Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle

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Campbell, S. E., and M. A. Febbraio. Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle. Am J Physiol Endocrinol Metab 281: E803–E808, 2001.—To examine the roles of 17β-estradiol (E2) and progesterone (Prog) in lipid metabolism, skeletal muscle enzyme activities were studied in female Sprague-Dawley rats. Groups included sham-operated rats (C) and ovariectomized rats treated with placebo (O), E2 (E), Prog (P), both hormones at physiological doses (P + E), or both hormones with a high dose of E2 (P + HiE). Hormone (or vehicle only) delivery was via time-release pellets inserted at the time of surgery, 15 days before metabolic testing. Results demonstrated that carnitine palmitoyltransferase maximal activity was 19, 21, and 19% lower (P < 0.01) in O, P, and P + E rats, respectively, compared with C rats. Conversely, activity in E and P + HiE rats was 14 and 19% higher (P < 0.01) than in C, β-Hydroxacyl-CoA dehydrogenase (β-HAD) maximal activity was 20% lower (P < 0.01) in O than in C rats; similarly, P and P + E rats were 18 and 19% lower, respectively (P < 0.01); however, treatment with E2 returned β-HAD activity to C levels. These results suggest that E2 plays a role in lipid metabolism by increasing the maximal activity of key enzymes in the fat oxidative pathway of skeletal muscle.

ovariectomy; estrogen; progesterone; lipid metabolism

It is well established that the ovarian hormones exert significant metabolic effects, particularly in lipid metabolism (11, 18–20, 31). Accordingly, treatment of male rats with estradiol (E2) significantly increased lipid availability during submaximal exercise (19). Furthermore, treatment of ovariectomized female rats with E2 stimulated fatty acid oxidation during prolonged exercise, whereas treatment with E2 and progesterone (Prog) in combination inhibited this oxidative effect (11). The mechanisms underlying these effects remain poorly understood; however, insight into these functional differences might be gained by examining some of the key enzymes of the major pathways for lipid metabolism in skeletal muscle. Enzymes representative of fatty acid flux in the mitochondria [carnitine palmitoyltransferase I (CPT I), the β-oxidation pathway [β-3-hydroxyacyl-CoA dehydrogenase (β-HAD)], and the tricarboxylic acid cycle [citrate synthase (CS)] were chosen for analysis, as these enzymes are important in the lipid oxidation pathways.

CPT I is considered to be the rate-limiting step in the oxidation of long-chain fatty acids (24), and in the liver its activity has previously been demonstrated to be influenced by estrogens (39). Although skeletal muscle and liver CPT I isoforms have exhibited many similarities, tissue-specific differences have been found in molecular masses (40) and sensitivity to inhibition by malonyl-CoA (26); therefore, further investigation of the effect of the ovarian hormones on skeletal muscle CPT I is warranted. Similarly, β-HAD is an important enzyme in the β-oxidation pathway in skeletal muscle (13), and its activity has been correlated with muscle fatty acid oxidative capacity (27). β-HAD activity is known to be altered in response to dietary manipulation and by exercise; the basis of this control is believed to be, in part, through substrate availability (12). Given that E2 seems to increase lipid availability (19, 31), it is possible that it may have some effect on β-HAD activity. Conversely, CS is thought to be a key regulatory enzyme in the tricarboxylic acid cycle, and thus of oxidative metabolism (23). Once through the β-oxidation pathway, fatty acid moieties enter the tricarboxylic acid cycle for further biochemical processing. Interestingly, Holt and Rhe (14) demonstrated that both β-HAD and CS activities in epithelial cells of the rat uterus increased with E2 treatment; however, this response was cell specific and was not seen in stromal or myometrial cells. This differential response demonstrates the necessity for tissue-specific assessment of the effect of E2 on enzyme activities.

The present study aimed to examine the effect of ovariectomy, as well as E2 and Prog replacement therapies, on the maximal activity of these key enzymes for lipid oxidation. Because the ovarian hormones have been demonstrated to have both individual (11, 18–20) and concerted (11, 18) metabolic effects, with Prog often inhibiting the effects of E2 (11, 18), replacement therapies used physiological levels of E2 and Prog separately and in combination. It was hypothesized that the maximal activities of fat oxidation enzymes would

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be negatively affected by the absence of the ovarian hormones, whereas treatment with E₂ would improve their function. Prog was expected to inhibit the effect of E₂ but to have little effect on its own. This study will help determine how the ovarian hormones affect lipid oxidation in skeletal muscle.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats 12–15 wk old and weighing 201.8 ± 4.9 (SE) g were used in these experiments. All animals were housed in a temperature-controlled room (21 ± 2°C) with a 12:12-h light-dark cycle. Water was available ad libitum, and rats were given 20 g of standard rat chow/day to control food intake. The amount of rat chow administered daily was selected on the basis of pilot work from our laboratory that demonstrated that food intake of 10% of body weight was sufficient to maintain body weight over the duration of the experiment. This experiment was approved by the Animal Research Ethics Committee of the University of Melbourne.

Experimental design. Rats were bilaterally ovariec- tomized or sham operated under sodium bretial anesthesia (60 mg/kg ip). Groups of ovariec- tomized rats were treated immediately with time-release hormone pellets (Innovative Research of America) inserted subcutaneously, with either E₂ (2.5 mg/day; E), Prog (1.5 mg/day; P), both hormones at the same doses (P + E), or both hormones with pharmacological levels of E₂ (25 µg/day; P + HiE). Groups of intact (C) and ovariec- tomized (O) rats were treated with vehicle-only placebo pe- llets. Animals were treated for 14 full days postoperation before metabolic testing. Efficacy of the ovariec- tomies and sex steroid treatments was confirmed by plasma E₂ and Prog levels. Animals were treated for 14 full days postoperation before metabolic testing. Efficacy of the ovariec- tomies and sex steroid treatments was confirmed by plasma E₂ and Prog levels.

Metabolic testing. Animals remained sedentary during the 14-day treatment period. Rats were fasted for 12 h before the commencement of metabolic testing. On the morning of day 15, animals were effectively suffocated with CO₂ (80:20, 15% CO₂-O₂), rendering them unconscious in <20 s. A midline incision was made, and the diaphragm was cut to ensure death. A cardiac puncture was performed, the blood was spun at 7,000 g for 2 min, and the plasma was removed and stored at −80°C for analysis of the sex steroids, insulin, free fatty acid (FFA), and glycerol. The muscles of the hindlimb were rapidly exposed, the gastrocnemius was removed and dis- sectected into white and red portions, and all visible connective tissue was removed. The red and white muscles were each divided into the following two portions: the first was placed on ice in precooled buffer for immediate isolation of mitochondria and determination of CPT I activity, and the second was frozen in liquid nitrogen and freeze-thawed three times to disrupt the mitochondrial membranes.

Isolation of intact mitochondria. The mitochondrial isolation method was as previously described by Jackman and Willis (15), and the entire procedure was performed at 0–4°C. The buffer solutions were as follows: solution I, 100 mM KCl, 40 mM Tris-methane (Tris-HCl), 10 mM Tris base, 5 mM MgCl₂, 1 mM EDTA, and 1 mM ATP, pH 7.4; solution II, 100 mM KCl, 40 mM Tris-HCl, 10 mM Tris base, 1 mM MgSO₄, 0.1 mM EDTA, 0.2 mM ATP, and 1.5% fatty acid-free BSA, pH 7.4; solution III, same as solution II without BSA; solution IV, 220 mM sucrose, 70 mM mannitol, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4.

Muscle samples (~50–70 mg wet muscle) were weighed, finely minced, gently homogenized with a hand-driven all- glass homogenizer in 20 vol/wt of solution I, and centrifuged at 700 g for 10 min. The supernatants were collected and further spun at 14,000 g for 10 min. The mitochondrial pellets were gently resuspended in 10 vol/wt of solution II and centrifuged at 7,000 g for 10 min. This pellet was resuspended in 10 vol/wt of solution III and spun at 3,500 g for 10 min. Finally, the pellet was resuspended in 1 vol/wt of solution IV, gently rendered homogenous, and subsequently kept on ice for enzymatic analysis. This procedure extracts mainly subsarcolemmal mitochondria that contain very low levels of contaminants, as previously described (2).

CPT I activity. CPT I (EC 2.3.1.21) activity measurements were carried out with the sensitive forward radioisotope assay, as previously described (25) and modified by Starritt et al. (37). Briefly, this assay measured the amount of labeled palmitoyl-CoA and labeled l-carnitine that were added to the medium surrounding the intact mitochondria. The standard incubation mixture contained the following in a volume of 90 µl: 117 mM Tris-HCl (pH 7.4), 0.28 mM reduced glutathione, 4.4 mM ATP, 4.4 mM MgCl₂, 16.7 mM KCl, 2.2 mM KCN, 40 mg/l rotenone, 0.5% BSA, 100 µM palmitoyl-CoA, and 2 mM l-carnitine with 1 µCi of L-[¹H]carnitine. The reaction was initiated by the addition of 10 µl of mitochondrial suspension (1:3 dilution) and was stopped 6 min later by the addition of 60 µl of ice-cold 1 M HCl. Palmitoyl-[¹H]carnitine formed during the reaction was extracted with 400 µl of water- saturated butanol in a process involving a series of washes with distilled water and subsequent centrifugations (1,000 g for 5 min) to separate the butanol phase. Radioactivity was measured in 100 µl of the butanol layer and 5 ml of liquid scintillation cocktail. All assays were performed in duplicate at 37°C.

CPT I activities were expressed in terms of whole muscle (µmol·min⁻¹·kg wet muscle⁻¹). This was achieved by refer- encing the CPT I activity in the mitochondrial suspension to the ratio of CS activity of the intact mitochondria in the suspension to that of the whole muscle homogenate.

Whole muscle homogenate preparation. Muscle samples (~20–30 mg wet muscle) were mechanically homogenized in 50 vol/wt of a 100 mM phosphate buffer solution containing 0.5 g/l BSA, pH 7.3. The homogenates were frozen in liquid nitrogen and freeze-thawed three times to disrupt the mitochondrial membranes.

CS and β-HAD activities. CS activity was measured spec- trophotometrically as previously described (36). CS activity was assayed in the whole muscle homogenate samples, in the intact mitochondrial suspension (1:20 dilution), and in the mitochondrial suspension after disruption of the mitochondria membranes by three freeze-thaw cycles and the addi- tion of 1% Triton X-100 to the assay medium. β-HAD was assayed spectrophotometrically as previously described (22). Both assays were performed in duplicate at 25°C.

Blood hormone and metabolite assays. Plasma E₂, Prog, and insulin were measured by commercially available double-antibody RIA kits (Diagnostic Products and Pharmacia & Upjohn, respectively). Plasma glycerol was measured by a fluorometric assay linked to NADH. Samples were deprotein- iated and incubated for 1 h in a solution containing the following: 1.0 M hydrazine, 0.2 M glycine, 1 mM EDTA, 2 mM MgCl₂, 0.2 mM NAD⁺, 0.5 mM ATP, pH 9.8, and the enzymes glycerokinase and glycerol 3-phosphate. Production of NADH is in a one-to-one ratio with glycerol.

Statistics. All statistical comparisons were made using one- or two-way ANOVA tables, as appropriate, with signif- icance set at the P < 0.05 level. Specific differences were located with Scheffe’s F-test post hoc comparison. All data statistics were compared using the Statistica software pack- age, and data are reported as means ± SE.
RESULTS

Characteristics of experimental animals. The animal characteristics are presented in Table 1. Plasma E2 and Prog concentrations confirmed the efficacy of the ovariectomies and sex steroid treatments. No significant differences were found among the mean values of either initial or final body weight. Plasma insulin values were significantly elevated in the P rats (P < 0.05) when compared with C, E, P + E, and P + HiE rats. There were no differences in insulin levels among any of the other treatment groups. Additionally, there were no significant differences in plasma FFA or glycerol values among any of the six groups of rats (Fig. 1).

Enzyme activities. Enzyme activities are presented in Table 2. In red gastrocnemius, CPT I maximal activity was 19, 21, and 19% lower (P < 0.01) in O, P, and P + E rats, respectively, compared with C rats. Activity in E and P + HiE rats was 14 and 19% higher (P < 0.05), respectively, compared with C. Findings were similar in white gastrocnemius, with CPT I maximal activity 19, 17, and 19% lower (P < 0.01) in O, P, and P + E rats, respectively, than C rats, and E and P + HiE rats were 20 and 25% higher (P < 0.01), respectively. Results indicated no between-group differences in CS maximal activity in either red or white gastrocnemius at physiological concentrations; however, CS maximal activity in red gastrocnemius was 19% higher (P < 0.02) in P + HiE compared with O.

Maximal activity of β-HAD was 20% lower (P < 0.01) in O than in C rats in red gastrocnemius. β-HAD activity was also reduced in P and P + E rats (18 and 19% lower, respectively, P < 0.01) compared with C rats; however, treatment with E2 (E) returned β-HAD activity to C levels. Interestingly, pharmacological doses of E2 restored levels to normal, despite the presence of Prog, as the P + HiE group was 19% (P < 0.01) higher than the P + E group. No significant differences were found in β-HAD maximal activity in the white gastrocnemius in physiological concentrations of E2; however, pharmacological levels of E2 increased the maximal activity of β-HAD to at least 25% above (P < 0.01) O, P, and P + E groups.

DISCUSSION

This study demonstrated that the ovarian sex steroids participate in the control of the maximal activity of several key enzymes of lipid oxidation in both red and white skeletal muscle. Ovariectomy decreased CPT I and β-HAD enzyme activities to below control levels. Maximal activities of these enzymes were restored by treatment with E2 (E) but not by treatment with Prog (P). Interestingly, the combination of E2 and Prog (P + E) produced similar maximal activities to those of ovariectomized animals, demonstrating that Prog inhibited E2 in physiological concentrations. However, this inhibitory effect was overridden in the pres-
groups. Additionally, with the exception of P rats, plasma insulin was also similar between treatment groups. Although previous studies have found differences in these parameters (5, 30), these groups did not control food intake and therefore had significant differences in weight gain, which is likely to affect insulin homeostasis. Insulin is a known inhibitor of lipolysis (41); thus, differences in plasma insulin levels would result in differences in plasma FFA and glycerol values. Of note, short-term administration (5 days) of E2 to male rats also resulted in elevated plasma FFA concentrations, with no change in body weight (8). Unfortunately, this study did not measure insulin levels, which can also be affected by short-term administration of the ovarian hormones (9), so it is unknown if this effect was independent of changes in plasma insulin. Furthermore, because of differences in receptor populations, males can respond differently to E2 administration (10), which could also account for the discrepancy in these results. Interestingly, despite elevated insulin levels in Prog-treated rats, plasma FFA and glycerol levels were not different from those of other groups, indicating that the insulin sensitivity had not yet become severe enough to alter lipid availability. This suggests that controlling for diet may help attenuate the negative effects of ovariectomy, or treatment with Prog, on insulin homeostasis and lipolysis, at least in the short term. This is not too surprising, given that the first intervention in the treatment of type II diabetes includes diet regulation.

Results from this study demonstrate that the ovarian hormones can alter the activity of CPT I; however, it is interesting to note that a recent study has reported no gender differences in this parameter (1). Although these results may at first seem paradoxical, several factors must be considered. First, there is no mention of the menstrual cycle by these authors, so it is unknown whether this variable was controlled for in their female subjects. In the present study, we observed that the ovarian hormones had divergent effects on the activity of CPT I and that these effects varied depending on their relative concentrations. Both the absolute and relative concentrations of estrogen and Prog change throughout the menstrual cycle and could therefore alter the activity of CPT I. Thus, if the menstrual cycle was not controlled for, this could result in a high variability that could mask the subtle effects of the ovarian hormones. Additionally, testosterone is also a steroid hormone that is capable of altering muscle metabolism and hence should be considered in gender comparisons as well. Therefore, although no gender differences were observed (1), this does not exclude the possibility of the ovarian hormones altering the activity of CPT I. Finally, differences in the regulation of lipid metabolism in the rat compared with human have previously been observed (35); thus, rat CPT I may respond differently to the ovarian hormones. Hence, interspecies differences remain a limitation in this study.

A possible mechanism by which the ovarian hormones alter the activities of CPT I and β-HAD is by...
upregulating their gene transcription, which would result in changes in the protein levels of these enzymes. This could have a potent effect on the overall activity of these enzymes, particularly when expressed per gram of tissue wet weight. Indeed, increases in hepatic CPT I activity as a result of dehydroepiandrostosterone were found to be closely correlated to increases in transcription rates and mRNA concentration (3). Dehydroepiandrostosterone is a precursor for steroid hormone synthesis and can result in elevated estrogen or testosterone levels. Additionally, the estrogen-signaling pathway has recently been recognized to play a pivotal role in constitutive hepatic expression of genes involved in β-oxidation (6, 28). Both the estrogen and Prog receptors are present in skeletal muscle (16); hence, this is a viable pathway for the ovarian hormones to exert their metabolic effects.

Of note was the difference in the enzyme activities between the P + E group, which received physiological concentrations of both E2 and Prog, and the intact C rats. This may best be explained by the fact that the intact rats were cycling normally and were therefore exposed to elevated Prog levels (40–50 ng/ml) for only ~12 h during the proestrous phase of their 4- to 5-day cycle (38). Furthermore, control rats were killed at various times throughout their estrous cycle, so that a more representative collection of data points could be obtained; therefore, these pooled results incorporate the changing levels of the ovarian hormones. Conversely, the P + E rats were exposed continuously to high levels of Prog; therefore, the P + E rats would have a greater tendency to exhibit any inhibitory characteristics of Prog.

It is tempting to associate the enhanced enzymatic activities seen in the presence of E2 with increased fatty acid oxidation in skeletal muscle. There is, however, some concern as to whether the maximal flux rate in a metabolic pathway depends on the absolute activities of the enzymes that catalyze the near-equilibrium reactions (29). Because fat oxidation was not measured in this study, this brings into question the physiological significance of the difference in the metabolic patterns observed with different hormone therapy treatments. The lower maximal activities of the fat oxidative enzymes would imply a lower maximal flux rate in the O, P, and P + E animals; however, such speculations must be treated with caution. Nonetheless, the data help offer a possible explanation for the increase in lipid oxidation seen with hormone replacement therapies in ovariectomized rats (11). It is interesting to note that the effect of E2 and that of E2 + Prog on enzyme activities seen in this study are both of a comparable magnitude to the altered lipid oxidation data from Hatta et al. (11). Furthermore, the activity of β-HAD has recently been correlated with whole body fat oxidation during exercise in humans, suggesting that changes in β-HAD activity result in changes in the capacity for fat oxidation capacity (27). Hence, it is likely that, by altering the maximal activity of CPT I and β-HAD, the ovarian hormones influence the ability of skeletal muscle to oxidize lipids, particularly in times of metabolic stress.

The importance of matching cellular fatty acid utilization rates to energy demand is emphasized by the dramatic consequence of genetic defects that result in a limited capacity for fatty acid oxidation. Clinical symptoms of mitochondrial enzymatic defects can include hypoglycemia, liver dysfunction, and cardiomyopathies (32). Hence, alteration in lipid homeostasis by the ovarian hormones may result in mild manifestations of these symptoms, particularly in cases with additional metabolic or cardiovascular complications, as are often prevalent in aging postmenopausal women. Interestingly, women treated with tamoxifen, a potent antiestrogen, are at increased risk of developing a fatty liver and nonalcoholic steatohepatitis (33). Furthermore, Djouadi et al. (6) have recently demonstrated that male peroxisome proliferator-activated receptor-α null mice treated with etomoxir, a CPT I inhibitor, have a 75% mortality rate compared with only 25% in female mice. Additionally, pretreatment of the males with E2 could reverse this effect, thereby implicating E2 in the maintenance of energy homeostasis. Correspondingly, pathologies such as obesity or diabetes could also be affected by altered metabolic capacity. In fact, decreased lipid oxidation in both of these conditions has been linked to an impaired capacity of lipolytic and oxidative enzymes in skeletal muscle (4, 17, 34) and therefore may also be influenced by the ovarian hormones. Consequently, consideration of hormonal status (amenorrhea, pregnancy, or menopause) could be helpful in the control of these and other metabolic diseases.

Of particular interest in these results is the inhibition of the lipolytic effects of E2 by Prog at physiological concentrations seen in both CPT I and β-HAD. This inhibition was abolished with pharmacological concentrations of E2, demonstrating that, in high enough concentrations, E2 can override the inhibitory effect of Prog. This could have significant clinical effects when considering long-term treatment of amenorrheic or postmenopausal women with oral contraceptive or hormone replacement therapy. In these situations, it may be prudent to treat with an estrogen-based therapy vs. either Prog or combination therapies. Conversely, if considering a combination hormone replacement therapy, our data suggest the utilization of elevated levels of E2 to offset any inhibition by Prog. It is interesting to note that similar results have been found with carbohydrate metabolism. Women using combination therapy demonstrated poorer glucose homeostasis and insulin sensitivity than those using estrogen alone (7, 21).

In conclusion, the absence of the ovarian hormones in female rats resulted in a decrease in the maximal capacity of skeletal muscle to oxidize lipids. Treatment with E2 restores this ability to at least control levels, whereas treatment with Prog alone has no effect. If ovariectomized animals are treated with both E2 and Prog in physiological concentrations, Prog inhibits the lipolytic effect of E2, and this is only restored with pharmacological concentrations of E2.
REFERENCES


