Time course of changes in markers of myogenesis in overloaded rat skeletal muscles

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Adams, Gregory R., Fadia Haddad, and Kenneth M. Baldwin. Time course of changes in markers of myogenesis in overloaded rat skeletal muscles. *J. Appl. Physiol.* 87(5): 1705–1712, 1999.—During the process of compensatory muscle hypertrophy, satellite cells are thought to proliferate, differentiate, and then fuse with existing myofibers. We hypothesized that early in this process changes occur in the expression of cellular markers indicative of the onset of myogenic processes. The plantaris muscles of rats were overloaded via the unilateral ablation of synergists. Groups of rats were killed at time points from 6 h to 12 days. Changes in muscle gene expression (mRNA) of cyclin D1, p21, myogenin, MyoD, and insulin-like growth factor I (IGF-I, mRNA and peptide) were measured. Cyclin D1 (a cell cycle marker) was increased after 24 h of overloading and corresponded with changes in muscle DNA content. In contrast, p21 and myogenin, markers of cellular differentiation, were increased after just 12 h. Muscle IGF-I peptide levels were also increased at early time points. The results of this study indicate that myogenic processes are activated in response to increased loading at very early time points (e.g., 12 h) and that IGF-I may be modulating this response. Furthermore, these findings suggest that some cells may have been differentiating very early in the adaptation process before events leading to cellular proliferation have been initiated.

muscle hypertrophy; cyclin D1; myogenin; insulin-like growth factor I

PREVIOUS STUDIES have reported findings to suggest that mature mammalian skeletal muscle fibers maintain a relatively finite, fiber type-specific relationship between the size of the myofiber (e.g., myofiber length, cross-sectional area, or cytoplasmic volume) and the number of myonuclei present in a given myofiber (5, 4, 19, 27, 39, 51, 73, 76, 77). The concept that there is a finite myonuclear domain size associated with myofibers predicts that hypertrophying myofibers must increase their myonuclear number to accommodate the enlargement process. However, shortly after birth, mammalian myofibers are permanently differentiated and thus cannot undergo mitotic division or directly increase their myonuclear number (i.e., myonuclear division) (18, 74). Therefore, myofibers undergoing hypertrophy appear to require an external source of new nuclei to maintain or reestablish a relatively constant myonucleus-to-fiber size ratio (18, 19, 80). There is a significant body of evidence to suggest that satellite cells are the probable source of new myonuclei in mature mammalian skeletal muscles. There is also some compelling evidence indicating that satellite cell proliferation is required to support the process of compensatory hypertrophy in mammalian skeletal muscle (4, 58, 63, 64, 68). These latter studies used radiation to prevent satellite cell proliferation before initiation of skeletal muscle functional overloading and found that this treatment prevented the hypertrophy response.

Satellite cells are small mononucleated skeletal muscle stem cells located between the basal lamina of the muscle and the sarcolemma of myofibers. Satellite cells are mobilized in response to increased loading conditions or after injury to muscle cells (4, 62). The initial events after satellite cell activation have been reported to be a proliferative response in which some or all of the activated satellite cells undergo at least one mitotic cycle (60, 69). After this initial phase, some of the activated cells and/or their progeny are thought to differentiate into myoblast-like cells. In regenerating muscle, these myoblasts can fuse with each other to form new myofibers or become incorporated into existing myofibers (12, 50, 52, 61, 62). In the case of the hypertrophy response, satellite cell-derived myoblasts are thought to fuse with existing myofibers, thereby providing additional myonuclei (i.e., DNA) (18, 53, 67, 68, 72). The inhibition of proliferation is thought to precede the expression of muscle-specific genes and the subsequent fusion of myoblasts into new or existing myotubes (57).

The mechanisms underlying the recruitment of satellite cells for regenerative or hypertrophic processes have not been established. On the basis of developmental models, such as those involving embryonic muscle or muscle cell line studies in culture, there appears to be a role for various growth factors, such as fibroblast growth factor (FGF), in the mobilization of satellite cells (6, 32, 44, 59, 81). However, most mitogenic growth factors have been found to inhibit the second phase of satellite cell responses (i.e., differentiation and fusion) (15, 31). In contrast, insulin-like growth factor I (IGF)-I [and possibly hepatocyte growth factor (HGF)] has been shown to promote the proliferation, differentiation, and fusion of satellite cells in primary cultures (18, 24, 78).

Using an avian muscle satellite primary cell culture preparation, Duclos et al. (25) reported that DNA synthesis can be initiated via binding of IGF-I and IGF-II and, to a lesser extent, insulin to the IGF-I receptor. We recently reported that the increase in IGF-I seen in rat skeletal muscles during compensatory hypertrophy was paralleled by an increase in muscle DNA content (2). Our analyses indicated a significant positive correlation between the increase in muscle DNA content and muscle IGF-I expression. In a separate study we found that the localized infusion of IGF-I

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into the skeletal muscles of rats in vivo resulted in hypertrophy and that the DNA-to-protein ratio of the hypertrophied muscles was unchanged from that of controls (3). These results support the hypothesis that IGF-I is mediating the hypertrophy response in part by stimulating the proliferation of satellite cells.

As noted above, it is generally assumed that new myonuclei are provided by satellite cells that enter the cell cycle and then differentiate. The proliferative phase of activated satellite cells is characterized by progression through successive steps of the cell cycle (18). Progression of quiescent cells from G_0 into G_1 and on through the mitotic process has been shown to be under the control of cyclin proteins that are complexed with various cyclin-dependent kinases (CDK). In particular, cyclin D1 expression is associated with the commitment to synthesize DNA (i.e., move through the restriction point in G_1 and into the S phase). Therefore, an increase in cyclin D1 expression in overloaded skeletal muscles would generally indicate that a population of cells has become mitotically active (57).

A second class of cell cycle proteins termed cyclin kinase inhibitors (CKIs) are known to mediate the withdrawal of cells from the cell cycle. One of these CKIs, cyclin kinase inhibitory protein, p21 [variously known as Cip1, Waf1, Sdi1, or Cap20 (55)], has also been associated with the differentiation of various cell types, including myogenic cells (57, 79, 86). Although CKIs such as p21 represent relatively universal markers of cell differentiation, the expression of myogenin, one of the MyoD family of muscle regulatory factors (MRFs), might serve as a muscle-specific marker of differentiation in vivo. A number of investigators have reported an apparent relationship between myogenin expression and differentiation in myocyte cell lines or satellite cell cultures (38, 48, 49, 65, 79, 86). For example, in satellite primary cell culture, Smith et al. (71) found that MyoD mRNA increased before proliferation, whereas the appearance of myogenin mRNA appeared to coincide with differentiation. As further evidence of its relationship to the differentiation step, the inhibition of myogenin expression has been shown to block the differentiation of myogenic cells (15, 38).

Although a number of growth factors have the potential to stimulate cell proliferation in various muscle cell types (41), the actions of growth factors other than IGF-I (and IGF-II) and HGF are generally antagonistic to differentiation (8, 31, 42, 46, 56). The IGFs and HGF are the only well-characterized growth factors that have been consistently shown to induce the processes of proliferation and differentiation in satellite primary cell culture and/or muscle cell lines. In addition, IGF-I has been reported to stimulate the expression of cyclin D1 and cyclin D2 (28).

On the basis of the foregoing information, we hypothesized that *1*) changes in gene expression indicative of myogenic processes occur in muscles undergoing compensatory hypertrophy in response to increased loading and *2*) changes in IGF-I expression are temporally regulated to mediate these responses. Our results indicate that increased loading results in changes in the level of expression of IGF-I, MyoD, myogenin, cyclin D1, and p21 in overloaded skeletal muscle and that these markers can provide useful indexes of the myogenic activity necessary for compensatory growth of muscle.

METHODS

Seventy-two female Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY). The initial body weight of these animals was 226 ± 5 g. All groups consisted of a minimum of six animals. All animals were housed in standard vivarium cages and allowed food and water ad libitum. All treatment protocols were approved by the institutional animal research committee.

Overload. Animals from the overload groups were anesthetized (80 mg/kg ketamine and 2 mg/kg acepromazine), and the plantaris muscles were overloaded via unilateral removal of the gastrocnemius and soleus muscles, as described previously (9). After the surgery the rats were generally ambulatory in ~2 h.

Tissue collection. At 6, 12, 24, and 48 h and after 3, 7, and 12 days of overloading, groups of rats were killed via an overdose of pentobarbital sodium. The overloaded and contralateral plantaris muscles were rapidly excised, cleaned of external connective tissue, weighed, freeze-clamped, and stored at −80°C.

Assessment of muscle hypertrophy. In addition to muscle wet weight, the muscle protein concentration was determined from whole muscle homogenate by the biuret method (35). Total muscle protein was calculated from the product of the concentration and the wet weight of the muscle sample recorded at the time the animals were killed.

mRNA analysis. Total RNA was extracted from frozen muscle samples by use of the TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the company's protocol, which is based on the method described by Chomczynski and Sacchi (20). Extracted RNA was precipitated from the aqueous phase with isopropanol, washed with ethanol, dried, and suspended in a small volume of 0.5% SDS in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The RNA concentration was determined by optical density at 260 nm (using a 260-nm optical density unit equivalent to 40 μg/ml). The RNA samples were stored frozen at −80°C until subsequently analyzed for myogenin, MyoD, p21, cyclin D, and IGF-I mRNA expression by Northern hybridization.

For Northern blot analysis, 10 μg of total cellular RNA was size fractionated by electrophoresis through a 0.8% agarose gel under formaldehyde denaturing conditions (75), transferred to a nylon membrane (Qiabrane, Qiagen, Valencia, CA) by the capillary method with 10× saline-sodium citrate as the transfer buffer, and fixed by ultraviolet irradiation. Blots were dried at 80°C for 30 min, then stored dry at 4°C until subsequent hybridization.

For *IGF-I* mRNA analyses, ^{32}P-labeled complementary RNA probes were generated using Ambion Maxiscript, as described previously (2), with the exception that ^{32}P-labeled UTP was used as the labeled nucleotide. For all other analyses, ^{32}P random primed cDNA probes were generated using the Rediprime labeling system (Amersham). Plasmids for the different cDNAs were gifts as follows: p21 from Dr. S. A. Belinsky (ITRI, Albuqurque, NM) (10), cyclin D1 from Dr. M. L. Brandi (University of Florence, Florence, Italy) (11), MyoD cDNA from Dr. A. B. Lassar (Harvard Medical School, Boston, MA) (23), and myogenin from Dr. W. E. Wright (University of Texas Southwestern Medical Center, Dallas, TX) (82). Prehybridization, hybridization, and washing were

performed using the NorthernMax reagents from Ambion (Austin, TX) according to the provided protocols. After exposure to X-ray film, band intensities on the autoradiograms were quantified using a laser scanning densitometer and Image Quant software (Molecular Dynamics). For normalization of the mRNA signals to the amount of RNA loaded, blots were washed in boiling 1% SDS to strip off the probe and rehybridized with an 18S oligoprobe, which detects the 18S ribosomal RNA unit (75). For each specific mRNA signal, the absorbance was normalized to its corresponding 18S signal.

Muscle IGF-I content. The IGF-I extraction was conducted as described previously (2). Briefly, muscle samples were pulverized under liquid nitrogen, and the resultant powder was transferred to tared precooled, microcentrifuge tubes for acid-ethanol extraction (14). The IGF-I RIA was conducted following the manufacturer's instructions with use of a rat-specific RIA kit (DSL, Webster, TX).

DNA determination. DNA concentration was measured in whole muscle homogenates with use of a fluorometric assay for the DNA binding fluorochrome bisbenzimide H-33258 (Calbiochem, San Diego, CA). Calf thymus DNA was used as a standard (47).

Statistical analysis. Values are means ± SE. For each time point, treatment effects were determined by ANOVA with post hoc testing (Student-Newman-Keuls) with use of the Prism software package (Graphpad). Pearson's correlation analyses of relationships comparing myogenin with p21 mRNA were performed using the Prism package. For all statistical tests the 0.05 level of confidence was accepted for statistical significance.

RESULTS

Muscle hypertrophy. Twelve days after surgery the overloaded plantaris muscles had increased in wet weight by ~66% compared with the contralateral muscles: 1.73 ± 0.18 vs. 1.07 ± 0.02 mg/g body wt. There appeared to be an increase in muscle wet weight as early as 12 h after the synergist ablation surgery (data not shown). However, this treatment is known to result in edema, and thus the wet weight values cannot be considered a reliable measure of muscle hypertrophy at the early time points. The plantaris muscle protein content was significantly increased 12 days (288 h) after the imposition of overloading (Fig. 1). Over the 12 days of the study the body weights of the rats continued to increase, such that the mean body weight had increased from 226 ± 5 to 257 ± 6 g for the 12-day

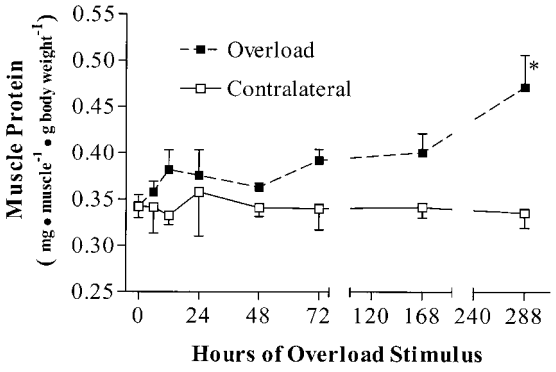

Fig. 1. Effects of unilateral ablation of soleus and gastrocnemius muscles on plantaris muscle protein. *$P < 0.05$ vs. contralateral muscle.

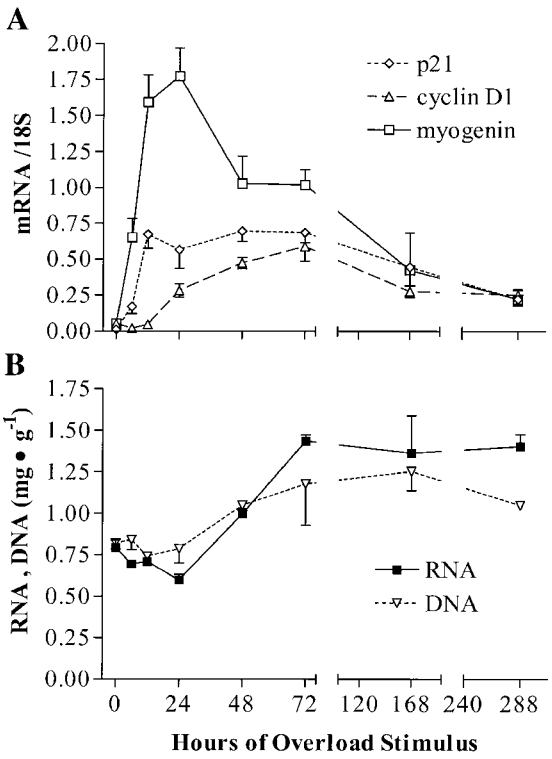

Fig. 2. Effects of unilateral functional overloading on markers of cellular proliferation and differentiation in plantaris muscles. As in Fig. 1, there were no changes in measured parameters in contralateral muscles at any time point. Accordingly, contralateral data are represented by "0"-h time point to facilitate presentation of data. *A*: myogenin and p21 mRNA were significantly increased before 24-h time point and declined toward baseline values thereafter. Cyclin D1 was significantly increased from 24-h time point onward. *B*: muscle DNA and RNA concentrations were significantly increased from 48-h time point onward. All mRNA values are expressed relative to 18S RNA, which was probed on same Northern blots.

groups. Consequently, we have expressed the muscle protein value as total muscle protein per muscle divided by body weight to allow comparison with the earlier time points and to maintain continuity with our previously published time course data (2).

Markers of proliferation and differentiation. Functional overloading of the plantaris muscle resulted in a rapid increase in the expression of mRNAs for myogenin and the CDK inhibitor p21, which were at or near their peak response 12 h after the initiation of overload (Fig. 2*A*). In contrast, cyclin D1 mRNA was not significantly increased until 24 h after surgery and did not peak until 72 h after surgery (Fig. 2*A*). There was a small but significant increase in MyoD1 mRNA at 12 h that returned to baseline by 24 h (data not shown). The DNA and RNA concentrations (mg/g) of the overloaded plantaris muscles were increased 48 h after the imposition of the overloading stimulus (Fig. 2*B*).

IGF-I. The IGF-I peptide concentration of overloaded plantaris muscles was increased from 12 h after surgery through the 12-day time point (Fig. 3). IGF-I mRNA expression was increased from the 48-h time point onward (Fig. 3). Despite relatively high plasma IGF-I concentrations (>500 μg/ml), we would not predict that plasma IGF-I contributes significantly to total

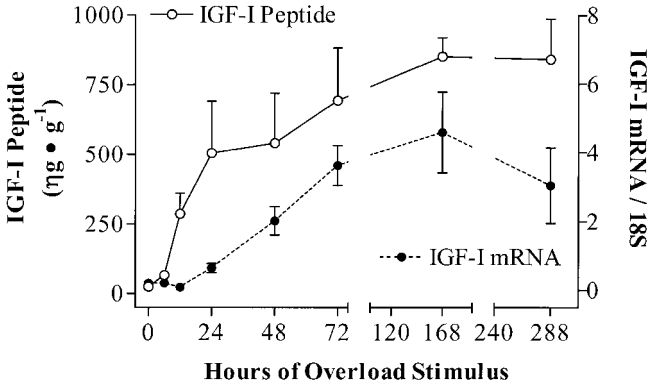

Fig. 3. Effects of functional overloading on insulin-like growth factor I (IGF-I) in plantaris muscle. Muscle IGF-I peptide levels were significantly elevated 12 h after imposition of overloading. Muscle IGF-I mRNA was significantly increased at 48 h and subsequent time points.

muscle IGF-I peptide because of the relatively small vascular volume (relative to total muscle volume) in unperturbed muscles. However, the imposition of functional overloading via the synergist ablation model is known to result in vascular congestion and edema at early time points. To determine whether plasma IGF-I might be contributing to the observed increase in muscle IGF-I at these early time points, we overloaded plantaris muscles in an additional group of rats for 8 h and subsequently perfused the lower limbs with physiological saline to remove the blood just before we killed the animals. During this process the muscles were observed under magnification and appeared to be blood free before excision. We found that saline perfusion did not alter the measured IGF-I concentration in the overloaded vs. nonperfused-overloaded muscles (data not shown). This result suggests that vascular accumulation did not account for the early increase in IGF-I seen in this study.

DISCUSSION

Numerous studies have reported that satellite cells proliferate and then fuse with existing myofibers as fully differentiated skeletal muscles adapt to increased loading (16, 61, 67, 68) and that this process appears to be obligatory for the development of compensatory hypertrophy (64, 58). The requirement for satellite cell proliferation and differentiation to support compensatory muscle hypertrophy suggests that it should be possible to detect cellular markers of these processes in overloaded skeletal muscles. The aim of the present study was to impose an increase in mechanical loading on a rat skeletal muscle to establish the temporal relationship between increased muscle loading and the expression of potential markers of myogenesis.

A unique finding of this study was that the earliest response to increased muscle loading appears to be related to the differentiation of a population of cells in the target muscle. Although the whole muscle analyses employed in the present study cannot definitively identify the cell types that are responsible for the increased expression of MRFs and cell cycle markers, a number of observations support the interpretation that satellite cells are the most likely candidates. The CDK inhibitor *p21* is expressed in cells that are preparing to exit the cell cycle and differentiate, whereas increased myogenin expression has been associated with, and may modulate, the differentiation of myocytes and/or muscle satellite cells (30, 37, 38, 86). Because existing myofibers in mammalian muscles are thought to be terminally differentiated and postmitotic in vivo, the increased expression of p21 is most likely to reside in nonmyofiber cells within the muscle. The expression of myogenin is thought to be confined to cells committed to become muscle. Therefore, the observation of concurrent increases in mRNA of myogenin and p21 at very early time points after the imposition of increased muscle loading strongly suggests that a myogenic cell population, e.g., satellite cells, was responding to this stimulus by initiating the expression of markers indicative of entrance into a differentiated state (70). In support of this suggestion, we found a significant, positive relationship between the observed increase in myogenin and p21 mRNA expression in this study (Fig. 4).

Alternatively, it is possible that mature myofibers may have been the source of increased myogenin and MyoD expression. In avian models an increase in myofiber MRF expression in response to stretch has been reported (29). In contrast, in a variety of mammalian models, increased myogenin or MyoD expression has generally been attributed to satellite cells and was seldom observed in myofibers (36, 40, 45). An exception to this finding involves studies using denervation in which increased myofiber MyoD or myogenin expression has been reported (26, 33). However, denervation represents a distinct atrophy model in which the myofibers degenerate and begin to express an undifferentiated sarcomeric phenotype, apparently including the reexpression of MRFs. Taken together, we propose that the findings from other mammalian muscle overload or injury models support the interpretation that the increased expression of myogenin and p21 reported here most likely reflects the differentiation of satellite cells.

Previous studies that have examined MRF expression in overloaded mammalian skeletal muscles have produced mixed results. For example, Mozdziak et al.

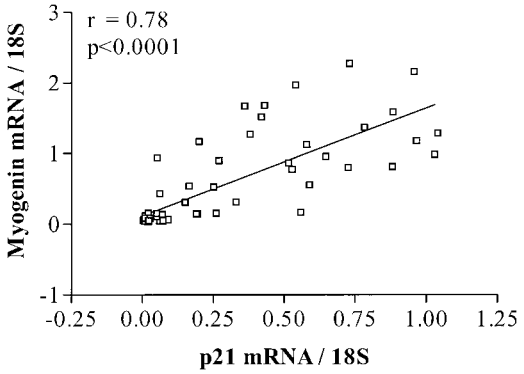

Fig. 4. Relationship between myogenin and p21 mRNA expression in functionally overloaded rat plantaris muscles.

(54) reported that 4 wk of functional overloading did not alter the relative expression of MyoD or myogenin mRNA. In the present study we found a significant increase in myogenin mRNA expression that began at 6 h after the imposition of increased loading that subsequently returned toward baseline values after 12 days of the initiating stimulus. Thus, at the 4-wk time point used in the study of Mozdziak et al., changes in myogenin expression most likely would not have been observed. Jacobs-el et al. (43) previously reported that myogenin mRNA was unchanged after a single 2-h bout of muscle stretching or stretching plus electrical stimulation in rat tibialis anterior muscles. In light of the present study, this observation suggests that some minimum amount of loading stimulus is required to activate the increased myogenin expression in mammalian muscles. In contrast to the two studies cited above, Carson and Booth (17) used a chronic avian compensatory hypertrophy model and found that myogenin mRNA levels were increased at the earliest time point measured (3 days) and remained elevated through 21 days of increased loading. These authors postulated that elevated myogenin expression may be important for the increase in muscle fiber number (i.e., hyperplasia), which is an important and unique adaptation seen in the avian model. In the present study the functional overload model would provide a chronic stimulus that is greater in magnitude and longer in duration than that used by Jacobs-el et al. and that is more similar to the intervention applied by Carson and Booth. Thus, despite potentially dissimilar resulting adaptive processes (myofiber hypertrophy vs. hyperplasia), the initiating mechanisms for the muscle hypertrophy response, e.g., satellite cell proliferation and differentiation, may be similar in the avian and mammalian models.

The finding that a population of cells within the muscle may be differentiating at the onset of adaptation is unique, in that much of the in vivo and satellite cell culture literature has suggested that activated satellite cells proliferate before they differentiate (7, 68, 69). For example, cell culture studies have found that myogenin expression does not become detectable until later time points (e.g., 48 h) after the provision of an activating (low mitogen) medium (7, 21, 48, 65, 71, 84). Although the time course of myogenin and MyoD expression reported here appears to be different from that seen in vitro, it is similar to that seen in vivo after muscle injury (36, 83).

The expression of cyclin D1 mRNA, a putative marker of the initiation of cellular proliferation processes, was not increased until 24 h after the imposition of increased loading, and it did not peak until the 72-h time point. This time course is similar to that reported for the exercise- or overload-induced activation of satellite cell proliferation in vivo (22, 68). In concert with the cyclin data, the DNA content of the overloaded muscles was not clearly elevated until the 48-h time point (Fig. 2). Thus these results lead us to propose that, in vivo, a population of satellite cells initially responded to increased loading not by proliferating, but by foregoing the ability to proliferate (increased p21 response) and initiating the expression of muscle-specific genes (myogenin response). A subsequent proliferation response appears to have been initiated after ~24 h of the overloading stimulus.

A number of studies have reported that IGF-I may be participating in the regulation of myogenic components of the compensatory hypertrophy responses in mature mammalian skeletal muscle (reviewed in Ref. 1). In the present study the observed overload-induced increases in muscle IGF-I peptide levels occurred with a time course similar to that of changes in myogenin and p21 (Figs. 2 and 3). This is consistent with a proposed role for this growth factor in the modulation of the skeletal muscle hypertrophy response. Because of the presence of IGF binding proteins, only a small fraction of IGF-I present in the circulation and in tissues is thought to be biologically available. Consequently, even a small local increase in free IGF-I might have a biologically significant effect, such as the induction of increased myogenin expression.

The finding that the increase in IGF-I peptide expression preceded that of the message for IGF-I indicates an accumulation of IGF-I peptide from sources outside the muscle or an increase in the translational efficiency of the IGF-I message. As reviewed by Booth et al. (13), there is evidence that a general increase in translational efficiency occurs at the onset of muscle hypertrophy. In the present study we found that muscle vascular accumulation of IGF-I peptide did not contribute measurably to the observed IGF-I levels in overloaded or control skeletal muscles (see RESULTS). Taken together these findings suggest that the observed disparity between IGF-I message and IGF-I peptide levels at very early time points may be a function of the increased translational efficiency that occurs in the early stages of compensatory overloading. However, it is possible that other cell types, including infiltrating immune cells, may have contributed to the early increase in muscle IGF-I (66).

In addition to IGF-I, HGF has been proposed as a candidate as an initiator of satellite cell activation. Recent studies have demonstrated that this growth factor can stimulate quiescent satellite cells to proliferate and possibly fuse to form myotubes in vitro, i.e., something that IGF-I may not be able to accomplish (78). We recently found that local infusion of HGF does in fact lead to a modest hypertrophy response in rat skeletal muscles (unpublished observations). However, the data supporting a role of HGF in the early stimulation of differentiation, as seen in the present study, are less certain. Although HGF has been shown to stimulate the differentiation of cells in culture, recent data suggest that it would be antagonistic to this process at early time points of satellite cell activation (8, 34). A recent report has indicated that FGF2 can stimulate the proliferation of satellite cells on isolated myofibers and that the continued presence of FGF2 did not prevent the differentiation of activated satellite cells, as was previously observed in vitro (85). Thus this

growth factor may play an important role in the myogenic response during hypertrophy.

In the present study the level of IGF-I peptide was observed to be elevated in overloaded muscles at later time points when the myogenin, p21, and cyclin D1 signals were declining. If the speculation that IGF-I is modulating myogenic processes is correct, then mechanisms such as target cell adaptations may be postulated. Such mechanisms may include the downregulation of IGF-I receptors or increased muscle IGF binding protein production/accumulation. Alternatively, elevated muscle IGF-I may be acting in concert with other growth factors such as FGF and HGF to initiate the myogenic cascade followed by alterations in the relative concentrations of growth factors, such that other nonmyogenic anabolic processes known to be associated with IGF-I are maintained.

In summary, the results of this study strongly support the concept that myogenic processes are part of the mechanisms by which skeletal muscle adapts to increased loading. The earliest response appears to be the differentiation of satellite cells followed by the proliferation and continued differentiation of this muscle precursor cell type. Changes in muscle IGF-I peptide levels occur on a time scale appropriate to the putative role of this growth factor in the stimulation of myogenesis during the adaptation to increased muscle loading.

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