|---|
| Arbitrary units (ph/tot) | 1.0 | 1.4 | 1.6 | 1.8 | 2.2 | 0.6 | 1.7 | 1.7 | 1.8 | 2.1 | 0.6 | 0.4 | 0.5 | 0.5 | 0.6 |

**B**

pcDNA3  Wild type  PKRΔ6

Phospho eIF2α

Total eIF2α

| Time (h) | 0 | 0.5 | 1 | 2 | 4 | 0 | 0.5 | 1 | 2 | 4 | 0 | 0.5 | 1 | 2 | 4 |
|----------|---|-----|---|---|---|---|-----|---|---|---|---|-----|---|---|---|
| Arbitrary units (ph/tot) | 0.3 | 0.4 | 0.6 | 0.7 | 0.9 | 0.3 | 0.6 | 0.7 | 0.9 | 1.4 | 0.4 | 0.5 | 0.4 | 0.5 | 0.3 |

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Figure 5

C

D
Figure 7

A

Proteasome activity vs. PIF (nM)

B

IkBα

Actin

C2C12 + - - - - + - - -
pcDNA3 - + - - - + - - -
Wild type - - + - - - + - -
PKR Δ6 - - - + - - - - +
PIF (4.2nM) - - - - + + + + +
Arbitrary units 100±0 100±0 100±0 100±0 34±4c 60±3c 53±2c 95±4
(% ctl)

C

IkBα

Actin

C2C12 + - - - - + - - -
pcDNA3 - + - - - + - - -
Wild type - - + - - - + - -
PKRΔ6 - - - + - - - - +
Ang II (0.5µM) - - - - + + + + +
Arbitrary units 100±0 100±0 100±0 100±0 61±7c 35±4c 29±4c 90±4
(% ctl)
Figure 7

### D

| Condition                  | C2C12 | pcDNA3 | Wild type | PKRΔ6 | PIF (4.2nM) | Ang II (0.5µM) | Arbitrary units | (% ctl) |
|----------------------------|-------|--------|-----------|-------|-------------|----------------|----------------|---------|
|                            | +     | -      | -         | -     | +           | -              | 100           | ±0      |
|                            | -     | +      | +         | -     | -           | +              | 100           | ±0      |
|                            | -     | +      | +         | -     | -           | +              | 100           | ±0      |
|                            | -     | -      | -         | -     | +           | +              | 100           | ±0      |
|                            | -     | -      | -         | -     | -           | +              | 100           | ±0      |
|                            | -     | -      | -         | -     | -           | +              | 100           | ±0      |

### E

| Condition                  | C2C12 | pcDNA3 | Wild type | PKRΔ6 | PIF (4.2nM) | Ang II (0.5µM) | Arbitrary units | (% ctl) |
|----------------------------|-------|--------|-----------|-------|-------------|----------------|----------------|---------|
|                            | +     | -      | -         | -     | +           | -              | 100           | ±0      |
|                            | -     | +      | +         | -     | -           | +              | 100           | ±0      |
|                            | -     | +      | +         | -     | -           | +              | 100           | ±0      |
|                            | -     | -      | -         | -     | +           | +              | 100           | ±0      |
|                            | -     | -      | -         | -     | -           | +              | 100           | ±0      |
|                            | -     | -      | -         | -     | -           | +              | 100           | ±0      |

*Arbitrary units: 100, 100, 100, 100, 195, 240, 213, 81*  
*(% ctl): ±0, ±0, ±0, ±0, ±8b, ±19c, ±30c, ±6*
Skeletal muscle atrophy: A link between depression of protein synthesis and increase in degradation
Helen L. Eley and Michael J. Tisdale

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