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Transforming growth factor βs are upregulated in the rat masseter muscle hypertrophied by clenbuterol, a β_2 adrenergic agonist

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1 The regulatory mechanism for the hypertrophy of skeletal muscles induced by clenbuterol is unclear. The purpose of the present study was to determine the extent to which transforming growth factor βs (TGFβs), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), and platelet-derived growth factors (PDGFs) are involved in the hypertrophy of rat masseter muscle induced by clenbuterol.

2 We measured the mRNA expression levels for TGFβs, FGFs, HGF, and PDGFs in rat masseter muscle hypertrophied by oral administration of clenbuterol for 3 weeks and determined correlations between the weight of masseter muscle and mRNA expression levels by regression analysis. We determined immunolocalizations of TGFβs and their receptors (TGFβRs).

3 The mRNA expression levels for TGFβ1, 2, and 3, and for PDGF-B demonstrated clenbuterol-induced elevations and positive correlations with the weight of masseter muscle. In particular, TGFβ1, 2, and 3 showed strong positive correlations (correlation coefficients >0.6). The mRNA expression levels for PDGF-A, FGF-1 and 2, and HGF showed no significant differences between the control and clenbuterol groups, and no significant correlations. TGFβ1, 2, and 3 were principally localized in the connective tissues interspaced among myofibers, and TGFβRI and II were localized in the periphery and sarcoplasm of the myofibers.

4 These results suggest that paracrine actions of TGFβ1, 2, and 3 *via* TGFβRI and II could be involved in the hypertrophy of rat masseter muscle induced by clenbuterol. This is the first study to document the involvement of TGFβs in the hypertrophy of skeletal muscles induced by clenbuterol.
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Abbreviations: Clen, clenbuterol; Con, control; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; s.d., standard deviation; TGFβ, transforming growth factor β; TGFβR, transforming growth factor β receptor

Introduction

Clenbuterol, a β_2-adrenergic agonist, is known to induce hypertrophy of skeletal muscles, such as the soleus, gastrocnemius, extensor digitorum longus, and masseter muscles (Emery *et al*., 1984; Benson *et al*., 1991; Rajab *et al*., 2000; Stevens *et al*., 2000a; Oishi *et al*., 2002; Wakana *et al*., 2003) and cardiac muscle (Wong *et al*., 1998; Soppa *et al*., 2005). The regulatory mechanism for the hypertrophy of skeletal and cardiac muscles induced by clenbuterol is unknown. However, insulin-like growth factors (IGFs) have been previously reported to play important roles in the clenbuterol-induced hypertrophy of skeletal muscles, such as the soleus, plantaris, and masseter muscles (Sneddon *et al*., 2001; Awede *et al*., 2002; Wakana *et al*., 2003). IGFs are well known to play essential roles in the development, growth, and regeneration of skeletal muscles (Jones & Clemmons, 1995; Florini *et al*., 1996; Adams, 1998; Yamane *et al*., 2002, 2003; Adams *et al*., 2004).

In addition to IGFs, other peptide growth factors, such as transforming growth factor βs (TGFβs), platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs), and hepatocyte growth factor (HGF) reportedly play important roles in the development, growth, and regeneration of skeletal muscles (Husmann *et al*., 1996; Tajbakhsh & Cossu, 1997; Sabourin & Rudnicki, 2000; Christ & Brand-Saberi, 2002; Buckingham *et al*., 2003; Charge & Rudnicki, 2004). There are several reports indicating that these growth factors are involved in the hypertrophy of skeletal muscles induced by mechanical loading, such as by stretching and by unilateral removal of the gastrocnemius and soleus muscles. TGFβ2 was reported to be upregulated and TGFβ receptor II (TGFβRII) to be downregulated in the hypertrophied rat plantaris muscle (Sakuma *et al*., 2000), and the expression levels for FGFs and HGF have been shown to be altered in hypertrophied rat plantaris and chicken anterior latissimus dorsi muscles, and in human cultured myotubes (Clarke & Feeback, 1996; Mitchell *et al*., 1999; Yamaguchi *et al*., 2004). However, there is, to date, no published result, which demonstrates the involvement of growth factors other than IGFs in the clenbuterol-induced hypertrophy of skeletal muscles.

On the basis of the above findings, we hypothesized that, in addition to IGFs, a complex network of cytokines including TGFβs, PDGFs, FGFs, and HGF functions to regulate the

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hypertrophy of skeletal muscles induced by clenbuterol. To test this hypothesis, the present study analyzed the mRNA expression levels for TGFβs, PDGFs, FGFs, and HGF and determined the correlation between the expression levels and the weight of masseter muscle by regression analysis. Furthermore, since stronger correlations were obtained between the expression levels for TGFβ mRNAs and the masseter wet weight than those between other growth factors and the masseter wet weight, we investigated the immunolocalization of TGFβs and TGFβRs.

Methods

Experimental animals

A total of 18 male Wistar rats were purchased from Clea Japan Inc., (Tokyo, Japan) and fed a hard diet (CE-2, Clea Japan Inc., Tokyo, Japan). They were divided into control and clenbuterol groups of nine rats each at 8 weeks of age. We orally administered 30 μg ml^{-1} of clenbuterol (C5423, Sigma-Aldrich Fine Chemicals, St Louis, MO, U.S.A.) to the rats in the clenbuterol group *via* their drinking water for 3 weeks, while pure water was given to the rats in the control group. We measured the daily consumption of pure water or water containing clenbuterol of a rat to estimate the daily dose of clenbuterol. Each animal was weighed daily while clenbuterol was administered. After 3 weeks, all the animals were killed by exsanguinations under ether anesthesia, and the left and right masseter muscles were dissected and weighed. The left masseter muscle was rapidly frozen and stored at −85°C until subsequent PCR analysis. The right masseter muscle was embedded in Tissue-Tek Oct Compound (Miles Laboratory, Elkhart, IN, U.S.A.), rapidly frozen, and stored at −85°C until subsequent histologic analysis. Experimental protocols concerning animal handling were reviewed and approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine.

RNA extraction, reverse transcription, and competitive polymerase chain reaction (competitive RT–PCR) amplification

Total RNA extraction, treatment with deoxyribonuclease I, and competitive RT–PCR amplification were performed as previously described (Ohnuki *et al*., 2000; Yamane *et al*., 2000). Briefly, total RNA was isolated from individual masseter samples according to the manufacturer's specifications (FastRNA™ Kit-GREEN, BIO 101, Vista, CA, U.S.A.). The isolated total RNA was treated with 2 U of ribonuclease-free deoxyribonuclease I. The RNA (1.5 μg) was reverse-transcribed to cDNA with 200 U of reverse-transcriptase.

Competitors for the competitive-PCR amplification were constructed according to the manufacturer's instructions (Competitive DNA Construction Kit, Takara, Shiga, Japan). The nucleotide sequences for primers and the competitive-PCR conditions for TGFβs, PDGFs, FGFs, HGF, and S16 (ribosomal protein) were identical to those used in our previous studies (Ohnuki *et al*., 2000; Yamane *et al*., 2004; Yoshida *et al*., 2005). The amplification products were isolated by electrophoresis on an agarose gel containing ethidium bromide. The fluorescent intensities of the bands of the target genes and their respective competitors were measured by an image analyzer (Molecular Imager FX, Bio-Rad, Hercules, CA, U.S.A.). We then calculated the ratios of the fluorescent intensities of the target gene bands to those of their respective competitor bands. The logarithmic value of the fluorescent intensity ratio was used to calculate the amount of endogenous target mRNA based on the formula of the regression line derived from a standard curve for each target gene. The standard curve was generated as described previously (Ohnuki *et al*., 2000; Yamane *et al*., 2000). Then, the quantity of endogenous S16 mRNA was quantified to normalize the quantity of the target mRNA relative to the quantity of S16 mRNA. The resulting ratio value was expressed as the percentage relative to the mean value of each target gene in the control group.

Histology

The middle portion of the right masseter muscle was cut at a microtome setting of 10 μm with a cryostat and was then air-dried for 1 h at room temperature. The sections were stained with nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) and observed under a light microscope. Pictures of the muscle tissue were taken with a digital camera (AxioCam, Carl Zeiss Japan Co., Tokyo, Japan) connected to a personal computer, and were then printed out. In all, 100 myofibers, the cross-section of which was oblong or as oblong as possible, were selected on the pictures from the middle portion of right masseter obtained from each rat. The minimal distance between the parallel sides of the oblong was measured by digital calipers as a minimal diameter of myofiber. The minimal diameters of 100 myofibers were then averaged to obtain the mean value for each rat. This mean value was further averaged to obtain the mean value for nine rats.

To detect the distribution of TGFβ1, 2, and 3, and of TGFβRI and II in the masseter muscle, their immunolocalizations were analyzed using a Vector Elite Immunodetection Kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.) as described previously (Urushiyama *et al*., 2004). Rabbit polyclonal antibodies against TGFβ1, 2, and 3, and against TGFβRI and II were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). For control staining, the primary antibody was replaced with normal rabbit IgG or PBS.

Statistical analyses

Student's *t*-test was used to compare the mean values between the clenbuterol and control groups. Regression analysis was used to examine the correlations between the expression levels for growth factor mRNAs and the wet weight of left masseter muscle or the fiber diameter of right masseter muscle. Differences were considered significant at $P<0.05$.

Results

Clenbuterol-induced hypertrophy of the rat masseter muscle

To determine effects of clenbuterol on body weight and muscle mass in the rat, we measured the body weight (Figure 1a), the wet weight of the left masseter muscle (Figure 1b) and the fiber

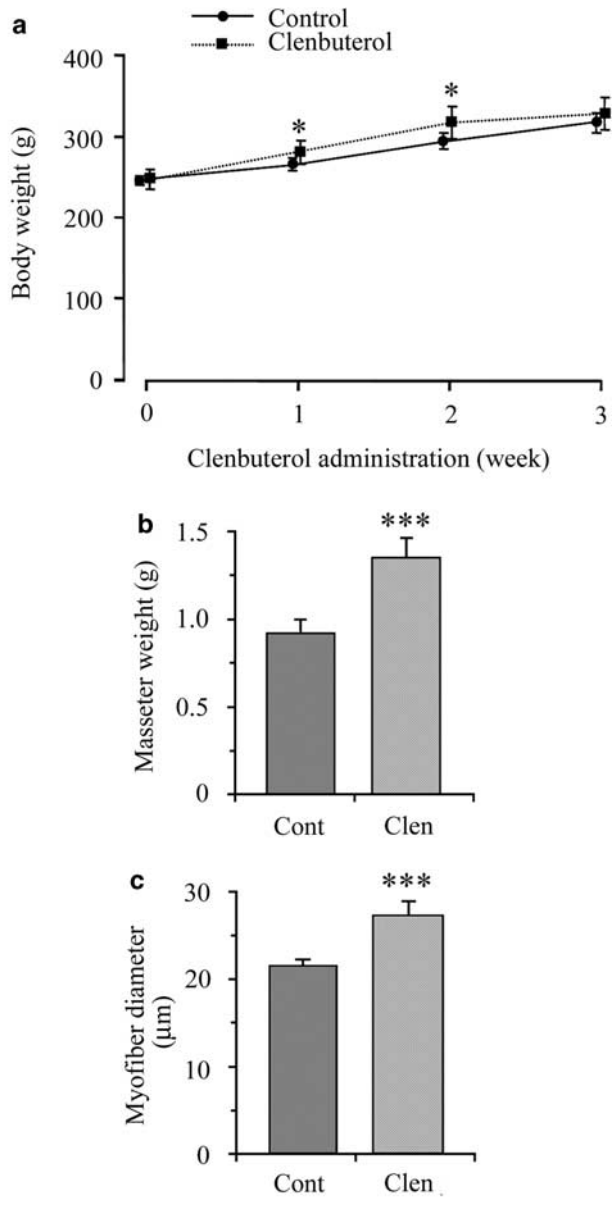

Figure 1 (a) Changes in body weight of rats in the control group (solid line) and clenbuterol group (dotted line) during the clenbuterol administration. Each point and its vertical bar represent the mean ± 1 s.d. of nine rats. (b, c) Bar graphs showing the wet weight of left masseter muscle (b) and the fiber diameter of right masseter muscle (c) in the control group (Cont) and clenbuterol group (Clen) at 3 weeks from the initiation of clenbuterol administration. Each column and its vertical bar represent the mean + 1 s.d. of nine rats. Significant difference between the control and clenbuterol groups, $*P<0.05$, $***P<0.001$.

diameter of the right masseter muscle in each rat (Figure 1c). The body weights of all animals in both the control and clenbuterol groups gradually increased during the entire experimental period (Figure 1a). At 1 and 2 weeks after the initiation of clenbuterol administration, the body weights in the clenbuterol group were significantly greater than those in the control group ($P<0.05$), but, at 3 weeks, there was no significant difference between the two groups. The wet weight of the left masseter muscles and the fiber diameter of the right masseter in the clenbuterol group were 1.35 ± 0.11 g (mean ± s.d.) and 27.4 ± 1.6 μm, which were 47 and 27% greater than those in the control group (0.92 ± 0.08 g and 21.6 ± 0.6 μm) ($P<0.001$), respectively, at 3 weeks after the initiation of clenbuterol administration. These results indicate that an oral administration of clenbuterol for 3 weeks induces hypertrophy of the rat masseter muscle. In addition, the correlation coefficient between the wet weight and fiber diameter of rat masseter muscle was strong (0.866, $P<0.001$), indicating that the wet weight was reliable and reflects a change in the myofibers.

Strong correlations between the expression levels for TGFβ mRNAs and the weight of the left masseter muscle

To determine whether TGFβ1, 2, and 3 are involved in the clenbuterol-induced hypertrophy of the rat masseter muscle, we analyzed the mRNA expression levels (Figure 2) and determined the correlations between the expression levels and the weight of the left masseter muscle (Figure 3, Table 1), and those between the expression levels and the fiber diameter of the left masseter muscle (data not shown). Figure 2a shows a gel electrophoretic pattern for TGFβ1 competitive PCR products of the rat masseter muscle of the control (upper panel) and clenbuterol (lower panel) groups. The ratios of the fluorescent intensities of the target gene bands (upper bands) to those of their respective competitor bands (lower bands) in the clenbuterol group appear to be greater than those in the control group. The image analysis of PCR bands indicated that clenbuterol significantly increased the mRNA expression levels for TGFβ1 from 100 ± 22 to 198 ± 34% ($P<0.001$) (Figure 2b), those for TGFβ2 from 100 ± 68 to 211 ± 90% ($P<0.05$) (Figure 2c), and those for TGFβ3 from 100 ± 22 to 180 ± 24% ($P<0.001$) (Figure 2d). The correlation coefficients between the mRNA expression levels for all TGFβs and the wet weight of the left masseter muscle were quite strong (0.611–0.833) and were statistically significant ($P<0.05$–0.001) (Figure 3, Table 1). The correlation coefficients between the mRNA expression levels for all TGFβs and the fiber diameter of the right masseter muscle were also quite strong (0.615–0.792) ($P<0.05$–0.001). These results suggest that TGFβs play a role in the clenbuterol-induced hypertrophy of masseter muscle in the rat.

Weak or no correlations between the expression levels for PDGF, FGF, and HGF mRNAs, and the weight of the left masseter muscle

To determine the extent to which PDGFs, FGFs, and HGF are involved in the clenbuterol-induced hypertrophy of the rat masseter muscle, we analyzed the mRNA expression levels (Figure 4) and determined the correlations between the expression levels and the wet weight of the left masseter muscle (Figure 5, Table 1) and those between the expression levels and the fiber diameter of the right masseter muscle (data not shown). The clenbuterol only induced a significant change in the mRNA expression levels for PDGF-B, which increased from 100 ± 37 to 151 ± 20% ($P<0.01$) in response to the clenbuterol (Figure 4b). The expression levels for growth factors other than PDGF-B showed no significant differences between the control and clenbuterol groups. The mRNA expression levels for PDGF-A, FGFs, and HGF did not show any significant correlations with the weight of masseter muscle

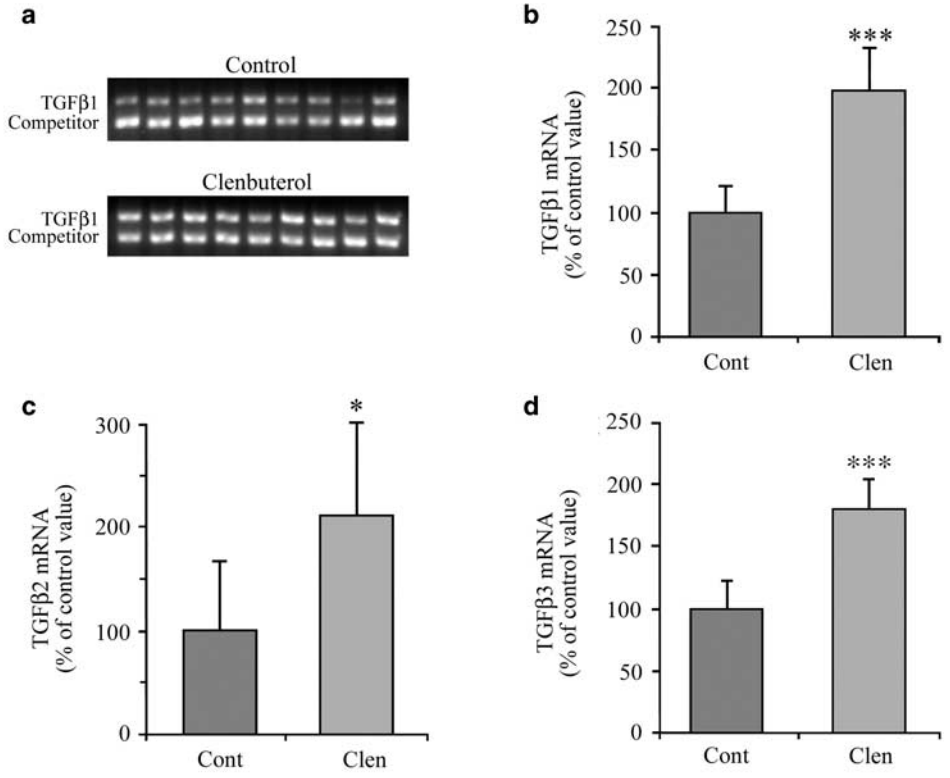

Figure 2 (a) Gel electrophoretic pattern for transforming growth factor β (TGFβ) 1 competitive RT–PCR products of the left masseter muscle of the control (Cont) and clenbuterol (Clen) groups. The target gene is shown in the upper bands, while the competitor is shown in the lower band. (b–d) Bar graphs showing the mRNA expression levels for TGFβ1 (b), 2 (c), and 3 (d) in the left masseter muscle of the control and clenbuterol groups. Each column and vertical bar represent the mean + 1 s.d. of nine rats. The vertical axis is expressed as a percentage of the mean control value set at 100. Significant differences between the control and clenbuterol groups, $^{*}P<0.05$; $^{***}P<0.001$.

and the fiber diameter of right masseter muscle. The mRNA expression level for PDGF-B showed weak significant correlations with the wet weight of left masseter muscle (correlation coefficient was 0.533, $P<0.05$) and the fiber diameter of right masseter muscle (correlation coefficient was 0.529, $P<0.05$). These results suggest that PDGF-A, FGFs, and HGF do not play important roles in the clenbuterol-induced hypertrophy of rat masseter muscles.

Immunolocalization of TGFβs and TGFβRs

The mRNA expression levels for all TGFβs exhibited a strong correlation with the weight of the rat masseter muscle. Thus, to further elucidate the role of TGFβs in the clenbuterol-induced hypertrophy of the rat masseter muscle, we examined the distributions of TGFβs (Figure 6) and TGFβRs (Figure 7) in the rat masseter muscle tissues immunohistochemically. Intensive immunostaining for TGFβ1 (arrows in Figure 6a and b) was observed in the connective tissues interspaced among the masseter myofibers of both the control (Figure 6a) and clenbuterol-treated (Figure 6b) rats. The number of regions immunostained for TGFβ1 in the connective tissues of the clenbuterol-treated rats was greater than that of the control rats. Faint dot-like staining for TGFβ1 was sporadically distributed in the myofibers of both the control and clenbuterol-treated rats. Immunostaining for TGFβ2 was localized in the connective tissues interspaced among the masseter myofibers of the clenbuterol-treated rats (Figure 6d) and very faint immunostaining for TGFβ2 was distributed in the connective tissues of the control rats (Figure 6c). Immunostaining for TGFβ2 was barely observable in the masseter myofibers of both the control and clenbuterol-treated rats. Immunostaining for TGFβ3 was localized in the connective tissues interspaced among the masseter myofibers of both the control (Figure 6e) and clenbuterol-treated (Figure 6f) rats, but the number and the area of the regions immunostained for TGFβ3 in the connective tissues of the clenbuterol-treated rats was greater than those of the control rats. Dot-like staining for TGFβ3 was observed in the myofibers of both the control and clenbuterol-treated rats.

The immunolocalization patterns for TGFβRI and II were similar (Figure 7). A number of dot-like immunostainings for both TGFβRI and TGFβRII were localized in the periphery of the myofibers of the both the control (Figure 7a and c) and clenbuterol-treated (Figure 7b and d) rats. This dot-like immunostaining for TGFβRI and TGFβRII was also found in the sarcoplasm of the myofibers. No marked difference in the patterns of immunostainings for TGFβRI and TGFβRII was found between the control and clenbuterol-treated rats.

Discussion

In the present study, we observed that the oral administration of clenbuterol for 3 weeks induced hypertrophy of the rat

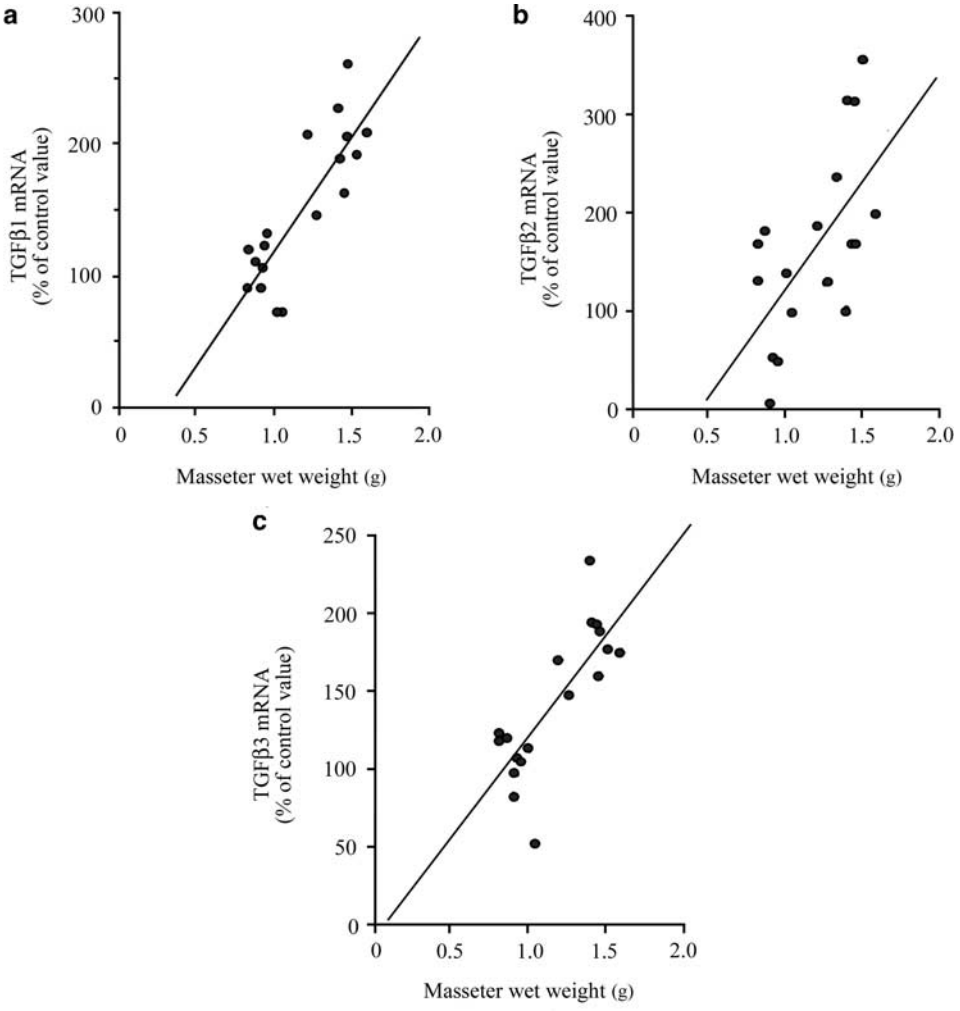

Figure 3 (a–c) Scatter diagrams and regression lines between the weight of left masseter muscle and the mRNA expression levels for TGFβ1 (a), 2 (b), and 3 (c). The vertical axis is expressed as a percentage of the mean control value set at 100. Values for both the control and clenbuterol groups were included.

Table 1 Formulae for the regression lines and correlation coefficients between the masseter wet weight (g) and the mRNA expression levels for growth factors (% of control value)

Growth factor	*Formula*	r	*Significance*
TGFβ1	$y=176x-54$	0.833	$P<0.001$
TGFβ2	$y=212x-91$	0.611	$P<0.05$
TGFβ3	$y=138x-22$	0.800	$P<0.001$
PDGF-A	$y=-37x+136$	−0.338	NS
PDGF-B	$y=77x+36$	0.533	$P<0.05$
FGF-1	$y=-15x+115$	−0.138	NS
FGF-2	$y=-38x+130$	−0.374	NS
HGF	$y=31x+77$	0.275	NS

x, the masseter weight (g); y, the mRNA expression levels for growth factor (% of control value); r, correlation coefficient; NS, not significant.

masseter muscle (Figure 1b), which is consistent with our previous study in the mouse masseter muscle (Wakana *et al*., 2003) and with studies in other muscles including the soleus, gastrocnemius, and extensor digitorum longus muscles (Emery *et al*., 1984; Benson *et al*., 1991; Stevens *et al*., 2000a; Sneddon *et al*., 2001; Awede *et al*., 2002; Oishi *et al*., 2002). Our PCR and immunohistochemical results demonstrated that clenbuterol can induce increases in the expressions of mRNAs (Figure 2) and proteins for three TGFβs (Figure 6) in the masseter muscle tissues of the rat. These results partially accord with the previous result that TGFβ2 is upregulated in the rat plantaris muscle hypertrophied by mechanical loading (Sakuma *et al*., 2000). Further, we observed that correlations between the wet weight of masseter muscle and the mRNA expression levels for TGFβ1, 2, and 3 (correlation coefficients were 0.833, 0.611, and 0.800, respectively) were as strong as those between the wet weight of masseter muscle and the mRNA expression levels for IGF-I and II (0.692 and 0.837, respectively; Yamane *et al*., unpublished data). Since IGFs are already known to play important roles in the clenbuterol-induced hypertrophy of skeletal muscles (Sneddon *et al*., 2001; Awede *et al*., 2002; Wakana *et al*., 2003), our present results suggest that TGFβ1, 2, and 3 could be also correlated with the clenbuterol-induced hypertrophy of skeletal muscles, although there is a possibility that a significant change in the mRNA expression level could not induce a change in net protein and hence no functional effect. To our knowledge, the present study is the first report to document the involvement of growth factors containing TGFβ, in addition to the known

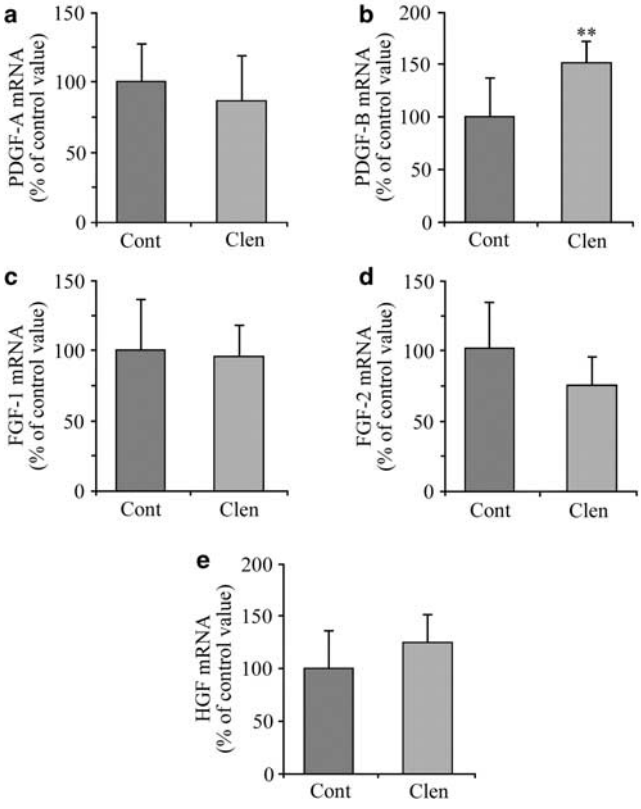

Figure 4 (a–e) Bar graphs showing the mRNA expression levels for platelet-derived growth factor (PDGF)-A (a) and B (b), fibroblast growth factor (FGF)-1 (c) and 2 (d), and hepatocyte growth factor (HGF) (e) in the left masseter muscle of the control (Cont) and clenbuterol (Clen) groups. Each column and vertical bar represent the mean + 1 s.d. of nine rats. The vertical axis is expressed as a percentage of the mean control value set at 100. Significant difference between the control and clenbuterol groups, $^{**}P<0.01$.

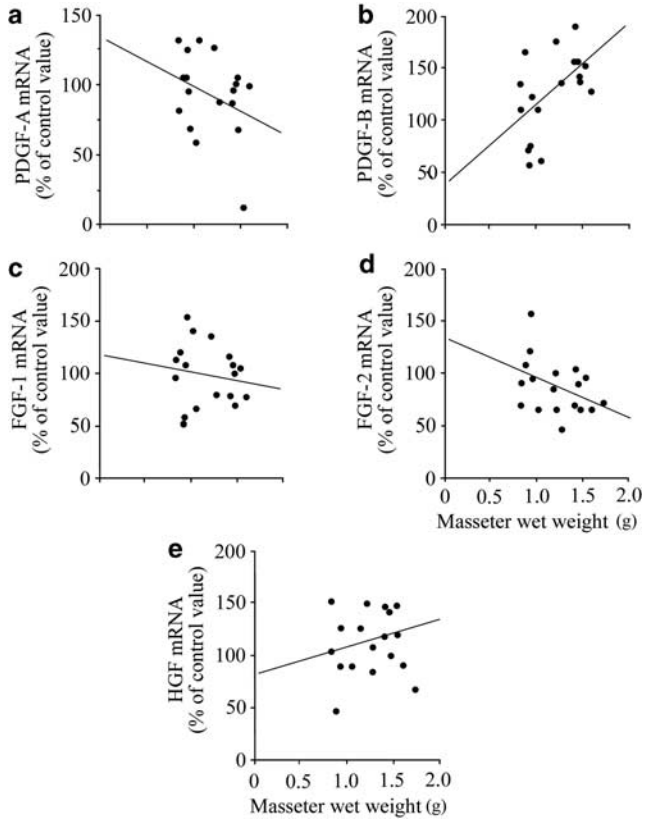

Figure 5 (a–e) Scatter diagrams and regression lines between the weight of left masseter muscle and the mRNA expression levels for PDGF-A (a) and B (b), FGF-1 (c) and 2 (d), and HGF (e). The vertical axis is expressed as a percentage of the mean control value set at 100. Values for both the control and clenbuterol groups were included.

involvement of IGFs, in the clenbuterol-induced hypertrophy of skeletal muscles.

Our immunohistochemical results demonstrated that TGFβs were principally expressed in the connective tissues interspaced among the myofibers (probably, nerves, blood vessels and tissues around them), but were minimally expressed in the myofibers of rat masseter muscles (Figure 6). These results are consistent with a previous result demonstrating the elevation of TGFβ2 protein expression in the connective tissues interspaced among the myofibers of the rat plantaris hypertrophied by mechanical loading (Sakuma *et al*., 2000). Furthermore, we showed that TGFβRI and II were expressed in the periphery and sarcoplasm of the myofibers (Figure 7). These results suggest that the paracrine signals of TGFβs secreted from connective tissues are predominant over the autocrine signals of TGFβs from the myofibers themselves in the clenbuterol-hypertrophy of the rat masseter muscle, and that the signals of TGFβs are transmitted into the myofibers through TGFβRI and II.

It is difficult to explain the mechanism for the clenbuterol-induced hypertrophy of the rat masseter muscle through upregulation of TGFβs, because a number of studies have indicated that TGFβs inhibit the proliferation and differentiation of cultured myoblasts and satellite cells (Husmann *et al*., 1996; Hawke & Garry, 2001; Charge & Rudnicki, 2004). However, it has been suggested that clenbuterol induces the hypertrophy of skeletal muscles by promoting protein synthesis and/or inhibiting protein degradation in the skeletal muscles of rats (Emery *et al*., 1984; Benson *et al*., 1991). To our knowledge, there is no published data regarding the effects of TGFβs on the total protein synthesis in adult myofibers. However, it is already reported that TGFβs inhibit the expression of calpains (Ca^{2+}-activated neutral protease) I and II in differentiating rat skeletal muscle cells (Poussard *et al*., 1993). In addition, calpain I and II are reportedly upregulated in the degrading muscles of progressive muscular dystrophy patients, such as in those with Duchenne and Becker muscular dystrophy, and amyotrophic lateral sclerosis (Kumamoto *et al*., 1995; Ueyama *et al*., 1998). On the basis of these data, we hypothesize that the locally elevated TGFβs induce hypertrophy of the rat masseter muscle by suppressing the expression of calpains and thereby inhibiting protein degradation in originally existing myofibers. There are supportive reports for our hypothesis that clenbuterol inhibited Ca^{2+}-dependent proteolysis in rat soleus and extensor digitorum longus muscles through β_2-adrenoceptor (Navegantes *et al*., 2000, 2001), but clenbuterol did not affect the rate of protein synthesis in rat soleus muscle (Navegantes *et al*., 2004). New studies are underway to test this hypothesis.

In the present study, we employed oral administration of clenbuterol *via* drinking water, because it was identified as

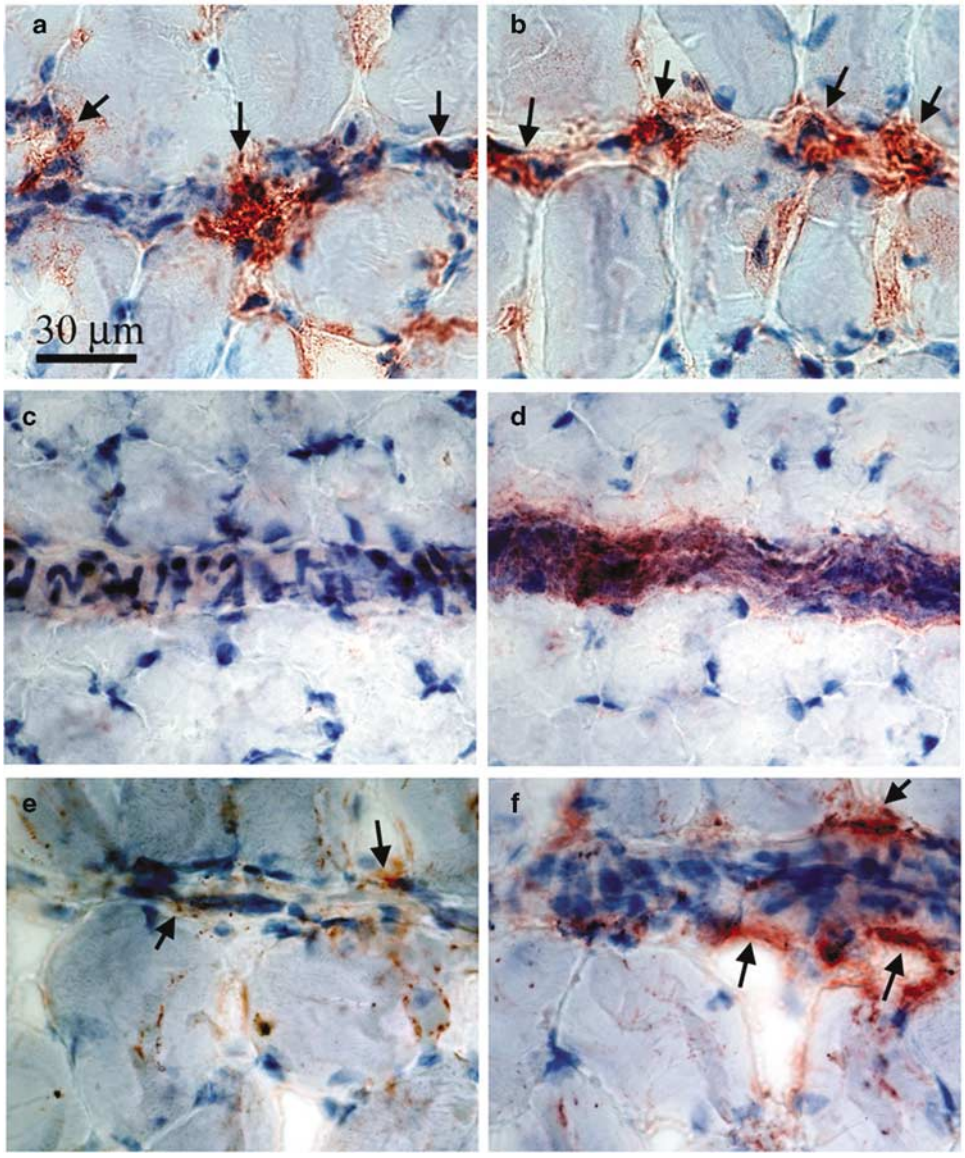

Figure 6 (a–f) Immunolocalization for TGFβ1 (a, b), 2 (c, d), and 3 (e, f) in the middle portions of the right masseter muscle in the control (a, c, e) and clenbuterol (b, d, f) rats. Arrows in (a and b), and those in (e and f) indicate the regions intensively immunostained for TGFβ1 and TGFβ3, respectively. (b–f) are at the same magnification as that in (a).

effective by our previous study (Wakana *et al*., 2003) and several other studies (Zeman *et al*., 1987; Ricart-Firinga *et al*., 2000; Stevens *et al*., 2000b; Awede *et al*., 2002; Oishi *et al*., 2002). We estimated the daily dose of clenbuterol by measuring the daily consumption of water containing clenbuterol of a rat. The daily dose of clenbuterol was $3.54 \pm 0.55\,mg\,kg^{-1}$ body weight (BW). Although this daily dose appears to be higher than those by subcutaneous injection ($1–2\,mg\,kg^{-1}$ BW) (Benson *et al*., 1991; Herrera *et al*., 2001; Deutsch *et al*., 2002; Wineski *et al*., 2002) and minipump ($1, 3\,mg\,kg^{-1}$ BW) (Hinkle *et al*., 2002), taking into account the loss of clenbuterol by spilling, degradation by light and first pass effect, the oral dose of clenbuterol by drinking water seem to be equivalent to those by other routes of administration such as subcutaneous injection and minipump.

The clenbuterol administration of this dose possibly induces myotoxic effects such as apoptosis and necrosis of myofibers (Burniston *et al*., 2002, 2005). It has been reported that TGFβ expression is elevated in injured muscles (Wright-Carpenter *et al*., 2004; Salvadori *et al*., 2005) and TGFβ1 is as a major stimulator which plays significant role in both the initiation of fibrotic cascades in skeletal muscle and the induction of myogenic cells into myofibroblastic cells in injured muscle (Li *et al*., 2004). Thus, it is possible that the elevations of TGFβs in the present study are related to the repairing activity of rat masseter muscle, such as fibrosis and scar formation in response to the myotoxic effects of clenbuterol.

In the present study, we found no significant changes in the mRNA expression levels for FGF-1 and 2, or for HGF in the masseter muscle hypertrophied by clenbuterol (Figure 4). However, significant alterations in these mRNA expression levels were reported in the rat plantaris and chicken anterior latissimus dorsi muscles hypertrophied by mechanical loading (Mitchell *et al*., 1999; Yamaguchi *et al*., 2004). In addition,

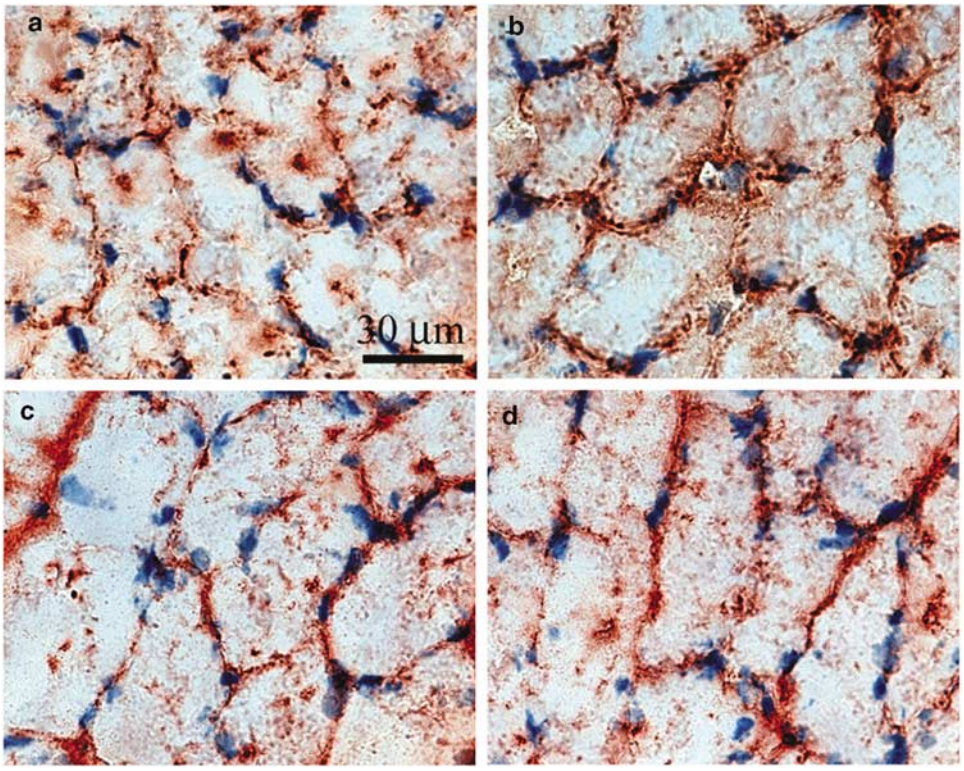

Figure 7 (a–d) Immunolocalization for transforming growth factor β receptor (TGFβRI) (a, b) and II (c, d) in the middle portions of the right masseter muscle in the control (a, c) and clenbuterol (b, d) rats. (b–d) are at the same magnification as that in (a).

there is no report that indicates a change in the expression levels of PDGFs in the hypertrophy of skeletal muscles induced by mechanical loading, although upregulation of PDGF-B was observed in the present study (Figure 4). These results suggest that growth factors, which take part in the cytokine network for regulation of the hypertrophy of skeletal muscles, do so differentially as a function of the animal muscle type, and means by which the hypertrophy is induced.

In conclusion, the mRNA expression levels for TGFβ1, 2, and 3 demonstrated clenbuterol-induced elevations and strong positive correlations with the weight of masseter muscle, similar to those for IGFs (correlation coefficients >0.6). Localizations of TGFβ1, 2, and 3 were principally observed in the connective tissues interspaced among the myofibers, and those of TGFβRI and II were observed in the periphery and sarcoplasm of the myofibers. These results suggest that the paracrine actions of TGFβ1, 2, and 3 *via* TGFβRI and II could be correlated with the hypertrophy of the rat masseter muscle induced by clenbuterol. This is the first study that suggests the involvement of TGFβs in the hypertrophy of skeletal muscles induced by clenbuterol. Further studies are necessary to elucidate the role of TGFβs in this clenbuterol-induced hypertrophy of skeletal muscle.

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