Sex differences in steroidogenesis in skeletal muscle following a single bout of exercise in rats

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Sex steroid hormones, such as testosterone and estradiol, play important roles in developing both strength and mass of skeletal muscle. Recently, we demonstrated that skeletal muscle can synthesize sex steroid hormones. Whether there are sex differences in basal steroidogenesis or acute exercise-induced alterations of steroidogenesis in the skeletal muscle is unknown. We examined sex differences in the levels of testosterone, estradiol, and steroidogenesis-related enzymes, such as 17β-hydroxysteroid dehydrogenase (HSD), 3β-HSD, and aromatase cytochrome P-450 (P450arom), in the skeletal muscle at rest and after exercise. We studied the gastrocnemius muscles of resting rats (10 wk old) and exercised rats (10 wk old, treadmill running, 30 m/min, 30 min). Basal muscular testosterone levels were higher in males than females, whereas estradiol did not differ between sexes. Additionally, 17β-HSD, 3β-HSD, and P450arom transcript and protein expression were greater in females. After acute exercise, testosterone levels and 17β-HSD expression increased in muscle in both sexes. By comparison, muscular estradiol levels increased in males following exercise but were unchanged in females. Expression of P450arom, which regulates estrogen synthesis, increased after acute exercise in males but decreased after exercise in females. Thus a single bout of exercise can influence the steroidogenic system in skeletal muscle, and these alterations differ between sexes. The acute exercise-induced alteration of steroidogenic enzymes may enhance the local steroidogenesis in the skeletal muscle in both sexes.

acute exercise; sex steroid hormone; 3β-hydroxysteroid dehydrogenase; aromatase cytochrome P-450 DHEAS levels in human adults are 100–500 times higher than testosterone, and 1,000–10,000 times higher than estradiol. The marked reduction in formation of DHEAs by the adrenals during aging results in a dramatic fall in the formation of androgens and estrogens in peripheral target tissues, a situation that is thought to be associated with diseases such as insulin resistance, obesity, and loss of muscle mass (20). Also, sex steroid metabolism is upregulated during sexual maturation, a time point at which sex steroids have an important role on longitudinal growth (44). Therefore, DHEA plays a critical physiological role in maintaining steroidogenesis in peripheral tissues in the process of development and aging (21, 22).

Sex steroid hormones are secreted not only by the ovary, testis, and adrenal gland, but also by various peripheral tissues, including bone, liver, and brain (44, 46, 51). Recently, we demonstrated that enzymes required for steroidogenesis are expressed in skeletal muscle and that the sex steroid hormones testosterone and estradiol are synthesized from DHEA and/or testosterone in the cultured muscle cells (1). Skeletal muscle is a sex steroid-sensitive tissue and expresses receptors for both androgen and estrogen (8, 24), considering that skeletal muscle can make use of testosterone and estrogen in both male and female. However, whether sex influences the gene expression of enzymes important for steroidogenesis or the local synthesis of sex steroids in skeletal muscle is unknown.

Chronic exercise induces increases in muscle size, strength, energy metabolism, and antioxidant capacity, as well as changes in muscle fiber type (2, 32, 37, 38). Sex steroid hormones partly contribute to these exercise-induced muscular adaptations (13, 14, 33, 36, 39, 41). In fact, administration of testosterone increases muscle strength and mass (36, 39). In contrast, administration of estrogen has little effect on muscle development and mass (13, 41). A single bout of acute exercise increases testosterone levels in plasma in males but not females (17, 18) although there are no sex differences in chronic exercise-induced adaptations by skeletal muscle (6, 38). As a reason of this adaptation, the skeletal muscle can synthesize sex steroid hormones from circulating DHEAs or testosterone and they would act as an autocrine and local paracrine muscle growth factor. However, it is unclear whether the muscular steroidogenetic system is changed by a single bout of acute exercise stimulation, and its response differs between both sexes. Accordingly, we hypothesized that basal muscular steroidogenesis may differ between males and females, and exercise may stimulate steroidogenesis differently in both sexes.
In the present study, we examined hormone levels and the expression of steroidogenesis-related enzymes in skeletal muscle of female and male rats at rest and after a single bout of exercise.

METHODS

Animals. Experimental protocols were approved by the Committee on Animal Research at the University of Tsukuba. Female and male 10-wk-old Sprague-Dawley (female, n = 16; male, n = 16) rats were obtained from Charles River Japan (Yokohama, Japan) and cared for according to the Guiding Principles for the Care and Use of Animals based on the Helsinki Declaration. All rats were maintained on a 12:12-h light-dark cycle and received food and water ad libitum. We used sexually mature male and female rats at 10 wk of age, which had reached mature body weight, body fat, and bone (3). Female and male rats were familiarized for 2 days by running on a motor-driven treadmill for 10-min intervals at a speed of 15 m/min without incline (0% grade). The body weight of each animal was measured 48 h before the experiment. On the day of the experiment, rats were randomly divided into two groups for each sex, an exercise group before the experiment. On the day of the experiment, rats were treadmill for 10-min intervals at a speed of 15 m/min without incline to reach mature body weight, body fat, and bone (3). Female and male 12-wk-old Sprague-Dawley (female, n = 16; male, n = 16) rats were obtained from Charles River Japan (Yokohama, Japan) and cared for according to our previous studies (11). Briefly, the tissue was homogenized in 50 mg tissue/1 ml Isogen (Nippon Gene; Toyama, Japan), according to our previous studies (11). The tissue was homogenized in 50 mg tissue/1 ml Isogen with a Polytron tissue homogenizer (model PT10/50K35, Kinematica, Lucerne, Switzerland). Total RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% (vol/vol) ethanol. Total RNA was treated with an Rnase-free DNase kit (QIAGEN, Tokyo, Japan) and further purified with an RNasey minikit (QIAGEN). The RNA concentration was determined spectrophotometrically at 260 nm. Total tissue RNA (2 μg) was primed with 0.05 μg of oligo(dT)12–18 and reverse transcribed with omniscript reverse transcriptase using a cDNA synthesis kit (QIAGEN) (12). The reaction was performed at 37°C for 60 min.

Real-time quantitative PCR analysis. Quantitative real-time PCR was used to measure relative mRNA expression (ABI-PRISMA 7700 Sequence Detector, Perkin-Elmer Applied Biosystems), as performed previously but with minor modifications (12). The gene-specific primers and TaqMan (FAM) probes were determined with Primer Sequence Detector, Perkin-Elmer Applied Biosystems), as performed previously but with minor modifications (12). The gene-specific primers and TaqMan (FAM) probes were determined with Primer Express v. 1.5 software (Perkin-Elmer Applied Biosystems). The sequences of the oligonucleotides for probes and forward and reverse primers for P450arom, 3β-HSD, 17β-HSD, and β-actin are shown in Table 1. Each PCR amplification was performed in triplicate with the following thermocycling profile: 1 cycle of 95°C 10 min and 40 cycles of 94°C for 15 s and 60°C for 1 min. The expression of β-actin mRNA was determined as an internal control. The quantitative values of P450arom, 3β-HSD, and 17β-HSD were determined by that of β-actin mRNA expression.

Immunoblot analysis. Tissues were homogenized with 20 mM Tris·HCl (pH 7.8), 300 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, 2% NP-40, 0.2% SDS, 0.2% sodium deoxycholate, 0.5 mM PMSF, 60 μg/ml aprotinin, and 1 μg/ml leupeptin. The homogenate was gently rotated for 30 min at 4°C and then centrifuged at 12,000 g for 15 min at 4°C. The protein concentration of the resulting supernatant was determined. Samples (50 μg protein) were then denaturated at 96°C for 7 min in Laemml buffer. Western blot analysis was performed to detect P450arom and 17β-HSD type I according to a previous report (1). Briefly, each sample was separated on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF, Millipore, Billerica, MA) membrane. The membrane was then incubated in blocking buffer, 3% skim milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) for 1 h at room temperature, followed by incubation with primary antibodies, a monoclonal anti-P450arom antibody (1:100 dilution with blocking buffer, Serotec, Kidlington, Oxford, UK), a polyclonal anti-17β-HSD type I (1:1,000 dilution with blocking buffer, Santa Cruz Biotechnology, Santa Cruz, CA), and a monoclonal anti-β-actin (1:1,000 dilution with blocking buffer, Santa Cruz Biotechnology) for 2 h at 4°C. Subsequently, the membrane was washed with PBS-T three times and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody, either an anti-mouse IgG (1:2,000 dilution with blocking buffer, Amersham Biosciences Piscataway) or an anti-goat IgG (1:4,000 dilution with blocking buffer, Amersham Biosciences, Piscataway) or an anti-goat IgG (1:4,000 dilution with blocking buffer, Santa Cruz Biotechnology), for 1 h at room temperature. After washing, as described above, binding was detected by chemiluminescence with the ECL plus system (Amersham Life Science) following exposure to Hyperfilm (Amersham Biosciences).

Sandwich-EIA. Tissues were homogenized by using the same homogenization method including buffers in the immunoblot analysis. Homogenated samples were diluted fourfold in total testosterone and diluted twofold in estradiol kits with each EIA assay buffer. Tissue concentrations of total testosterone and estradiol in skeletal muscle extracts were determined using a sandwich-EIA Kit (total testosterone: R&D systems, Minneapolis, MN; total estradiol: Cayman Chemical, Ann Arbor, MI) (1). All techniques and materials used in this analysis were in accordance with the manufacturer’s protocol. The immobilized antibodies were monoclonal and raised against total testosterone and estradiol, while each secondary HRP-coupled antibody was monoclonal or polyclonal. Optical density was quantified on a microplate reader using a BioLumin 960 (Molecular Dynamics, Tokyo, Japan). All samples were assayed in duplicate. Minimal detectable concentrations of total testosterone and estradiol were 3.8 pg/ml and 8 pg/ml, respectively. Intra-assay variance for total testosterone and estradiol was 8.9% and 5.6%, respectively. Interassay variance for total testosterone and estradiol was 9.3% and 6.1%, respectively.

Statistics. Values were expressed as means ± SE. Differences between groups were assessed by unpaired t-tests and by two-way ANOVA. When the ANOVA F-ratio was significant, a post hoc

### Table 1. Sequences of oligonucleotides

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>P450arom Forward</td>
<td>5′-CAGATGCAGAATCTTCTGGGAAAAG-3′</td>
</tr>
<tr>
<td>P450arom Reverse</td>
<td>5′-CTCTGGAGGAAACTTT-3′</td>
</tr>
<tr>
<td>3β-HSD Forward</td>
<td>5′-GGCCCAACCTCTCTAGAAGATCAT-3′</td>
</tr>
<tr>
<td>3β-HSD Reverse</td>
<td>5′-TCCTGGTCTGTTGGATGACT-3′</td>
</tr>
<tr>
<td>17β-HSD type I Forward</td>
<td>5′-GCCGACCAAGCAGAATGTC-3′</td>
</tr>
<tr>
<td>17β-HSD type I Reverse</td>
<td>5′-TTTTGACATCCAATTTCC-3′</td>
</tr>
<tr>
<td>β-Actin Forward</td>
<td>5′-GGGGGGAACAGTCAAGAC-3′</td>
</tr>
<tr>
<td>β-Actin Reverse</td>
<td>5′-GCTGGGCTGTTGGAACCTGTA-3′</td>
</tr>
</tbody>
</table>

P450arom, aromatase cytochrome P-450; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.
analysis were performed using Dunn’s method. $P < 0.05$ was accepted as significant.

**RESULTS**

We examined sex differences in the basal mRNA levels of 17β-HSD, 3β-HSD, and P450arom mRNAs in skeletal muscle tissue (gastrocnemius) from male and female rats (Fig. 1). The expression of these transcripts was significantly higher in females (Fig. 1A: $P < 0.05$, Fig. 1B: $P < 0.01$, Fig. 1C: $P < 0.01$). In addition, we confirmed the basal expression of 17β-HSD and P450arom by examining protein levels (Fig. 2). The basal levels of 17β-HSD and P450arom protein in skeletal muscle reflected the differences observed in mRNA expression ($P < 0.05$, Fig. 2A; $P < 0.01$, Fig. 2B).

To investigate whether sex differences in the expression of steroidogenic enzymes affects the production of sex steroid hormones in muscle, we measured the levels of testosterone and estradiol in male and female rats (Fig. 3). The concentration of testosterone in skeletal muscle from males was significantly higher than that in females ($P < 0.01$, Fig. 3A), whereas the concentration of estradiol did not differ between sexes.

Next, we examined whether a single bout of acute exercise altered either the mRNA or protein expression of steroidogenic enzymes (Fig. 4). The mRNA expression of 17β-HSD was significantly higher in exercised female rats ($P < 0.05$, Fig. 4A), but there were no detectable differences in male rats. In comparison, the mRNA expression of 3β-HSD was signifi-

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**Fig. 1.** Sex differences in the mRNA expression of steroidogenesis-related enzymes 17β-hydroxysteroid dehydrogenase (17β-HSD; A), 3β-HSD (B), and aromatase cytochrome P-450 (P450arom; C) in skeletal muscle of male and female rats. Transcript levels were determined by real-time quantitative PCR. β-Actin mRNA was employed as an internal control for normalization. Data are expressed as means and SE.

**Fig. 2.** Protein expression levels of 17β-HSD (A) and P450arom (B) in the skeletal muscle of male and female rats. β-Actin protein was employed as an internal control for normalization. Data are expressed as means and SE.
significantly higher in exercised male rats relative to their control “resting” counterparts \( (P < 0.01, \text{Fig. 4B}) \), but there were no detectable differences in female rats. The mRNA expression of P450arom was significantly higher in exercised male rats \( (P < 0.05, \text{Fig. 4C}) \), while in female rats, the level of P450arom was significantly lower in exercised animals \( (P < 0.01, \text{Fig. 4C}) \). In addition, mRNA expression of β-actin, which was an internal control, did not differ between sexes and before and after exercise.

We also examined if these exercise-induced changes in enzyme mRNA expression resulted in associated changes in protein expression for 17β-HSD and P450arom (Fig. 5). The protein expression of 17β-HSD was significantly higher in exercised male rats \( (P < 0.01, \text{Fig. 5A}) \), and that was significantly higher in exercised female rats \( (P < 0.05, \text{Fig. 5A}) \). Interestingly, the P450arom protein levels were significantly higher in exercised relative to resting male rats \( (P < 0.01, \text{Fig. 5B}) \), while protein levels were significantly lower in exercised relative to resting females \( (P < 0.01, \text{Fig. 5B}) \). In addition, protein expression of β-actin, which is an internal control, did not differ between sexes or before and after exercise.

Last, we examined if there were sex differences in the levels of testosterone and estradiol in skeletal muscle following acute exercise. The muscular testosterone concentration was significantly higher in exercised male rats relative to resting male controls \( (P < 0.01, \text{Fig. 6A}) \) and was significantly higher in exercised female rats relative to resting female controls \( (P < 0.05, \text{Fig. 6A}) \). By comparison, the muscular estradiol concentrations were significantly higher in exercised relative to resting male rats \( (P < 0.01, \text{Fig. 6B}) \) but were unchanged in female rats (Fig. 6).

In this study, we identify sex differences in the expression of steroidogenesis-related enzymes and in the concentration of the sex steroid hormones testosterone and estradiol at rest and after a single bout of exercise. Interestingly, under resting conditions, three enzymes, 17β-HSD, 3β-HSD, and P450arom, are expressed at higher levels in females. In males, the expression of these three enzymes increased with exercise, whereas in females, only the expression of 17β-HSD was increased with exercise, while 3β-HSD was unchanged, and P450arom decreased. Muscular testosterone levels were higher in males but were elevated in both sexes by exercise. Basal muscular...
estrogen levels did not differ between sexes and were elevated by exercise only in males. Thus exercise acutely induced alterations in the levels of different steroidogenic enzymes in each sex. These different responses may lead to the production of sex steroids in skeletal muscle of both sexes.

Although the concentration of circulating testosterone is 20 times higher in males (35), tissue concentrations of testosterone were only 3 times higher. Thus the sex difference in muscular testosterone levels is smaller than that of plasma testosterone levels. Recently, we demonstrated that skeletal muscle can synthesize sex steroid hormones from circulating DHEA or testosterone (1). As DHEAs, which are precursors of sex steroid hormones, are the most abundant circulating steroid hormones in both males and females (19, 21, 22), the higher expression of steroidogenic enzymes in females may permit greater local testosterone production from DHEA by sex steroid metabolism in the skeletal muscle.

In males, we demonstrated that expression of 17β-HSD, 3β-HSD, and P450arom in the skeletal muscle was increased by exercise. In contrast, in females only the expression of 17β-HSD was increased by exercise, while P450arom expression decreased. Thus a single bout of exercise induced sex-specific changes in steroidogenic enzyme expression. Additionally, females displayed an increase in muscular testosterone levels after exercise, while males exhibited an increase muscular estrogen and testosterone levels. Previous studies have demonstrated that circulating DHEAs in blood are also increased by a single bout of exercise (43). Thus sex-specific changes in muscular sex steroid hormone levels induced by exercise may reflect a differing capacity for metabolizing DHEAs in skeletal muscle. P450arom is an important enzyme for metabolizing androgen to estrogen (4, 5). Interestingly, we observed that muscular P450arom expression was increased in males but decreased in females by a single bout of exercise. Thus P450arom expression in the skeletal muscle was inversely correlated with the response by the two sexes to exercise. The physiological balance between sex steroid hormones is largely controlled by aromatase (5). Therefore, in exercising skeletal muscle, estrogen synthesis may increase in males while testosterone synthesis may increase in females. In fact, tissue estrogen levels in males were increased by acute exercise, as were tissue testosterone levels in female. Thus sex differences in the regulation of P450arom by exercise may contribute to compensating for insufficient local levels of sex steroid hormones in skeletal muscle for each sex.

In the present study, we examined muscular steroidogenesis-related enzymatic expression and hormonal levels using whole gastrocnemius muscle, which is an intermediate muscle fiber type, including both fiber types of red and white. In soleus and gastrocnemius muscle of rats, acute running exercise induced decrease in glycogen concentration (40). Furthermore, endurance running training induced in increase of citrate synthase activity in both gastrocnemius muscle and soleus muscle (26).
Thus these observations considered that the gastrocnemius muscle is constantly used by running exercise, and this muscle is dramatically adapted by running exercise training. We recently reported that the gastrocnemius muscle is capable of local steroidogenesis (1). In addition, skeletal muscle tissues possess receptors for both androgen (AR) and estrogen (ER-α and ER-β). Acute exercise induced increase in expression of AR mRNA in skeletal muscle (50). Endurance training also elevates the expression of the ER-α gene in gastrocnemius muscle (26). Moreover, exercise-induced alteration in androgen binding capacity in skeletal muscle fibers of rats differs between endurance and resistance exercise (7). Future studies need to investigate expression of steroidogenesis-related enzymes in various exercise types and muscular fiber types, i.e., slow- and fast-twitch fibers of muscle.

The mechanisms underlying these sex differences in steroidogenesis-related enzyme expression in response to acute exercise remains to be elucidated. Several studies have reported that nuclear factors, such as steroidogenic factor-1 (SF-1) (23) and adrenal 4-binding protein (Ad4BP) (25, 29), are transcriptional regulators of the cytochrome P-450 steroidogenic enzymes. In addition, other transcriptional regulating factors, pituitary peptide hormones, such as adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), and follicle-stimulating hormone, act via G protein-coupled receptors and adenylate cyclase to increase cAMP and increase steroidogenic P-450 enzyme levels (48, 49). As the levels of circulating ACTH and LH increases with exercise (42, 43), these pituitary hormones may contribute to increasing sex steroid metabolism in muscle. Future studies are required to identify the transcription factors that mediate these sex-specific differences in the expression of steroidogenesis-related enzymes.

There are major differences between female and male skeletal muscle (9, 15, 16, 34). The mechanisms behind these sex-related differences in the skeletal muscle may involve a consequence of different sex hormonal status. In postmenopausal women, the secretion of estradiol by the ovaries is impaired, decreasing to levels comparable to those in men (35). This age-induced reduction in steroidogenesis increases the risk for brain, bone, and cardiovascular disorders, as well as affecting muscular function (10, 28); in particular, a dramatic reduction of hormone production after menopause in females accelerates sarcopenia (45). Villareal and Holloszy (47) reported that DHEA replacement has the additive effect of enhancing the increases in muscle mass and strength induced by resistance exercise in elderly subjects. In the present study, acute exercise induced alteration in the expression of sex steroid hormone-related enzymes in skeletal muscle using young rats (10 wk old) at developing stage but that of steroidogenesis is unknown in aged rats. Sex steroid hormones are involved in the regulation of longitudinal growth process in skeletal muscle at each stage of maturation, development, and aging. Therefore, it will be necessary to make further research at some stages during aging.

The present results demonstrated that the expression of steroidogenesis-related enzymes and intracellular sex steroid hormone altered immediately after a single bout of exercise in both sexes. Thus the alteration of steroidogenesis-related enzymes in the muscle tissue may lead to its local conversion to testosterone and estrogens from circulating DHEA or testosterone. Moreover, the highly effective local synthesis of sex steroid hormones in the skeletal muscle would be helpful in differentiating the synthetic capacity of sex steroid hormones. However, the source of exercise-induced alteration of sex steroid hormones is unclear.

Several studies reported that various hormonal and metabolic factors change during and after exercise (27, 40, 43). Moreover, we measured only the point immediately after the exercise. Further studies should examine the mRNA and protein expression of these enzymes in the time course of alteration pattern after the exercise and need to increase the number of samples.

In conclusion, the present study characterizes for the first time the expression of three enzymes contributing to steroidogenesis and examines how their expression is altered by exercise. A single bout of exercise increases the expression of two of these enzymes, 17β-HSD and 3β-HSD, in both male and female rats. In addition, a sex difference in the expression of P450arom in skeletal muscle was induced by exercise. Thus the exercise induces acute changes in the expression of steroidogenesis-related enzymes that may contribute to increasing local steroidogenesis in skeletal muscle.
SEX DIFFERENCES IN STEROIDOGENESIS IN MUSCLE

GRANTS
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