Expression of steroidogenic enzymes and synthesis of sex steroid hormones from DHEA in skeletal muscle of rats

Katsuki Aizawa,1 Motoyuki Iemitsu,1,2 Seiji Maeda,1,2 Subrina Jesmin,2 Takeshi Otsuki,2 Chishimba N. Mowa,3 Takashi Miyachi,2 and Noboru Mesaki1

1Graduate School of Comprehensive Human Sciences and 2Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Ibaraki, Japan; and 3Department of Biology, Appalachian State University, Boone, North Carolina

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SEX STEROID HORMONES ARE SECRETED mainly by the ovary, testis, and adrenal cortex and regulate diverse physiological processes of target tissues, including those of the reproductive tissues, bone, liver, cardiovascular tissues, brain, and skeletal muscles (37). Dehydroepiandrosterone (DHEA) and its sulfate-bound form (DHEA-S), secreted mainly by the adrenal gland and ovary, are the most abundant hormones in humans and other mammals and, in peripheral tissues, are important precursors of sex steroid hormones (19, 23). Thus DHEA plays a critical physiological role for peripheral tissues in maintaining steroidogenesis in that it is one of the available precursors converted to testosterone and estrogens (19, 22, 23). The biosynthesis of active androgens and estrogens from DHEA is achieved by three steroidogenesis-related enzymes (22). Testosterone is synthesized through metabolism of DHEA by 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD, and in turn, estrogens are converted from androgens catalyzed by aromatase cytochrome P-450 (P450arom). The physiological balance between sex steroid hormones is largely controlled by aromatase (8). P450arom is a product of a single gene, whereas there are several types of 17β-HSDs, which are each a product of a distinct gene. Twelve different 17β-HSDs sites on the chromosome have been identified and in humans; so far, 10 different 17β-HSDs have been cloned, 1–5, 7, 8, 10, 11, and 12 (29). The 17β-HSDs differ in tissue distribution, catalytic preferences, substrate specificity, subcellular localization, and mechanisms of regulation. Rat 17β-HSD type I also efficiently converts androstenedione to testosterone (34).

The skeletal muscle is a sex steroid-sensitive tissue in that it expresses receptors for both androgen and estrogen (11, 25). Testosterone and estradiol affect growth, strength, metabolism, and antioxidants in the skeletal muscle (16, 17, 35, 39, 40). Administration of testosterone has been reported to induce an increase in muscle strength with accretion of protein synthesis in the muscle (46). Deficiency of sex steroid hormones leads to retardation in skeletal muscle development, and long-term administration of DHEA promotes muscle strength and mass in humans (30, 48). Reports on the effects of estrogen replacement therapy on muscular strength (40) are contradictory; whereas some studies have attributed it to increased muscle strength, others report that it (estrogen) does not (3) increase muscle strength. To date, it is unclear whether sex steroid hormones acting on skeletal muscle are locally produced by steroidogenesis-related enzymes. Because DHEA is present in high amounts in circulation and is peripherally converted to testosterone and estrogens in most tissues, it is likely also converted locally by steroidogenesis-related enzymes (3β- and 17β-HSD and P450arom) into active sex steroid hormones in skeletal muscles (21).

For this reason, we hypothesized that steroidogenesis-related enzymes, including 3β-HSD, 17β-HSD, and P450arom, are present in the skeletal muscle and locally synthesize sex steroid hormones from circulating DHEA. Thus, in the present study, we examined whether these enzymes (mRNA and protein) are 1) expressed, 2) active, and 3) capable of synthesizing sex steroid hormones in the skeletal muscle of adult male rats in vivo and in vitro. Moreover, we investigated whether mRNA levels of steroidogenic enzymes are differentially expressed in different skeletal muscle types, i.e., gastrocnemius (intermediate fiber), soleus (mainly slow fiber), and plantaris (mainly fast fiber) muscles of...
rat, because there are three types of skeletal muscles, namely type I (slow-twitch fiber), type IIA (intermediate of slow and fast-twitch fiber), and type IIB (fast-twitch fiber), which have distinct contractile, metabolic, and vascular properties.

**METHODS**

**Animals.** Ten 10-wk-old Sprague-Dawley (SD) male rats were obtained from Charles River Japan (Yokohama, Japan) and used in this study according to the Guiding Principles for the Care and Use of Animals, based on the Helsinki Declaration. After the rats had been adequately anesthetized with diethyl ether, the gastrocnemius, soleus, and plantarisk skeletal muscles and testis, brain, liver, and kidney were rapidly excised and washed thoroughly with cold saline to remove contaminating blood, processed, and snap-frozen in liquid nitrogen. These tissue samples were stored at −80°C and were later analyzed for 1) mRNA expression of P450arom, 3β-HSD, and 17β-HSD type I by semiquantitative RT-PCR and real-time quantitative RT-PCR analysis; 2) protein expression of P450arom and 17β-HSD type I by Western blotting analysis and immunofluorescence staining; and 3) total testosterone and estradiol levels by sandwich enzyme immunoassay (EIA) analysis. All of the above and subsequent experimental protocols in the present study were approved by the Committee on Animal Research at the University of Tsukuba.

**Cells.** Myoblasts of skeletal muscle were isolated from 2- to 3-day-old SD rats, as described previously (42). After confluent proliferation for 2 days, myoblast cells were incubated on fibronectin-coated dishes in DMEM-Ham’s F12 medium supplemented with 2 ml of DMEM + 2% horse serum (Cell Garage, Hokkaido, Japan) for 7 days, differentiated to develop myotubes, and then used for further experiments. Briefly, muscle cells were exposed to either vehicle-only control, in 95% air-5% CO2; DHEA, or testosterone for 24 h and then harvested for analysis. DHEA (Wako Pure Chemical Industries, Osaka, Japan) and testosterone (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide and then harvested for analysis. DHEA, or testosterone for 24 h and then centrifuged at 12,000 g at 4°C, and the protein concentration of the resulting supernatant was determined. The samples (50 µg of protein) were then subjected to Western blot analysis at 96°C for 30 min and 40 g centigrade of 94°C for 15 s and 60°C for 1 min. The expression of β-actin mRNA was used as an internal control. The quantitative values of P450arom, 3β-HSD, and 17β-HSD type I mRNA were normalized by that of β-actin mRNA expression.

**Immunoblot analysis.** Tissues and cells were homogenized with 20 mM Tris·HCl (pH 7.8), 300 mM NaCl, 2 mM EDTA, 2 mM DTT, 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 at 4°C, and the protein concentration of the resulting supernatant was determined. The samples (50 µg of protein) were then subject to heat denaturation at 96°C for 7 min with Laemmli buffer. Western blot analysis for detection of P450arom and 17β-HSD type I was performed according to previous reports (27, 44), with minor modifications. Briefly, each sample was separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (Millipore, Billerica, MA) membrane. The membrane was then incubated in blocking buffer of 3% skimmed milk in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) for 1 h at room temperature followed by incubation with primary antibody, monoclonal anti-P450arom antibody (1:1,000 dilution with blocking buffer; Serotec, Kidlington, Oxford, UK), or polyclonal anti-17β-HSD type I (1:1,000 dilution with blocking buffer, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. The membrane was washed with PBS-T three times and incubated with horseradish peroxidase-conjugated secondary antibody, which was an anti-mouse IgG (1:2,000 dilution with blocking buffer; Amersham Biosciences, Piscataway, NJ) and an anti-goat IgG (1:4,000 dilution with blocking buffer, Santa Cruz Biotechnology) for 1 h at room temperature. After a washing as described above, the expression level of each molecule was detected by the ECL Plus system (Amersham Life Sciences), followed by exposure to Hyper film (Amersham Biosciences).

**Immunofluorescence staining.** Immunofluorescence staining was performed, according to our previous report, with minor modifications (18). For immunohistochemical studies of P450arom, frozen tissue samples (gastrocnemius muscle) were cut by cryostat into 10-µm sections, fixed in acetone, and air-dried. To prevent nonspecific staining by secondary antibody, the sections were blocked with nonimmune serum (1% bovine albumin in Tris) for 30 min at room temperature. After an overnight incubation at 4°C with primary antibody, the sections were rinsed in phosphate buffer solution and then exposed to the fluorescein secondary antibody. Cy3-conjugated AffiniPure anti-mouse IgG or fluorescein-conjugated AffiniPure rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), for 2 h according to the manufacturer’s instructions. The samples processed with primary antibodies

**PCR.** Semiquantitative PCR was performed according to the method described in our previous report (14) with minor modifications. Each PCR reaction contained 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, dNTP at 200 mM each, and gene-specific primers at 0.5 µM each, and 0.25% (v/v) ExTq polymerase (Takara). The cycle profile included denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 60 s at 72°C. The amplified PCR products were electrophoresed on 1.2% agarose gels stained with ethidium bromide, visualized with an ultraviolet transilluminator, and photographed (M085D; Polaroid, Waltham, MA).

**Real-time quantitative PCR analysis.** Quantitative real-time PCR was used for measurement of relative mRNA expression (ABI-PRISM 7700 Sequence Detector; PerkinElmer Applied Biosystems), according to our previous report, with minor modifications (15). The gene-specific primers and TaqMan (FAM) probes were designed by Primer Express v. 1.5 software (PerkinElmer Applied Biosystems). The sequences of the oligonucleotide were as follows: P450arom forward: 5’-CGAATTCGATGCTCGTGGAGGAAAG-3’, reverse: 5’-TGCAAATCTCATACTATGTTGTTT-3’, probe: 5’-CTGCGAGAGATTATGCTG-3’, 3β-HSD forward: 5’-GCCCAACTCTCACAAGAGGATC-3’, reverse: 5’-CTCGCCCTCTTCTGTGAT-3’, probe: 5’-ATGTGCTTCTGATTGCTT-3’, 17β-HSD type I forward: 5’-CCTGGGCTCCTCAGGATCT-3’, reverse: 5’-GCAGACCGCAAGGATCC-3’, probe: 5’-TCTCTGACATCCATTTCC-3’, β-actin forward: 5’-GCCCGGAGCCTGCA-3’, reverse: 5’-GCTTGGTGTTGAAGCTG-3’, probe: 5’-AATCCATTGAGGAGCGA-5’.

Each PCR amplification was performed in triplicate using the following profile: 1 cycle of 95°C for 10 min and 40 cycles of 94°C for 15 s and 60°C for 1 min. The expression of β-actin mRNA was used as an internal control. The quantitative values of P450arom, 3β-HSD, and 17β-HSD type I mRNA were normalized by that of β-actin mRNA expression.

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served as negative controls. Immunofluorescent images were observed under a Laser Scanning Confocal Imaging System (MRC-1024; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). In our hands, the intensity of aromatase immunoreactivity in frozen tissue sections was stronger compared with paraffin-embedded tissues. Furthermore, using CYP19 C-16 (Santa Cruz Biotechnology), an affinity-purified goat polyclonal antibody raised against a peptide at the carboxyl terminus of human CYP19, we were able to generate good immunofluorescence staining.

**Sandwich EIA.** Concentrations of total testosterone and estradiol in the skeletal muscle and muscle cell tissue extracts were determined using a sandwich EIA kit (total testosterone: R&D Systems, Minneapolis, MN; estradiol: Cayman Chemical, Ann Arbor, MI) (9, 28, 31). All techniques and materials used in this analysis were in accordance with the manufacturer’s protocols. The immobilized antibodies were monoclonal raised against total testosterone and estradiol, whereas each secondary horseradish-peroxidase-coupled antibody was monoclonal or polyclonal. Optical density was quantified on a microplate reader using BioLumin 960 (Molecular Dynamics, Tokyo, Japan). All samples were assayed in duplicate. The sensitivities of intra- and interassay coefficients of variation for the hormone assays were as follows: total testosterone 0.01–50 ng/ml, 8.9 and 9.3%, respectively; estradiol 0.05–1 μg/l, 5.6 and 6.1%, respectively.

**Statistics.** Values are expressed as means ± SE. Statistical analyses of DHEA and testosterone were carried out by analysis of variance followed by Scheffe’s F-test for multiple comparisons. \( P < 0.05 \) was accepted as significant.

**RESULTS**

To investigate whether or not skeletal muscle cells possess steroidogenic capabilities, we examined the existence of 3β-HSD, 17β-HSD type I, and P450arom mRNAs in skeletal muscle tissue (gastrocnemius) in the rat and compared their levels with various tissues that are known to express these genes, namely the ovary, testis, liver, kidney, and brain (Fig. 1). Consistent with our hypothesis, mRNA expression of 3β-HSD, 17β-HSD type I, and P450arom were detected in the skeletal muscle of rats in vivo. However, the levels of these genes tended to be lower in skeletal muscle than in ovary, testis, and brain tissues. We also sought to examine whether mRNA levels of steroidogenic enzymes in different skeletal muscle types are differentially expressed in the rat (Fig. 2). The mRNA level of 3β-HSD in gastrocnemius was significantly higher than in plantaris (\( P < 0.05 \)), and that of P450arom in soleus was significantly higher than in plantaris (\( P < 0.05 \)). However, the mRNA level of 17β-HSD type I was not significantly different between these fiber types.

We sought to examine changes in protein levels of 17β-HSD type I and P450arom in different tissue types and skeletal muscle. Figure 3 shows immunoblotting data for 17β-HSD type I and P450arom protein expression in ovary, testis, liver,
Protein expressions of 17β-HSD type I and P450arom were detected in the skeletal muscle of rats in vivo. The levels of these proteins tended to be lower in skeletal muscle than in ovary, testis, liver, kidney, and brain (Fig. 3).

Additionally, using immunohistochemistry, we examined the localization pattern of P450arom protein expression in the skeletal muscle, which is characteristically synthesized and localized in the endoplasmic reticulum (Fig. 4). P450arom protein was localized in the cytoplasm and cytoplasmic membrane of skeletal muscle.

To determine whether the detected steroidogenic enzymes in skeletal muscle were capable of generating sex steroid hormones, we measured tissue concentrations of testosterone and estradiol and compared them with various tissues, i.e., ovary, testis, liver, kidney, and brain (Fig. 5). We found that tissue concentration of testosterone in the skeletal muscle (gastrocnemius) was significantly lower than in those of the ovary and testis (P < 0.05). Tissue concentration of estradiol in the skeletal muscle was also significantly lower than in those of the ovary (P < 0.05), whereas those were significantly higher than in those of liver and kidney (P < 0.05).

We confirmed the existence of 3β-HSD, 17β-HSD type I, and P450arom mRNAs in cultured muscle cells in vitro (Fig. 6). Consistent with our hypothesis, mRNA expressions of 3β-HSD, 17β-HSD type I, and P450arom were detected in cultured muscle cells of rats in vitro.

Finally, having detected steroidogenic enzymes in skeletal muscle, we sought to investigate whether skeletal muscles are capable of using circulating DHEA as a substrate or metabolite.

Fig. 3. Protein expression of steroidogenic enzymes in various tissues of rat. Representative images of immunoblotting for 17β-HSD type I and P450arom protein in rat skeletal muscle (gastrocnemius) compared with positive control tissues, namely ovary, testis, liver, kidney, and brain.

Fig. 4. Localization of steroidogenic enzymes protein expression in skeletal muscle of rat. Representative images of immunofluorescence for P450arom in skeletal muscle (gastrocnemius) of male rats (A–D). Aromatase stained red, and immunoreactivity is clearly seen in the cytoplasm and cytoplasmic membrane (A–D) magnification (×200). E: positive staining was also observed in blood vessels (magnification ×200). F: omission of primary antibody showed no immunoreactivity in the skeletal muscle, indicating the specificity of the antibody (magnification ×200).
precursor for local steroidogenesis, as previously reported in other peripheral tissues, by adding increasing concentration of DHEA or testosterone to muscles in culture (Fig. 7). Interestingly, the intracellular levels of testosterone and estradiol in muscle cells increased in a dose-dependent manner by addition of DHEA at 10–500 μM (Fig. 7, A and B). Peak levels of testosterone and estradiol were seen when 500 μM DHEA was added; similarly, when 10–500 μM testosterone was added, the intracellular levels of testosterone and estradiol in muscle cell increased in a dose-dependent manner (Fig. 7, C and D). Peak levels of testosterone and estradiol were seen when 500 μM testosterone was added.

DISCUSSION

The present study revealed expression of mRNA of three steroidogenic enzymes, namely 3β-HSD, 17β-HSD, and P450arom in the skeletal muscle cells of rats both in vivo and in culture (in vitro). We also demonstrated the presence of 17β-HSD and P450arom proteins and an intracellular formation of testosterone and estradiol from DHEA or testosterone in cultured muscle cell in a dose-dependent manner. These findings are novel and important in that they provide the first evidence showing that skeletal muscles are capable of locally synthesizing sex steroid hormones from circulating DHEA, namely 3β-HSD, 17β-HSD, and P450arom, in the skeletal muscle. Levels of these enzymes in the skeletal muscle were comparable to those found in the kidney and liver, implying that skeletal muscle is potentially an important extragonadal source of sex steroid hormones.

Interestingly, we demonstrated, for the first time, a dose-dependent intracellular formation of testosterone and estradiol from DHEA or testosterone in cultured muscle cell. The functional significance of gonadal derived sex steroid hormones has been extensively studied; however, it is only recently that the importance of local extragonadal derived sex steroid hormones in cell physiology and pathophysiology is beginning to be appreciated in tissues such as the nervous, adipose, and adrenal gland (38). In the skeletal muscle cell, interest and evidence supporting a role for local sex steroid hormones, namely testosterone and estrogen, has recently been widely reported (16, 17, 35, 41, 43, 49). However, the exact source of these hormones is unclear. The present report is the first to clearly demonstrate presence and activity of a local metabolic machinery or pathway for the substrate DHEA and the subsequent synthesis of testosterone and estrogen in skeletal muscle tissue. On the basis of earlier reports that skeletal muscle tissues possess receptors for both androgen (AR) and estrogen (ERα and, the most recently isolated subtype, ERβ) in various species (human, bovine, mouse, and rat) and that, as revealed here, they are capable of generating sex steroids locally, we propose the existence of a local autocrine and/or paracrine system of testosterone and estrogen action in skeletal muscle cells. The functional significance of the androgen-to-estrogen ratio and interaction is a concept that is increasingly

Fig. 5. Tissue levels of sex steroid hormones in various tissues of rat. Tissue concentrations of total testosterone and estradiol in skeletal muscle (gastrocnemius) in male rats compared with positive control tissues, namely ovary, testis, liver, kidney, and brain (n = 10). Data are expressed as means ± SE. Statistical analysis was done with post hoc Scheffé’s F-test for multiple comparisons.

Fig. 6. mRNA expression of steroidogenic enzymes in cultured muscle cells. Representative images showing expression of 3β-HSD, 17β-HSD type I, and P450arom mRNA in cultured muscle cell, as revealed by RT-PCR. Rat ovary was used as positive control tissue, and β-actin mRNA was used as an internal control.
becoming more important than single hormone action (7, 38). This ratio is thought to be controlled by aromatase, whose levels are regulated in a complex and tissue-specific manner. Admittedly, at this juncture, the functional significance of the androgen/estrogen ratio in the skeletal muscle is not known.

DHEA, and DHEA-S, is the most abundant endogenous hormone in humans and other mammals, with 50% of the hormone secreted by the adrenal cortex from the classical biosynthetic pathway of pregnenolone and 17β-hydroxypregn- nenolone. The remaining 50% is synthesized by the testis, ovary, and brain (5). For instance, plasma DHEA-S levels in human adults are 100–500 times higher than testosterone and 1,000–10,000 times higher than estradiol (23). Because no receptor for DHEA to date has been identified, it is thought to be more of a metabolic precursor of other steroids, such as testosterone and estrogen (30). Alternatively, it is believed to exert its influence via nongenomic mechanisms. Consistent with the earlier proposal, namely metabolic precursor, the present study demonstrates conversion of DHEA substrate to testosterone and estradiol by steroidogenic enzyme-expressing skeletal muscle cells in culture. Levels of the generated or synthesized hormones in vitro were much higher than those found in intact skeletal muscle (in vivo). This finding is in agreement with previous proposals suggesting that uptake of circulating DHEA by peripheral tissues leads to its conversion to testosterone and estrogens by 3β-HSD, 17β-HSD, and P450arom (7); i.e., DHEA is converted to Δ4-androstenedione and Δ3-androstenediol by 3β-HSD and 17β-HSD, respectively, which are in turn both converted to testosterone. Ultimately, P450arom irreversibly converts testosterone to estrogens. These data are important in that they reveal that circulating DHEA-S as well as testosterone are important substrates or precursors for local production of sex steroid hormones in skeletal muscle.

In postmenopausal women, the secretion of estradiol by the ovaries is impaired, decreasing to levels comparable to those in men (38). This age-induced reduction in steroidogenesis increases the risk for brain, bone, and cardiovascular disorders in postmenopausal women (2, 4, 20). The highly effective local synthesis of sex steroid hormones from DHEA in skeletal muscle tissue, as demonstrated in the present study, would be helpful in differentiating the synthetic capacity of sex steroid hormones between the sexes or dysfunction of steroidogenesis by sexual organ diseases.

The present study reveals that levels of basal mRNA of steroidogenic enzymes, i.e., 3β-HSD and P450arom, are differentially expressed in different skeletal muscle type but not that of 17β-HSD. Slow-twitch fibers, with a rich supply of mitochondria, are highly dependent on oxygen and use fatty acids as their major energy source. On the other hand, fast-twitch fibers are glycolytic and movement muscles. Thus, this difference in gene expression of steroidogenesis-related enzymes may account for differences in contractility and metabolic and vascular properties between slow- and fast-twitch fibers of muscle.

Aromatase was localized in the cytoplasm of the skeletal muscle cells, but not in the nucleus, consistent with its expected location, i.e., endoplasmic reticulum (8, 24). Overall, the detection of the three enzymes of interest by immunohistochemistry correlated with the data that we generated using quantitative techniques, namely Western blot and real-time PCR analysis. These techniques revealed significant levels of protein and gene expression comparable to those reported previously, including PCR (13), immunoblot analysis, immunofluorescence staining, and EIA (18). Importantly, the fact that the protein levels of these enzymes and concentrations of the respective hormones that they synthesize were strikingly similar, as revealed by enzyme immunoassay, implies that they are not only transcribed and translated locally but are functionally active. On Western blots, we detected distinct bands for each enzyme, with molecular masses that were very close to the expected location, i.e., mitochondria, are highly dependent on oxygen and use fatty acids as their major energy source. On the other hand, fast-twitch fibers are glycolytic and movement muscles. Thus, this difference in gene expression of steroidogenesis-related enzymes may account for differences in contractility and metabolic and vascular properties between slow- and fast-twitch fibers of muscle.

DHEA-S is activated to DHEA after hydrolysis of the sulfate group by the microsomal enzyme steroid sulfatase. Thus steroid sulfatase is the key enzyme with important roles for


In conclusion, the novel findings of the present study, demonstrating for the first time the presence of local and active metabolic machinery or pathway (mRNA and protein expressions of 3β-HSD, 17β-HSD, and P450arom) for synthesis of sex steroid hormones (testosterone and estrogen) from circulating DHEA in the skeletal muscle cells, provides further evidence for the presence of an autocrine/paracrine system for sex steroid hormones and their roles in skeletal muscle function and adaptation.

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