Elevated IGF-II mRNA and phosphorylation of 4E-BP1 and p70S6k in muscle showing clenbuterol-induced anabolism

A. A. SNEDDON, M. I. DELDAY, J. STEVEN, AND C. A. MALTIN

The Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, Scotland, United Kingdom

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Elevated IGF-II mRNA and phosphorylation of 4E-BP1 and p70S6k in muscle showing clenbuterol-induced anabolism. Am J Physiol Endocrinol Metab 281: E676–E682, 2001.—Muscle wasting affects large numbers of people, but few therapeutic approaches exist to treat and/or reverse this condition. The β2-adrenoceptor agonist clenbuterol produces a muscle-specific protein anabolism in both normal and cachectic muscle and has been used to limit muscle wasting in humans. Because clenbuterol appears to interact with or mimic innervation, its effect on the expression of the neurotrophic agents insulin-like growth factor (IGF)-II and H19 and their putative pathways was examined in normal rat plantaris muscle. The results showed that the well-documented early effects of clenbuterol on protein metabolism were preceded by elevated levels of IGF-II and H19 transcripts together with increased phosphorylation of eukaryotic initiation factor (eIF)4E binding protein-1 (4E-BP1) and p70S6k. By 3 days, transcript levels for IGF-II and H19 and 4E-BP1 and p70S6k phosphorylation had returned to control values. These novel findings indicate that clenbuterol-induced muscle anabolism is potentially mediated, at least in part, by an IGF-II-induced activation of 4E-BP1 and p70S6k.

Hypertrophy; β2-agonist; growth factor; protein translation; insulin-like growth factor II; eukaryotic initiation factor 4E binding protein-1

MUSCLE WASTING AFFECTS more than 3 million people per annum in Europe and the United States. At present, there are few therapeutic approaches to the reversal of this condition, but advances toward new drug development are being made by identifying the precise cellular signaling mechanism(s) that drives muscle hypertrophy. Many current strategies involve subjecting normal muscle to stimuli that induce a hypertrophic response, so that the underlying changes in gene expression may be examined. For example, increased mechanical or electrical activity in muscle provokes changes in transcript levels for a number of genes (7). Similar changes in gene expression (22) occur in muscles treated with the anabolic β2-adrenoceptor agonist clenbuterol, which promotes muscle-specific hypertrophy in both normal and pathological states of muscle and has clinical utility to treat muscle wasting diseases in humans (23). However, presently, the precise mechanism whereby clenbuterol mediates its effects is unknown.

Evidence suggests that skeletal muscle hypertrophy is associated with transient activation of insulin-like growth factor (IGF) gene transcripts; the hypertrophic stimuli of work overload and passive stretch are both associated with increased levels of IGF-I transcripts in muscle (39). A similar role has been proposed for IGF-II, but in this instance it has been suggested that the production of IGF-II may be autocrine or paracrine (36) and that innervation status (12) together with muscle activity (6) may operate or cooperate to influence the level of IGF-II gene transcripts. Because it has been suggested that the action of clenbuterol may be dependent on both innervation status (22) and activity level (20), it was speculated that one possible mechanism through which the drug could elicit the observed effects on muscle mass and protein metabolism was via the IGF-II axis. Consequently, the present study was designed to examine the potential role for IGF-II in clenbuterol-induced muscle protein anabolism. In addition, the expression of H19 was also examined, since IGF-II has been implicated as exhibiting a muscle-specific function (28), and recent evidence suggests it may regulate IGF-II expression (40); hence, its potential role in hypertrophy was investigated. Because clenbuterol-induced protein accretion appears to be initially mediated via a stimulation of muscle protein synthesis (24), the effect and timing of clenbuterol action on eukaryotic initiation factor (eIF)4E binding protein-1 (4E-BP1) and p70S6k, proteins involved in IGF-induced activation of protein translation, were also investigated. 4E-BP1 binds to and regulates the availability of eIF4E, which is thought to be one of the rate-limiting factors for translational initiation (29). When phosphorylated at the appropriate site(s), 4E-BP1 dissociates from eIF4E, allowing the factor to participate in translational initiation. p70S6k mediates the phosphorylation of the 40S ribosomal S6 protein leading to an upregulation of translation of mRNAs encoding ribosomal proteins and elongation factors (15). By investi-
gation of the effect of clenbuterol on the temporal expression of IGF-II/H19 in muscle and nonmuscle tissues, and the activation of 4E-BP1 and p70^S6K in relation to the increase in the fractional rate of protein synthesis, a further insight into the mechanism of action of the drug would be attained.

METHODS

Animals and treatments. Male Rowett hooded Lister rats were used throughout. All procedures and animal husbandry complied with the Animals (Scientific Procedures) Act 1986. At 22 days of age, rats were introduced to a semisynthetic, nutritionally complete control diet (PW-3) (32) for 4 days and then divided into 16 groups of equal mean weight. Groups 1, 3, 5, 7, 9, 11, 13, and 15 were offered PW-3 as before, whereas groups 2, 4, 6, 8, 10, 12, 14, and 16 were offered PW-3 containing clenbuterol (3 mg/kg diet). The number of animals in each group is shown in RESULTS. Previous similar studies have shown that the food intake of either control or medicated diet is between 10 and 12 g/day (Ref. 33 and unpublished observations). At intervals after starting the experimental dietary regimen, animals from the control and clenbuterol groups were euthanized (groups 1 and 2, 1 day; groups 3 and 4, 2 days; groups 5 and 6, 3 days; and groups 7 and 8, 7 days), and plantaris muscles were rapidly removed bilaterally, weighed, and, after appropriate preparation, rapidly frozen in liquid nitrogen before analysis. One muscle from each animal was used for Northern hybridization, and the other muscle was used for either Western blotting or immunohistochemistry. Samples of gut, liver, and heart were also removed and frozen in nitrogen before Northern analysis. In addition, after 1 day (groups 9 and 10), 2 days (groups 11 and 12), 3 days (groups 13 and 14), and 7 days (groups 15 and 16), plantaris muscle protein synthesis was measured. Muscles from these animals were also used to estimate total protein and RNA and DNA content.

Measurement of protein synthesis. Previous studies demonstrated that protein synthesis rates in clenbuterol-treated animals increase between 1.5 and 3 days (24). Between 1 and 7 days of treatment, the fractional rate of protein synthesis in plantaris muscle in both control and clenbuterol-treated animals (n = 6) was measured using the flooding dose technique (10). Briefly, [2,6-3H]phenylalanine (100 μCi/100 g body wt; Amersham) combined with 150 mmol/100 g body wt of unlabeled phenylalanine was administered to each rat by the tail vein (10). After 10 min, animals were killed (10), and the hindlimbs were removed, stripped of skin, and rapidly plunged into iced water. After being cooled, the plantaris muscles were removed bilaterally, with one muscle being used for measurement of protein synthesis and the other being used for determination of total protein (19). RNA (27), and DNA (3) content. Phenylalanine specific activity was measured (10), and the fractional rate of protein synthesis (k_s) expressed as the percentage of protein synthesized per day) was calculated using the following relationship (10):

\[ k_s = 100 \times \frac{S_b}{S_p} \times t \]

where \( S_b \) and \( S_p \) are the specific radioactivities of free and protein-bound phenylalanine, respectively, and \( t \) is the incorporation time in days. For statistical comparison between groups, the data were analyzed by analysis of variance and Student's t-test with statistical significance set at the 5% probability level.

RNA extraction and Northern hybridization analysis. Total RNA was extracted from tissues (n = 5/group) using TRIzol reagent (GIBCO-BRL). Northern blot analysis of RNA was performed using standard techniques (34). 32P-labeled probes were generated by random primed labeling by use of the following DNA templates: a rat H19 cDNA fragment (nucleotides 483–1098) (31), a rat IGF-II cDNA fragment (nucleotides 978–1434) (8), and a 1.2-kb EcoRI-PstI fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (30). Each Northern blot was probed sequentially with IGF-II, H19, and GAPDH probes with stripping in between. The intensities of the 3.8-kb IGF-II and the 2.5-kb H19 mRNAs were quantified (Instant Imager, Packard Instrument) after loading variations were corrected for by GAPDH expression levels. Results were expressed on a within-blot basis as a percentage of the 1-day control. Data were analyzed using Student's t-test, and statistical significance was set at the 5% probability level.

Immunocytochemical detection of IGF-II. Plantaris muscles from control and 1-day clenbuterol-treated animals (groups 1 and 2; 4 animals/group) were prepared for cryostat sectioning before being stored in liquid nitrogen. Serial transverse sections (6 μm) were cut, air dried for 30 min at room temperature, and fixed in fresh ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The sections were washed 3 × 10 min in PBS, treated for 5 min with 0.1% Saponin in PBS, washed 2 × 10 min in PBS, and incubated overnight at 4°C with primary antibody (1:50 dilution; R&D Systems). After 3 × 10-min washes with PBS, secondary antibody (anti-goat-FITC; 1:1,000; Sigma, St. Louis, MO) was applied, and the sections were incubated for 60 min at room temperature in a humidified chamber. The slides were then washed as before, treated with 4,6-diamidino-2-phenylindole (DAPI, 1 ng/ml; Sigma) for 5 min, washed thoroughly in PBS, and mounted in Vectashield (Vekta Labs). For negative controls, the primary antibody was incubated with a 1:10 molar ratio excess of IGF-II protein (R&D Systems) for 1 h at room temperature before being added to the section.

Western immunoblotting. Samples of plantaris muscle (n = 4/treatment) from 1, 2, 3, and 7 days were rapidly crushed between aluminum blocks that had been precooled to −80°C and then homogenized on ice for 30 s in 20 mM Tris, pH 7.0, 0.27 M sucrose, 5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium β-glycerophosphate, 1 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM 1,4-dithiothreitol (DTT), 22 μM leupeptin, 8 μM apro- tinin, 16 μM trans-epoxysuccinyl-l-leucylamido(4-guanidinobutane (E64), 40 μM bestatin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and 15 μM pepstatin A. The protein concentrations were assayed [bicinchoninic acid (BCA) assay kit; Pierce, Rockford, IL], and 150 μg (for 4E-BP1) and 20 μg (for p70^S6K) of total protein from each treatment group were combined with Laemmli sample buffer and subjected to SDS-PAGE analysis. Gels were electrotransferred onto Immobilon-P membrane (Millipore, Bedford, MA) and blocked for 1 h at room temperature in TBST (Tris-buffered saline, pH 7.6, with 0.1% Tween 20) containing 5% (wt/vol) dried milk (Marvel, Stafford, UK) and then incubated for 1 h at room temperature in TBST (Tris-buffered saline, pH 7.6, with 0.1% Tween 20) containing 5% (wt/vol) dried milk (Marvel, Stafford, UK) and then incubated for 1 h at room temperature. Membranes were washed (6 × 5 min) in TBST and then incubated for 30 min in secondary antibody conjugated to horseradish peroxidase (Chemicon International, Harrow, UK). After an additional six washes (see above), the membranes were left in Tris-buffered saline (TBS), and chemiluminescent detection was carried out (Pierce). Specificity of the bands was assessed by the inclusion of an appropriate positive control from the manufacturer.
RESULTS

Muscle composition and protein synthesis. The well-known effects of clenbuterol on the weight of plantaris muscles were clearly documented in the present study and are shown in Table 1. RNA content and the fractional rate of protein synthesis were significantly increased in clenbuterol-treated muscles after 2 days (Table 1). By 7 days of treatment, rates of protein synthesis had returned to control values, although RNA contents remained elevated. Changes in RNA and protein synthesis rates preceded the increase in muscle protein content, which was evident by 3 days of treatment and was persistent at 7 days (Table 1). No significant changes in body weight were observed (data not shown), but muscles from the animals offered the clenbuterol-containing diet showed a significant increase in wet weight after 3 days, concomitant with the increase in protein content. The changes in RNA and protein content, which were evident by 3 days of treatment, are shown in Table 1. RNA content and the fractional rate of protein synthesis occurred roughly at the same time and preceded the increases in protein and wet weight as might be expected.

IGF-II and H19 mRNA expression in plantaris muscle. Three major IGF-II mRNA transcripts of 4.6, 3.8, and 1.2 kb and several minor transcripts of 3.2, 2.7, and 2.2 kb were detected in plantaris muscle. After 1 day of clenbuterol treatment, the expression of the most abundant 3.8-kb IGF-II message was increased 80% over control levels (Fig. 1, A and B). Concomitant increases in the expression of the other IGF-II transcripts were also evident. The level of induction of the 3.8-kb message was increased 50% over control after 2 days, but levels fell thereafter, such that by 7 days after clenbuterol treatment, levels were downregulated by 60% compared with control. A single H19 mRNA transcript of 2.5 kb was detected in plantaris muscle. Clenbuterol treatment resulted in a 50% increase of H19 mRNA transcript levels at 1 and 2 days, with a return to control levels by 3 days (Fig. 1C). GAPDH transcript levels did not change (Fig. 1C).

IGF-II and H19 expression in nonskeletal muscle tissues. The administration of clenbuterol did not induce expression of IGF-II mRNA in nonskeletal muscle tissues such as liver, gut, or heart muscle (Fig. 2).

Additionally, no effect of clenbuterol was detected on the expression levels of H19 mRNA in these tissues (data not shown).

Immunolocalization of IGF-II in plantaris muscle. IGF-II immunopositive staining was associated with some, but not all, myonuclei in muscles from all groups (Fig. 3, A and B, m+ and m−). In addition, nonmuscle cell nuclei stained positive with the DNA dye DAPI (Fig. 3A, nm) but in general did not show IGF-II positivity (Fig. 3B, nm). The specificity of the IGF-II immunopositive staining was validated by demonstrating that no signal was obtained by first preincubating the primary antibody with an excess amount of recombinant IGF-II protein (Fig. 3C).

Western blot analysis of 4E-BP1 phosphorylation in plantaris muscle. 4E-BP1 can be multiply phosphorylated, leading to reduced electrophoretic mobility, and typically three bands (α, β, and γ) are resolved. For skeletal muscle, the γ- and β-forms appear to predominate, whereas the unphosphorylated α-form is very often of low intensity or absent (37). Bands corresponding to γ- and β-forms were detected in both control and treated muscles at 1, 2, 3, and 7 days using an anti-phospho-4E-BP1 antibody (Fig. 4A). The more highly phosphorylated form of the protein, the γ-form, does not bind eIF4E (18). After 1 and 2 days of treatment with clenbuterol, the abundance of the γ-form was significantly enhanced (Fig. 4A, B), but by 3 and 7 days, the signal strength for both β- and γ-forms was similar. A very similar banding pattern was obtained with an anti-4E-BP1 antibody that recognizes both phosphorylated and nonphosphorylated forms (not shown).

Western blot analysis of p70S6k phosphorylation in plantaris muscle. p70S6k is activated by insulin and growth factors through phosphorylation at multiple sites in the protein. Phosphorylation of two sites (Thr252 and Thr412) has been shown to contribute the most to activation of p70S6k (38). With the use of an anti-phospho-p70S6k (Thr412) antibody, phosphorylated (p70S6k) was increased 500% after 1 day of clenbuterol treatment (Fig. 5, A and B). By 3 days, p70S6k activation, as assessed by Thr412 phosphorylation, was down-

### Table 1. Effect of clenbuterol on protein metabolism in rat plantaris muscle

<table>
<thead>
<tr>
<th></th>
<th>Wet Wt, mg</th>
<th>Protein, mg</th>
<th>RNA, μg</th>
<th>kₜ, %/Day</th>
<th>kₛ·RNA⁻¹·Protein⁻¹ × 10³</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
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<tr>
<td>1 Day</td>
<td>62.2±1.3</td>
<td>11.5±0.3</td>
<td>145.2±7.1</td>
<td>18.9±0.7</td>
<td>1.5±0.6</td>
</tr>
<tr>
<td>2 Days</td>
<td>57.5±5.7</td>
<td>11.1±1.2</td>
<td>122.7±5.0</td>
<td>19.2±2.8</td>
<td>1.7±0.2</td>
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<tr>
<td>3 Days</td>
<td>59.5±5.6</td>
<td>11.6±0.8</td>
<td>125.3±9.6</td>
<td>15.3±3.5</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>7 Days</td>
<td>79.8±7.5</td>
<td>13.3±1.1</td>
<td>148.0±13.4</td>
<td>13.2±0.9</td>
<td>1.2±0.1</td>
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<tr>
<td><strong>Clenbuterol</strong></td>
<td></td>
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<tr>
<td>1 Day</td>
<td>65.3±3.7</td>
<td>12.4±0.8</td>
<td>150.0±9.7</td>
<td>20.5±2.6</td>
<td>1.7±0.8</td>
</tr>
<tr>
<td>2 Days</td>
<td>58.2±1.8</td>
<td>10.8±0.5</td>
<td>145.5±2.9</td>
<td>26.1±3.3†</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>3 Days</td>
<td>68.5±4.8*</td>
<td>13.9±0.9†</td>
<td>173.2±11.4</td>
<td>23.0±2.5†</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>7 Days</td>
<td>92.3±7.4*</td>
<td>15.8±1.5†</td>
<td>179.0±10.6</td>
<td>11.7±0.6</td>
<td>1.0±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SD, with 6 animals in each group. kₜ, Fractional rate of protein synthesis. The effect of clenbuterol treatment was assessed by use of analysis of variance and Student’s t-test: *P < 0.05, †P < 0.01, and ‡P < 0.001.

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regulated to control levels (Fig. 5B). As a loading control, the Western blot was probed with an antibody that recognizes both phosphorylated and nonphosphorylated p70S6k protein (Fig. 5A).

**DISCUSSION**

Many studies (24, 26, 33) have indicated that clenbuterol-induced muscle anabolism is mediated by at least two mechanisms: an initial transient increase in the fractional rate of protein synthesis (up to 3–4 days) followed by a more prolonged depression in the fractional rate of protein degradation. The mechanism involved in the clenbuterol-stimulated transient increase in protein synthesis is unknown. The current study addressed the role of IGF-II and H19 and 4E-BP1 and p70S6k in this process.

In this study, clenbuterol induced muscle protein gain at 3 and 7 days, which was preceded by an increase in total RNA content and an increase in the fractional rate of protein synthesis, which waned after day 3. Both IGF-II and H19 transcript levels were elevated by clenbuterol at day 1 and 2 before the increase in the fractional rate of protein synthesis in innervated plantaris muscle. Additionally, concomitant elevated phosphorylation of both 4E-BP1 and p70S6k was also observed at days 1 and 2. The increased protein gain observed at day 7, occurring at a time when the fractional rate of protein synthesis had

**Fig. 1.** The effect of clenbuterol on insulin-like growth factor (IGF)-II and H19 transcript levels in plantaris muscle. Rats were either untreated or treated with clenbuterol for 1, 2, 3, or 7 days (1d, 2d, 3d, or 7d, respectively). A: autoradiograph showing transcript sizes and levels for IGF-II from control (−) and clenbuterol-treated (+) rats. B: histogram showing mean values ± SE (n = 5) for the IGF-II 3.8-kb transcript level from control (open bars) and clenbuterol-treated (solid bars) rats expressed as a percentage of 1d control value. C: autoradiograph showing transcript size and levels for H19 (top) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; bottom) from control (−) and clenbuterol-treated (+) rats. D: histogram showing mean values ± SE (n = 5) for H19 transcript levels from control (open bars) and clenbuterol-treated (solid bars) rats expressed as a percentage of 1d control values. Significance of difference from control (**P < 0.01 and *P < 0.05) was determined by Student’s t-test.

**Fig. 2.** The effect of clenbuterol on IGF-II transcript levels in nonmuscle tissues. Rats were either untreated (−) or treated (+) with clenbuterol for 1 day, and IGF-II levels were then measured by Northern blotting of total RNA from plantaris muscle (SkM), liver (Liv), gut, and heart (Hrt). The ethidium bromide (EtBr)-stained gel is shown below the autoradiograph as a loading control. Representative Northern blots from 3 separate experiments are shown.
returned to basal level, is most likely explained by the subsequent decrease in the fractional rate of protein degradation induced by the drug (33).

In muscle, the anabolic actions of insulin and IGF-I on protein metabolism are well documented (29). Downstream signaling from insulin and IGF-I receptors involves multiple pathways leading to the phosphorylation of a number of translation initiation factors, regulatory proteins, and the 40S ribosomal subunit. The phosphorylation of the 4E-BP1 is a key
Clenbuterol activates 4E-BP1, p70S6K, and IGF-II mRNA

4E-BP1 and p70S6K are key regulators of protein synthesis. Clenbuterol increases cAMP levels in muscle, suggesting that the observed effects on 4E-BP1 and p70S6K phosphorylation are not mediated via the classical B2-adrenoceptor signaling process. However, whether activation of this latter receptor may play a role in the induction of IGF-II mRNA is not known.

IGF-II and H19 genes show a very similar spatial/temporal expression pattern during development (11, 13), and their reciprocal imprinting has been proposed to be controlled by the possession of common sets of regulatory elements (17). The transient changes in H19 gene expression after clenbuterol treatment, occurring over a time scale very similar to those for IGF-II transcripts, support this proposal. Because the expression of H19 is downregulated in most adult tissues except skeletal muscle (28), this implicates H19 as harboring a muscle-specific function. The finding of increased H19 transcript levels is the first to be reported in skeletal muscle hypertrophy in vivo; however, the precise role of H19 in this case is unclear.

Previous studies implicating IGF-II in the regulation of hypertrophy have also suggested that autocrine or paracrine production is important (36). The lack of an effect of clenbuterol on IGF-II expression in nonmuscle tissues such as liver and gut, together with the myonuclear localization of IGF-II in the present study, would be consistent with this suggestion. Furthermore, the association between elevated IGF-II expression and increased fractional rate of protein synthesis in muscle is strengthened by the above finding that clenbuterol does not induce IGF-II expression in nonmuscle tissues (such as gut or liver), where the fractional rate of protein synthesis and protein accretion are not affected (1, 26). The immunolocalization of IGF-II to the nucleus is unclear; however, it does demonstrate that IGF-II protein is present in muscle and not in nonmuscle cells. Receptor-bound IGF-II (as, say, occurring at the membrane or binding with IGF binding proteins) may result in a masking of the epitope for the anti-IGF-II antibody and hence remain undetected. The localization of IGF-II to satellite cells during regeneration (14) and studies showing clenbuterol-induced satellite cell activation (21) could reflect localized production of IGF-II in a manner similar to that proposed in regenerating muscle. Such local production of growth factors would then lead to receptor-ligand interactions driving downstream consequences such as increased protein synthesis, accretion, and the observed hypertrophy. Future studies will no doubt address the precise signaling pathway(s) involved in the phosphorylation of 4E-BP1 and activation of p70S6K and the role that IGF-II plays in the clenbuterol-mediated process of skeletal muscle anabolism.

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