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 I (CPT I) activity in E and P + HiE rats was 14 and 19% higher (P < 0.01) than in C, β-Hydroxyacyl-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 isozymes 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 E\textsubscript{2} would improve their function. Prog was expected to inhibit the effect of E\textsubscript{2} 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 ovariectomized or sham operated under sodium brietal anesthesia (60 mg/kg ip). Groups of ovariectomized rats were treated immediately with time-release hormone pellets (Innovative Research of America) inserted subcutaneously, with either E\textsubscript{2} (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 E\textsubscript{2} (25 mg/day; P; + HiE). Groups of intact (O) and ovariectomized (O) rats were treated with vehicle-only placebo pellets. Animals were treated for 14 full days postoperation before metabolic testing. Efficacy of the ovariectomies and sex steroid treatments was confirmed by plasma E\textsubscript{2} and Prog RIA kits (Diagnostic Products) at the end of the treatment period.

**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 CO\textsubscript{2} (80:20, 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 spectrophotometrically 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 mitochondrial membranes by three freeze-thaw cycles and the addition 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 E\textsubscript{2}, Prog, and insulin were measured by commercially available double-antibody RIA kits (Diagnostic Products and Pharmacia & Upjohn, respectively). Plasma glyceral was measured by a fluorometric assay linked to NADH. Samples were deproteinated and incubated for 1 h in a solution containing the following: 1.0 M hydradine, 0.2 M glycine, 1.0 mM EDTA, 2.0 mM MgCl\textsubscript{2}, 0.2 mM NAD\textsuperscript{+}, 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 glyceral.

**Statistics.** All statistical comparisons were made using one- or two-way ANOVA tables, as appropriate, with significance 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 package, 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 < 0.01), 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-

Table 1. Animal characteristics

<table>
<thead>
<tr>
<th></th>
<th>C (n = 10)</th>
<th>O (n = 11)</th>
<th>E (n = 9)</th>
<th>P (n = 9)</th>
<th>P + E (n = 9)</th>
<th>P + HiE (n = 10)</th>
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<tbody>
<tr>
<td>Final body wt, g</td>
<td>216 ± 5</td>
<td>209 ± 5</td>
<td>212 ± 5</td>
<td>210 ± 6</td>
<td>212 ± 7</td>
<td>208 ± 8</td>
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<tr>
<td>Plasma E2, pg/ml</td>
<td>36.3 ± 3.7b–f</td>
<td>16.9 ± 0.9a,c,e,f</td>
<td>46.0 ± 3.4a,b,d–f</td>
<td>16.8 ± 0.8a,c,e,f</td>
<td>50.4 ± 4.6a–d,f</td>
<td>1.594 ± 90–w</td>
</tr>
<tr>
<td>Plasma Prog, ng/ml</td>
<td>35.6 ± 4.7b,c</td>
<td>9.5 ± 1.0a,d–f</td>
<td>10.3 ± 0.7a,d–f</td>
<td>45.8 ± 1.5b,c</td>
<td>49.6 ± 1.9e,c</td>
<td>52.3 ± 35b,c</td>
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<tr>
<td>Plasma insulin, pmol/l</td>
<td>45.4 ± 3.2z</td>
<td>54.7 ± 3.2</td>
<td>45.2 ± 3.4d</td>
<td>58.1 ± 3.1a,c,e,f</td>
<td>45.9 ± 1.9d</td>
<td>44.9 ± 1.6d</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. C, intact placebo group; O, ovariectomized rats treated with placebo; E, ovariectomized estradiol (E2)-treated group; P, ovariectomized progesterone (Prog)-treated group; P + E, ovariectomized E2 + Prog-treated group; P + HiE, ovariectomized high E2 (pharmacological) + Prog. P < 0.05 compared with C (a), O (b), E (c), P (d), P + E (e), and P + HiE (f). *P < 0.05, main effect of exercise compared with rest.

Table 2. Maximal enzyme activities

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>O</th>
<th>E</th>
<th>P</th>
<th>P + E</th>
<th>P + HiE</th>
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<tbody>
<tr>
<td>Red gastrocnemius</td>
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<tr>
<td>CPT I, nmol·min⁻¹·g⁻¹ wet muscle⁻¹</td>
<td>496.0 ± 8.5b–f</td>
<td>410.2 ± 7.0a,c,f</td>
<td>574.4 ± 9.0b,d–f</td>
<td>390.2 ± 4.6a,c,f</td>
<td>403.5 ± 6.1a,c,f</td>
<td>614.2 ± 11.3a–w</td>
</tr>
<tr>
<td>CS, nmol·min⁻¹·g⁻¹ wet muscle⁻¹</td>
<td>34.4 ± 0.8</td>
<td>30.0 ± 1.4f</td>
<td>34.0 ± 1.3</td>
<td>34.2 ± 1.3</td>
<td>33.3 ± 0.9</td>
<td>36.8 ± 1.4b</td>
</tr>
<tr>
<td>β-HAD, nmol·min⁻¹·g⁻¹ wet muscle⁻¹</td>
<td>15.6 ± 0.4b–e</td>
<td>12.5 ± 0.8a,c,e,f</td>
<td>15.4 ± 0.2d,e</td>
<td>13.6 ± 0.6a,c,f</td>
<td>13.5 ± 0.4a,b,c</td>
<td>15.6 ± 0.4b–e</td>
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<tr>
<td>White gastrocnemius</td>
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<tr>
<td>CPT I, nmol·min⁻¹·g⁻¹ wet muscle⁻¹</td>
<td>255.1 ± 6.9b–f</td>
<td>206.5 ± 3.4a,c,e,f</td>
<td>320.4 ± 7.2b,d,e</td>
<td>212.7 ± 4.8a,d,e</td>
<td>204.6 ± 5.8a,c,f</td>
<td>344.5 ± 5.9b,d,e</td>
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<tr>
<td>CS, nmol·min⁻¹·g⁻¹ wet muscle⁻¹</td>
<td>11.5 ± 1.0</td>
<td>10.0 ± 0.6</td>
<td>11.6 ± 0.8</td>
<td>10.8 ± 0.8</td>
<td>11.5 ± 0.8</td>
<td>12.9 ± 0.8</td>
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<tr>
<td>β-HAD, nmol·min⁻¹·g⁻¹ wet muscle⁻¹</td>
<td>3.5 ± 0.2</td>
<td>2.9 ± 0.3f</td>
<td>3.7 ± 0.2</td>
<td>3.3 ± 0.2f</td>
<td>2.9 ± 0.1f</td>
<td>4.4 ± 0.2b–e</td>
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</table>

Values are means ± SE. CPT I, carnitine palmitoyltransferase; CS, citrate synthase; β-HAD, β-hydroxacyl-CoA dehydrogenase. P < 0.05 compared with C (a), O (b), E (c), P (d), P + E (e), and P + HiE (f).
ence of pharmacological concentrations of E2 (P + HiE). These data suggest that the ovarian hormones are capable of altering the capacity for skeletal muscle to oxidize FFAs. Although this study did not directly measure fatty acid oxidation, E2 has previously been demonstrated to increase fat oxidation during exercise in ovariecotomized rats, whereas E2 and Prog together had no effect (11). Hence the alteration in CPT I and b-HAD maximal activity observed in the present study provides a possible mechanism for these changes in oxidative capacity. Of note, the b-HAD enzyme measured in this study catalyzes the oxidation of short-chain fatty acids, whereas CPT I is involved in long-chain fatty acid oxidation. Because both enzymes were affected by ovariectomy and hormone replacement therapy, this suggests that the ovarian hormones can influence fat oxidation regardless of chain length. Accordingly, the absence of E2 has recently been demonstrated to negatively affect the activity of very long-, long-, and medium-chain acyl-CoA dehydrogenases in the liver (28), indicating the E2 has a similar effect on all of the acyl-CoA dehydrogenase enzymes, regardless of the fatty acyl chain length they bind.

By controlling the food intake, and hence weight gain, of the rats in this study, we maintained similar plasma FFA and glycerol levels across all treatment 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 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 b-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 dehydroepiandrosterone were found to be closely correlated to increases in transcription rates and mRNA concentration (3). Dehydroepiandrosterone 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


