MECHANISM OF ATTENUATION OF SKELETAL MUSCLE ATROPHY BY D-MYO-INOSITOL 1,2,6-TRIPHOSPHATE

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ABSTRACT

D-Myo-inositol 1,2,6-triphosphate (alpha trinositol, AT) has been shown to attenuate muscle atrophy in a murine cachexia model through an increase in protein synthesis and a decrease in degradation. The mechanism of this effect has been investigated in murine myotubes using a range of catabolic stimuli, including proteolysis-inducing factor (PIF), angiotensin II (Ang II), lipopolysaccharide, and tumour necrosis factor-α / interferon-γ. At a concentration of 100μM AT was found to attenuate both the induction of protein degradation and depression of protein synthesis in response to all stimuli. The effect on protein degradation was accompanied by attenuation of the increased expression and activity of the ubiquitin-proteasome pathway. This suggests that AT inhibits a signalling step common to all four agents. This target has been shown to be activation (autophosphorylation) of the dsRNA-dependent protein kinase (PKR) and the subsequent phosphorylation of eukaryotic initiation factor 2 on the α-subunit, together with downstream signalling pathways leading to protein degradation. AT also inhibited activation of caspase-3/-8, which is thought to lead to activation of PKR. The mechanism of this effect may be related to the ability of AT to chelate divalent metal ions, since the attenuation of the increased activity of the ubiquitin-proteasome pathway by PIF and Ang II, as well as the depression of protein synthesis by PIF, were reversed by increasing concentrations of Zn^{2+}. The ability of AT to attenuate muscle atrophy by a range of stimuli suggests that it may be effective in several conditions.

1. Introduction

A number of chronic and end-stage diseases including cancer, chronic heart failure (CHF), chronic obstructive pulmonary disease (COPD), sepsis and end-stage renal failure are associated with muscle atrophy, leading to weakness and eventually death [1]. For muscle atrophy to occur protein degradation must exceed protein synthesis, but usually there is an increase in protein degradation together with a depression of protein synthesis. A number of agents have been shown to induce muscle atrophy including proteolysis-inducing factor (PIF) [2], angiotensin II (Ang II) [3], tumour necrosis factor-α [4] and lipopolysaccharide (LPS) [5]. These agents cause a depression of protein synthesis in skeletal muscle, as well as an increase in protein degradation, and recent results suggest that they all work through the same cellular signalling pathway to induce muscle atrophy. Thus the depression of protein synthesis induced by PIF [6], Ang II, TNF-α and LPS [7] has been attributed to activation of the dsRNA-dependent protein (PKR), with subsequent phosphorylation of eukaryotic initiation factor 2 (eIF2) on the α-subunit. This blocks translation initiation by inhibiting binding of initiator methionyl tRNA to the 40S ribosomal subunit [8]. They also increase phosphorylation of the elongation factor (eEF2), decreasing its affinity for the ribosome and contributing to the depression of global protein synthesis [9].

The predominant pathway responsible for protein degradation in muscle in a range of catabolic conditions is the ubiquitin proteolytic pathway [10]. There is increased expression of this pathway in response to PIF [6], Ang II, TNF-α [11] and LPS [5], mediated by activation of PKR leading to increased formation of reactive oxygen species (ROS), via activation of p38 mitogen-activated protein kinase (p38MAPK) [11]. The increased ROS leads to activation of nuclear factor-κB (NF-

κB), which is responsible for increased expression of proteasome subunits and the ubiquitin ligase MuRF1 [12]. Activation (autophosphorylation) of PKR was shown to occur through an increase in activity of caspases-3 and -8 [11]. Caspases-3, -7, and -8 have been reported [13] to cleave PKR at Asp^{251}, releasing the kinase domain from the control of the regulatory amino-terminal domain leading to autophosphorylation.

We have recently identified a polyanionic metal binding agent D-myo-inositol-1,2,6-triphosphate (alpha trinositol, AT) which attenuated muscle atrophy in mice bearing a cachexia-inducing tumour [14]. Muscle mass increased due to an increase in protein synthesis and a decrease in protein degradation. The decrease in protein degradation was associated with a decrease in activity of both the ubiquitin-proteasome pathway and caspase-3 and -8, while protein synthesis was increased due to reduced phosphorylation of both PKR and eIF2α. In vitro studies also showed AT to attenuate the increased protein degradation in murine myotubes in response to PIF and Ang II.

The current study investigates the mechanism of this effect, as well as protein degradation induced by TNF-α and LPS and the ability of AT to attenuate the depression of protein synthesis induced by PIF, Ang II, TNF-α and LPS.

2. Materials and Methods

2.1 Materials Foetal calf serum (FCS), horse serum (HS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Paisley, UK). L-[2,6-^{3}H] Phenylalanine (sp.act.2.2TBq/mmole) was purchased from American Radiochemicals (Cardiff, UK). Hybond A nitrocellulose membranes, and ECL development kits were from Amersham Biosciences Ltd (Bucks, UK). Mouse monoclonal antibodies to 20S proteasome α-subunits and p42 were from Affiniti Research Products (Exeter, UK). Rabbit monoclonal antibodies to phospho and total PKR were purchased from New England Biolabs (Herts, UK). Rabbit polyclonal antisera to phospho eIF2α (Ser^{51}) and to total eIF2α was from Santa Cruz Biotechnology (CA). Rabbit polyclonal antisera to myosin heavy chain was from Novocastra (Newcastle, UK). Rabbit polyclonal antisera to mouse β-actin, LPS from Ecoli O111:B4, TNF-α, IFN-γ, Ang II, the chymotrypsin substrate succinyl LLVY-7-amino-4-methylcoumarin (LLVY-AMC) and dichlorodihydrofluorescein diacetate, were purchased 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). Electrophoretic mobility shift assay (EMSA) kits were from Panomics (Redwood City, CA). 1-D-Myo-inositol 1,2,6-triphosphate (alpha trinositol, AT) was supplied by Bioneris (Helsinki, Finland). Phosphosafe™ extraction reagent and rabbit polyclonal antisera to mouse PKCα was from Merck Eurolab Ltd (Leicestershire, UK). The caspase-3 and -8 substrates and inhibitors were purchased from Biomol International (Devon, UK). PIF was purified from solid MAC16 tumours using affinity chromatography with an anti-PIF monoclonal antibody as previously described [15].

2.2 Myogenic cell culture C_2C_{12} myoblasts were routinely propagated in DMEM supplemented with 10% FCS, glutamine and 1% penicillin-streptomycin under an atmosphere of 10% CO_2 in air at 37°C. When the myoblasts reached confluence they were allowed to differentiate into myotubes by replacing the growth medium with DMEM containing 2% HS, with media changes every two days. Differentiation was complete within 3 to 5 days and the myotubes were used for experimentation within a 4 day period.

2.3 Measurement of protein synthesis and degradation in myotubes Experiments were carried out on myotubes formed in 6-well multiwell dishes, as described previously [6]. Briefly for protein degradation myotubes were labelled with L-[2,6-^{3}H] phenylalanine for 24h prior to experimentation, washed extensively, and chased for 2h in fresh DMEM without phenol red, to allow degradation of short-lived proteins. The myotubes were then incubated with either PIF or Ang II, at the concentrations and additions detailed in the figure legends, for 24h in the presence of excess (2mM) phenylalanine, to prevent reincorporation of radioactivity. The extent of protein degradation was determined from the radioactivity released into the medium, as a fraction of the total radioactivity incorporated into the myotubes. Protein synthesis was determined by the incorporation of L-[2,6-^{3}H] phenylalanine into myotubes over a 4h period in the presence of the additions described in the figure legends. Protein synthesis was calculated as the radioactivity incorporated into acid (0.2M perchloric acid) insoluble material.

2.4 Measurement of proteasome activity Functional 20S proteasome activity was determined as the “chymotrypsin-like” enzyme activity by the release of 7-amino-4-

methylcoumarin (AMC) from the fluorogenic peptide succinyl-LLVY-7-AMC as described by Orino et al [16]. We have previously described the method in detail for murine myotubes [17]. Activity was measured in the absence and presence of the specific proteasome inhibitor lactacystin (10μM). Only lactacystin suppressible activity was considered to be proteasome specific.

2.5 Caspase activity The activity of caspase 3 was determined by the relase of AMC from the specific substrate AcDEVD-AMC in the presence or absence of the caspase 3 inhibitor AcDEVD-CHO, as described [11]. Myotubes were homogenised in lysis buffer (150mmol/L NaCl, 1% NP40, 50mmol/L Tris HCl, pH7.4, 0.25% sodium deoxycholate, 2mmol/L EGTA, 1mmol/EDTA, 0.2mmol/L sodium orthovanadate, 20mmol/L NaF and 1% proteasome inhibitor mixture), left at 4°C, and then room temperature for 10min, followed by centrifugation at 15,000g for 15 min. The supernatant (50μg protein) was incubated with the caspase 3 substrate for 1h, and the increase in fluorescence due to AMC was determined at an excitation wavelength of 370nm and an emission wavelength of 430nm. The difference in values in the absence and presence of the caspase-3 inhibitor was a measure of activity. The method for caspase 8 was similar with the substrate being Z-IEFTD-AFC and the inhibitor IETD-CHO. The increase in fluorescence due to the release of 7-amino-4-trifluoro-methylcoumarin (AFC) was measured with an excitation wavelength of 400nm and an emission wavelength of 505nm.

2.6 Measurement of ROS formation in myotubes The method has previously been described in detail [18], and measures the conversion of the cell-permeable probe 2,7-dichlorodihydrofluorescein diacetate, to 2,7-dichlorofluorescein upon oxidation by

ROS. Myotubes were incubated with PIF for 30min, washed with PBS and sonicated at 4°C after scraping from the plate. An aliquot of the supernatant formed by centrifugation at 2800g was read on a fluorimeter at an excitation wavelength of 480nm and an emission wavelength of 510nm. In all experiments the protein concentration of the samples was determined using the Bio-Rad reagent.

2.7 Western blot analysis After the treatments as described in the figure legends, myotubes were sonicated in Phosphosafe™ Extraction Reagent and centrifuged at 18000g for 5 min. Samples of cytosolic protein (10-15μg) were resolved on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6% for eIF2α) at 180V for approximately 1h. The protein on the gels was then transferred to 0.45μm nitrocellulose membranes, which were then 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). The secondary antibodies were used at a dilution of 1:1000. Incubation was either for 1h at room temperature, or overnight, and development was by ECL. Blots were scanned by a densitometer to quantitate differences.

2.8 Electrophoretic mobility shift assay (EMSA) DNA binding proteins were extracted from myotube nuclei by hypotonic lysis, followed by high salt extraction as described [19]. 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'AGTTGAGGGGACTTTCCCAGGC3'). Unlabeled probe was added to negative controls, while a positive probe was also

supplied by the manufacturer. Protein-DNA complexes were separated using nondenaturing PAGE.

2.9 Statistical analysis All results are shown as mean ± S.E. for at least three replicate experiments. Differences in means between groups were determined by one-way analysis of variance followed by Tukey-Kramer multiple comparison test. p values less than 0.05 were considered ‘significant’.

3. Results

The effect of AT on total protein degradation in murine myotubes induced by LPS over a 24h period is shown in Fig. 1A. LPS produced a dose-dependent increase in protein degradation at concentrations up to 100ng/ml, and this was completely attenuated by AT (100μM), at all concentrations of LPS. LPS induced an increase in expression of both the 20S proteasome, α-subunits and p42, an ATPase subunit of the 19S regulator, at the same concentrations as that inducing protein degradation (Fig. 1B), suggesting that it was mediated through the ubiquitin-proteasome pathway. The increase in expression of both the 20S proteasome and p42 in response to LPS was completely attenuated by AT, although basal levels were not affected (Fig. 1B). LPS also depressed protein synthesis in myotubes by 50-60% at the same concentrations as that inducing protein degradation, and this was also completely attenuated by AT (Fig. 1C).

TNF-α (50ng/ml) either alone, or in combination with interferon-g (IFN-γ; 10ng/ml), also induced protein degradation in myotubes over a 24h period, and this was also completely attenuated by AT down to basal levels (Fig. 2A). TNF-α alone produced a 40% decrease in protein synthesis in myotubes after 2h incubation, and this was increased up to 60% depression in the presence of IFN-γ. Treatment with AT, not only attenuated the depression of protein synthesis by TNF-α or TNF-α+ IFN-γ, but also caused a significant increase in protein synthesis above basal levels (Fig. 2B).

We have previously shown [14] that protein degradation in myotubes in response to PIF and Ang II was also attenuated by AT. Protein degradation induced by PIF and Ang II is also mediated by the ubiquitin-proteasome pathway, and both induced an increase in proteasome functional activity, as measured by the

'chymotrypsin-like' enzyme activity (Fig. 3A and B). As with total protein degradation, the induction of 'chymotrypsin-like' enzyme activity by PIF and Ang II was attenuated by AT, suggesting inhibition of the ubiquitin-proteasome pathway. This was confirmed by measurement of expression of the 20S proteasome α-subunits (Fig. 3C) and p42 (Fig. 3D), which were increased in the presence of PIF and completely attenuated by AT. These results show that AT attenuates protein degradation induced by LPS, TNF-α, PIF and Ang II by down-regulation of the increased expression of the ubiquitin-proteasome pathway. It also suggests that AT inhibits a common mechanism linking protein synthesis and degradation in skeletal muscle.

One potential target is the activation (autophosphorylation) of PKR and the subsequent phosphorylation of eIF2 on the α-subunit [6]. The immunoblots in Fig. 4 show that PIF induced phosphorylation of both PKR (Fig. 4A) and eIF2α (Fig. 4B), and that this was maximal at the same concentration (4.2nM) as that inducing proteasome activity (Fig. 3A), and was completely attenuated by AT. Other signalling pathways activated by PIF and leading to protein degradation including activation of protein kinase C (PKC) (Fig. 5A) [20], formation of ROS (Fig. 5B) [18], and nuclear accumulation of NF-κB (Fig. 5C) [21], were all attenuated by AT. Since these pathways are thought to be downstream of PKR [11], this suggests that AT inhibits a signalling pathway upstream of PKR. One possible candidate could be activation of caspase-3/-8, which has been shown to activate PKR in response to TNF-α/IFN-γ and Ang II [11]. PIF also induced an increase in activity of caspase-3 (Fig. 6A) and caspase-8 (Fig. 6B) in myotubes, which was completely attenuated by AT. The mechanism by which this effect is manifested is not known, but could be related to the ability of AT to chelate divalent metal ions. Thus the inhibition by AT

of the increase in chymotrypsin-like enzyme activity induced by both PIF (Fig. 6C) and Ang II (Fig. 6D) were completely attenuated by concentrations of Zn^{2+} of 150μM, or above, while the AT-induced attenuation of protein synthesis inhibition by PIF was also reversed by Zn^{2+} at concentrations of 100μM or above (Fig. 6E).

4. Discussion

AT is a polyanionic compound capable of chelating divalent metal ions, such as Ca^{2+} and Zn^{2+}, which bind to phosphates P1 and P6 in the inositol ring structure [22]. A previous study [14] showed that AT attenuated muscle atrophy in a murine cachexia model and the current study suggests that it may be a potential therapy for a range of catabolic conditions, since it attenuates both the depression of protein synthesis and increase in protein degradation induced not only by PIF, but by Ang II, LPS and TNF-α/IFN-γ. Since all four agents follow the same cellular signalling pathway inside of the target tissue, to induce muscle atrophy it suggests that AT inhibits a common step. Since AT attenuates both the depression of protein synthesis and increase in protein degradation in muscle, and since activation of PKR is thought to be the key signal leading to both of these processes [6], it is likely that AT inhibits a common step leading to activation of PKR. Indeed downstream signals from PKR involved in both the depression of protein synthesis (phosphorylation of eIF2α) and increase in protein degradation (activation of PKC, ROS formation and nuclear accumulation of NF-κB) are inhibited by AT. Activation of PKR is thought to arise from the caspase-3/-8 cleavage of the regulatory domain leading to autophosphorylation [13] and the specific caspase-3 and -8 inhibitors were found to attenuate activation of PKR by TNF-α and Ang II, as well as the increase in ROS formation [11].

The mechanism for activation of caspase-3/-8 is not known, but activation of caspase-3 has been suggested to be triggered by Ca^{2+} release from the endoplasmic reticulum (ER) [23]. An increase in Ca^{2+} concentration has also been reported to lead to activation of caspase-3 [24], while depletion of ER Ca^{2+} stores has also been shown to activate PKR and induce phosphorylation of eIF2α [25]. We have also shown

(unpublished results) that the membrane-permeable Ca^{2+} chelator BAPTA-AM attenuated the PIF-induced activation of caspase-3/-8. These results suggest a role for Ca^{2+} in caspase activation.

While AT can chelate Ca^{2+} its affinity for Zn^{2+} is higher. Also the polyanionic nature of AT would suggest that it would not readily enter cells, and the previous studies suggest that mobilization of Ca^{2+} from the ER is involved in the activation of caspase-3/-8, rather than Ca^{2+} influx from the extracellular space. The ability of Zn^{2+} to reverse the attenuation by AT of both the depression in protein synthesis by PIF, and the increased activity of the ubiquitin-proteasome pathway induced by PIF and Ang II, suggests a role for Zn^{2+} in the signalling process. Ca^{2+} release from intracellular pools in the coloncytic cell line HT29 has been linked to extracellular Zn^{2+} through a zinc sensing receptor [26]. The Ca^{2+} release requires the formation of inositol 1,4,5-triphosphate and is zinc specific. The Ca^{2+} response is mediated by a Gq-coupled receptor that activates the inositol phosphate pathway and was inhibited by phospholipase C (PLC) inhibitors [26, 27]. The Zn^{2+} receptor has also been reported to be present on prostate cancer cells [27], but it is not known if it is present on skeletal muscle. If it were it would link the ability of AT to modify intracellular processes, even though the high negative charge would preclude its entry into the cell. Certainly induction of protein degradation induced by both PIF [28] and Ang II [29] was inhibited by both U-73122, which inhibits agonist-induced PLC activation, and D609, a specific inhibitor of phosphatidylcholine-specific PLC. This suggests that the zinc-receptor pathway may be operating, although PLC may form another part of the signalling cascade. We have shown a clear inhibitory effect against muscle atrophy, but we have yet to clarify the specific mode of action by, for example, identifying a specific receptor which can be blocked by AT. Further studies are required to

investigate the possible, still unidentified, zinc receptor and to explain the Ca^{2+} release from intracellular pools in muscle atrophy.

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Figure Legends

Fig. 1 (A) Effect of LPS alone (▲), or in the presence of AT (100μM;■), added 2h prior to LPS, on total protein degradation in murine myotubes over a 24h period. (B) Western blots showing expression of the 20S proteasome α-subunits and p42 in murine myotubes 24h after addition of LPS, either alone, or in the presence of AT (100μM). The densitometric analysis is an average of 3 separate Western blots. (C) Effect of LPS on protein synthesis in murine myotubes after 4h incubation, either alone (□), or in the presence of AT (100μM) (■). Differences from control are indicated as c, $p<0.001$, while differences in the presence of AT are indicated as f, $p<0.001$.

Fig. 2 Effect of TNF-α (50ng/ml) alone, or in combination with IFN-γ (10ng/ml) on total protein degradation in murine myotubes over a 24h period (A) and protein synthesis over 2h (B), either alone (□), or in the presence of AT (100μM) (■), added 2h prior to the TNF-α. Differences from control are indicated as c, $p<0.001$, while differences in the presence of AT are shown as f, $p<0.001$.

Fig. 3 Chymotrypsin-like enzyme activity in murine myotubes 24h after addition of PIF (A), or Ang II (B), alone (▲), or in the presence of AT (■) (100μM). Western blotting of 20S proteasome α-subunits (C) and p42 (D) in murine myotubes 24h after addition of PIF alone, or in the presence of AT (100μM). Actin was used as a loading control. The densitometric analysis is the average of three separate Western blots. Differences from control are indicated as c, $p<0.001$, while differences in the presence of AT are shown as f, $p<0.001$.

Fig. 4 Western blots showing the effect of PIF on phosphorylation of PKR (A) and eIF2α (B) after 4h, alone and in the presence of AT (100μM) added 2h prior

to PIF. The densitometric analysis represents the average of three separate Western blots. Differences from control are indicated as c, $p<0.001$, while differences in the presence of AT are shown as f, $p<0.001$.

Fig. 5 Western blot of cytosolic and membrane bound PKC in murine myotubes 2h after addition of PIF in the absence of presence of AT (100μM). (B) Dose-response curve for ROS production in murine myotubes 30min after addition of PIF alone (▲), or in the presence of AT (■). The results shown are an average of three separate experiments, and are expressed taking basal fluorescence of control cells as 100%. (C) Nuclear binding of NF-κB determined by EMSA after treatment of myotubes for 30min with either PIF alone or in combination with AT. The lane marked +ve was a positive control for NF-κB (HeLa nuclear extract supplied by the manufacturer), while the lane marked –ve contains the positive control for NF-κB, plus a 100-fold excess of unlabelled NF-κB probe. Differences from control are indicated as c, $p<0.001$, while differences in the presence of AT are shown as e, $p<0.01$ or f, $p<0.001$.

Fig. 6 Activity of caspase 3 (A) and caspase 8 (B) in murine myotubes after treatment with PIF alone (▲), or in the presence of AT (100μM) (■) for 1h. The chymotrypsin-like enzyme activity in murine myotubes treated with either 4.2nM PIF (C), or 0.5μM Ang II (D), with or without AT (100μM) for 24h is shown in the presence of various concentrations of $ZnSO_4$. (E) Protein synthesis in murine myotubes after 4h incubation with PIF (4.2nM), with or without AT (100μM), and in the presence of various concentrations of $ZnSO_4$. 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 AT are shown as f, $p<0.001$ and differences from AT alone are shown as h, $p<0.01$ or i, $p<0.001$.