Estrogen Effects on Skeletal Muscle Insulin-Like Growth Factor–1 and Myostatin in Ovariectomized Rats

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Previous work showed that estrogen replacement attenuates muscle growth in immature rats. The present study examined muscle insulin-like growth factor–1 (IGF-1) and myostatin expression to determine whether these growth regulators might be involved in mediating estrogen's effects on muscle growth. IGF-1 and myostatin message and protein expression in selected skeletal muscles from 7-week-old sham-ovariectomized (SHAM) and ovariectomized rats that received continuous estrogen (OVX/E2) or solvent vehicle (OVX/CO) from an implant for 1 week or 5 weeks was measured. In the 1-week study, ovariectomy increased IGF-1 mRNA expression in fast extensor digitorum longus and gastrocnemius muscles; the increase was reversed by estrogen replacement. A similar trend was observed in the slow soleus muscle, although the change was not statistically significant. In contrast to mRNA, muscle IGF-1 protein expression was not different between SHAM and OVX/CO animals in the 1-week study. One week of estrogen replacement significantly decreased IGF-1 protein level in all muscles examined. Myostatin mRNA expression was not different among the 1-week treatment groups. One week of estrogen replacement significantly increased myostatin protein in the slow soleus muscle but not the fast extensor digitorum longus and gastrocnemius muscles. There was no treatment effect on IGF-1 and myostatin expression in the 5-week study; this finding suggested a transient estrogen effect or upregulation of a compensatory mechanism to counteract the estrogen effect observed at the earlier time point. This investigation is the first to explore ovariectomy and estrogen effects on skeletal muscle IGF-1 and myostatin expression. Results suggest that reduced levels of muscle IGF-1 protein may mediate estrogen's effect on growth in immature, ovariectomized rats. Increased levels of muscle myostatin protein may also have a role in mediating estrogen's effects on growth in slow but not fast skeletal muscle. Exp Biol Med 232:1314–1325, 2007

Key words: estrogen; myostatin; IGF-1; ovariectomy; skeletal muscle; growth

Introduction

Estrogen not only is important for reproductive tissue functions but also influences several peripheral tissues including bone and cardiovascular and skeletal muscles (1). This notion is further supported by the discovery of estrogen receptors (ERs) in the peripheral tissues in several species (2–4). Although the protective nature of estrogen on bone density in young and aged animals is well established, the effect of estrogen on skeletal muscles is not yet clear. Using mature rodent models, several laboratories have shown beneficial effects of estrogen against injury caused by oxidative stress, modified muscle usage, and exercise (5, 6). Other researchers, in contrast, found no beneficial effects of estrogen replacement on muscle mass and strength in postmenopausal women (7, 8).

Although there is a considerably large body of literature dealing with the effects of hormone replacement therapy (HRT) on skeletal muscle function in elderly women, there is limited research addressing estrogen's effect on skeletal muscle in girls and young women. Our laboratory previously reported a decrease in hindlimb muscle size in young ovariectomized (OVX) rats following estrogen replacement (9, 10). The molecular mechanism governing estrogen effects on skeletal muscle size has not been established. We propose that estrogen may change muscle size by altering levels of hypertrophy/atrophy pathway

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modulators, more specifically, insulin-like growth factor–1 (IGF-1) and myostatin.

IGF-1 was first discovered as a downstream mediator of the growth-promoting actions of growth hormone (GH). IGF-1 is essential for embryonic and postnatal development of several tissues, including skeletal muscles (11). Previous work has shown that serum IGF-1 concentration increases following ovariectomy (12), but circulating IGF-1 may not have a primary role in regulating skeletal muscle growth. Although the liver is the major site for circulating IGF-1 synthesis, specific hepatic IGF-1 inactivation in mice does not affect postnatal body growth, and this lack of effect suggests that locally synthesized IGF-1 has an autocrine or paracrine function within peripheral tissues (13). In the skeletal muscle, IGF-1 stimulates muscle growth by activating the calcium-dependent calcineurin signaling pathway (14, 15).

Myostatin, a member of the TGF-β superfamily, is a negative regulator of muscle size and muscle growth (16). Like other TGF-β family members, myostatin is secreted and proteolytically processed, and this proteolysis is followed by the formation of a disulfate-linked dimer of the C-terminal region (25–30 kDa) (17). Myostatin is almost exclusively expressed in skeletal muscles, although a lower level of myostatin has been detected in the heart and adipose tissue (18). Myostatin-null mice have a 2- to 3-fold increase in muscle mass because of muscle-fiber hypertrophy (increase in size) and hyperplasia (increase in cell number) (16). Myostatin overexpression causes muscle wasting in mice (19). Muscle myostatin expression and protein levels respond to various atrophic stimuli, including denervation (20), hindlimb unloading (21), and glucocorticoids (22). There also appears to be a gender-related effect on muscle myostatin protein levels. The amount of processed myostatin protein isolated from the limb muscles is significantly higher in female mice than in male mice (23). This is consistent with the observation that females are generally less muscular than males.

Given that IGF-1 and myostatin are important growth factors in regulating muscle size and our previous observations that estrogen replacement alters muscle size, we hypothesized that estrogen may affect muscle IGF-1 and myostatin expression. This hypothesis was tested by comparing muscle IGF-1 and myostatin mRNA and protein expression in animals which were sham-ovariectomized (SHAM), ovariectomized with a corn oil implant (OVX/CO), and ovariectomized with an implant of 17β-estradiol dissolved in corn oil (OVX/E2). Seven-week-old female Sprague-Dawley rats were assigned to each treatment group and sacrificed 1 or 5 weeks after surgery to assess short-term and long-term estrogen effects. Three hindlimb skeletal muscles—the extensor digitorum longus (EDL), the soleus (SOL), and the gastrocnemius (GAS) muscles—were selected for our investigation. The SOL muscle is comprised of mostly slow-twitch fibers (type I), whereas the EDL and GAS muscles have predominantly more fast-twitch fibers (24). Our selection enabled us to explore muscle-specific effects of estrogen on IGF-1 and myostatin expression.

Materials and Methods

Animal Care and Study Design. Female Sprague-Dawley rats (6 weeks old, 125–150 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and accommodated to their housing environment for 1 week. Seven-week-old animals were divided into three groups: the SHAM group, the sham-ovariectomized control; OVX/CO, ovariectomized rats with a vehicle-only implant; OVX/E2, ovariectomized rats with an implant that contained 17β-estradiol in corn oil (4 mg/ml) as discussed previously (9). To investigate the short-term estrogen effects, we monitored the food intake and body weight of six animals from each group throughout a 1-week period. Animals were sacrificed by exsanguination following anesthesia at the end of the 1-week study, and the leg skeletal muscles and the EDL, GAS, and SOL muscles were spotted dry, weighed, immediately frozen in liquid nitrogen, and transferred to a –80°C freezer until further analysis.

Another batch of animals (eight per group) was treated for 5 weeks to study possible long-term effects of estrogen. It is well known that long-term ovarian hormone depletion following ovariectomy causes hyperphagia (25). As such, differential food intake among groups could be a confounding factor when experimental results are interpreted. Therefore, the food consumption among groups was controlled by feeding the SHAM and OVX/CO animals the same amount of food consumed by the OVX/E2 animals. The body weight and food intake were recorded throughout the study. The EDL, GAS, and SOL muscles along with the heart, liver, and diaphragm were processed and stored at the end of the 5-week study as described above.

The uterine weight from each animal was also recorded at the end of both studies because the uterine weight has been shown to be a robust and more reliable biomarker for estrogen exposure than plasma estrogen concentration. All the study protocols were approved by the Institutional Animal Care and Use Committee at the University at Buffalo and were in compliance with the National Institutes of Health guidelines.

Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR). Frozen skeletal muscle (approximately 50 mg per sample) was powdered in liquid nitrogen by using a pestle and mortar and then transferred to a 1.5-ml microtube. The total RNA from each muscle sample was extracted by using a SV Total RNA Isolation Kit (Promega, Madison, WI). All of the RNA samples were subjected to DNase I treatment as described in the manufacturer's manual. The RNA concentration was determined by absorbance at 260 nm, and the 260-nm to 280-nm ratio of each sample was greater than 1.8 to ensure RNA integrity. The RNA samples were stored at a –80°C freezer until all

the RNA samples were collected for the Q-RT-PCR analysis.

The target mRNA content was quantified by a two-step method that consists of a reverse transcription reaction and quantitative real-time PCR. Each total RNA sample (1.5 μg) was converted to cDNA by adding 1 μl StrataScript reverse transcriptase (Stratagene, La Jolla, CA), 1 μl oligo-$(dT)_{12–18}$ (1 μg/μl), 2 μl dNTPs (5 m*M* each) and double-distilled water to make up a 50-μl reaction. The reaction mixtures were incubated at 37°C for 50 mins and then at 70°C for 15 mins to inactivate the transcriptase enzyme. The cDNA samples were stored at –20°C until further analysis.

PrimerExpress software (Perkin Elmer, Boston, MA) was used to design primer sets for rat IGF-1 and myostatin mRNA detection. All the primer sequences and reaction conditions used are summarized in Table 1. The primer sequences were blasted against the rat genome to ensure target sequence specificity. For Q-RT-PCR, an absolute quantitation of the target mRNA quantity using a set of target cDNA standards was performed. The target sequence was first PCR-amplified and then cloned by using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). The cloned amplicon was then sequenced and confirmed to be the target sequence. A serial dilution of the cloned amplicon served as standards to allow for the estimation of copy number of target mRNA for each sample by using standard curves generated from each run. The Q-RT-PCR for each sample was performed in triplicate with standards and controls.

For each run of Q-RT-PCR, the standards spanned the complete range of observed target copy numbers. The Ct threshold and the standard curve was automatically selected and generated by the Mx3005P v3.00 program (Stratagene). The amplification of the target was detected by using a fluorescence dye, SYBR Green I. Included in each run was a reference dye, ROX, for normalization of background fluorescence. After optimization, PCR was performed in a 50-μl reaction mix. Five microliters of cDNA from each sample was added to a total volume of 45 μl that consisted of 5 μl of 10× buffer (containing 15 m*M* $MgCl_2$), 0.4 μl dNTP (25 m*M* each), a designated volume of each primer (10 μ*M*), 0.5 μl ROX (1:500 dilution), 0.5 μl SYBR green (1:750 dilution), 0.3 μl TAQ DNA polymerase (5 units/μl), and DNase-free water. The thermal profile was the following: 95°C for 4 mins, then 40 cycles of 94°C for 30 secs, a designated annealing temperature for each target gene for 33 secs, 72°C for 1 min, a subsequent dissociation curve detection at 95°C for 1 min, and 41 cycles starting at 55°C at an increment of 1°C for 33 secs per cycle.

Q-RT-PCR Data Normalization. The expression levels of three internal control genes (GAPDH, cyclophilin, and β-actin) were quantified for the purpose of normalizing the data. An internal control gene-stability measure (*M*) was calculated to identify the most stable internal control gene for data normalization (26). In short, for each control gene the pair-wise variation with all other control genes, expressed as the standard deviation of the logarithmically

Table 1. Real-Time PCR Primers and Optimized Reaction Conditions[a]

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature	$MgCl_2$ concentration	Primers concentration
IGF-1	GCT TGC TCA CCT TTA CCA GC	AGT GTA CTT CCT TCT GAG TCT	60°C	1.5 m*M*	0.3 μ*M*
Myostatin	ACT CAT CTT GTG CAC CAA GCA A	GGT CTA CTA CCA TGG CTG GAA TTT	66°C	1.5 m*M*	0.36 μ*M*
β-actin	ACC AAC TGG GAC GAT ATG GAG AAG	TAC GAC CAG AGG CAT ACA GGG ACA	60°C	1.5 m*M*	0.1 μ*M*
GAPDH	AAC GAC CCC TTC ATT GAC	TCC ACG ACA TAC TCA GCA C	57°C	4.0 m*M*	0.1 μM
Cyclophilin	CCA AAC ACA AAT GGT TCC CAG TT	TGC CTT CTT TCA CCT TCC CAA A	60°C	1.5 m*M*	0.3 μ*M*

[a] The primer sets for the Q-RT-PCR were selected by using PrimerExpress. The primer sequences were blasted against the rat genome to ensure target sequence specificity. The thermal profile was the following: 95°C for 4 mins, then 40 cycles of 94°C for 30 secs, the designated annealing temperature for each target gene for 33 secs, and 72°C for 1 min, subsequent dissociation curve detection at 95°C for 1 min, and 41 cycles starting at 55°C at an increment of 1°C for 33 secs per cycle.

Table 2. Internal Control Gene-Stability Measure (*M*) Values[a]

	SOL		EDL		GAS	
M	1 week	5 weeks	1 week	5 weeks	1 week	5 weeks
GAPDH	0.009	0.067	0.013	0.097	0.011	0.138
Cyclophilin	0.386	0.620	0.853	0.866	0.830	1.330
β-actin	0.296	0.943	0.793	1.342	0.893	1.089

[a] The expression levels of three internal control genes were quantified for purpose of normalizing the data. An internal control gene-stability measure (*M*), proposed by Vandesompele *et al.*, was calculated to identify the most stable internal control gene for data normalization (26). Genes with the lowest *M* values have the most stable expression. GAPDH was found to have the lowest *M* value across muscle types and experiment conditions and was therefore used to normalize the Q-RT-PCR data.

transformed expression ratios, was determined. The variation of a particular gene with all other control genes was averaged to obtain an *M* value. Genes with the lowest *M* values have the most stable expression. The *M* values for GAPDH, cyclophilin, and β-actin were calculated for each muscle for the 1-week and 5-week studies. GAPDH had the lowest value of *M* (i.e., the most suitable internal control gene) and was therefore used to normalize the results (Table 2).

IGF-1 ELISA. Skeletal muscle (approximately 50 mg) was minced in a homogenizing solution that consisted of T-PER tissue protein extraction reagent (Pierce, Rockford, IL) and 10 μl of the protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO) on ice. The solution was then subjected to three 15-sec pulses at a setting of 4 on ice by using a polytron PT 10–35 homogenizer; the supernatant was collected after a 10-minute centrifugation (10,000 *g*) at 4°C. The total protein concentration for each muscle sample was determined by using a BCA protein assay kit (Pierce). Each protein sample was then diluted accordingly to a final concentration of 2 μg/μl and stored at –20°C. The muscle IGF-1 protein was detected by using the Quantikine Mouse IGF-1 Immunoassay (R&D Systems, Minneapolis, MN). This assay also recognizes rat IGF-1 as indicated by the manufacturer. The results were expressed as the mouse IGF-1 equivalent because only the recombinant mouse IGF-1 protein was provided with the kit. Each muscle protein sample had a 1:4 dilution into Calibrator Diluent ED5–38, and duplicate determinations were made as recommended by the manufacturer. The total amount of protein in each well was 2.5 μg, and the IGF-1 protein for each muscle sample was expressed as mouse IGF-1 protein equivalent (in picograms) per microgram of muscle protein.

Myostatin Western Blot Analysis. Protein extracts (15 μg) were resolved on a precast 14% Tris-glycine gel (Invitrogen), transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NY), and immunodetected by adding the SuperSignal West Dura Extended Duration Substrate (Pierce). The following primary antibodies were used: mouse GAPDH monoclonal antibody, 1:2000 dilution (Imgenex, San Diego, CA) and rabbit GDF8/myostatin polyclonal antibody, 1:450 (Novus Biologicals, Littleton, CO). Secondary antibodies for GAPDH and myostatin were peroxidase-conjugated anti–mouse IgG, 1:2000 (Pierce) and anti–rabbit IgG, 1:16,000 (Sigma). Recombinant human myostatin (Cell Sciences, Canton, MA) was incorporated in each Western blot analysis and served as a positive control. The digital images were captured on a Kodak Image Station and analyzed by using Kodak 1D image analysis software (Eastman Kodak Co., Rochester, NY). The myostatin protein levels were normalized against the loading control, GAPDH, and expressed as a ratio of myostatin to GAPDH.

Statistical Analysis. All values were expressed as means ± SE. The total food intake and uterine, body, organ, and muscle weights for the 1-week and 5-week studies were analyzed separately by using one-way ANOVA ($P < 0.05$). For the mRNA and protein data, two-way ANOVA was used to detect treatment and study duration effects and the statistical significance was set at $P < 0.05$. Whenever ANOVA revealed a significant difference, the Scheffe *post hoc* test was used to detect pairwise differences.

Results

Estrogen Exposure and Physiologic Measurements. Because of the cyclical endogenous variability in estrogen concentrations, uterine weight has been used as a surrogate for estrogen exposure. Ovariectomy-induced uterine atrophy in the OVX/CO animals was apparent in the 1-week study. The uterine weight was reduced by 70% as compared with that of the SHAM animals, indicating a successful reduction in endogenous estrogens (Fig. 1A). The uterine weights for the SHAM and OVX/E2 animals were similar, indicating effective estrogen delivery in the OVX/E2 group (Fig. 1A). A similar reduction (50%) in the OVX/CO uterine weight and restoration by estrogen replacement were also observed in the 5-week study (Fig. 1B).

There was no significant difference in food intake among the experimental groups in both studies (Tables 3 and 4). Despite similar food intake, the mean OVX/E2 body weight was significantly lower than those of the SHAM and OVX/CO animals in the 1-week and 5-week studies. The SHAM and OVX/CO animals gained similar amounts of weight (%), whereas the OVX/E2 animals lost weight immediately after the surgery and did not reach the weights of the other two groups in the 1-week study (Fig. 2A). In the 5-week study, a similar trend was observed up to 18 days

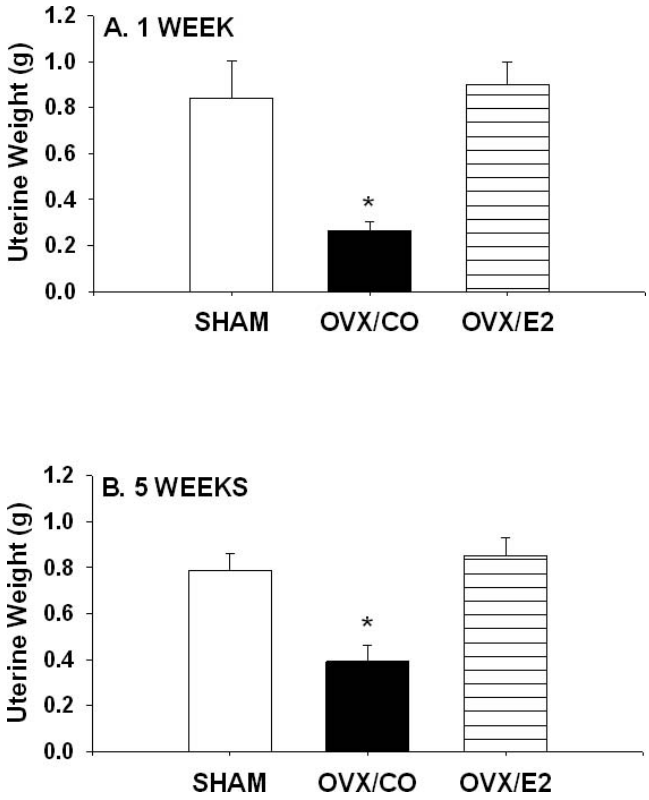


Figure 1. Uterine weight as a biological marker of estrogen exposure. (A) Uterine weights of the (A) 1-week study SHAM ($n = 4$), OVX/CO ($n = 5$), and OVX/E2 ($n = 6$) animals. (B) Uterine weights of the 5-week study SHAM ($n = 8$), OVX/CO ($n = 7$), and OVX/E2 ($n = 8$) animals. Results are expressed as mean ± SE. (*$P < 0.05$; one-way ANOVA with the Scheffe *post hoc* test indicated that the uterine weight of OVX/CO animals was significantly different than those of the SHAM and OVX/E2 animals.

after surgery, after which the OVX/CO animals gained more weight than did the SHAM animals (Fig. 2B).

Skeletal Muscle and Organ Weights. In the 1-week study, the GAS muscle weights were significantly lower in the OVX/E2 group than in the SHAM and OVX/CO treatment groups. There was no difference in the EDL and SOL muscle weights among experimental groups (Table 3). In the 5-week study, the SOL and the EDL muscle weights in the OVX/CO group were significantly higher than those in the SHAM and OVX/E2 groups. The GAS muscle weight in the OVX/E2 group was lower than that in the other two treatment groups (Table 4). The heart, liver, and diaphragm were also collected in the 5-week study (four per group). Whereas weight-bearing muscles were affected by the treatment, there was no weight difference in the diaphragm, liver, and heart among treatment groups. The changes in weight-bearing muscle weights were proportional to the change in body weight as the muscle-to-body weight ratio was similar among groups.

IGF-1 mRNA and Protein in the EDL Muscles. Two-way ANOVA detected significant treatment and study duration effects ($P = 0.0028$ and $P = 0.047$, respectively) on IGF-1 mRNA expression (Fig. 3A). There was a slight increase in the IGF-1 message in the 5-week study versus the 1-week study ($P = 0.02$). At the end of the 1-week study, the IGF-1 mRNA level in OVX/CO animals was 30% higher than that in the SHAM animals ($P = 0.03$). The increase in IGF-1 mRNA expression was reversed with estrogen treatment (Fig. 3A). A similar trend was observed in the 5-week study as the EDL muscles of OVX/CO animals had 36% more IGF-1 mRNA than did those of the SHAM animals. Estrogen treatment for 5 weeks, in contrast, did not significantly change the IGF-1 message level (Fig. 3A).

Interestingly, despite a slight increase in the message level during the 5-week study but not in the 1-week study, the IGF-1 protein level was lower in the 5-week study animals (two-way ANOVA showed $P < 0.0001$). At the end of the 1-week study, the IGF-1 protein level in EDL muscles was similar between the SHAM and OVX/CO animals (Fig. 3B). With estrogen replacement, however, the IGF-1 protein level was 40% less than that in the SHAM and OVX/CO ($P < 0.05$). No treatment effect was observed in the 5-week study (Fig. 3B).

IGF-1 mRNA and Protein in the GAS Muscles. Two-way ANOVA detected significant treatment and study duration effects ($P = 0.02$ and $P = 0.05$, respectively) on the IGF-1 mRNA level (Fig. 4A). There was a slight

Table 3. One-Week Study: Total Food Intake and End Body and Muscle Weights[a]

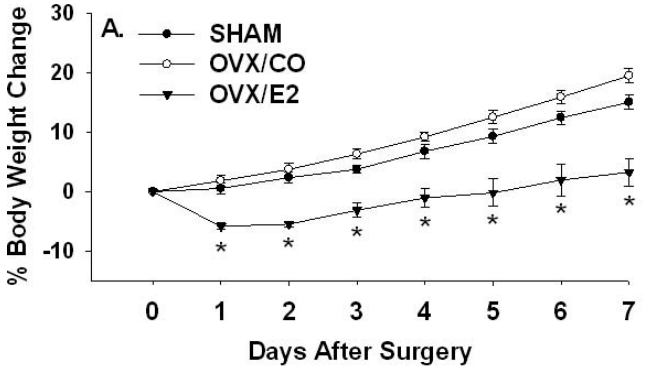
	SHAM	OVX/CO	OVX/E2
Total food intake (g)	101 ± 3	105 ± 1	91 ± 8
Final body weight (g)	175 ± 3	182 ± 1	158 ± 4*
Muscle weight			
EDL weight (mg)	82 ± 3	84 ± 3	74 ± 4
EDL-to-body weight ratio	0.47 ± 0.01	0.46 ± 0.01	0.47 ± 0.03
GAS weight (g)	1.09 ± 0.05	1.10 ± 0.03	0.92 ± 0.03*
GAS-to-body weight ratio	6.21 ± 0.20	6.05 ± 0.15	5.79 ± 0.17
SOL weight (mg)	87 ± 2	81 ± 3	76 ± 4
SOL-to-body weight ratio	0.50 ± 0.01	0.44 ± 0.02	0.48 ± 0.03

[a] Values represent means ± SE.
* $P < 0.05$; one-way ANOVA and the Scheffe test showed the indicated values were significantly different than those of the SHAM and OVX/CO groups.

Table 4. Five-Week Study: Total Food Intake and End Body, Organ, and Muscle Weights[a]

	SHAM	OVX/CO	OVX/E2
Total food intake (g)	506 ± 13	493 ± 7	520 ± 15
Final body weight (g)	208 ± 6	227 ± 6	192 ± 6*
Organ weight			
Heart weight (mg)	818 ± 39	918 ± 45	787 ± 16
Heart-to-body weight ratio	4.01 ± 0.14	3.81 ± 0.05	3.82 ± 0.08
Liver weight (g)	5.92 ± 0.17	6.32 ± 0.31	6.89 ± 0.33
Liver-to-body weight ratio	29.54 ± 2.50	30.30 ± 1.34	28.92 ± 1.95
Muscle weight			
Diaphragm weight (mg)	680 ± 39	753 ± 41	630 ± 41
Diaphragm-to-body weight ratio	3.18 ± 0.20	3.02 ± 0.21	3.34 ± 0.15
EDL weight (mg)	96 ± 3	106 ± 4**	94 ± 3
EDL-to-body weight ratio	0.46 ± 0.01	0.47 ± 0.01	0.49 ± 0.01
GAS weight (g)	1.36 ± 0.03	1.36 ± 0.04	1.22 ± 0.02*
GAS-to-body weight ratio	6.73 ± 0.25	6.28 ± 0.13	6.77 ± 0.25
SOL weight (mg)	89 ± 3	100 ± 3**	86 ± 2
SOL-to-body weight ratio	0.43 ± 0.02	0.44 ± 0.01	0.45 ± 0.01

[a] Values represent means ± SE.
* $P < 0.05$; one-way ANOVA and the Scheffe test showed the values to be significantly different than those of the SHAM and OVX/CO animals.
** $P < 0.05$; one-way ANOVA and the Scheffe test showed the values to be significantly different than those of the SHAM and OVX/E2 animals.



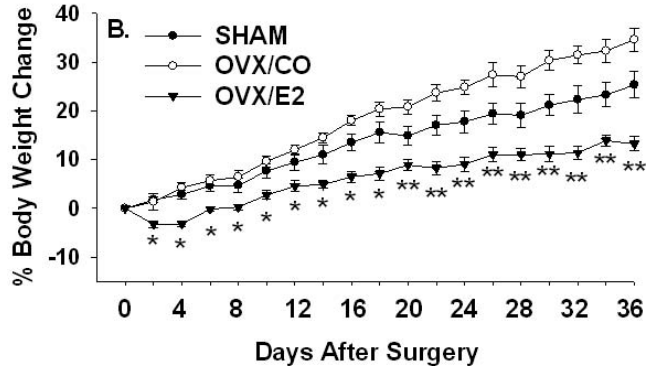
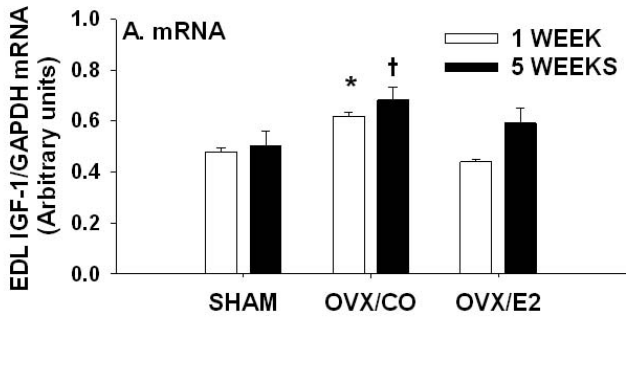


Figure 2. Body weight record. (A) The body weights of the 1-week study SHAM (solid circle, $n = 4$), OVX/CO (open circle, $n = 5$), and OVX/E2 (triangle, $n = 6$) animals. (B) The body weights of the 5-week study SHAM (solid circle, $n = 8$), OVX/CO (open circle, $n = 7$), and OVX/E2 (triangle, $n = 8$) animals. Results are expressed as mean ± SE. (*$P < 0.05$; one-way ANOVA with the Scheffe *post hoc* test found the body weights of the OVX/E2 animals to significantly differ from those of the SHAM and OVX/CO animals. Double asterisks (**) indicate that values for three experimental groups were significantly different.



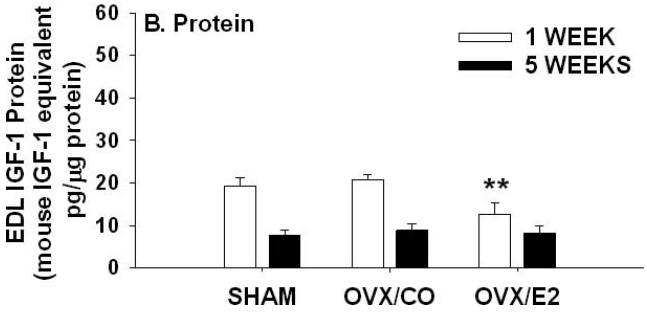


Figure 3. IGF-1 mRNA and protein expression in EDL muscles. Open bars and black bars represent mean ± SE data from the 1-week and 5-week studies, respectively. (A) The overall IGF-1 mRNA was lower in the 1-week study than in the 5-week study ($P < 0.05$). The EDL IGF-1 mRNA in the OVX/CO group was significantly higher than that in the other two groups in the 1-week study (*$P = 0.03$, Scheffe *post hoc* analysis). The EDL IGF-1 mRNA in the OVX/CO group was significantly higher than that in the SHAM group in the 5-week study (†$P < 0.05$). (B) The EDL IGF-1 protein was significantly lower in the 5-week study. The IGF-1 protein in the OVX/E2 group was significantly lower than that in the other two groups in the 1-week study (**$P < 0.05$). No difference in EDL IGF-1 protein was found among groups in the 5-week study.

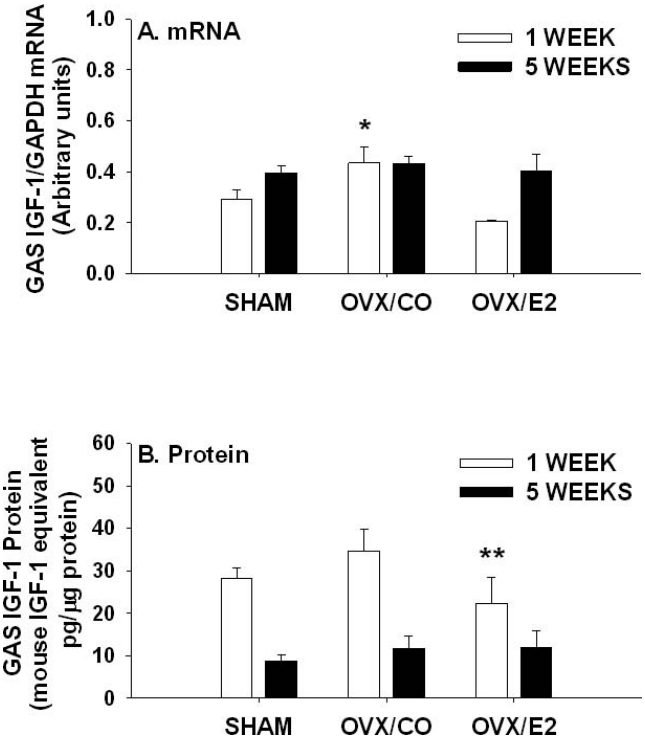


Figure 4. IGF-1 mRNA and protein expression in the GAS muscles. Open bars and black bars represent mean ± SE data from the 1-week and 5-week studies, respectively. (A) The IGF-1 mRNA was lower in the 1-week study than in the 5-week study ($P < 0.05$). IGF-1 mRNA in the GAS muscles of the OVX/CO group was significantly higher than that in the other two groups in the 1-week study (*$P < 0.05$, Scheffe *post hoc* analysis). There was no difference in IGF-1 mRNA among groups in the 5-week study. (B) The overall IGF-1 protein was significantly lower in the 5-week study. The IGF-1 protein in the GAS muscles of the OVX/E2 group was significantly lower than that in the OVX/CO group in the 1-week study (**$P < 0.05$). No difference in GAS IGF-1 protein was found among groups in the 5-week study.

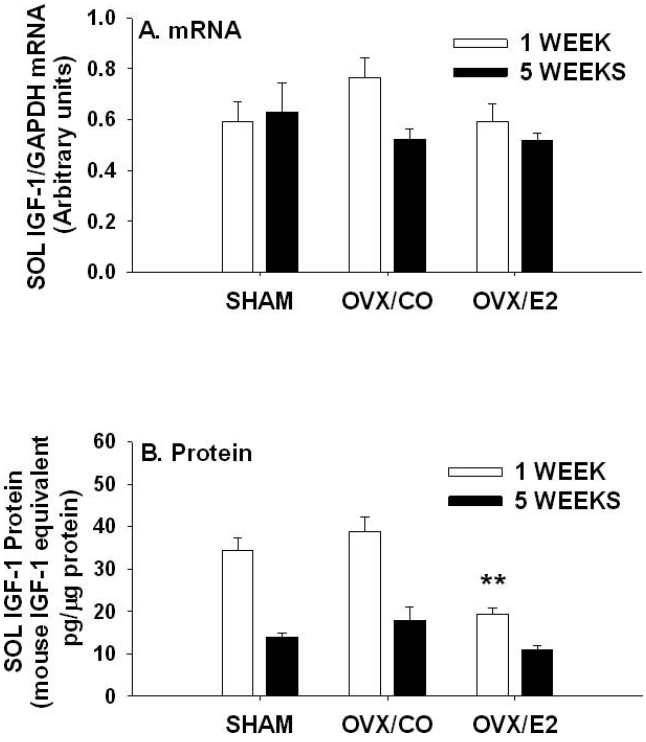


Figure 5. IGF-1 mRNA and protein expression in the SOL muscles. Open bars and black bars represent mean ± SE data from the 1-week and 5-week studies, respectively. (A) No difference in SOL IGF-1 mRNA was found regardless of treatment or study duration. (B) The IGF-1 protein was significantly lower in the 5-week study. The IGF-1 protein in the SOL muscles of the OVX/E2 group was significantly lower than that in the other two groups in the 1-week study (**$P < 0.001$, Scheffe *post hoc* analysis). No difference in IGF-1 protein was found among groups in the 5-week study.

increase in IGF-1 mRNA in the 5-week versus the 1-week study ($P < 0.05$). GAS muscles of OVX/CO animals had 50% and 100% more IGF-1 message than those of SHAM animals ($P = 0.04$) and OVX/E2 animals ($P = 0.0009$), respectively. The treatment effects on IGF-1 message in the 1-week study were not observed in the 5-week study.

The IGF-1 protein level, in contrast, was lower in the 5-week study animals than in the 1-week study animals (Fig. 4B). In the 1-week study, the OVX/E2 GAS muscles had 44% less IGF-1 protein than did the OVX/CO ($P = 0.04$). No treatment effect on IGF-1 protein expression was found in the 5-week study.

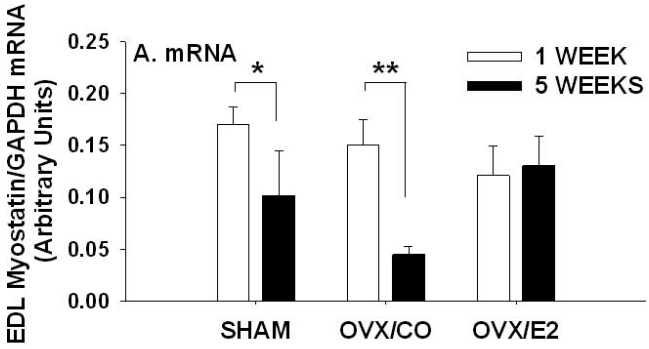
IGF-1 mRNA and Protein in the SOL Muscles. The IGF-1 message levels in the SOL muscles did not differ among treatment groups in both studies (Fig. 5A). There was no difference in the overall IGF-1 message level between the 1-week and 5-week studies.

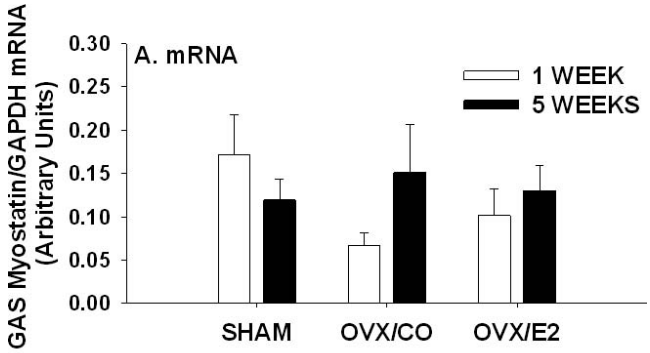
The overall SOL IGF-1 protein level was lower in the 5-week study than in the 1-week study ($P < 0.05$, Fig. 5B). Despite no difference in message level at the end of the 1-week study, the IGF-1 protein level was 40% and 50% lower in SOL muscles from OVX/E2 animals than in the SHAM and OVX/CO animals ($P < 0.001$). There was no treatment effect observed in the 5-week study.

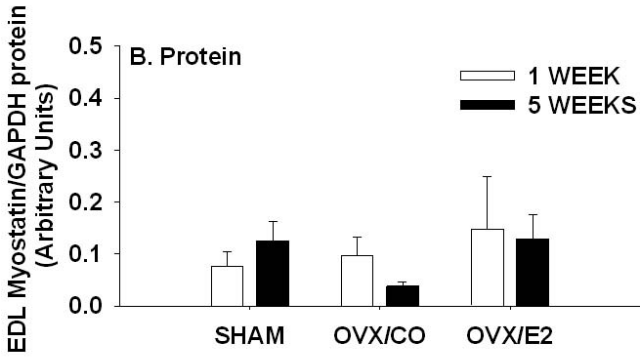
Myostatin mRNA and Protein in the EDL Muscles. Two-way ANOVA showed the overall EDL myostatin mRNA expression in the 5-week study was less than that in the 1-week study ($P < 0.005$). *Post hoc* analysis revealed a trend toward decreased myostatin expression ($P = 0.056$) in SHAM animals and a significant decrease in the OVX/CO animals ($P < 0.001$) in the 5-week study versus the 1-week study (Fig. 6A). The time-dependent decline in EDL myostatin mRNA was not observed in the OVX/E2 animals (Fig. 6A). There was no difference in myostatin protein expression among treatment groups in either study (Fig. 6B and C).

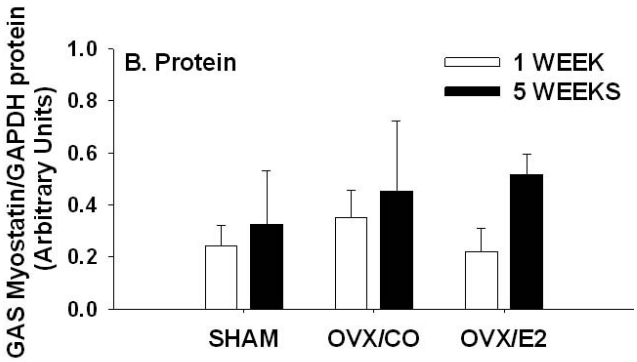
Myostatin mRNA and Protein in the GAS Muscles. Myostatin mRNA and protein expression data from the GAS muscles are shown in Figure 7. Two-way ANOVA did not detect any treatment or study duration effects on the IGF-1 mRNA and protein expression in the GAS muscles.

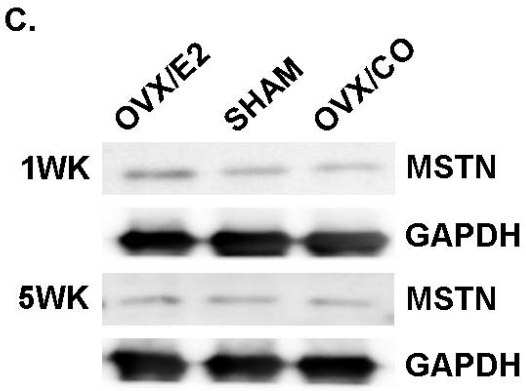
Myostatin mRNA and Protein in the SOL Muscles. The SOL myostatin mRNA expression was not different between treatment groups (Fig. 8A). There was a











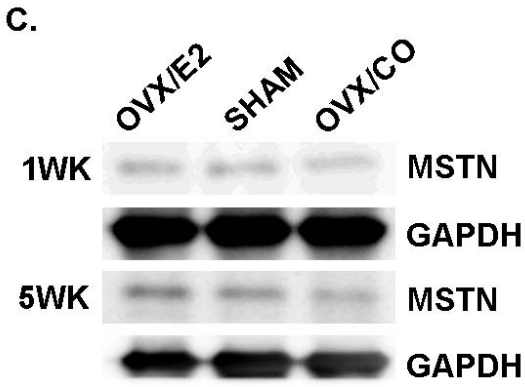


Figure 6. Myostatin mRNA and protein expression in the EDL muscles. Open bars and black bars represent mean ± SE data from the 1-week and 5-week studies, respectively. (A) The myostatin mRNA was lower in the 5-week study than in the 1-week study in the OVX/CO group (**$P < 0.001$, Scheffe *post hoc* analysis). A similar trend was observed in the SHAM group; however, the change was not significant (*$P = 0.056$). (B) Myostatin protein in the EDL muscles was not different among treatment groups. (C) Representative myostatin Western blots for the EDL muscles in the 1-week and 5-week studies. GAPDH was used as a gel-loading control.

Figure 7. Myostatin mRNA and protein expression in the GAS muscles. Open bars and black bars represent mean ± SE data from the 1-week and 5-week studies, respectively. (A) Myostatin mRNA was similar in the GAS muscles among all groups. (B) Myostatin protein in the GAS muscles was also similar among all groups. (C) Representative myostatin Western blots for the GAS muscles in the 1-week and 5-week study. GAPDH was used as a gel-loading control.

significant increase in the myostatin protein level in the SOL muscles of SHAM animals in the 5-week when compared with the same in the 1-week study ($P < 0.01$, Fig. 8B and C). The myostatin protein in the SOL muscle of animals that received estrogen replacement was significantly greater than that in the SHAM and OVX/CO animals in the 1-week study ($P < 0.005$, Fig. 8B and C). However, the estrogen effect observed in the 1-week study was not observed in the 5-week study.

Discussion

Ovariectomy, Body Weight, and Muscle Weight. An ovariectomized rodent model was employed in this present study to investigate the effect of estrogen on skeletal muscle IGF-1 and myostatin expression and growth in immature animals. Studies have shown that long-term

ovariectomy causes hyperphagia in rodents (27–29). There is limited information, however, on the onset of hyperphagia after ovariectomy. In the current 1-week study, food intake did not differ among the experimental groups, a finding indicating that ovariectomy-induced hyperphagia does not begin until at least 1 week after surgery.

Effects of long-term estrogen manipulation on body weight regulation have been confounded by differential food intake between OVX and control animals. To rule out the potential confounding factor on body weight regulation, we matched the food intake so the three groups consumed equal amounts during the 5-week study. Despite similar food intake, the OVX/E2 animals showed a significant initial decrease in body weight and remained smaller than the SHAM and OVX/CO animals throughout this study period. The OVX/CO animals weighed more than the SHAM animals, and the differences became significant after day 20. Body weight is determined by the balance between total energy intake and total energy expenditure. The total energy expenditure is composed primarily of basal metabolic rate, diet-induced thermogenesis, and effect of physical activity (30). Because there was no difference in energy intake among groups, differences in body weight among groups suggest that total energy expenditure was altered in the OVX and OX/E2 animals. Ovariectomy in rodents has been found to reduce spontaneous cage activity (27, 31), increase feeding patterns (32), and reduce lean body mass (33), which in turn changes physical activity, diet-induced thermogenesis, and basal metabolic rate, respectively. Little is known about the underlying mechanism of the ovariectomy-induced behavioral and metabolic changes, and this area warrants further investigation.

We found small weight changes in the weight-bearing muscle in response to ovariectomy and/or estrogen replacement in the 1-week study. The change in muscle weight became more apparent after 5 weeks of treatment. The change in wet muscle weight was proportional to the change in body weight, and no difference was found when the data were expressed as muscle-to-body weight. The observed changes in absolute weight of the limb muscles cannot be overlooked because the weight of other organs (heart and liver) and the weight of the non–weight-bearing diaphragm muscle were unchanged among treatment groups. The observed weight changes in the limb muscles are consistent with previous reports of estrogen replacement reducing myofiber cross-sectional area and altering muscle contractile function but not necessarily changing the muscle-to-body weight ratio (9, 10, 34, 35).

Estrogen and Muscle IGF-1 Expression. The main objective of this work was to begin to understand the underlying cause of the observed changes in muscle weight. Two major pathways for muscle size regulation, IGF-1 and myostatin, have been identified. In this current study, estrogen, when unopposed by progesterone, decreased IGF-1 protein in all muscles examined. This finding is in accordance with our and other researchers' previous findings that estrogen replacement in ovariectomized animals decreases skeletal muscle cell size (9, 34, 35). In contrast to our findings, in domestic meat–producing animals, estrogens in combination with trenbolone acetate have long been used to stimulate skeletal muscle growth, which is accompanied by an increase in the plasma and muscle IGF-1 level (36). Nonetheless, it is important to note the estrogen was given in combination with an androgenic

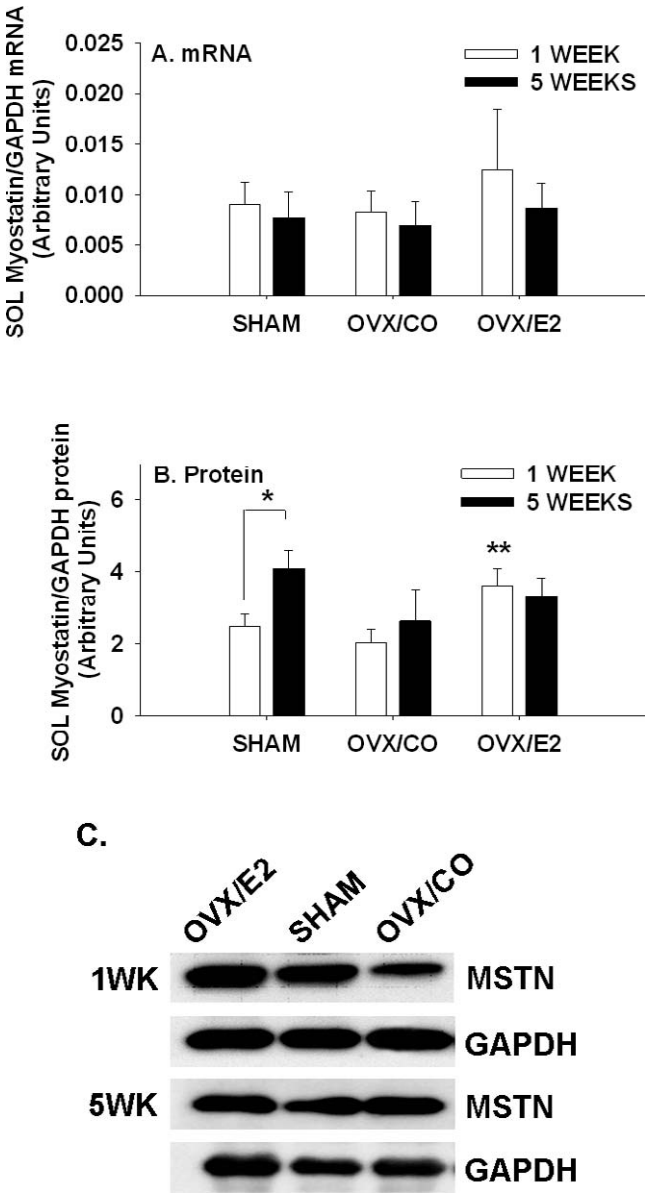


Figure 8. Myostatin mRNA and protein expression in SOL muscle. Open bars and black bars represent mean ± SE data from the 1-week and 5-week studies, respectively. (A) Myostatin mRNA was similar in SOL muscles among all groups. (B) Myostatin protein in the SOL muscles of SHAM animals was higher in the 5-week study than in the 1-week study (*$P < 0.01$, Scheffe *post hoc* analysis). The SOL muscles of the OVX/E2 group had higher myostatin protein than did the other two groups in the 1-week study (**$P < 0.005$). No treatment effect was observed in the 5-week study. (C) Representative myostatin Western blots for the SOL muscles in the 1-week and 5-week studies. GAPDH was used as a gel-loading control.

agent and the study employed only male animals; therefore, the results may not necessarily be comparable to the present findings.

How estrogen signals are transduced to modulate IGF-1 expression remains unanswered. It has been speculated the effect of estrogen on IGF-1 was mediated through the recruitment of ERs in neurons, breast tumors, and the heart (37–39). Nonetheless, there is very little information available on ER expression and distribution in the skeletal muscles. Lemoine and colleagues reported higher ERα mRNA expression in the slow SOL muscle than in the fast GAS and EDL muscles (40). Despite different ERα content in the slow and fast muscles, estrogen replacement had similar effects on the IGF-1 in the slow and fast muscles. As such, the data do not support a predominant role of ERα in the estrogen regulation of IGF-1. Another isoform, ERβ, has also been identified in various skeletal muscles (2). The ERβ distribution among different muscle types is unknown and may be important for this regulation.

Another important consideration is the estrogen effects on the GH/IGF-1 axis. Estrogen has been shown to antagonize several metabolic actions of GH (41). Because estrogen was administered systematically, we cannot rule out the possibility that the decreased IGF-1 expression is secondary to the estrogen-attenuated GH actions. Further studies will be needed to determine whether the estrogen effect on skeletal muscle IGF-1 is direct or indirect.

Estrogen and Muscle Myostatin Expression. The GAS and EDL muscles had higher myostatin mRNA content than the SOL muscle (note the scale for SOL is much lower than for GAS and EDL). This finding is consistent with the observation that myostatin mRNA expression is highly correlated with the myosin heavy chain IIb (42), which is found predominantly in the GAS and EDL muscles but not in the SOL muscles (24). The active myostatin protein content (26 kDa), on the other hand, was highest in the SOL muscles rather than in the EDL and GAS muscles. In close agreement with previous reports (18, 43–45), the Western blot analysis detected the active form of myostatin as a dimer, but not as the monomer, despite attempts to dissociate the dimer by the addition of reducing agents and acid treatments. There are confounding reports as to whether myostatin protein content is more abundant in the slow-twitch muscles or in the fast-twitch muscles (18, 21, 45–47). Kirk and colleagues used immunohistochemistry to show that myostatin protein is only expressed in fast-twitch fibers and that myostatin was detected in slow-twitch fibers when the muscles were damaged by myotoxin treatments (47). Sakuma and colleagues, however, demonstrated that the SOL muscle has the most abundant endogenous myostatin protein content when compared with EDL and GAS muscles (45).

In the present study, estrogen replacement increased myostatin protein level in the SOL muscle. The myostatin protein content in the EDL and GAS muscles, in contrast, did not vary as a function of ovarian hormonal status. As such, the data support myostatin as an important mediator of estrogen's effects on growth in slow but not in fast muscles. Studies have shown that ER-α mRNA and ER binding site density are higher in slow-twitch muscle (SOL) than in fast muscles (40, 48), and this finding might, at least partially, explain the differential effect of estrogen on myostatin protein expression. Estrogen may also have differential effects on these muscles in the context of age. Myostatin protein content in the SHAM SOL muscle was higher in the 5-week study than in the 1-week study, a result suggesting an age-related increase. In the SHAM EDL muscle, myostatin mRNA level was lower in the 5-week study than in the 1-week study, a result suggesting an age-related decline. Estrogen replacement abolished this age-related decline. This estrogen effect, however, was not observed at the protein level, and the absence of this effect indicates that other factors may offset estrogen-mediated transcriptional regulation of myostatin in the EDL muscle.

The differential effects of estrogen on myostatin expression in fast and slow muscles may be related to growth patterns in these muscles. In comparing the 1-week and 5-week data from this study, it appears that the EDL and GAS muscles grew more than the SOL muscle (17%, 24%, and 2%, respectively). Consistent with this finding, other investigators have shown fast muscles, such as the EDL and tibialis anterior, achieved their final adult size later than the SOL muscle (49, 50). Findings from the present study suggest that these differences in the growth patterns of fast and slow muscles may be related to myostatin protein levels; myostatin protein levels were at least 10 times higher in the SHAM SOL muscle than in the SHAM GAS and EDL muscles.

Transcriptional and Post-Transcriptional Regulation by Estrogen. It is important to note that many genes show a discordance in the mRNA and protein content in skeletal muscles (51–54). Here we observed a discrepancy between mRNA and protein levels for both IGF-1 and myostatin. We are not the first to observe discordance between myostatin mRNA and protein. McMahon and colleagues demonstrated that females have more active myostatin protein expression than males despite there being no difference in mRNA expression between the genders (23). The mismatch between mRNA and protein content could result from alternative splicing of the mRNA, post-transcriptional regulation, altered translational efficiency, as well as modification of mRNA and protein stability. The muscle IGF-1 protein may be affected by the serum IGF-1 level, and this could also explain the discordance between the IGF-1 mRNA and protein expression in muscles. Recently, estrogen has been shown to affect mRNA stability in ovarian cancer cells (55). The mechanism of hormonal regulation of mRNA stability is largely unknown, but estrogen upregulates or downregulates transcriptional factors that bind to adenylate/uridylate-rich elements, which mediate the turnover of mRNAs encoding proliferation/

differentiation-regulating proteins (55). Further work will be required to understand the underlying mechanisms.

Transient Induction of IGF-1 and Myostatin by Estrogen. In this study, we observed estrogen effects on skeletal muscle IGF-1 and myostatin expression after 1 week but not 5 weeks of treatment. This initial fluctuation in these growth regulators seems to be sufficient to maintain smaller muscles or prevent catch-up growth in the estrogen replacement group because the reduction in muscle size (9, 10) and muscle wet weight was observed at times long after 1 week of treatment. Several reports using muscle atrophy models also showed an initial change in myostatin and/or IGF-1 expression, which later returned to levels seen in control conditions (20, 22, 56, 57). We suspect that the effect of estrogen is transient or there is an upregulation of a compensatory mechanism to counteract the estrogen effects and return growth factor expression to control levels.

Conclusions. This work is the first to report IGF-1 and myostatin expression, at both mRNA and protein levels, in response to ovariectomy and estrogen replacement in growing animals. Estrogen had a universal blunting effect on IGF-1 expression in the weight-bearing skeletal muscles examined in this study. The estrogen effect on myostatin, in contrast, was muscle-specific with the predominant effect observed in the slow SOL muscle. Our results suggest the need for a greater understanding of the mechanisms of action of estrogen in skeletal muscles. The alternation in growth factor expression by estrogen could be of great importance when the muscles are subjected to challenging conditions such as exercise or casting and warrants further investigation. Knowledge of ER content in different muscles as well as the effects of estrogen on the downstream effectors of the IGF-1 signaling pathway will add to our understanding of the actions of estrogen on skeletal muscle growth.

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