Expression of fibroblast growth factor family during postnatal skeletal muscle hypertrophy

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Mitchell, Pamela, Tom Steenstrup, and Kevin Hannon. Expression of the fibroblast growth factor family during postnatal skeletal muscle hypertrophy. J. Appl. Physiol. 86(1): 313–319, 1999.—The potential role of the fibroblast growth factor (FGF) family during stretch-induced postnatal skeletal muscle hypertrophy was analyzed by using an avian wing-weighting model. After 2 or 11 days of weighted stretch, anterior latissimus dorsi (ALD) muscles were, on average, 34 (P < 0.01) and 85% (P < 0.01) larger, respectively, than unweighted ALD control muscles. By using quantitative RT-PCR, FGF-1 mRNA expression was found to be significantly decreased in ALD muscles stretched for 2 or 11 days. In contrast, FGF-4 and FGF-10 mRNA expression was significantly increased 2 days after initiation of stretch. FGF-2, FGF-10, fibroblast growth factor receptor 1, and FREK mRNA expression was significantly increased at 11 days poststretch. Increases in FGF-2 and FGF-4 protein could be detected throughout the myofiber periphery after 11 days of stretch. On a cellular level, FGF-2 and FGF-4 proteins were differentially localized. This differential expression pattern and protein localization of the FGF family in response to stretch-induced hypertrophy suggest distinct roles for individual FGFs during the postnatal hypertrophy process.

MATERIALS AND METHODS

Animals. Four-month-old Cornish-cross broilers were housed in groups of 10 at the Purdue Poultry Farm. Birds received food and water ad libitum. Experimental procedures were approved by the Purdue Animal Care Committee.

Hypertrophy. Hypertrophy of the right ALD muscle was induced as previously described (16). A lead band that was equivalent to 10% of body weight was wrapped around the right humerus, thereby stretching and loading the right ALD muscle. The left ALD muscle served as an intra-animal control. Wings were weighted for 2 (n = 5) or 11 (n = 5) days. After the weighting period, birds were euthanized, and the right and left ALD muscles were weighed and dissected. The distal one-third of each muscle was immediately frozen on dry ice. The remaining proximal portion of the muscle was embedded in OCT compound (Miles Laboratories) and immediately frozen in crushed dry ice. These samples were stored at –70°C.

RNA isolation. An RNA pool that represented the distal one-third of each ALD muscle was generated. We propose that the distal one-third of the ALD muscle is representative of the entire muscle, because McCormick and Schultz (16) have demonstrated that the growth response of the ALD in response to stretch appears to occur throughout the length of

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the muscle. To generate these RNA pools, we separated the distal one-third of each ALD muscle into 250-µg aliquots. Total RNA was isolated from these aliquots, as previously described (6), with the following modifications. Samples were homogenized (Polytron, setting no. 4) in 8 ml of 4 M guanidine isothiocyanate for 15 s. Homogenate was then centrifuged for 5 min at 200 g at 4°C. Supernatant from this spin was layered over 4 ml of 5.7 M CsCl and was ultracentrifuged for 21.5 h at 174,000 g at 20°C. RNA pellets were recovered and pooled. Quantity of RNA was determined by absorbance at 260 nm. RNA integrity and quantity were confirmed by electrophoresis through a 1% agarose gel stained with ethidium bromide.

RT-PCR. Total RNA (6.25 µg) was added to reverse transcriptase (RT) buffer (GIBCO-BRL Life Technologies) containing 0.94 mM deoxynucleoside triphosphate (dNTP), 94 pmol of random hexamer primer (Pharmacia LKB Biotechnology), and 0.141 mM dithiothreitol in a total volume of 24 µl. After an incubation at 65°C for 10 min, 19 µl of this mixture was removed, added to 200 U of RT (Superscript, GIBCO-BRL Life Technologies), and incubated at 25°C for 15 min, 37°C for 90 min, 42°C for 15 min, and 90°C for 1 min. cDNAs were diluted to 100 ng/µl with distilled, deionized H2O and stored at −20°C. The remaining 5 µl of the original RT mixture was diluted to 25 µl and was used as a nonreverse-transcribed control in the PCR reaction. To minimize RT reaction variability, the integrity and quantity of all muscle RNA samples were confirmed by gel electrophoresis, and all RNA samples were reverse transcribed simultaneously by using a RT “master mix” (12). A cDNA and no-RT control were made from RNA isolated from each control and each weighted ALD muscle. Therefore, a total of 20 cDNAs and 20 no-RT controls were synthesized. These cDNAs and no-RT controls supported all the subsequent PCR analyses for all genes considered.

For amplification, a PCR master mix containing 1× PCR buffer (Boehringer Mannheim), 250 µM dNTP, 0.25 µM forward and reverse primer, and 0.05 U/µl of Taq polymerase (Boehringer Mannheim) was prepared just before use. From this mixture, 18 µl were removed and added to 2 µl of cDNA or no-RT control and then placed in a heating block (MJ Research). Cycling parameters were as follows: denaturation at 95°C for 45 min, annealing at various temperatures (50–65°C) for 30 s, and elongation at 72°C for 1 min. Specific primers and accession numbers are shown in Table 1. The members of the FGF family chosen for PCR amplification were determined by availability of chicken-specific cDNA sequences. For each primer set, each cDNA was amplified for various cycle numbers (cycle titration) to ensure that PCR products were being obtained and analyzed during the logarithmic phase of PCR amplification. This allowed quantitative comparisons of PCR product between weighted and control ALD muscles (8, 12). The range of cycle numbers included the logarithmic phase of PCR amplification for each primer set are shown in Table 1. In situations in which a PCR product could not be detected in adult ALD skeletal muscle cDNAs, a positive control cDNA was used to ensure that PCR amplification was functioning properly. These positive control cDNAs are identified in Table 1. After amplification, PCR products were electrophoresed through 1% agarose gels, stained with ethidium bromide, and photographed. To validate PCR product identification, PCR products were digested with specific diagnostic restriction enzymes (see Table 1). Quantitation of PCR products was performed by using the densitometry functions of Kodak image-analysis software (Kodak Digital Science 1D). Densitometry values from the area under the right ALD muscle were expressed as a percentage of the value obtained from the control ALD muscle (assigned a value of 100%). Statistical analysis was performed by using a t-test on two paired samples for means (Microsoft Excel). Other statistical analyses were performed by using ANOVA.

To isolate a chicken FREK cDNA sequence, primers encoding the chicken homologue of FREK, and subsequently used to design primers for amplification of chicken FREK (Table 1).

Immunolocalizations. By using a cryostat, 20-μm sections from ALD muscles embedded in OCT compound were generated, fixed to glass slides (Super Frost Plus, Fisher), allowed to dry for 30 min, and stored at −20°C until further use.

Primary antibodies used in these experiments were 148.2.1.1 [a mouse monoclonal anti-FGF-2 antibody (23)] and C-18 [a goat polyclonal anti-FGF-4 antibody (Santa Cruz Biotechnologies)]. These are FGF-specific antibodies and do not cross react with other cloned and characterized members of the FGF family (23). The secondary antibodies used were a biotinylated horse anti-mouse secondary antibody (South

Table 1. Primers, gene accession numbers, range of logarithmic cycle numbers, positive control cDNAs, and diagnostic restriction enzymes used in PCR reactions and on PCR products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Accession No.</th>
<th>Logarithmic Range of Cycle Nos.</th>
<th>Positive Control cDNA</th>
<th>Diagnostic Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF-1</td>
<td>gccatgcgccgaggggag</td>
<td>tcgtaacgccgacaccgg</td>
<td>S63263</td>
<td>38–43</td>
<td></td>
<td>BamHI</td>
</tr>
<tr>
<td>FGF-2</td>
<td>gaatcgaacgacaccg</td>
<td>agaatcgcacacccct</td>
<td>M95707</td>
<td>38–43</td>
<td></td>
<td>PstI</td>
</tr>
<tr>
<td>FGF-4</td>
<td>ggctgatcgcagggggag</td>
<td>tcgagcgtaccgccg</td>
<td>U14654</td>
<td>40–45</td>
<td></td>
<td>BamHI</td>
</tr>
<tr>
<td>FGF-8</td>
<td>agaagtgcgatgttcggg</td>
<td>agaatgcacgtgtggttg</td>
<td>U55189</td>
<td>Stage 24 limb bud</td>
<td></td>
<td>PstI</td>
</tr>
<tr>
<td>FGF-10</td>
<td>ggagctaaacttcacctcc</td>
<td>gccctctgctgcttcc</td>
<td>D68333</td>
<td>35–40</td>
<td>M24637</td>
<td>TaqI</td>
</tr>
<tr>
<td>FGF-1 (Cek-1)</td>
<td>atgaactctgtgctgta</td>
<td>ggtggtgctggtggttg</td>
<td>M35196</td>
<td>Stage 24 limb bud</td>
<td>M35195</td>
<td>BstXI</td>
</tr>
<tr>
<td>FGF-2 (Cek-3)</td>
<td>tgaactcaaggtcctcc</td>
<td>ggactctaatctaatata</td>
<td>M35195</td>
<td>Stage 24 limb bud</td>
<td></td>
<td>EcoRI</td>
</tr>
<tr>
<td>FGF-3 (Cek-2)</td>
<td>cattgggaagacattcct</td>
<td>gcgtgctatgcctaccc</td>
<td>M35195</td>
<td>Stage 24 limb bud</td>
<td></td>
<td>EcorI</td>
</tr>
<tr>
<td>FGF-9</td>
<td>gcccagctgtagccaga</td>
<td>gcgtctctgtagttagtc</td>
<td>AF809063</td>
<td>30–40</td>
<td>K01458</td>
<td>NcoI</td>
</tr>
</tbody>
</table>

Forward and reverse primers used during PCR amplifications and gene accession nos. are shown. FGF, fibroblast growth factor; FGF, fibroblast growth factor receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Cek, chicken embryo kinase. Range of cycle nos. that included the logarithmic phase of PCR amplification for each primer set are also indicated. Quantitative comparisons of PCR products can be performed if the products are obtained during the logarithmic phase of PCR amplification (10). In situations in which a PCR product could not be detected in the adult anterior latissimus dorsi skeletal muscle cDNAs, a positive control cDNA, indicated above, was used to ensure that PCR amplification was functioning properly. Diagnostic restriction enzymes used for validation of PCR product identification are shown.
Western Biotechnologies) with the FGF-2 primary antibody and a biotinylated horse anti-goat secondary antibody (Fisher Scientific) with the FGF-4 primary antibody.

For immunolocalizations, sections were fixed with 4% paraformaldehyde in 1× PBS (in mM: 138 NaCl, 2.7 KCl, 8.1 Na2HPO4, 1.2 KH2PO4, pH 7.2, for 5 min at 22°C. After two rinses in 1× PBS at 22°C for 5 min each, sections were incubated with blocking buffer (20 mM Tris base, pH 7.4; 100 mM NaCl (Tris-buffered saline)) + 2.5% horse serum, containing 0.3% H2O2 and 0.1% Triton X-100, for 30 min at 22°C. After two rinses in blocking buffer at 22°C for 5 min each, sections were incubated with primary antibodies diluted in blocking buffer (anti-FGF-2, 1:1,000 vol/vol; anti-FGF-4, final concentration 5 mg/ml) for 1 h at 22°C. Sections were then rinsed twice in blocking buffer at 22°C for 30 min and incubated with secondary antibody diluted 1:400 vol/vol in blocking buffer for 1 h at 22°C. After two rinses in 1× Tris-buffered saline for 30 min at 22°C, antibody visualization was performed by using ABComplex and 3,3'-diaminobenzidine (DAB) substrate according to the manufacturer’s instructions (Vector Laboratories). After the DAB reaction, slides were coverslipped, and images were obtained by using a Sony charge-coupled-device digital camera. To obtain quantitative results, extreme care was taken to incubate the slides with the DAB substrate reaction for identical periods of time, and comparisons between stretched and control muscle sections were done from anatomically similar areas of the ALD muscle. As a negative control, sections were treated as described above, with the exception of the primary-antibody-incubation step. Immunolocalization analyses were done on sections obtained from three animals from both periods of stretch.

**RESULTS**

To induce hypertrophy of the ALD muscle, a lead band equivalent to 10% of body weight was wrapped around the humerus for either 2 or 11 days. After 2 days of stretch, the wet weight of the ALD muscle was increased from an average 4.1 to 5.5 g (n = 5 for each mean; SE = 0.22; P < 0.01) for an average 34% increase. The increases in ALD muscle weight after 2 days of stretch ranged from 17 to 71%. After 11 days of stretch, the wet weight of the ALD muscle was increased from an average of 4.1 to 7.4 g (n = 5 for each mean; SE = 0.26; P < 0.01) for an average 85% increase. The increases in ALD muscle weight after 11 days of stretch ranged from 35 to 129%.

![Fig. 1. Fibroblast growth factor (FGF)-family member RNA expression in adult avian anterior latissimus dorsi (ALD) muscles with or without stretch for 2 or 11 days. Total RNA was isolated from stretched and control ALD muscles, reverse transcribed, and PCR-amplified by using primers coding for specific FGF-family members as described in MATERIALS AND METHODS. A: representative PCR products obtained from weighted (W) or control (C) ALD muscles from 2 birds representing 2 days of stretch (left) or 2 birds representing 11 days of stretch (right). These products are from an equivalent cycle of amplification, obtained during logarithmic phase of amplification (see Table 1). FGF, fibroblast growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. A quantitative comparison was performed between PCR products obtained from all 5 ALD muscles weighted for either 2 (B) or 11 (C) days, with those obtained from the unweighted contralateral control ALD muscles, by using densitometry and statistical analysis as described in MATERIALS AND METHODS; n = 5 muscles for each mean. *Statistical probability, P < 0.05; **statistical probability, P < 0.01. Bars, SE.](image-url)
11-day weighting experiments were temporally performed such that the muscles from these two experiments were isolated 11 days apart. Therefore, although they were isolated 11 days apart, there was no difference between weights of control ALD muscles dissected from birds during the 2- or 11-day weighting studies. This result demonstrates that there was minimal growth occurring in the nonweighted ALD muscles.

To analyze FGF-family expression, total RNA was isolated from stretched and control ALD muscles. Due to an extensive amount of connective tissue, it is difficult to obtain large amounts of RNA from adult skeletal muscle. However, we found that adding a 5-min centrifuge procedure between the homogenization and ultracentrifugation steps of our RNA isolation protocol removed a substantial amount of dense connective tissue from the homogenate, thereby increasing RNA yields from the adult skeletal muscle samples from 71.1 to 192 µg of total RNA per gram of wet weight muscle (n = 5 for each mean; SE = 30.8; P < 0.01). Using this modified procedure, we found that total RNA yields from control ALD muscles (192 µg of total RNA per gram of wet weight muscle) were significantly less than 2-day weighted (385 µg of total RNA per gram of
wet weight muscle) and 11-day weighted (543 µg of total RNA per gram of wet weight muscle) ALD muscles (n = 5 for each mean; SE = 31.2; P < 0.05).

Isolated RNA was reverse transcribed and subjected to quantitative PCR amplification. RT-PCR was used for gene analysis because expression levels of the FGF mRNAs in skeletal muscle are very low, and, as mentioned above, it is difficult to obtain large amounts of RNA from adult skeletal muscle. Of the FGF ligands and receptors tested, FGF-1, FGF-2, FGF-4, FGF-10, FGFR-1 (Cek-1), and FREK mRNAs were detectable in adult ALD skeletal muscle. FGF-8, FGFR-2 (Cek-3), and FGFR-3 (Cek-2) mRNAs were not detected in adult ALD skeletal muscle (Fig. 1). In response to stretch for 2 or 11 days, FGF-1 mRNA expression was significantly decreased, 2.5-fold (Fig. 1). FGF-2, FGFR-1, and FREK mRNA expression was not consistently changed after 2 days of stretch but was significantly increased after 11 days of stretch (Fig. 1). FGF-4 mRNA expression was significantly increased in ALD muscles 2 days after initiation of stretch but was not consistently changed after 11 days of stretch (Fig. 1). FGF-10 mRNA expression levels were significantly increased in ALD muscles 2 and 11 days after wing weighting (Fig. 1). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was unchanged after 2 or 11 days of stretch. (Fig. 1). Because only a fraction of each cDNA is used for each RT-PCR, one cDNA from each muscle sample supported all of the subsequent PCR analyses. Therefore, the unchanged nature of the GAPDH mRNA levels acted as an internal control for the RT reactions. Each one of the PCR amplifications was performed twice with similar results.

Sections from stretched and control ALD muscles were examined immunohistochemically for FGF-2 and FGF-4 protein. In response to 2 days of stretch, no obvious changes in localization or quantity of FGF-2 or FGF-4 protein were detected (data not shown). FGF-2 and FGF-4 immunostaining was similar to that observed in the control ALD muscles from the 11-day weighting experiments (Figs. 2, A and C). In these sections, FGF-2 and FGF-4 protein was detected in the perimysium and endomysium of the myofiber periphery (Fig. 2, A and C). No FGF-2 or FGF-4 protein was detected within the myofibers. However, after 11 days of stretch, dramatic increases in both FGF-2 and FGF-4 protein could be detected throughout the endomysium and greatly enlarged perimysium of the myofiber periphery (Fig. 2, B and D; Fig. 3, A and B). Low-magnification micrographs demonstrate that increases in FGF protein are spread throughout the ALD muscle and not localized to a specific region within the muscle (Fig. 2, A-D). Whereas the localization of FGF-2 and FGF-4 proteins was quite similar, one major difference was noted in the ALD muscles stretched for 11 days. Nuclear FGF-2 immunostaining was observed in a specific population of cells localized within the myofiber periphery (Fig. 3A). This nuclear staining was confirmed by using a fluorescent secondary antibody to the FGF-2 antibody and a fluorescent nuclear stain (Hoechst 33342 dye; data not shown). This type of nuclear staining was not detected with the FGF-4 antibody (Fig. 3B). Similar results were observed in ALD sections obtained from two other animals from each time period of stretch.

**DISCUSSION**

The mechanisms by which stretch is translated into skeletal muscle hypertrophy are presently unknown. We have begun to examine the role of FGFs during stretch-induced postnatal skeletal muscle hypertrophy. We have analyzed 1) which members of the avian FGF-family are expressed in adult skeletal muscle and 2) how their mRNA and protein expression are affected by stretch-induced hypertrophy.

We detected FGF-1, FGF-2, FGF-4, FGF-10, FGFR-1, and FREK mRNA expression in adult ALD muscle. Among the FGF-family members expressed in adult ALD muscle, there was considerable variation among
the animals in levels of expression. However, we still found two distinct, significant expression patterns in response to stretch. The first pattern observed was a decrease in expression of FGF-1 mRNA. To our knowledge, this is the first report of a decrease in growth factor expression in response to stretch hypertrophy. A decrease in mRNA expression in this type of skeletal muscle hypertrophy model is difficult to interpret, because we and others have found that total RNA content in stretched skeletal muscles is increased (29). Therefore, a decrease in FGF-1 mRNA expression could be a simple dilution effect caused by the increase in total RNA or a specific downregulation in response to stretch. It will be important to localize FGF-1 mRNA and protein to clarify the significance of the decreased FGF-1 expression. Our preliminary attempts to localize FGF-1 protein with available FGF-1 antibodies were unsuccessful, presumably due to the low level of FGF-1 protein within ALD muscle.

In contrast to the pattern with FGF-1, the other significant expression pattern that we observed in response to stretch was an increase in mRNA levels, observed with FGF-2, FGF-4, FGF-10, FGF-1, and FREG. Along with increased mRNA levels, we also observed a dramatic increase in FGF-2 and FGF-4 protein. The amount of FGF-2 protein mimicked the observed changes in FGF-2 mRNA expression. Increases in FGF-4 mRNA were detected after 2 days of stretch. However, the levels of FGF-4 protein did not dramatically increase until after 11 days of stretch. Although FGF-4 mRNA concentrations were still slightly elevated at this time point, the differences were no longer statistically significant. The reasons for this discordance between FGF-4 protein and mRNA are unknown. One possibility is that increases in FGF-4 protein levels occurred 2 days poststretch but were beyond the sensitivity of our immunolocalization studies. It is also possible (and is our favored hypothesis) that significant increases of steady-state concentrations of FGF protein within a skeletal muscle are ultimately dependent on enlargement of the extracellular matrix component. Contained within this matrix are heparan sulfate proteoglycans, proteins that sequester and protect FGFs from degradation (5, 21, 22). We observed an enlargement of the perimysium of the ALD muscle after 11 days of stretch. It was only at this point that we could detect elevations of FGF-4 protein, although increases in FGF-4 mRNA had occurred after 2 days of stretch. Increasing the capacity of the FGF reservoir in skeletal muscle could function to keep FGF protein concentrations elevated, even beyond periods of increased mRNA expression, and to ensure FGF availability and function during the hypertrophic process.

Regardless of the mechanism, FGF-2 and FGF-4 protein levels were both elevated in ALD muscles at 11 days of stretch. As in localization studies of FGF-2 protein in dystrophic skeletal muscle (4), the majority of FGF-2 and FGF-4 protein was localized to the myofiber periphery in both control and stretched muscles. This type of localization directly contrasts that reported for insulin-like growth factor-1, which, like FGF-2 and FGF-4, is also upregulated during skeletal muscle hypertrophy but is expressed by and predominantly localized to the myofiber during skeletal muscle hypertrophy (28–30). The classical role attributed to FGF-2 and FGF-4 during postnatal skeletal muscle cell development is stimulation of myoblast proliferation and inhibition of differentiation (1, 11, 14, 15). However, this hypothesis is derived solely from studies that added exogenous FGF-2 and FGF-4 to cultures of satellite cells. The exact role that these two FGFs play, or even whether they are endogenously present, during postnatal skeletal muscle development in vivo, is unknown. Our results demonstrate that these two FGF-family members are 1) expressed by adult skeletal muscle, 2) upregulated during stretch-mediated hypertrophy, and 3) localized to the myofiber peripheral matrix during skeletal muscle hypertrophy. The myofiber periphery is an area of extensive satellite cell proliferation in response to injury or other growth stimuli (17, 24, 27). The upregulation and localization of FGF-2 and FGF-4 within this region support the classical notion of these two proteins expanding the satellite cell population in mature skeletal muscle in response to a growth stimulus. In contrast to FGF-4, FGF-2 protein was localized to the nucleus of a population of cells residing within the myofiber periphery. The functional roles of differentially sequestered FGF proteins are unknown. Although our data support the concept of FGF-2 and FGF-4 stimulating satellite cell proliferation, it is conceivable that these two FGFs accomplish this function through different mechanisms, as shown by their differential localization.

In summary, we have found that several members of the FGF family are expressed in adult skeletal muscle and that they exhibit differential expression patterns in response to stretch-induced hypertrophy. These results implicate certain FGFs as potential activators and mediators of postnatal skeletal muscle hypertrophy and suggest distinct roles for individual FGFs during postnatal skeletal muscle development.

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