ABSTRACT: Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) and immunohistochemistry were performed to demonstrate whether a correlation exists between insulin-like growth factors (IGFs)—positive regulators of growth—and myostatin, a negative regulator of muscle growth. IGF-I, -II, and IGF-receptor-1 (IGF-R1) mRNA and IGF-II protein expressions were determined in control and myostatin knockout mice tissues. IGF-I gene expressions were similar between control and knockout mice tissues, whereas IGF-II mRNA levels were significantly higher in myostatin knockout mice kidney and soleus muscles than those of control mice ($P < .01$). IGF-R1 mRNA levels from control mice heart ($P < .05$) and kidney ($P < .01$) were significantly higher than in myostatin knockout mice, whereas levels were lower in pectoralis muscle of control mice than knockout mice ($P < .01$). The strongly IGF-II–positive cells in soleus muscle were more common in myostatin knockout mice and were seen in a few foci in control mice. IGF–II immunoreactivity in both control and myostatin knockout mice kidneys was localized to the epithelium of renal tubules and collecting ducts. Reciprocal changes in the expression of myostatin and IGF-II and IGF-R1 may underlie normal growth of skeletal muscle and other organs in mammals, and the changes in these tissues associated with disease.
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IGF-I, IGF-II, AND IGF-RECEPTOR-1 TRANSCRIPT AND IGF-II PROTEIN EXPRESSION IN MYOSTATIN KNOCKOUT MICE TISSUES

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Insulin-like growth factors (IGFs)–I and –II are produced by several different tissues and are essential for both embryonic and postnatal development.[5,13] Severe disruption of tissue development, particularly in skeletal muscles, was observed in IGF-I or IGF-II knockout mice. For instance, IGF-I knockout mice were significantly smaller than their control littermates and had reduced development of muscle tissue,[18] whereas transgenic mice overexpressing IGF-I showed enhanced body growth with an increase in muscle mass.[14] Additionally, IGF-II expression has been positively associated with skeletal muscle development in double-muscled cattle[11] and in pig breeds with exceptional muscle mass.[16] In vitro, both IGFs inhibited apoptosis[22] and promoted proliferation and differentiation of skeletal muscle cells.[1] As evidenced by these in vivo and in vitro findings, IGFs are undoubtedly important components of skeletal muscle development. When the myostatin gene (also known as growth differentiation factor 8), a recently identified member of the TGF-β family, was disrupted by homologous recombination in mice, skeletal muscle mass significantly increased, up to three times the normal size.[15] Increased muscle mass in these mice was due predominantly to hyperplasia but also involved hypertrophy. Additionally, myostatin mutation resulting in functional loss of the protein has been linked to double-muscled cattle

Abbreviations: IGF, insulin-like growth factor; IGF-R1, insulin-like growth factor–receptor-1; PCR, polymerase chain reaction; RT, reverse transcription; TBS, tris-buffered saline

Key words: immunohistochemistry; insulin-like growth factor; insulin-like growth factor receptors; myostatin knockout mice; RT-PCR

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breeds.[8,10] Therefore, it has been proposed that myostatin is a negative regulator of skeletal muscle growth. We postulated that the ratio of mRNA and protein levels between IGFs, positive regulators of muscle growth, and myostatin, a negative regulator, may serve as indicators of skeletal muscle mass. Also, based on the gene knockout observations, IGFs, in addition to being a pivotal regulator of muscle growth, appear to be involved in controlling overall growth, whereas myostatin seems to be acting only on muscle growth. Thus, to demonstrate whether a correlation exists between IGFs and myostatin, IGF-I and -II and IGF–receptor-1 mRNA (IGF-R1) and protein levels were determined in a wide variety of myostatin knockout mice tissues.

MATERIALS AND METHODS

Animals. Nine-month-old male myostatin knockout ($n = 3$; SVJ/129) and control mice ($n = 3$; C57Bl/6) were generously provided by Metamorphix Inc., (Baltimore, Maryland). Euthanasia of mice and subsequent tissue collections were performed in compliance with an approved West Virginia University Animal Care and Use Committee protocol.

RNA Extraction and RT-PCR. Total RNA was extracted separately from myostatin knockout and control mouse brain, heart, liver, kidney, and skeletal muscle tissues (soleus, gastrocnemius, and pectoralis) using the Tri-Reagent (Sigma, St. Louis, Missouri) modification of the guanidine isothiocyanate/phenol-chloroform method.[4] The RNA concentration was estimated by absorbance at 260 nm in a Shimadzu spectrophotometer (model UV-1201, Columbia, Maryland). The integrity of the RNA was assessed by UV visualization of ethidium-bromide–stained intact 28S and 18S bands on a mini-agarose gel. Samples of RNA were stored at –80°C.

Reverse transcription (RT) was performed by adding 2 μg of total RNA to 2 μg of oligo dT primers and sterilized nuclease-free dd H_2O in a final volume of 15 μl. The samples were heated at 70°C for 5 min and then immediately cooled to 4°C for 2 min. Reverse transcription buffer containing dNTPs (final concentration of each was 10 mM), 25 units of RNase inhibitor, and 200 U of murine maloney leukemia virus reverse transcriptase (Promega, Madison, Wisconsin) were added to each sample. The sample, with a final volume of 40 μl, was incubated at 37°C for 1 h followed by a 5-min incubation at 95°C. For the polymerase chain reaction (PCR), 2 μl of RT reaction mixture were added to 50 μl of solution containing 5 μl of Taq buffer, 1 μl Taq DNA polymerase (Display Systems Biotech, Vista, California), 1 μl deoxyribonucleoside triphosphates (final concentration of each was 10 mM), 1 μl each of forward and reverse primers, and 41 μl sterile nuclease-free dd H_2O. The PCR reaction started with one cycle consisting of 94°C for 5 min, an annealing step of 65°C for IGF-I or 55°C for IGF-II and β-actin for 1 min, and extension at 72°C for 1 min. The first cycle was followed by 30 cycles (25 cycles for β-actin) consisting of 45-s intervals of 94°C, followed by 65°C for IGF-I or 55°C for IGF-II and β-actin, followed by 72°C. For IGF-R1, touchdown PCR was run. This program consisted of a 5-min, 94°C denaturation step, followed by 5 cycles in which the initial annealing temperature of 72°C was reduced by 1°C per cycle, then 30 cycles in which the annealing temperature was 68°C. Denaturation, extension, and annealing time were programmed as described above. To establish a linear range of amplification for each gene, several different cycle numbers of PCR (10, 15, 20, 25, and 30 cycles) were run. As a control, a PCR reaction without complementary DNA (c-DNA) was run, and no contamination was found in the reaction mixtures (data not shown).

PCR Primers. All PCR primers were synthesized by Gibco BRL (Grand Island, New York). Primers for IGF-I were designed on the basis of published sequences of chicken IGF-I.[2] The sequence of the forward primer was 5′ GCTGAGCTGGTGGATGCTCTTCAGTTC 3′, and the reverse primer was 5′ CTTCTGAGTCTTGGGCATGTCAGTGTG 3′. Forward and reverse primers predicted a PCR product of 215 base pairs (bp), which corresponds to bases (160–265) of the sequence. Primers for IGF-II were designed on the basis of published sequences of chicken IGF-II.[19] Primers for IGF-II amplified a PCR product of 356 bp, which corresponded to bases (1041–1397) of the sequence. The sequence of the forward primer for IGF-II was 5′ GAGCTTGTTGACACGCTTCAGTTTGTC 3′, and the reverse primer was 5′ ACGTTTGGCCTCTCTGAACTCTTTGAG 3′. Primers for IGF-R1 were designed on the basis of published sequence of mouse IGF-R1.[21] The sequence of the forward primer was 5′ GACATCCGCAACGACTATCAG 3′, and the reverse primer was 5′ GTAGTTATTGGACACCGCATC 3′. Primers for IGF-R1 amplified a PCR product of 395 bp that corresponded to bases (114–509) of the sequence. Forward and reverse primers for β-actin were predicted to amplify a 285 bp product as previously published,[24] as an internal standard to verify the level of amplification. The sequence of the forward primer was 5′ TCATGAAGTGTGACGTTGACATCCGT 3′,

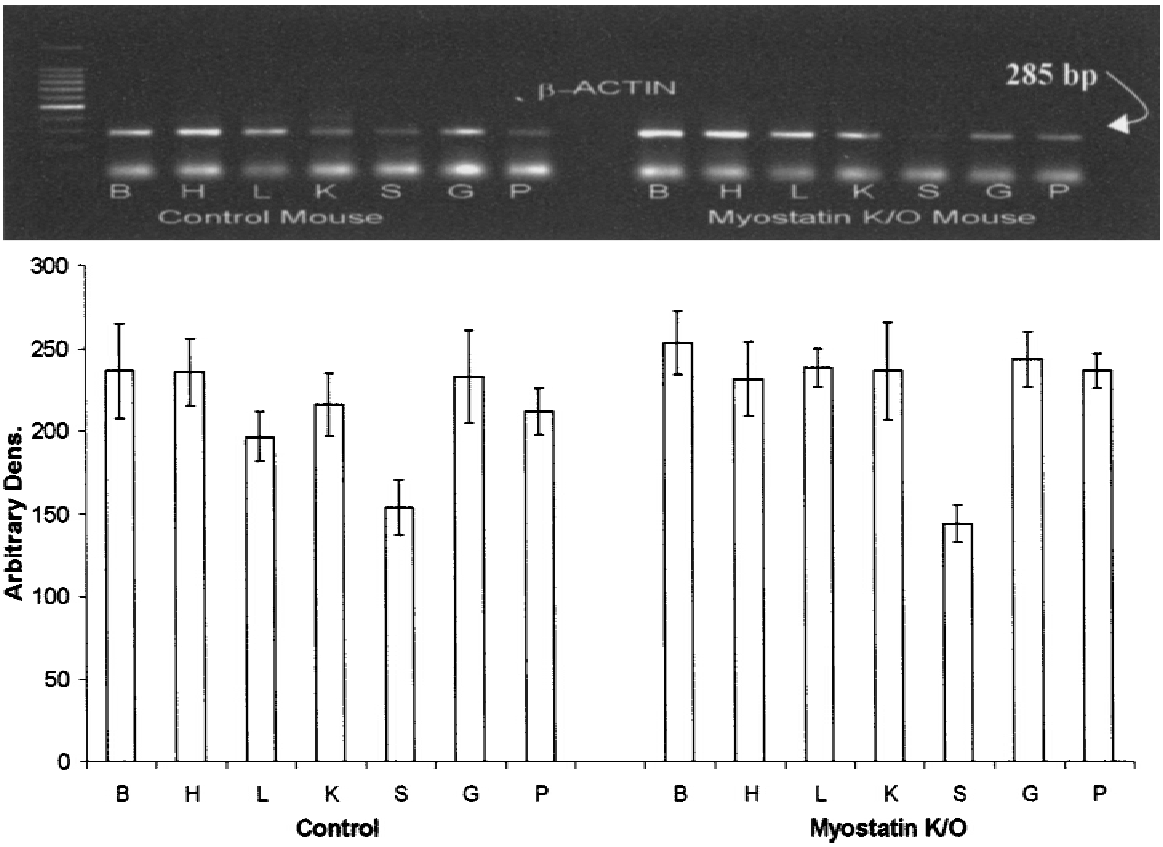

FIGURE 1. Steady-state levels of β-actin mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control ($n = 3$) and myostatin knockout mice ($n = 3$) tissues. The bands for β-actin were analyzed by densitometry, and the integration values (mean ± SD) were expressed in arbitrary units for each tissue.

and the reverse primer was 5′ CCTAGAAGCATTTGCGGTGCACGATG 3′.

The amplified PCR products for each gene were visualized on 1.5 % agarose gels stained with ethidium bromide. Products were analyzed by densitometry of stained gels, and data should therefore be considered to be semiquantitative. The identity of all PCR products was confirmed by sequence analysis.

Immunohistochemistry. Kidney and soleus muscles from adult myostatin knockout and control mice were immersion-fixed in Prefer fixative (Anatech, Battle Creek, Michigan). Sections were processed overnight and embedded in paraffin. Unstained sections were cut at 5 μm and incubated at 60°C for 20 min. Sections were then deparaffinized in three baths of xylene (6 min each), a bath of 100% alcohol (3 min), a bath of 95% alcohol (3 min), a bath of 80% alcohol (3 min), and a final bath of distilled water for 5 min. The sections were then placed in a coplin jar with 0.01M edetic acid (EDTA, pH 8; Fisher Scientific, Pittsburgh, Pennsylvania) for antigen retrieval. The coplin jar was placed in microwave on high for 1 min and 45 s, to bring temperature up to boiling point. A defrost cycle was set for 6 min, to keep the solution just below boiling. After 6 min, the coplin jar was removed from the microwave and allowed to sit for 20 min. The slides were then rinsed and incubated in distilled water for 5 min. Slides were incubated in 3% hydrogen peroxide for 10 min at room temperature. After rinsing with distilled water, slides were incubated in Tissue Conditioner (Biomeda, Foster City, California) for 10 min at 37°C. Slides were rinsed with distilled water and placed in tris-buffered saline (TBS; Dako, Carpinteria, California) for 5 min. After diluting with antibody diluent (1/100; Dako), IGF-II goat anti-human antibody (Sigma) was applied as a drop on the section for 1 h at room temperature. Slides were then rinsed with distilled water and incubated in TBS for 5 min at room temperature. Vectastain biotinylated secondary antibody (antigoat IgG) was applied as outlined by the manufacturer (Vectastain ABC kit, Vector Laboratories, Burlingame, California) for 30 min at room temperature. Slides were then rinsed with distilled water and incubated in TBS for 5 min

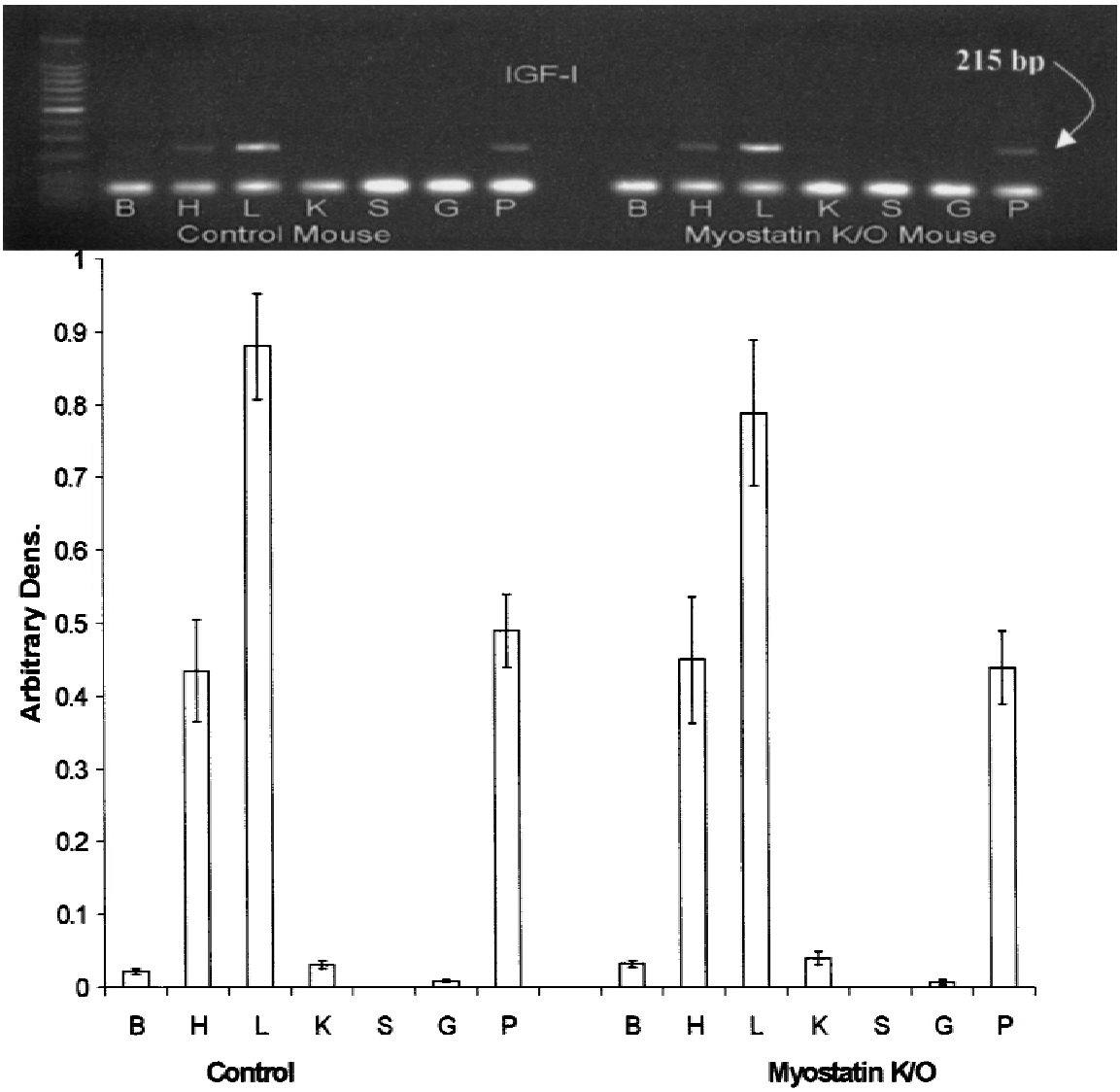

FIGURE 2. Steady-state levels of IGF-I mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control ($n = 3$) and myostatin knockout mice ($n = 3$) tissues. The bands for IGF-I were analyzed by densitometry, and the integration values (mean ± SD), after normalization to β-actin, were expressed in arbitrary units for each tissue.

at room temperature. Vectastain ABC (avidin-biotinylated enzyme complex) reagent was applied for 30 min at room temperature. Slides were rinsed with distilled water and incubated in for 5 min at room temperature. Aqueous hematoxylin (Biomeda) was applied as a drop to the section for 2 min to counterstain. Then slides were rinsed in tap water and coverslipped with Crystal Mount (Biomeda). After crystal mount was dried, the slides were post-mounted with Permount (Fisher Scientific) and a glass coverslip was placed over the Permount.

Whole-section digital images were captured using a Sprint Scan slide scanner (Polaroid, Cambridge, Massachusetts) and a PathScan Enabler (Myer Instruments, Houston, Texas). Digital photomicrographs were captured using a Quantix digital camera (Photometrics, Tucson, Arizona). For digital capture, images from control and knockout mice were captured in the same session using identical settings. Western blotting was used to validate the quality of the primary antibody. Rabbit Super Sensitive Control Serum (Biogenex, San Ramon, California) in place of primary antibody was used as a negative control on one section for each run.

Statistical Analysis. Probability of differences of least-square means was used to compare densitometric values (General Linear Model procedure of SAS, SAS Institute Inc., Cary, North Carolina).

RESULTS

β-Actin, IGF-I, IGF-II, and IGF-R1 mRNA Expression. β-Actin mRNA expression was not different for the same tissues between control and myostatin knockout mice (Fig. 1). IGF-I gene expression for brain, heart, liver, kidney, and pectoralis muscles were similar between control and knockout mice, and no IGF-I mRNA was detected in either control or knockout mice soleus muscles (Fig. 2). IGF-II mRNA levels were significantly higher in kidney and soleus muscles of myostatin knockout mice than in control mice ($P < .01$; Fig. 3). No IGF-II gene expression was observed in liver of control and myostatin knockout mice (Fig. 3). Levels of IGF–receptor-1 mRNA from control mice heart ($P < .05$) and kidney ($P < .01$) were significantly higher than from myostatin knockout mice, whereas levels were lower in pectoralis muscle of control mice than in knockout mice ($P < .01$; Fig. 4). IGF-R1 gene expression was similar in brain, liver, soleus, and gastrocnemius muscles of both control and myostatin knockout mice.

IGF-II Immunohistochemistry. An IGF-II immunohistochemistry negative control is shown in Figure 5. IGF-II was principally localized to small cells located adjacent to soleus muscle myofibers (Fig. 6). The strongly IGF-II–positive cells were more common in myostatin knockout mice and were seen in a few foci in control mice. The IGF-II–positive cells are believed to be satellite cells based on location and size. The IGF-II immunoreactivity in both control and myostatin knockout mice kidneys was localized to the epithelium of renal tubules and collecting ducts

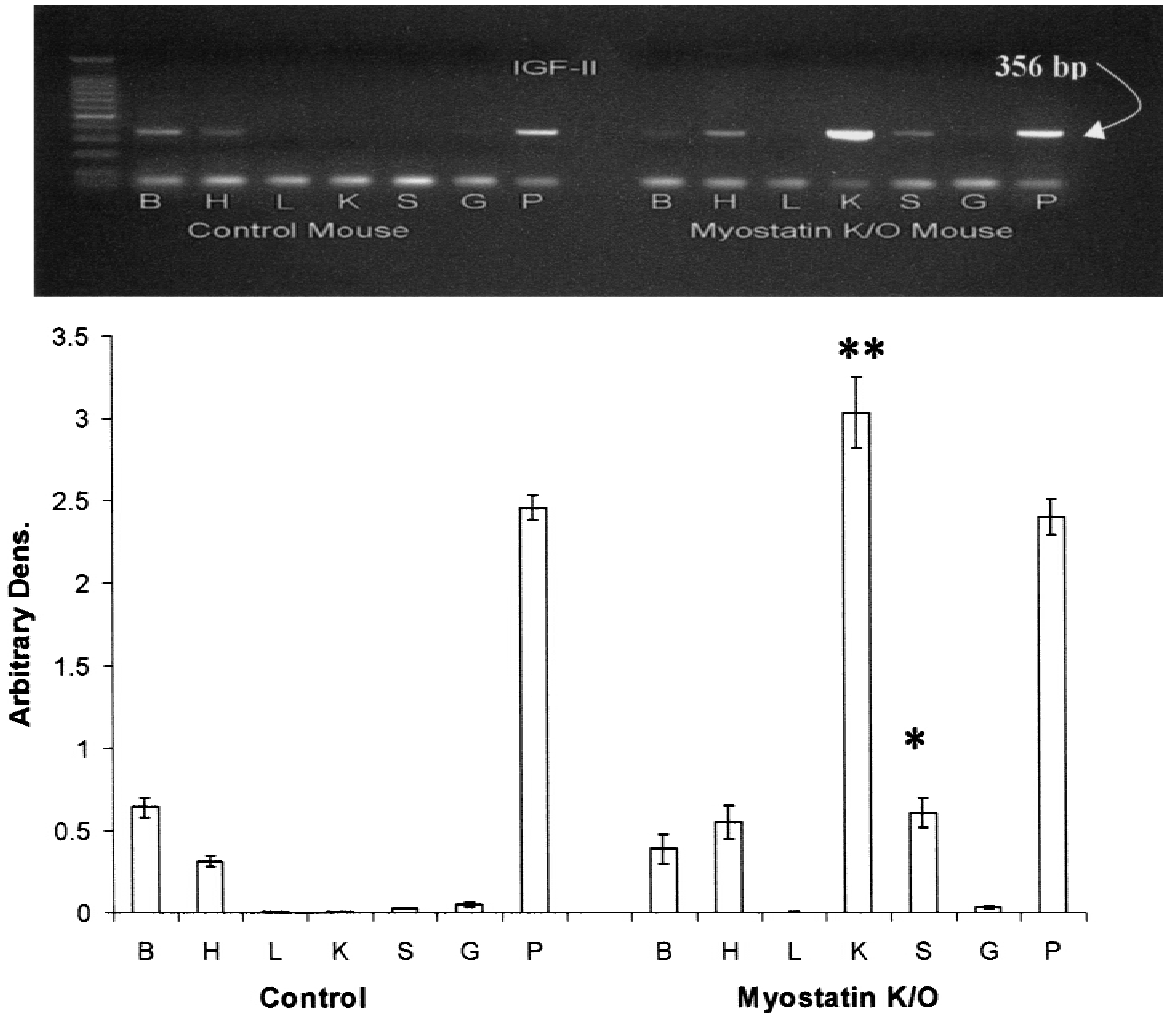

FIGURE 3. Steady-state levels of IGF-II mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control ($n = 3$) and myostatin knockout mice ($n = 3$) tissues. The bands for IGF-II were analyzed by densitometry, and the integration values (mean ± SD), after normalization to β-actin, were expressed in arbitrary units for each tissue. Means with asterisks were significantly different (*P < .05, **P < .01).

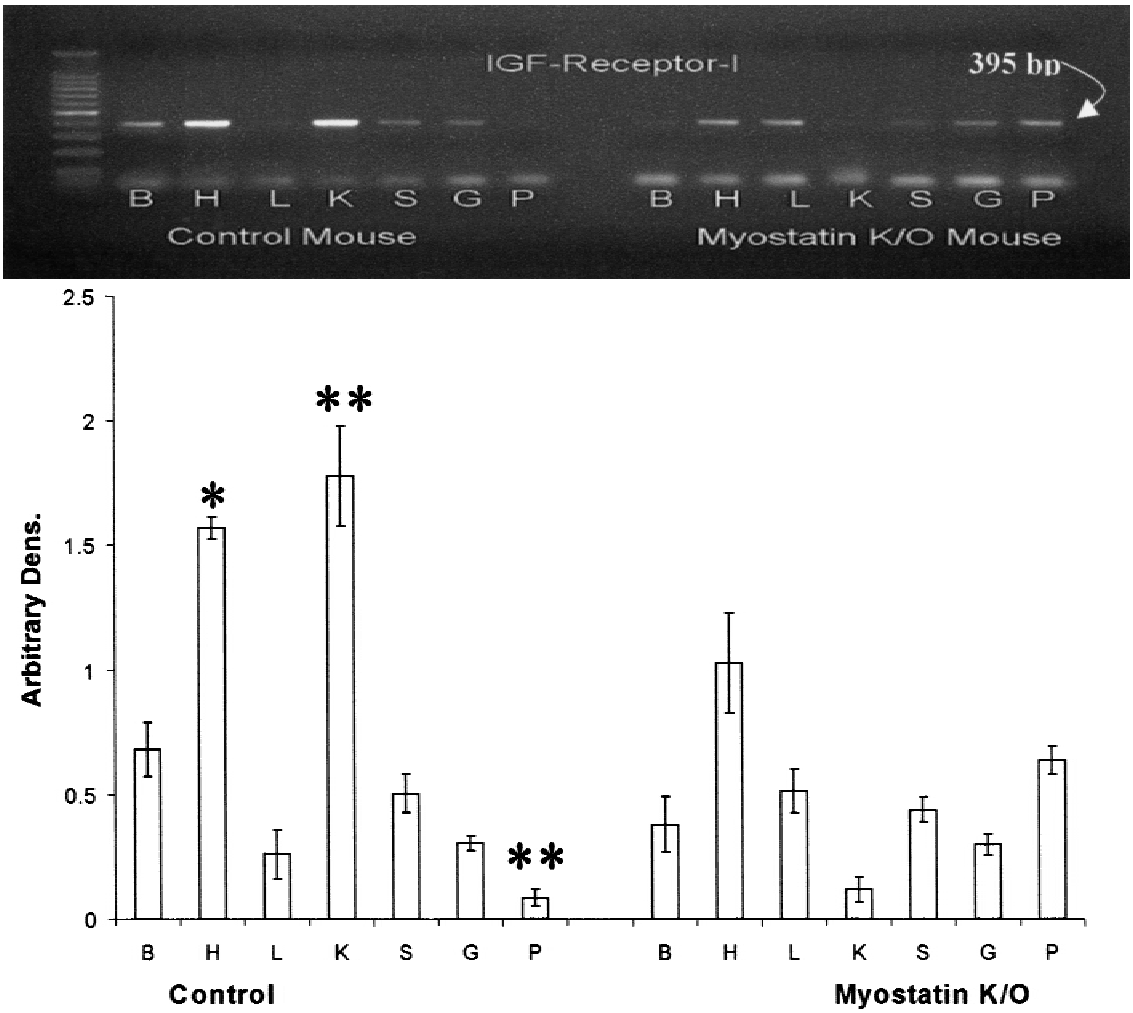

FIGURE 4. Steady-state levels of IGF-R1 mRNA in brain (B), heart (H), liver (L), kidney (K), soleus (S), gastrocnemius (G), and pectoralis (P) muscle of control ($n = 3$) and myostatin knockout mice ($n = 3$) tissues. The bands for IGF-R1 were analyzed by densitometry, and the integration values (mean ± SD), after normalization to β-actin, were expressed in arbitrary units for each tissue. Means with asterisks were significantly different (*P < .05, **P < .01).

(Fig. 7). No consistent differences in expression were noted between the two groups of mice.

DISCUSSION

Although both IGF-I and IGF-II are well-known positive regulators of muscle growth, their gene expressions in the current study demonstrated different patterns between muscle tissues of myostatin knockout and control mice. The IGF-I mRNA levels from soleus (predominantly red fibers), gastrocnemius (predominantly white fibers), and pectoralis (white and red fibers) muscles were not different between myostatin knockout and control mice (Fig. 2), but IGF-R1 mRNA levels from pectoralis muscles were significantly higher in myostatin knockout mice than

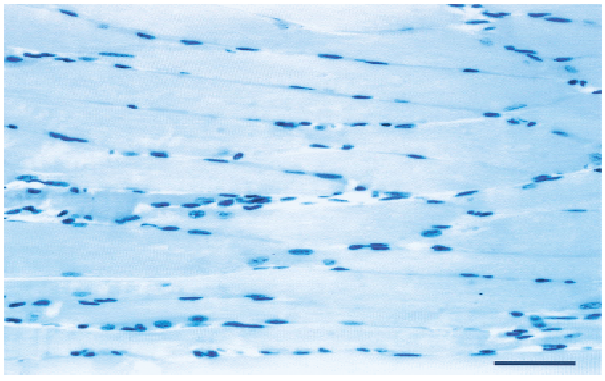

FIGURE 5. Rabbit Super Sensitive Control Serum (Biogenex) in place of primary antibody was used as negative control on one section for each run. Note the absence of red staining. (Bar = 50 μm.)

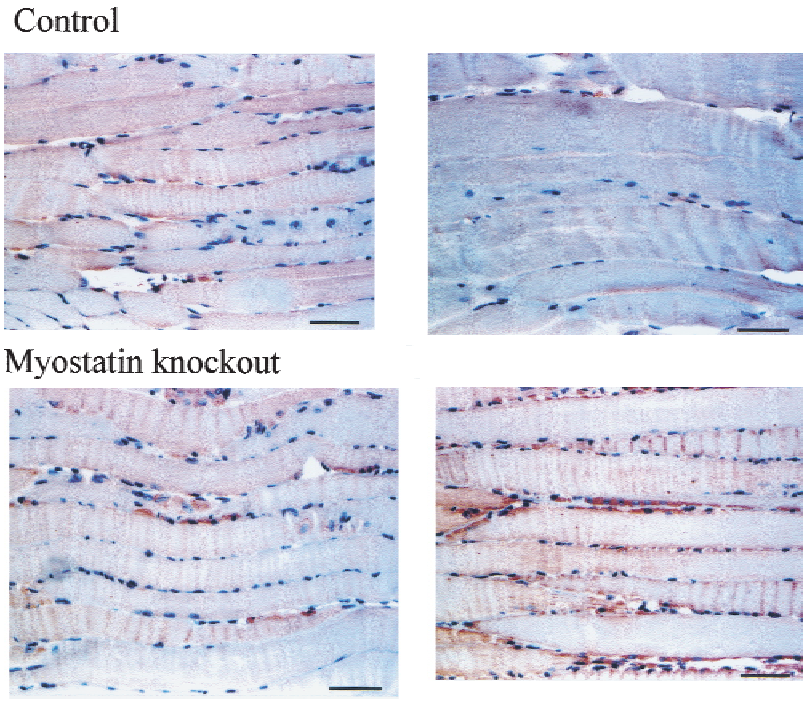

FIGURE 6. IGF-II immunohistochemistry in control and myostatin knockout mice soleus muscles. The IGF-II immunoreactivity (red staining) is principally localized in satellite cells. (Bar = 50 μm.)

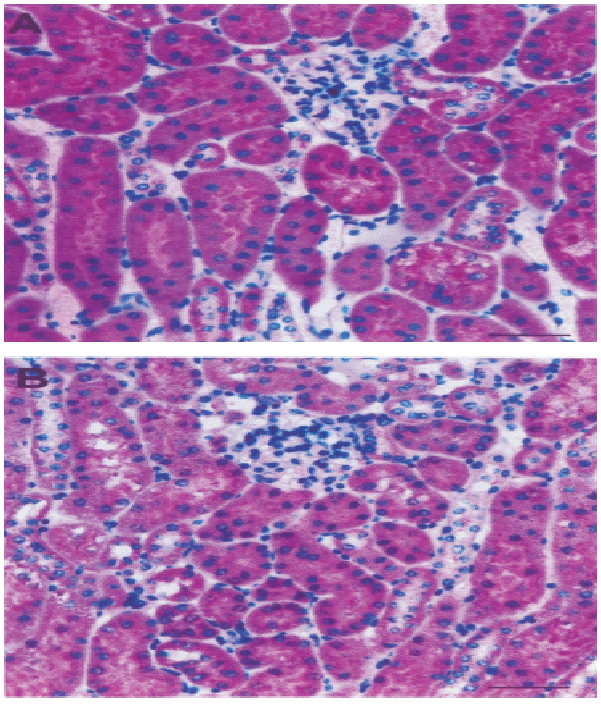

FIGURE 7. IGF-II immunohistochemistry in control **(A)** and myostatin knockout mice **(B)** kidneys. The IGF-II immunoreactivity (red staining) is localized to the epithelium of renal tubules and collecting ducts. (Bar = 50 μm.)

in control mice (Fig. 4). By contrast, IGF-II mRNA levels from soleus muscles were higher in myostatin knockout mice than in control mice, and no difference was observed between myostatin knockout and control mice gastrocnemius and pectoralis muscles (Fig. 3).

Based on the immunohistological findings of the present study, elevated IGF-II in the soleus of myostatin knockout mice is believed to originate from activated satellite cells. Varying expression patterns of IGF-I and IGF-II in myostatin knockout mice soleus and gastrocnemius muscle may be explained as follows. First, because myostatin mRNA and protein levels were higher in gastrocnemius than in soleus muscle of normal adult mice,[3] complete absence of myostatin in the knockout mice may have triggered distinct and novel muscle regulatory pathways in these two different fiber-type–dominated muscles, thereby causing the observed differences in the expression patterns of IGF-I and -II. Second, Semsarian et al.[20] demonstrated that myostatin mRNA level was not affected by IGF-I overexpression in C2C12 cell cultures. Along the same line, the loss of skeletal muscle mass induced during space flight of rats was associated with increased myostatin mRNA and protein levels in the skeletal muscle and decreased IGF-

II mRNA, whereas no change in IGF-I mRNA level was observed.[12] Based on these in vitro and in vivo findings, it is possible to expect that complete absence of myostatin should increase IGF-II mRNA levels without affecting IGF-I levels. Indeed, our finding of strong IGF-II mRNA and protein expression in soleus muscle of myostatin knockout mice and no difference in IGF-I mRNA levels of soleus, gastrocnemius, and pectoralis muscle between these mice partially supports the in vitro and in vivo findings. Even though no changes in either IGF-I or IGF-II levels from pectoralis muscles were observed between myostatin knockout and control mice, IGF-R1 expression from the same muscle was significantly increased in myostatin knockout mice (Fig. 4). Because both IGFs use IGF-R1 for their mitogenic and myogenic signal transduction pathways,[7] the significance of increased IGF-R1 expression without corresponding increases of ligands from pectoralis muscles of myostatin knockout mice remains to be further explored. Additionally, the local or systemic production of the IGF binding proteins may play a critical role in regulating the activity of these growth factors and therefore merits further investigation.

In the present study, strong IGF-II gene expression was observed in myostatin knockout mice kidney, whereas IGF-R1 expression was significantly lowered when compared with control mice (Figs. 3 and 4, respectively). However, no consistent differences in IGF-II immunoreactivity were observed between the two groups of mice. This was not totally unexpected, as the enzymatic immunohistochemical techniques use amplification cascades designed to maximize chromagen precipitation resulting from low levels of antibody binding. Also, the similar distribution of IGF-II in control and knockout mice (Fig. 7) suggests that changes in IGF-II expression result from increased expression in cells that normally express IGF-II rather than changes in the type of cells expressing IGF-II. Body weight of transgenic mice overexpressing IGF-II was not different from control mice but had increased kidney weight without any change in other organs.[23] Although myostatin knockout kidney weight was not measured in the current and previous studies,[15] increased IGF-II levels may have caused the decline in IGF-R1 mRNA expression to maintain proper size of myostatin knockout mice kidney. Several studies, however, have suggested that metabolic actions of IGF-II, unlike its mitogenic actions, are not mediated through IGF-R1 but through insulin receptors, to which IGF-II can bind with low affinity.[6,9] Additionally, IGF-II, but neither insulin nor IGF-I, stimulated Na^+–H^+ exchange across the brush-border membrane of proximal tubular cells.[17] Therefore, it is possible that increased IGF-II levels from myostatin knockout mice kidneys may be due to the disturbed metabolic balance in response to excess muscle growth of those mice. Further studies regarding the measurements of both metabolic parameters, such as blood glucose and fatty acids, and hormones such as insulin and growth hormone should be conducted to clarify this speculation.

Because tissue samples were obtained only at one age, we do not know whether the changes in IGF-I, IGF-II, and IGF-R1 gene and protein expression were the same throughout the neonatal and adult life of the mice. Reciprocal changes in the expression of myostatin and IGF-II and IGF-R1 may be important not only for skeletal muscle growth but also for other organ development in mammals as well as in leading to the changes of these tissues associated with disease.

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