Induction of protein catabolism in myotubes by 15(S)-hydroxyeicosatetraenoic acid through increased expression of the ubiquitin–proteasome pathway

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The potential role of 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) as an intracellular signal for increased protein catabolism and induction of the expression of key components of the ubiquitin–proteasome proteolytic pathway induced by a tumour cachectic factor, proteolysis-inducing factor has been studied in murine C2C12 myotubes. 15(S)-HETE induced protein degradation in these cells with a maximal effect at concentrations between 78 and 312 nM. The effect was attenuated by the polyunsaturated fatty acid, eicosapentaenoic acid (EPA). There was an increase in 'chymotrypsin-like' enzyme activity, the predominant proteolytic activity of the proteasome, in the same concentration range as that inducing total protein degradation, and this effect was also attenuated by EPA. 15(S)-hydroxyeicosatetraenoic acid also increased maximal expression of mRNA for proteasome subunits C2 and C5, as well as the ubiquitin-conjugating enzyme, E2 14k, after 4 h incubation, as determined by quantitative competitive RT–PCR. The concentrations of 15(S)-HETE affecting gene expression were the same as those inducing protein degradation. Western blotting of cellular supernatants of myotubes treated with 15(S)-HETE for 24 h showed increased expression of p42, an ATPase subunit of the regulatory complex at similar concentrations, as well as a decrease in expression of myosin in the same concentration range. 15(S)-hydroxyeicosatetraenoic acid activated binding of nuclear factor-κB (NF-κB) in the myotube nucleus and stimulated degradation of I-κBα. The effect on the NF-κB/I-κBα system was attenuated by EPA. In addition, the NF-κB inhibitor peptide SN50 attenuated the increased chymotrypsin-like enzyme activity in the presence of 15(S)-HETE. These results suggest that 15(S)-HETE induces degradation of myofibrillar proteins in differentiated myotubes through an induction of an increased expression of the regulatory components of the ubiquitin–proteasome proteolytic pathway possibly through the intervention of the nuclear transcription factor NF-κB, and that this process is inhibited by EPA.

British Journal of Cancer (2003) 89, 737–745. doi:10.1038/sj.bjc.6601184 www.bjcancer.com
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Keywords: 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE); protein catabolism; proteoscore induction; nuclear factor-κB (NF-κB)

Proteolysis through the ubiquitin–proteasome pathway has an important role in the regulation of the cell cycle and division, differentiation and development, as well as a variety of other basic cellular processes (Giechanover et al, 2000). In addition, this pathway plays an important role in the wasting of skeletal muscle seen in a range of catabolic processes, including starvation, sepsis, metabolic acidosis, weightlessness, severe trauma, denervation atrophy and cancer cachexia (Lecker et al, 1999). In this process, proteins are degraded into peptides within the 20S proteasome, a cylindrical structure of four stacked rings, each composed of seven subunits surrounding a central cavity. The inner β rings contain proteolytic enzymes, while the two outer α rings surround a small cavity through which protein substrates must enter. Cellular proteins are marked for degradation by attachment of a polyubiquitin chain. While the ubiquitin-conjugating enzyme (E2 14k) was suggested to be the rate-limiting step in the pathway in starvation (Wing and Banville, 1994), it has recently been shown that E2 14k knockout is not associated with decreased protein catabolism (Adegoke et al, 2002). Instead, ubiquitin-protein ligases (E3) may play a more important role in muscle atrophy and E3 knockout is clearly associated with increased protein catabolism (Bodine et al, 2001; Gomes et al, 2001).

Despite the importance of the ubiquitin–proteasome pathway, there is little information on intracellular signals leading to an increased gene transcription of the rate-limiting enzymes. It has been suggested that glucocorticoids and a low insulin level in tandem activate the ubiquitin–proteasome proteolytic system (Mitch et al, 1999). Glucocorticoids increase protein catabolism by opposing the suppression of the transcription of the proteasome C3 α-subunit by nuclear factor-κB (NF-κB), by antagonising the interaction of NF-κB with an NF-κB response element in the C3 subunit promoter region (Du et al, 2000). Also physiological levels of glucocorticoids are necessary, but not sufficient for the catabolic response in rats with metabolic acidosis (Price et al, 1994), starvation (Wing and Goldberg, 1993) and sepsis (Tiao et al, 1996). However, chronic excessive glucocorticoid production, as occurs in Cushing’s syndrome is not associated with an increased mRNA for ubiquitin, the ubiquitin-conjugating enzyme, E2 14k, or proteasome subunits (Ralliere et al, 1997), suggesting that other factors may be involved.

We have isolated a sulphated glycoprotein of Mr 24 000 produced by cachexia-inducing murine and human tumours.
(Todorov et al., 1996), which induces proteolysis directly in isolated skeletal muscle (Lorite et al., 1997) and in C2C12 murine myoblasts in the absence of glucocorticoids (Smith et al., 1999). This factor, named proteolysis-inducing factor (PIF), induces release of arachidonic acid from muscle cells with an increased production of prostaglandin (PG) E2 and F2α, together with metabolism through the lipoygenase pathways to 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs) (Smith et al., 1999). Release of arachidonic acid and its subsequent metabolism was shown to be directly related to the induction of protein catabolism, since it was completely attenuated in the presence of eicosapentaenoic acid (EPA), which also completely inhibited protein degradation induced by PIF. Of the metabolites of arachidonate only 15(S)-HETE produced a significant increase in protein degradation alone, suggesting that it acts as an intracellular signal for the action of PIF. Since PIF has been shown to upregulate the ubiquitin–proteasome pathway (Lorite et al., 1998), this suggests that 15(S)-HETE should also induce the key regulatory components of this pathway.

This study utilizes an in vitro model of skeletal muscle (C2C12 myotubes) to investigate the effect of 15(S)-HETE on protein degradation and protein and mRNA levels of the key enzymes of the ubiquitin–proteasome pathway, as well as the involvement of potential transcription factors.

**MATERIALS AND METHODS**

**Materials**

L- [2,6-3H] Phenylalanine (sp. act. 2.00 TBq mmol⁻¹) was purchased from Amersham International (Bucks, UK). Foetal calf serum (FCS), horse serum (HS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Life Technologies (Paisley, Scotland). Mouse anti-p42 antibody was a gift from Dr Jane Arnold, UK. Mouse monoclonal antibody to myosin heavy chain was purchased from Novocastra (Newcastle-upon-Tyne, UK). Rabbit polyclonal antisera to residues 1–317 of human k-Bz was purchased from Santa Cruz Biotechnology Inc., CA, USA. Peroxidase-conjugated rabbit antimouse antibody was purchased from Dako Ltd, Cambridge, UK and peroxidase-conjugated goat anti-rabbit antibody from Sigma Chemical Co., Dorset, UK. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA, USA. Oligonucleotides for EMSA, T4 polynucleotide kinase 10ơC2HETE was purchased from Biomol Research Laboratories Inc., PA, USA. 15(S)-HETE was purchased from Biomol Research Laboratories Inc., PA,

**Measurement of total protein degradation**

This was measured essentially as previously described for murine myoblasts (Lorite et al., 2001). Myotubes were formed in six-well multiswatches containing 2 ml DMEM and 2% HS. Cells were labelled for 24 h with 1-[2,6-3H] phenylalanine (0.67 mCi mmol⁻¹), and washed three times in PBS. They were then incubated for 2 h in DMEM without phenol red until no more radioactivity appeared in the supernatant, and then for a further 24 h period with various concentrations of 15(S)-HETE in fresh DMEM without phenol red to reduce quenching of counts, and in the presence of 2 mCi cold phenylalanine to prevent reincorporation of radioactivity. The amount of radioactivity released into the medium was determined using a 2000CA Tri-Carb liquid scintillation analyzer and is expressed as a percentage of control cultures determined from myotubes incubated with the [3H]phenylalanine for 24 h, but not treated.

**Measurement of proteasome activity**

The functional activity of the β-subunits of the proteasome was determined as the ‘chymotrypsin-like’ enzyme activity determined fluorimetrically according to the method of Orino et al., (1991). Cells were sonicated in 20 mM Tris-HCl, pH 7.5, 2 mM ATP, 5 mM MgCl2 and 1 mM dithiothreitol (DTT) at 4°C and enzyme activity was determined on the supernatant fraction, formed by centrifugation for 10 min at 18000 g, using the fluorogenic substrate succinyl-LLVY-AMC (0.1 mM) in the presence or absence of 10 μM lactacystin, a specific proteasome inhibitor (Fenting and Schreiber 1998). Only lactacystin suppressible activity was considered to be proteasome specific. The reaction was terminated by the addition of 80 mM sodium acetate, pH 4.3, and the fluorescence determined with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The activity was adjusted for the protein concentration of the sample, determined using the Bradford assay (Sigma Chemical Co., Dorset, UK). Both substrate and enzyme concentrations were optimal and the results were similar to those obtained using purified proteasomes (Lorite et al., 2001).

**Western blot analysis**

Myotubes were treated with various concentrations of 15(S)-HETE, with or without 2 h preincubation with 50 μM EPA. The medium was removed and the cells were washed with PBS, followed by scraping from the plastic surface and sonication in 500–2000 μl of the buffer used to measure proteasome activity. After centrifugation (18000 g for 5 min), the supernatant was assayed for protein concentration using a colourimetric protein assay (Biorad, UK). Samples of cytosolic protein (2.5 μg) were resolved on 12% sodium dodecylsulphate, polyacrylamide gels (SDS/PAGE) and transferred to 0.45 μm nitrocellulose membranes (Hybond A, Amersham, UK), 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 1:1500 dilution, except for myosin heavy chain used at 1:250 dilution and 1-kBz at 1:400. The secondary antibodies were used at 1:2000 dilution. Incubation was for 1 h at room temperature and development was by enhanced chemiluminescence (ECL) (Amersham, UK). Blots were scanned by a densitometer to quantitate differences, and a parallel gel was silver stained to ensure equal loading.

**Quantitative competitive RT–PCR**

Total RNA was extracted from muscle using TRI-reagent and the RNA concentration was determined from the absorbance at 260 nm. To quantitate the mRNA of interest, increasing amounts of competitor RNA, which differed from the mRNA of interest by containing a short deletion, were added to 250 ng of total RNA and

**Cell culture**

The C2C12 myoblast cell line was grown in DMEM supplemented with 10% FCS plus 1% penicillin and streptomycin under an atmosphere of 10% CO2 in air at 37°C. Myotubes were formed by allowing confluent cultures of myoblasts to fuse in DMEM containing 2% HS over a 7-day period, with changes of medium every 2 days.
complemented by RT–PCR. The amount of specific mRNA was calculated from the amount of competitor, when equal amounts of PCR product were obtained from the competitor and target RNA, as previously described (Wang et al., 1989; Taylor et al., 1998).

The C2 competitor was obtained from the mouse gene using the primer pairs: 5’GGGAGTTCAACCTGAGGATGGAG–3’ and 5’GGTACAGTGGCTTTGCCA–3’. The competitor was obtained from the mouse gene using the primer pairs: 5’TCAACGGAGTACTGATGATTGGG–3’ and 5’GCATGGAAGTGGAGCTGAGAC–3’. All amplicons were excised from the agarose gel and gel purified using a QIAquick Gel Extraction kit (Qiagen). The amplicons were then fragmented using a Covaris flex system (Covaris, Woburn, MA) using a preset protocol with 20 cycles to yield an average size of 50 nt. The competitors were then ligated into the pET30a vector, which had been blunt-end cut with BglII using the TA cloning kit (Invitrogen, Carlsbad, CA). The ligated vector was used to transform competent cells. Plasmid DNA was prepared from PCR-positive clones using Wizard-mini-prep. The amount of competitor was quantitated using the positive clones using Wizard-mini-prep. Competitor RNA was obtained from the rat gene using the primer pairs: 5’GCTGATTGAGAACGG–CATAACCAGCAATGAGCAGC–3’ and 5’GGGATTTCAAGCG–3’. The E2 14k competitor was obtained from the rat gene using the primer pairs: 5’GTACGAGCTGATTGAGAAC–3’ and 5’GGAGATTCAACCGG–3’, together with 1 U Taq polymerase. For the C2 gene, 20 pmol of each of the primers 5’CTCATGC GGGATTTCGACCGG–3’ and 5’CTCTCTCTCATATCCGTTG–3’ were used. The temperature-cycling profile for amplification of the cDNA by PCR, 50 μl of PCR mix was added to each tube. The PCR mixture contained 1 × PCR buffer (without magnesium) together with 3 mM MgCl2 (E2), 3.5 mM MgCl2 (C5) and 2.5 mM MgCl2 (C2) together with 1 U Taq polymerase. For the E2 gene, 10 pmol of each of the primers 5’TCTCATGCGGGA–3’ and 5’GCTGATTGAGAAC–3’ were used, while for the C5 gene 10 pmol of each of the primers 5’TCAACGGAGTACTGATGATTGGG–3’ and 5’GCATGGAAGTGGAGCTGAGAC–3’ were used. The temperature-cycling profile for amplification was as follows: 95°C for 2 min for one cycle followed by 95°C for 30 s, 58°C annealing for 1 min and 72°C extension for 1 min for 30 cycles. Control reactions containing all components except reverse transcriptase and another without template were carried out alongside each experiment to show that the RNA (both target and competitor) had no DNA contamination, while the second control showed that there was no contamination in the PCR mixture. Complification of E2 target and competitor produced DNA fragments of 395 and 284 bp, respectively; C2 target and competitor produced 385 and 309 bp fragments and C5 target and competitor produced 414 and 297 bp fragments, respectively.

For analysis of results, 15 μl of PCR products were separated on a 2% (w/v) agarose gel containing ethidium bromide. The gel images were visualised on a UV transilluminator and photographed. The intensity of the bands was quantitated using a Phoretix photo-imager programme. The volumes of the competitor and target bands were plotted against the known serial dilutions of the competitor used in the experiment. The amount of the sample RNA corresponds to the amount of competitor when the ratio of competitor to target is 1.0. A representative gel showing competitor and target RNA, as well as a dose–response curve is shown in Figure 1.

Preparation of nuclear proteins

Cell monolayers were rinsed, scraped and pelleted in 10 mM HEPES, pH 7.5, 10 mM KCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM EDTA, 0.4 mM PMSF, 0.2 mM NaF, 0.2 mM sodium orthovanadate and 0.3 mg ml−1 leupeptin. The pellets were resuspended in 300 μl of the same wash buffer and incubated on ice for 15 min. Lysis was achieved by the addition of 30 μl of 1% Triton X-100, followed by vortexing, and nuclei were pelleted by a 30 s centrifugation at 14 000 × g, and the supernatant was thoroughly removed. The nuclear pellet was resuspended in 50 μl ice-cold lysis buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 0.3 mM NaCl, 0.1 mM EDTA and 1 mM DTT) and the suspension was kept on ice for 20 min with a 30 s vortex every 3–5 min. Centrifugation at 14 000 × g, for 5 min yielded the supernatant containing the protein extract.

Electrophoretic mobility shift assay

The transcription factor consensus oligonucleotides for the NF-kB-responsive element (5’AGT TGA GAG TTT CCC AGG C3’) was purchased from Promega (Southampton, UK). The probes were labelled with [γ-32P]ATP by using T4 polynucleotide kinase (Promega) for 10 min at 37°C. For the binding reaction, nuclear extract (10 μg protein) was incubated in a 10 μl volume with 2 μl EMSA binding buffer (20% glycerol; 5 mM MgCl2; 250 mM NaCl; 2.5 mM EDTA; 2.5 mM DTT; 50 mM Tris-HCl, pH 7.5 and 0.25 mg ml−1 poly (dl-dc)-poly (dl-dc) and 1 μl γ-32P-labelled probe for 20 min at room temperature following a 10 min preincubation in the absence of the labelled probe. The specificity of the binding reaction was determined by coincubating duplicate samples with unlabelled probes. A negative control reaction was also included which contained everything except sample. Protein–nucleic acid complexes were resolved using an 8% nondenaturing polyacrylamide gel and a native 5% polyacrylamide stacking gel in 25 mM Tris, pH glycine for 30 min at 150 mV. Gels were dried.
between Whatman 3 M filter paper (Whatman Inc., Clifton, NJ, USA), dried under vacuum at 80 °C for 1 h and exposed to ‘Hyperfilm MP’ (Amersham Pharmacia Biotech) for 48 h at −70 °C.

Statistical analysis

Results are presented as mean ± s.e.m. Differences were determined by one-way ANOVA, followed by Tukey’s test.

RESULTS

The effect of 15(S)-HETE on total protein degradation in C2C12 myotubes, as measured by [3H]phenylalanine, is shown in Figure 2A. The concentrations of 15(S)-HETE chosen were based on those produced by PIF in murine myoblasts (Smith et al, 1999). In this study PIF increased 15-HETE production from 25 to 70 nmol per 5 × 10⁶ cells per 2 h, that is, an increase of 9 nmol per 10⁶ cells. If all the 15-HETE entered the cells in the current experiments, this would give a value between 1.4 and 6 nmol per 10⁶ cells. As previously reported in C2C12 myoblasts, 15(S)-HETE produced a significant increase in [3H]phenylalanine release with a bell-shaped dose–response curve, although the concentrations producing maximal protein catabolism (78–156 nM) were higher than required in myoblasts (Smith et al, 1999). A similar dose–response curve is observed with PIF (Smith et al, 1999), and with protein breakdown in muscle of tumour-bearing animals (De-Blaauw et al, 1997), and may result from downregulation of receptors, or receptor desensitisation as occurs in the stimulation of lipolysis by isoprenaline. The effect was attenuated down to control values in the presence of 50 μM EPA at all concentrations of 15(S)-HETE (Figure 2A), as well as the highly specific and irreversible proteasome inhibitor, lactacystin (data not shown), suggesting that the action of 15(S)-HETE may be mediated through the ubiquitin–proteasome proteolytic pathway. This concentration of EPA has been previously shown to attenuate PIF-induced protein catabolism in murine myoblasts (Smith et al, 1999). Lower concentrations of EPA had no effect. The plasma concentration of EPA in humans consuming large doses would be in the range 100–200 μM. To determine if proteasome proteolytic activity was also increased by 15(S)-HETE, the ‘chymotrypsin-like’ enzyme activity, the predominant proteolytic activity of the proteasome, was determined using the fluorogenic substrate Suc LLVY-MCA. The results presented in Figure 2B show that 15(S)-HETE induced upregulation of the ‘chymotrypsin-like’ activity of the proteasome, with maximal effect in the concentration range 78–312 nM, as for total protein catabolism (Figure 2A). As with protein catabolism, proteasome proteolytic activity was attenuated in cells pretreated with 50 μM EPA.

To determine whether the increased proteasome proteolytic activity arose from an increased gene expression mRNA levels of proteasome subunits C2(α) and C5(β), as well as the C2(α′) non-conjugating enzyme, E2α, were determined in C2C12 myotubes exposed to various concentrations of 15(S)-HETE for various times to determine the earliest time of gene induction and how long this persisted, using quantitative competitive RT–PCR (Figure 3). Expression of the mRNA for the 20S proteasome subunits C2 (Figure 3A) and C5 (Figure 3B) was significantly increased with 15(S)-HETE concentrations in the range 78–312 nM after 4 h, as for protein catabolism with a similar bell-shaped dose–response curve. The level of expression of mRNA for C5 was approximately 2000-fold greater than for C2. Expression of mRNA for E2α was also significantly increased 4 h after treatment with 15(S)-HETE (Figure 3C), although the concentration range was somewhat broader than for proteasome subunits (78–780 nM). The extent of induction of C2, C5 and E2α by 15(S)-HETE was less than that observed at 4 h. The increased expression of proteasome subunits in the presence of 15(S)-HETE was attenuated by prior treatment with EPA (Figure 4). There was also evidence for an increased expression of mRNA for both C2 (Figure 6A) and C5 (Figure 6B) proteasome subunits after 24 h. As with studies on PIF (Gomes-Marcondes et al, 2002), higher concentrations of 15(S)-HETE produced increases in proteasome mRNA expression at 24 h than at 4 h (Figure 3).

To determine whether increased mRNA expression for proteasome subunits C2 and C5 was reflected in higher protein levels of other subunits, cellular supernatants of C2C12 myotubes treated with 15(S)-HETE for 24 h were immunoblotted for 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 (Tanahashi et al, 1999) (Figure 7A). Maximal expression was seen at concentrations of 15(S)-HETE between 156 and 312 nM, which is in the range where an increased proteasome mRNA expression was seen (Figure 3). Activation of proteasome expression was associated with a decrease in myosin expression in the same concentration range (78–312 nM) (Figure 7B). These results suggest that 15(S)-HETE leads to an increased expression of the ubiquitin–proteasome pathway, with an increase in overall protein degradation and depletion of myofibrillar proteins.

The NF-κB transcriptional pathway was investigated as a possible mediator of the increased expression of the ubiquitin–proteasome pathway, since NF-κB has been shown to be an
essential mediator of TNF-α-induced protein catabolism in differentiated muscle cells (Li and Reid, 2000). As shown in Figure 8A, 15(S)-HETE induced elevated levels of NF-κB DNA binding activity in the myotube nucleus, after a 30 min incubation, with a maximal effect between 156 and 234 nM. Increased nuclear accumulation of NF-κB was not seen after pretreatment with EPA. Depletion of I-κBα from the cytosol was determined by immunoblotting of cytoplasmic extracts from the same cells in which NF-κB translocation was measured. Rapid breakdown of I-κBα was evident 30 min after treatment with 156 nM 15(S)-HETE (Figure 8B), and this was not seen in cultures pretreated with 50 μM EPA, suggesting a mechanism by which EPA could downregulate the increased proteasome expression seen in the presence of 15(S)-HETE. The NF-κB inhibitor peptide, SN50 (Lin et al, 1995) attenuated the increased chymotrypsin-like enzyme activity in the presence of 15(S)-HETE (Figure 8C), suggesting the NF-κB may be involved in transcriptional activation induced by 15(S)-HETE.

**DISCUSSION**

There are three main pathways for the intracellular catabolism of proteins in skeletal muscle; lysosomal proteases, a nonlysosomal calcium-activated process involving the cysteine proteases calpains.
ubiquitin–proteasome proteolytic pathway (Smith et al., 1999), responsible for the increased transcription of the genes of the ubiquitin–proteasome proteolytic pathway. Of the three, the latter is considered to be most important (Lecker et al., 1999), since the former two processes contribute less than 15–20% of total protein breakdown and do not catabolise myofibrillar proteins (Lowell et al., 1986). In cancer cachexia, where there is extensive and specific loss of skeletal muscle, tissue levels of mRNA for ubiquitin, 20S proteasome α and β subunits and the ubiquitin-conjugating enzyme, E2ubk, have been found to be increased in mouse (Lorite et al., 1998), rat (Temparis et al., 1994) and humans (Williams et al., 1999). Knowledge of the mechanism of this increased gene transcription is important for the design of therapeutic agents, because muscle wastage leads to weakness, fatigue and eventually death through impairment of respiratory muscle function (Windsor and Hill, 1998).

Previous studies on protein degradation, induced by PIF in vitro, suggested 15(S)-HETE as a possible intracellular signal responsible for the increased transcription of the genes of the ubiquitin–proteasome proteolytic pathway (Smith et al., 1999). This suggestion is supported by recent observations (Whitehouse et al., 2001) showing that EPA, an inhibitor of 15(S)-HETE, produced in response to PIF, attenuated the development of muscle catabolism in the MAC16 murine cachexia model by downregulation of the increased expression of proteasome α subunits and chymotrypsin-like enzyme activity. In addition, we have recently found (Whitehouse and Tisdale, 2001) that both EPA and a 12/15-lipoxygenase inhibitor, CV-6504, were also effective in attenuating protein degradation in acute starvation mediated through the ubiquitin–proteasome pathway. This suggests that in both models of muscle wasting the same intracellular-mediator is involved, possibly 15(S)-HETE. The aim of the present study has been to determine whether 15(S)-HETE alone is capable of upregulation of the key enzymes of the ubiquitin–proteasome pathway using C2C12 myotubes.

Using this in vitro system, 15(S)-HETE has been shown to induce total protein degradation at concentrations between 78 and 312 nm, with a bell-shaped dose–response curve similar to that previously reported in C2C12 myoblasts (Smith et al., 1999), although the concentration to produce a maximal effect was higher in myotubes. Since the effect of PIF on protein catabolism in myotubes has been shown to be mediated through an
upregulation of the ubiquitin–proteasome proteolytic pathway (Gomes-Marcondes et al, 2002), if 15(S)-HETE was acting as an intracellular transducer of PIF action then the effect on protein degradation would also be mediated by this pathway. Accordingly 15(S)-HETE was shown to increase the 'chymotrypsin-like' enzyme activity of the \( \beta \) subunit of the proteasome, suggested to be the rate-determining step in protein catabolism (Chen and Hochstrasser, 1996; Rock et al, 1994), in the same concentration range as that stimulating total protein degradation. Further confirmation that

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\text{15(S)-HETE stimulated expression of the rate-limiting components of the ubiquitin–proteasome pathway was provided by an increased mRNA level for proteasome subunits C2 and C5, as well as E2\(_{14k}\), and increased protein levels of proteasome subunit, p42, over the same concentration range as that inducing total protein degradation. Previous studies have reported increased proteasome subunits (Temparis et al, 1994; Lorite et al, 1998) MSSI (Combaret et al, 1999) and E2\(_{14k}\) with increased muscle catabolism in cancer cachexia, suggesting that they play important roles in protein degradation. Indeed, cellular levels of the myofibrillar protein, myosin, were reduced in myotubes treated with 15(S)-HETE over the same concentration range as that inducing the regulatory components of the ubiquitin–proteasome proteolytic pathway.}

We have suggested (Whitehouse et al, 2001) that EPA attenuates protein degradation and the increased expression of the key regulatory components of the ubiquitin–proteasome pathway as a result of the inhibition of 15(S)-HETE production through the PIF-induced activation of phospholipase A\(_2\) and arachidonate release (Smith et al, 1999). It was therefore surprising that EPA was able to attenuate protein degradation and the increase in proteasome expression induced by 15(S)-HETE. This suggests that EPA also affects a downstream pathway involved in induction of proteasome expression by 15(S)-HETE, possibly a nuclear transcription factor, since Ross et al (1999) have shown that in the pancreatic cancer cell line, MiaPaCa 2, exposed to EPA for 2h prior to a pulse of TNF-\( \alpha \), I-\( \beta \)B was preserved, suggesting an effect of EPA in stabilising the NF-\( \kappa \)B complex in the cytoplasm.

Watchorn et al (2001) have shown PIF to activate both the transcription factors NF-\( \kappa \)B and STAT3 in hepatic cells. Li et al (1998) showed that TNF-\( \alpha \) stimulated protein loss in C\(_{6}C_{12}\) myotubes involved the ubiquitin–proteasome proteolytic pathway and was accompanied by nuclear translocation of NF-\( \kappa \)B to its targeted DNA sequence by stimulating phosphorylation, ubiquitin conjugation and proteolysis of I-\( \kappa \)Bz. Total protein content and myosin heavy chain levels in C\(_{6}C_{12}\) myotubes were found to be unaltered in cells transfected with viral plasmid constructs that overexpress mutant I-\( \kappa \)Bz proteins that are insensitive to degradation by the ubiquitin–proteasome pathway (Li and Reid, 2000). These data suggest that NF-\( \kappa \)B is an essential mediator of TNF-\( \alpha \)-induced protein catabolism in differentiated muscle cells. However, Du et al (2000) found that NF-\( \kappa \)B is a repressor of C3 proteasome subunit transcription in muscle cells and that

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British Journal of Cancer (2003) 89(4), 737 – 745

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**Figure 8** (A) Activation of NF-\( \kappa \)B binding to DNA as demonstrated by EMSA. Nuclear extracts of C\(_{6}C_{12}\) myotubes treated with 0\( \mu \)M (lane 1), 31\( \mu \)M (lane 2), 156\( \mu \)M (lane 3), 234\( \mu \)M (lane 4) or 312\( \mu \)M 15(S)-HETE (lane 5) for 30 min were compared with cells treated with 0\( \mu \)M (lane 6), 31\( \mu \)M (lane 7), 156\( \mu \)M (lane 8), 234\( \mu \)M (lane 9) or 312\( \mu \)M 15(S)-HETE (lane 10) pretreated with 50\( \mu \)M EPA for 2h. The gel shown is a representative example of three separate determinations and the average densitometric data are presented below the blot. **(B)** Western blot of I-\( \kappa \)Bz levels in cytoplasmic extracts of myotubes treated with 0\( \mu \)M (lane 1), 3.1\( \mu \)M (lane 2), 15.6\( \mu \)M (lane 3), 31\( \mu \)M (lane 4), 156\( \mu \)M (lane 5) and 312\( \mu \)M 15(S)-HETE (lane 6) for 30 min or after pretreatment for 2h with EPA (50\( \mu \)M) and treated with 0\( \mu \)M (lane 7), 3.1\( \mu \)M (lane 8), 15.6\( \mu \)M (lane 9), 31\( \mu \)M (lane 10), 156\( \mu \)M (lane 11) and 312\( \mu \)M 15(S)-HETE (lane 12). Detection was by rabbit polyclonal antisera to residues 1–317 of human I-\( \kappa \)Bz. The blot shown is representative of three separate determinations and the average densitometric data are provided below the blot. **(C)** The effect of the NF-\( \kappa \)B inhibitor peptide SN50 on 15(S)-HETE induced upregulation of the 'chymotrypsin-like' activity of the proteasome. Myotubes were treated with the cell-permeable NF-\( \kappa \)B inhibitor peptide, SN50 (18\( \mu \)M) for 20 min prior to the addition of 15(S)-HETE. The activity shown represents the lactacystin (10\( \mu \)M) suppressible proteolytic activity. Differences are indicated as (a) \( P<0.05 \) and (b) \( P<0.01 \) from those in the absence of 15(S)-HETE and (c) \( P<0.01 \) and (d) \( P<0.001 \) from those in the absence of SN50.
The mechanism by which 15(S)-HETE could activate NF-κB is not known. One possibility is that as 15(S)-HETE is oxidised it could result in the generation of reactive oxygen species, which might regulate the redox sensitive NF-κB, possibly through oxidation of constituent proteins, which could augment DNA-binding activity or promote the release from, or degradation of I-κB. Mild oxidative stress has been shown to induce 20S proteasome subunits and E2α in murine myotubes (Gomes-Marcondes and Tisdale, 2002). However, it is not clear why 5- and 12-HETE would not have the same properties as 15-S-HETE if this were the mechanism. Further studies are required to determine the role of transcription factors and cellular signalling pathways in the action of 15(S)-HETE.

ACKNOWLEDGEMENT

This work was supported by the Lustgarten Foundation for Pancreatic Cancer Research.

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15(S)-HETE and protein catabolism
AS Whitehouse et al