Effect of a Cancer Cachectic Factor on Protein Synthesis/Degradation in Murine C2C12 Myoblasts: Modulation by Eicosapentaenoic Acid

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ABSTRACT

The effect of a proteolysis inducing factor (PIF) on protein synthesis and degradation and the modulation of this effect by the polyunsaturated fatty acid, eicosapentaenoic acid (EPA), have been examined using a surrogate model system, C2C12 myoblasts in vitro. After 90 min of incubation, PIF produced a significant inhibition of protein synthesis in a dose-dependent manner, with maximal inhibition at a concentration of 4 nM. The effect was attenuated both by treatment with a monoclonal antibody to PIF and by treatment with insulin at physiological concentrations (1 nM) and below (0.1 nM), but not by EPA (50 μM). The inhibitory effect on protein synthesis was transitory and was not seen after prolonged incubation with PIF. An increased rate of protein degradation was observed in C2C12 myoblasts after addition of PIF, which was also maximal at a concentration of PIF of 4 nM. Higher concentrations of PIF did not produce an increase in protein degradation. Unlike the effect on protein synthesis, the enhanced protein degradation was completely abolished by pretreatment with 50 μM EPA, suggesting that the two effects are mediated by different mechanisms. PIF produced an increased release of [3H]arachidonic acid from prelabelled myoblasts with a dose-response curve parallel to that of protein degradation and with a maximum at 4 nM PIF. Release of [3H] arachidonic acid was completely blocked in cells pretreated with 50 μM EPA, suggesting that the effect was related to protein degradation. The [3H]arachidonic acid was rapidly metabolized to prostaglandins E2 and Fα, and to 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs). Production of all eicosanoids was attenuated in cells pretreated with EPA. Of all the metabolites, only 15-HETE produced a significant increase in protein degradation in C2C12 myoblasts with a maximal effect at 30 nM and with a bell-shaped dose-response curve similar to that produced by PIF. These results suggest that PIF enhances protein degradation as a result of an increased production of 15-HETE.

INTRODUCTION

Analyses of the changes in body composition of cachectic cancer patients have shown that the principal components of weight loss were skeletal muscle and fat (1). In patients with lung cancer, skeletal muscle protein reserves were shown to be depleted up to 75%, while nonmuscle protein stores remained the same. Although cytokines such as tumor necrosis factor-α (2), interleukin 1 (3), and ciliary neurotrophic factor (4) produce muscle catabolism when administered to animals, in most cases, evidence for a direct catabolic effect on skeletal muscle has not been obtained. We have recently isolated a sulfated glycoprotein of apparent Mr 24,000 from the urine of patients with cancer cachexia (5, 6) that, when administered to mice, produced a state of cachexia, with specific loss of the nonfat carcass mass (7). There was a decrease in protein synthesis and an increase in protein degradation in the skeletal muscle of such treated animals, an effect similar to that observed in mice bearing a cachexia-inducing tumor (8). Unlike the cytokines, the glycoprotein was capable of initiating protein degradation directly in isolated gastrocnemius (6) and soleus (9) muscles and for this reason has been referred to as PIF (1). Induction of protein degradation in vitro by PIF was associated with a significant elevation of PGE2, which may be causally related to the process of muscle catabolism, because it was attenuated by both a monoclonal antibody to PIF and the polyunsaturated fatty acid EPA, agents that also attenuated the enhanced protein degradation.

To further examine the mechanism for the changes in protein synthesis and degradation in skeletal muscle induced by PIF and the effect of EPA on this process, we now report studies using the mouse myoblast cell line C2C12 derived from the satellite cell population of the thigh muscle of a 2-month-old mouse (10). This cell line has receptors for insulin and responds to physiological stimuli in a manner similar to muscle fibers in vivo (11). Although the ultimate in vivo target is mature myotubes, the myoblast cell line provides a surrogate model system for testing the effect of PIF on protein synthesis and degradation.

MATERIALS AND METHODS

Materials. L-[2, 6-3H]Phenylalanine (specific activity, 2.00 TBq/mmol) was purchased from Amersham International (Buckinghamshire, United Kingdom), and [5, 6, 8, 9, 11, 12, 14, 15-3H]arachidonic acid (specific activity, 7.6 TBq/mmol) was purchased from DuPont (UK) Ltd. (Herts, United Kingdom)

Bovine fetal serum and DMEM were purchased from Life Technologies (Paisley, Scotland). All other chemicals were purchased from Sigma Chemical Co. (Dorset, United Kingdom). Optiphase Hisafe 3 scintillation fluid was supplied by Fisons (Loughborough, United Kingdom).

Cell Culture. The C2C12 mouse myoblast cell line was grown in 60 × 15-mm Petri dishes in 3 ml of DMEM supplemented with 12% FCS, 1% non-essential amino acids, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO2 in air at 37°C. All experiments with myoblasts were performed on cells in the subconfluent state.

Purification of PIF. Solid MAC16 tumors, excised from mice with a weight loss between 20 and 25%, were homogenized, followed by ammonium sulfate (40% w/v) precipitation, and the supernatant was subjected to affinity chromatography as described (5, 6). The immunogenic fractions were concentrated and used without further purification, because the major impurity was albumin (5), which was present in the tissue culture media.

Measurement of Protein Synthesis. Protein synthesis in C2C12 myoblasts was measured as described by Southorn and Palmer (12), during the final 60 min of the incubation with PIF, by adding 10 μCi of [3H]phenylalanine (75 μmol l-phenylalanine and 50 μCi of l-2,6-3Hphenylalanine/ml). The incubation was terminated by washing the cells three times with ice-cold PBS (1 ml; pH 7.4), and after removal of any residual PBS, incubation was continued at 4°C for 20 min with 0.2 μl peroxidase (1 ml). The peroxidase activity was removed and replaced with 1 ml of 0.3 M NaOH, and the Petri dish was placed at 4°C for an additional 30 min, followed by an additional incubation at 37°C for 20 min. The NaOH solution, containing the dissolved cellular protein, was transferred to clean tubes, and an additional 1 ml of 0.3 M NaOH was used to rinse the dishes. To precipitate cellular protein, 2 ml peroxidase (0.5 ml) was added, mixed, and then placed on ice for 20 min. After this time, the samples were centrifuged at 3000 × g for 10 min at 4°C, and the supernatant was used for measuring RNA content. The pellet, which comprised DNA and protein, was dissolved in 1 ml of 0.3 M NaOH, and an aliquot (20 μl) was used to measure protein concentration using Bio-Rad protein assay reagent.

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The abbreviations used are: PIF, proteolysis inducing factor; EPA, eicosapentaenoic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid.
reagent. An additional aliquot (0.5 ml) was mixed with scintillation fluid, and the radioactivity was determined using a 2000CA Tri-Carb liquid scintillation analyzer. The ratio of protein synthesis were calculated as the specific radioactivity (Sr) in dpm/µg protein/h as described (12).

Measurement of Total Protein Breakdown. C2C12 myoblasts were seeded at 2 × 10⁴ cells/well in 2 ml of DMEM in six-well multidishes. After 24 h, cells were labeled with l-[2,6-³H]phenylalanine (10 µCi/ml) and the experiment was repeated three times; bars, SE. a, differences from cells incubated in the absence of PIF (P < 0.05, as determined by Student's t test with Bonferroni correction).

Effect of PIF on Arachidonate Release and Metabolism. C2C12 myoblasts were seeded at 2 × 10⁴ per ml in 2 ml of medium in six-well multidishes, left for 24 h, and were labeled with 10 µM arachidonic acid (containing 1 µCi of [³H]arachidonic acid/ml). After 24 h, cells were washed with PBS, and fresh medium was added with or without EPA (50 µM), and the cells were left for an additional 2 h prior to the addition of PIF. After 24 h, 1 ml of medium was removed for determination of the radioactivity released, and cell bound radioactivity was determined as for protein synthesis.

Analysis of Metabolites of Arachidonate Formed in the Presence of PIF. C2C12 myoblasts were seeded at 2 × 10⁴ cells/ml in 120 ml of medium, left for 24 h, and then incubated with various concentrations of PIF for an additional 24 h. The cells were washed in PBS and resuspended in fresh medium containing 2.5 µCi of [³H]arachidonic acid, mixed with unlabeled arachidonic acid, to give a final concentration of 10 µM. After 2 h, the cells were washed three times with ice-cold PBS, trypsinized, washed, and resuspended in PBS (0.9 µl on ice) and sonicated for three 15-s pulses with 10-s intervals. The pH was adjusted to 3.5 with IN HCl, and chloroform:methanol (1:2, v/v; 5 ml) was added, followed by vigorous mixing for 1 min. After 30 min at room temperature, chloroform (2 ml) was added, and after vigorous mixing, was followed by the addition of 0.001 N NaOH (1 ml) and vortexing for another 10 s. After centrifugation at 2000 × g for 20 min, the chloroform layer was removed and evaporated under a stream of nitrogen. The residue was dissolved in acetonitrile (0.1 ml) and analyzed by RP-HPLC with a Waters µ Bondapak C₁₈ column (3.9 × 300 mm) by an isocratic elution at 1.0 ml min⁻¹ with 58% acetonitrile:water:acetic acid (100:3:0.05) and 42% acetonitrile:acetic acid (100:0.05), as described (13). Radioactivity and UV absorbance at 237 nm were monitored. Peaks were identified based on the retention times of authentic standards, and the eicosanoid concentration was calculated from the radioactivity present in the fractions.

RESULTS

After a 90-min incubation of C2C12 myoblasts with PIF, inhibition of protein synthesis, measured over the final 60 min of incubation, was observed in a dose-dependent manner with concentrations between 0.5 and 10 nM (Fig. 1). A maximum decrease in protein synthesis of 28% was achieved with 4 nM PIF (Fig. 1). This depression in protein synthesis was transient, and incubation of C2C12 myoblasts with PIF for longer periods of time did not significantly affect the rate of protein synthesis. The depression in protein synthesis in C2C12 myoblasts induced by PIF was attenuated by prior treatment with a murine monoclonal antibody (Table 1), which we have shown previously to be specific for PIF (14). Interestingly, the rate of protein synthesis in cells treated with PIF plus the monoclonal antibody was found to be significantly higher than in cells treated with the monoclonal antibody alone. Treatment of myoblasts with insulin also completely blocked the inhibition of protein synthesis by PIF (Table 2). Insulin was effective in suppressing the effect of PIF at physiological concentrations (1 nM) and below (0.1 nM) and caused significant increases in the rate of protein synthesis at all concentrations above physiological levels. Incubation of myoblasts with EPA (50 µM) for 24 h alone caused a small stimulation in protein synthesis but did not attenuate the inhibitory effect of PIF (Fig. 1).

Protein degradation was measured by the release of l-[2,6-³H]phenylalanine into the culture medium, in the presence of excess phenylalanine from C2C12 myoblasts, labeled previously for a 24-h period,

Table 1 Effect of murine anti-PIF monoclonal antibody on PIF-induced inhibition of protein synthesis in C2C12 myoblasts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sr ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11.00 ± 0.40</td>
</tr>
<tr>
<td>PIF</td>
<td>10.16 ± 0.24¹</td>
</tr>
<tr>
<td>Anti-PIF antibody</td>
<td>11.60 ± 1.03¹</td>
</tr>
<tr>
<td>PIF + anti-PIF antibody</td>
<td>12.96 ± 0.48¹</td>
</tr>
</tbody>
</table>

¹ Cells were pretreated with anti-PIF antibody (10 µg/ml) for 1 h prior to addition of PIF (1.4 nM). Protein synthesis rates were determined after 90 min as described in “Materials and Methods.”
ALTERATIONS IN PROTEIN SYNTHESIS BY A CANCER CACHECTIC FACTOR

Table 2. Effect of insulin on PIF-induced inhibition of protein synthesis in C₂C₁₂ myoblasts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sr ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15.60 ± 0.32</td>
</tr>
<tr>
<td>PIF (1.4 nM)</td>
<td>13.77 ± 0.37 b</td>
</tr>
<tr>
<td>Insulin (0.1 nM)</td>
<td>15.89 ± 0.39</td>
</tr>
<tr>
<td>Insulin (0.1 nM) + PIF (1.4 nM)</td>
<td>15.37 ± 0.29 c,d</td>
</tr>
<tr>
<td>Insulin (1 nM)</td>
<td>17.36 ± 0.63 d</td>
</tr>
<tr>
<td>Insulin (1 nM) + PIF (1.4 nM)</td>
<td>17.05 ± 0.43 d,e</td>
</tr>
<tr>
<td>Insulin (10 nM)</td>
<td>19.46 ± 0.26 f</td>
</tr>
<tr>
<td>Insulin (10 nM) + PIF (1.4 nM)</td>
<td>19.46 ± 0.79 b,c,d,e</td>
</tr>
</tbody>
</table>

* Cells were preincubated with insulin for 90 min prior to the addition of PIF. Protein synthesis rates were determined 90 min after addition of PIF as described in “Materials and Methods.”

** From the no-treatment group: a P < 0.01; b P < 0.05; c P < 0.005.
** From the PIF group as determined by Student’s t test, where n = 5, and the experiment was repeated at least three times: a P < 0.05; b P < 0.005.

and from the decrease in protein-bound radioactivity. An increased rate of protein degradation was observed 24 h after the addition of PIF, which was maximal at a concentration of 4 nM (Fig. 2), with increasing concentrations of PIF producing a decrease in protein degradation. As with protein synthesis, the increased protein degradation induced by PIF was attenuated with a murine monoclonal antibody specific to PIF (Fig. 3). However, unlike the effect on protein synthesis, preincubation of C₂C₁₂ myoblasts with EPA (50 μM) for 24 h prior to PIF completely abolished the increase in protein degradation (Fig. 2).

In vitro studies with isolated muscle have implicated PGE₂ production in the process of protein degradation induced by PIF (9). The first step in PGE₂ production is the release of arachidonic acid from membrane phospholipids, catalyzed by phospholipase A₂, which then becomes available for oxidation by the cyclooxygenase and lipoxygenase enzyme system. This suggests that activation of phospholipase A₂ may be the first step in the induction of protein degradation in skeletal muscle by PIF. Treatment of C₂C₁₂ myoblasts with PIF has been shown to inhibit protein synthesis and increase protein degradation, an effect similar to that found in gastrocnemius muscles of mice 24 h after administration of PIF (9). This suggests that this cell line responds in a manner similar to that of skeletal muscle in vivo, and therefore that it is an appropriate model in which to investigate the mechanism of this effect. This view is supported by the selective attenuation of PIF-induced protein degradation by EPA in this cell line, without an effect on the inhibition of protein synthesis.

DISCUSSION

Treatment of C₂C₁₂ myoblasts with PIF has been shown to inhibit protein synthesis and increase protein degradation, an effect similar to that found in gastrocnemius muscles of mice 24 h after administration of PIF (9). This suggests that this cell line responds to PIF in vitro in a manner similar to that of skeletal muscle in vivo, and therefore that it is an appropriate model in which to investigate the mechanism of this effect. This view is supported by the selective attenuation of PIF-induced protein degradation by EPA in this cell line, without an effect on the inhibition of protein syn-
thesis. This effect is the same as that observed in skeletal muscle of mice bearing the MAC16 tumor, which had been administered EPA by gavage, where EPA significantly reduced the enhanced protein degradation, without an effect on the reduced protein synthesis (8). Thus, EPA can be used to distinguish second messenger pathways for the effect of PIF on protein synthesis and degradation.

Inhibition of protein synthesis by PIF might result from an ability to block translation, because no significant changes in protein synthesis were observed beyond 6 h, which would have reflected changes at the transcriptional level. An effect on translation is also suggested from the fact that pretreatment of cells with insulin for 90 min prior to the addition of PIF abolished the inhibitory effect on protein synthesis. The increase in protein synthesis in C2C12 myoblasts after short-term incubation with insulin has been attributed to an increase in the level of translational efficiency, because actinomycin D has been shown to inhibit this response (15). Further studies are required to establish the mechanism for the inhibition of protein synthesis by PIF, but the effect is specific, because it was completely abolished after pretreatment of C2C12 cells with a monoclonal antibody, which we have shown previously to be specific for PIF (14). A similar short-term inhibition of protein synthesis has been observed in hepatocytes after incubation with vasopressin, and this effect has been attributed to a reduction in eIF-2B activity arising from an increase in phospho-

Fig. 3. Effect of anti-PIF monoclonal antibody on PIF-induced protein degradation in C2C12 myoblasts. Cells were incubated with increasing concentrations of PIF alone (×) or after pretreatment with monoclonal antibody (10 μg/ml) 2 h before the addition of PIF (■). Results are shown as means, where n = 9; bars, SE. a, differences from controls in the absence of PIF (P < 0.005); b, differences from PIF-treated cells (P < 0.005); both a and b, as determined by Student’s t test with Bonferroni correction.

Fig. 4. Effect of PIF on the release of [³H]arachidonate from C2C12 cells without (●) or with (○) 2 h of preincubation with 50 μM EPA. The radioactivity associated with cells is also shown in the absence (×) and presence (■) of EPA. Results are shown as means for four samples/data point, and the experiment was repeated three times; bars, SE. Differences from controls in the absence of EPA are: a, P < 0.05; b, P < 0.01; and c, P < 0.005, as determined by Student’s t test with Bonferroni correction.
Fig. 5. Effect of PIF on production of arachidonic acid (○), PGE$_2$ (×), PGE$_{2α}$ (■), 5-HETE (○), 12-HETE (▲), and 15-HETE (△) by C$_2$C$_{12}$ cells. Cells were incubated with the indicated concentrations of PIF for 24 h, labeled with [H]arachidonate, and the amount of radioactivity in eicosanoid metabolites was determined after separation by RP-HPLC, as described in “Materials and Methods.” Results are shown as means for four samples/data point, and the experiment was repeated three times; bars, SE. Differences from controls in the absence of PIF are: a, $P < 0.05$; b, $P < 0.01$; and c, $P < 0.005$, as determined by two-way ANOVA, followed by Tukey’s test.

Fig. 6. Effect of EPA on the eicosanoids produced by C$_2$C$_{12}$ myoblasts in response to PIF. Cells were incubated either alone (□) or with EPA (△), or with (■) or without (□) EPA (50 μM) for 2 h prior to the addition of PIF (4 nM). After 24 h, cells were labeled with 10 μM [H]arachidonate acid, and the formation of prostaglandins and HETEs was determined after an additional 2 h, as described in “Materials and Methods.” Differences from controls in the absence of PIF are: a, $P < 0.05$; and b, $P < 0.005$. Differences from cells incubated with PIF are: c, $P < 0.01$; and d, $P < 0.005$. a–d, as determined by two-way ANOVA, followed by Tukey’s test.

rlation of eIF-2α (16). Future studies will evaluate whether similar mechanisms are operative with PIF.

Protein degradation rates in C$_2$C$_{12}$ myoblasts increased by 50–90% in response to PIF, and maximal stimulation was achieved at a concentration of 4 nM, which also produced maximal inhibition of protein synthesis. Higher concentrations of PIF did not enhance protein degradation, giving a bell-shaped dose-response curve. A similar dose-response relationship for protein degradation has been obtained for purified PIF added to soleus muscle in vitro (9) and with serum from cachectic mice with increasing weight loss added to gastrocne-
muscle in vitro (17). Similar results have been reported for the increased protein breakdown of muscle in tumor-bearing animals, which reduces with increases in tumor growth (18). The effect is similar to hormone-induced desensitization of lipolysis in adipocytes and may result from down-regulation of receptors.

Previous results from our own laboratory (7, 9, 17) and those of others (19, 20) suggest a role for PGE2 in the induction of protein degradation in skeletal muscle. In addition, PGE2 has been shown to directly increase protein degradation in diaphragm and soleus muscle (21). However, the role of prostaglandins in the regulation of muscle protein breakdown is controversial, and some studies (22–24) have failed to confirm an increased protein degradation when PGE2 was incubated with rat or mouse muscles. In addition, the cyclooxygenase inhibitor indomethacin inhibited muscle PGE2 production by incubated muscles from septic rats but did not lower proteolytic rates (24).

In the present study, we have shown release of arachidonic acid in C2C12 myoblasts in response to PIF. This step appears to be related to the increase in protein degradation, because it was inhibited in cells pretreated with EPA, which also inhibited the increase in protein degradation in response to PIF. Release of arachidonic acid will lead to metabolism through both the cyclooxygenase and lipoxygenase pathways. This study shows that PIF caused a rise, not only in PGE2 and PGF2α, but also in the lipoxygenase products 5-, 12-, and 15-HETE. Such lipoxygenase metabolites, rather than PGE2, may be the signal for protein degradation. Studies in which the individual eicosanoids were added to C2C12 myoblasts showed no significant stimulation by PGE2, PGF2α, 5-HETE, or 12-HETE or arachidonic acid. However, 15-HETE produced a dose-dependent stimulation of protein degradation with a bell-shaped profile similar to that produced by PIF and with a maximal 2-fold stimulation at a concentration of 30 nM.
This suggests that 15-HETE rather than PGE2 is the intracellular mediator for the stimulation of protein degradation by PIF. This would explain why CV-6504, a 15-lipoxygenase inhibitor (13), was capable of attenuating the development of cachexia in mice bearing the MAC16 tumor (25). The coordinated release of PGE2 and 15-HETE would explain the apparent correlation between PGE2 production and protein degradation observed previously (7, 17, 19, 20). The mechanism by which 15-HETE stimulates protein degradation will be the subject of additional studies.

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