Effect of Ovariectomy and Estradiol Replacement on Skeletal Muscle Enzyme Activity in Female Rats

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In female rats, ovariectomy (OVX) is associated with increased body fat and insulin resistance, and estradiol replacement prevents these alterations. These metabolic changes related to the estrogen-deficient state might be due, in part, to alterations in skeletal muscle substrate metabolism. We tested the hypothesis that estradiol affects the regulation of enzymes involved in substrate oxidation and storage within skeletal muscle. Specifically, we examined enzymes involved in the regulation of glycogen synthesis (glycogen synthase [GS]), glycolysis (phosphofructokinase [PFK]), tricarboxylic acid cycle activity (citrate synthase [CS]), and β-oxidation (β-hydroxyacyl-CoA dehydrogenase [β-HADH]). Twenty-two, female Sprague-Dawley rats (7 to 8 weeks old) were separated into 3 groups: OVX + placebo (P; n = 8), OVX + estradiol (E_2; n = 8), and sham-operated (S; n = 6). Rats from E_2 and P groups were pair-fed to the S group to control for OVX-induced changes in food intake. After 16 days, activities of GS, PFK, CS, and β-HADH were measured in vastus medialis muscle. GS fractional velocity was significantly lower ($P < .05$) in P (mean ± SE; 39.7% ± 6.2%) compared with both S (61.9% ± 8.8%) and E_2 (65.8% ± 8.4%) rats. In addition, E_2 rats (41.4 ± 2.0) had significantly higher ($P < .05$) CS activity than P (34.9 ± 2.0) and S (33.9 ± 1.4 μmol/min/g) groups. There was no effect of OVX or estradiol replacement on β-HADH or PFK. Our results suggest that, independent of alterations in food intake, estradiol availability affects the regulation of enzymes involved in nonoxidative glucose disposal (GS) and oxidative metabolism (CS) in skeletal muscle.

OVARIAN HORMONE deficiency has profound effects on energy and substrate metabolism. In rodents, ovariectomy (OVX) is associated with body fat accumulation and insulin resistance.[1-6] Conversely, estradiol replacement after OVX prevents such changes.[4-6] These results suggest that ovarian hormones, estradiol, in particular, regulate energy and substrate metabolism. However, because hyperphagia is a well-recognized response to OVX and is prevented if estradiol is replaced, many of the effects attributed to estradiol may be explained primarily by changes in food intake.[6] The possibility that estradiol regulates substrate metabolism independent of changes in food intake has not been examined directly.

Skeletal muscle is of primary importance in the regulation of energy and substrate metabolism. Alterations in enzymatic pathways of substrate oxidation or storage in skeletal muscle may contribute to changes in adiposity and glucose metabolism.[7-9] Reduced activity of β-hydroxyacyl-CoA dehydrogenase (β-HADH), the enzyme controlling β-oxidation of fatty acids is associated with increased fat gain.[9] Moreover, reduced glycogen synthase (GS) activity, together with reduced glucose transport and phosphorylation, are of central importance in skeletal muscle insulin resistance.[8] Thus, estradiol may partially regulate energy and substrate metabolism through its effects on skeletal muscle enzyme activity. Relatively few studies, however, have examined the effect of estradiol on enzymatic pathways of substrate utilization and storage in skeletal muscle.[5,10-12] Moreover, only one of these studies has controlled for the confounding effects of changes in food intake.[10] Thus, it is difficult to differentiate between the effects of estradiol and OVX-induced hyperphagia on skeletal muscle enzyme activities.

In the present study, we examined the effects of OVX with and without estradiol replacement on the enzymatic profile of skeletal muscle. The enzymes measured participate in the regulation of glycogen synthesis (GS), glycolysis (phosphofructokinase [PFK]), β-oxidation of fatty acids (β-HADH), and tricarboxylic acid cycle activity (citrate synthase [CS]). We pair-fed all OVX animals to sham-operated rats to control for the effect of estradiol on food intake. This experimental paradigm permitted us to examine the effect of estradiol deficiency and replacement, per se, on the regulation of skeletal muscle enzyme activity independent of changes in food intake.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats weighing 175 to 200 g (7 to 8 weeks old) were purchased from Taconic (Germantown, NY) and were housed singly in wire-bottom cages. Rats were maintained on a 12-hour:12-hour light/dark cycle in a temperature-controlled (21.1°C ± 0.2°C) room. Tap water and chow (Harlan-Teklad LM-485; Harlan-Teklad, Madison, WI) were available ad libitum before initiation of the study. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Vermont and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC). We have previously reported data from these animals regarding the effects of ovarian hormones on skeletal muscle protein synthesis and lipoprotein lipase activity.[13]

Protocol

Rats were divided into 3 groups: sham-operated (S), OVX plus placebo (P), and OVX plus estradiol (E_2). Four days after their delivery to the laboratory, baseline body composition was measured by total body conductivity under light methoxyflurane anesthesia. Bilateral

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ovariectomy or sham operations were performed the following morning under acepromazine (2.5 mg/kg) and ketamine (75 mg/kg) anesthesia. In the OVX groups, placebo or 17 β-estradiol (0.1 mg, 21-day release pellets; Innovative Research of America, Sarasota, FL) were placed subcutaneously in the subscapular region. These pellets were chosen to produce plasma 17β-estradiol levels between 10 and 30 pg/mL, (personal communication, Innovative Research of America, October 2000). Buprenorphine was administered directly after surgery (0.025 mg/kg) and every 12 hours thereafter for 36 hours.

S animals were permitted ad libitum access to food and water for the next 16 days. Food intake was monitored daily by weighing the amount of chow (powdered to facilitate food intake measurement) consumed over each 24-hour period. Food intake for the S group for each 24-hour period was averaged and this amount fed to the 2 OVX groups. The average daily food intake during the 16-day study period was similar among S (19 g $\pm$ 3 g), P (18 g $\pm$ 1 g) and E_2 (18 g $\pm$ 1 g) groups. On the afternoon of day 16, body composition was measured under light methoxyflurane anesthesia.

On day 17, rats were anesthetized using acepromazine (2.5 mg/kg) and ketamine (75 mg/kg). Approximately 20 minutes after injection of anesthesia, rats were decapitated, blood was collected, and their right hindlimb stripped of skin, removed, and placed in ice-cold saline. The vastus medialis muscle of the right thigh (primarily type II fibers)[14] was dissected, blotted dry, and frozen in liquid nitrogen. All samples were kept at -80°C until analysis.

Body Composition

Fat mass and fat-free mass were measured by total body electrical conductivity using an EM-SCAN Model SA-2 small animal body composition analyzer (EM-SCAN, Springfield, IL). The average of 4 measurements was used to calculate fat-free mass (g) from the following equation: $22.69 + (0.1185 * E^{0.513}) L^{1.42}$, where E is the electrical conductivity measure and L is the nasoanal length (cm). Body fat was calculated by subtracting fat-free mass from body mass. Prior studies show no effect of steroid hormones on body water content or the hydration of lean tissue.[15,16] Moreover, body composition estimated using electrical conductivity correlates closely with similar measurements derived from densitometry, total body water, and chemical analysis.[17] Thus, we believe that total body electrical conductivity provides reliable estimates of body composition.

Tissue Homogenization

Samples of frozen muscle tissue ($\approx$ 10 mg wet muscle) were homogenized with a Potter Elvejem homogenizer for 2 minutes in an aliquot of enzyme-specific homogenizing buffer. The homogenate was centrifuged for 10 minutes at 700 g and 4°C, and the resulting supernatant was decanted off. This supernatant was kept on ice until use. Muscle enzyme assays were performed with minor modifications from the methods of Passonneau and Lowry,[18] and activities were expressed as μmol/min/g wet muscle.

GS

GS activity was assessed by measuring the rate of uridine diphosphate formation (end product of glycogen synthesis released from uridine diphosphate glucose in the elongation of glycogen) using an enzymatic cascade sequence that ultimately produced fluorescent nicotinamide adenine dinucleotide (NAD). GS activity was assayed under 2 concentrations of glucose-6-phosphate: (1) subsaturated (GS I + D_{subsat}); 0.03 mmol/L glucose-6-phosphate and (2) saturated (GS I + D_{sat}); 10 mmol/L glucose-6-phosphate. These conditions measure the glucose-6-phosphate independent (I) and dependent form (D) of GS at subsaturated and saturated concentrations of glucose-6-phosphate, respectively. Fractional velocity of GS was calculated as the ratio of GS activity at a physiologic (subsaturated) glucose-6-phosphate concentration (GS I + D_{subsat}) to the total activity at a saturating glucose-6-phosphate concentration (GS I + D_{sat}).[8,19,20]

Muscle samples were homogenized with a 1:50 dilution of buffer (40 mmol/L $KHPO_4$, 0.5 mmol/L EDTA, 5 mmol/L 2-mercaptoethanol, 0.02% bovine serum albumin (BSA), 50% glycerol, pH 7.4). Aliquots of the supernatant were added to a reagent solution (45 mmol/L imidazole, 18 mmol/L HCl, 0.03% glycogen, 210 mmol/L KCl, 2.3 mmol/L EDTA, 9 mmol/L NaF, 0.5 mmol/L dithiorthreitol [DTT], pH 7.4) in the 2 conditions and incubated for 60 minutes at 37°C. After the incubation, a second reagent solution (25 mmol/L imidazole, 75 mmol/L HCl, 30 mmol/L KCl, 0.06% BSA, 0.25 mmol/L phosphoenolpyruvate, 25 U/mL pyruvate kinase, 3.25 U/mL lactic dehydrogenase, 0.16 mmol/L NADH, pH 7.0) was added to each tube and allowed to incubate for 20 minutes at 37°C. This reaction was then stopped with the addition of 1 N HCl and allowed to stand for 10 minutes. NaOH-imidazole was added, and the tubes were incubated for 20 minutes at 60°C. The fluorescence was read at 360/460 nm. Intra- and interassay coefficients were 11.1% and 8.5% for I + D_{subsat} and 3.4% and 9.7% for I + D_{sat}.

CS

Measurement of CS activity was based on the reduction of 5,5'-dithiobis-2-nitrobenzoate by the CoA-SH released in the cleavage of acetyl-CoA during the formation of citrate. Muscle samples were homogenized with a 1:15 dilution of buffer (0.18 mol/L KCl, 2 mmol/L EDTA, pH 7.4). This homogenate underwent 2 freeze-thaw cycles to ensure freeze-fracture of the mitochondria and was added to reagent cocktail (76 mmol/L Tris, 0.1 mmol/L 5,5'-dithiobis-2-nitrobenzoate, 0.2 mmol/L acetyl-CoA, 0.5 mmol/L oxaloacetate). Homogenate was added to the tube, and the change in absorbance over time was monitored at 412 nm for 4 minutes. Intra- and interassay coefficients were 1.0% and 16.2%, respectively.

PFK

Activity of PFK was measured using an indirect assay based on the rate of appearance of fructose-1,6-diphosphate (F-1,6-P_2). The F-1,6-P_2 produced was used in a specific enzymatic cascade to produce fluorescent NAD. Muscle samples were homogenized with a 1:250 dilution of buffer (same as GS). Homogenate or standard was added to reagent solution (25 mmol/L Tris Base, 25 mmol/L Tris HCl, 1 mmol/L adenosine triphosphate [ATP], 1 mmol/L fructose-6-phosphate, 1 mmol/L adenosine monophosphate [AMP], 10 mmol/L K_2HPO_4, 2 mmol/L $MgCl_2$, 0.04% BSA, 1 mmol/L 2-mercaptoethanol, 0.2 mmol/L NADH, 2.7 U/mL aldolase, 1 U/mL α-glycerophosphate dehydrogenase-triosephosphate, pH 8.1) and incubated 60 minutes at 25°C. The reaction was stopped with the addition of 0.7 N HCl and allowed to stand for 20 minutes. NaOH–imidazole was added, and the tubes were incubated for 20 minutes at 60°C. Fluorescence was read at 360/460 nm. Intra- and interassay coefficients were 5.0% and 4.6%, respectively.

β-HADH

Activity of β-HADH was measured by monitoring the rate of NAD appearance in the conversion of acetoacetyl-CoA to β-hydroxyacetyl-CoA. Muscle samples were homogenized with a 1:150 dilution of buffer (40 mmol/L $KHPO_4$, 0.5 mmol/L EDTA, 0.02% BSA, 50% glycerol, pH 7.4). Homogenate or standard was added to reagent solution (135 mmol/L imidazole–HCl, 15 mmol/L imidazole, 0.05% BSA, 1 mmol/L EDTA, 200 μmol/L acetoacetyl-CoA, 0.2 mmol/L NADH, pH 6.1) and incubated for 60 minutes at 25°C. The reaction was stopped with the addition of 0.5 N HCl and allowed to stand for 10 minutes. NaOH–imidazole was added, and the tubes were incubated for

Table 1. Body Size and Composition

Variable	S (N = 6)		P (N = 8)		E_2 (N = 8)	
	Pre	Post	Pre	Post	Pre	Post
Length (cm)	19.5 ± 0.2	20.5 ± 0.1	19.5 ± 0.1	20.7 ± 0.1	19.0 ± 0.1	19.8 ± 0.1*
Body mass (g)	191 ± 4.6	231 ± 5.8	185 ± 3.2	227 ± 3.6	188 ± 2.7	204 ± 2.0*
Fat mass (g)	43 ± 2.7	53 ± 1.8†	44 ± 1.4	38 ± 2.7	47 ± 1.3	40 ± 2.0
Fat-free mass (g)	149 ± 4.3	178 ± 5.7	141 ± 2.2	190 ± 3.3	142 ± 2.7	164 ± 2.5*

NOTE. Values are mean ± SE.

Abbreviations: S, sham-operated; P, ovariectomized + placebo; E_2 ovariectomized + estradiol.

*$P < .05$ P and S greater than E_2.

†$P < .05$ S greater than P and E_2.

20 minutes at 60°C. The fluorescence was read at 360/460 nm. Intra- and interassay coefficients were 2.1% and 1.6%, respectively.

Substrate Measurements

Plasma free fatty acid (FFA) concentrations were measured by a colorimetric assay using a commercially available kit (Wako Chemicals, Richmond, VA). Plasma glucose concentrations were measured using a colorimetric assay (Wako Chemicals). Intra- and interassay coefficients were 0.9% and 6.8% for FFA and 3% and 0.4% for glucose.

Statistical Analysis

Analysis of variance (ANOVA) was used to determine differences among groups. If a significant group effect was found, a Fisher post hoc test was used to identify the location of differences among groups. Because GS fractional velocity had an abnormal distribution (Shapiro-Wilk test; $P < .05$), $\log_{10}$-transformed values were used. Analysis of covariance (ANCOVA) was used to examine differences among groups after controlling for changes in fat mass. All data are expressed as mean ± SE, unless otherwise specified.

RESULTS

Nasoanal length, body mass, fat mass, and fat-free mass data at baseline and day 16 are shown in Table 1. No differences at baseline were found among groups. Differences were noted, however, in the change in body composition during the study. The S group gained ($P < .001$) fat mass (10.4 g ± 2.4 g), while both P (-5.7 g ± 2.2 g) and E_2 (-6.3 g ± 2.8 g) groups lost fat mass. In addition, the P group gained more ($P < .05$) fat-free mass (48.2 g ± 2.4 g) than either S (29.4 g ± 7.1 g) or E_2 (22.3 g ± 2.1 g) groups. Collectively, these changes resulted in a lower ($P < .05$) increase in body mass in the E_2 (16.0 g ± 2.3 g) than either S (39.8 g ± 5.2 g) or P (42.5 g ± 2.4 g) groups. The pattern of differences among groups was similar when body size and composition variables were expressed as a relative change from baseline (data not shown).

Activities of skeletal muscle enzymes are shown in Table 2. There were no differences among groups in GS activity at subsaturated or saturated glucose-6-phosphate concentrations. Activity of CS was greater ($P < .05$) in E_2 than both S and P groups. No differences were found in PFK or β-HADH activity. Differential changes in fat mass among groups may partially contribute to differences in skeletal muscle enzyme activity. Thus, we adjusted CS activity for changes in fat mass. After adjustment for changes in fat mass, group differences in CS persisted between E_2 (40.6 ± 2.0) and P (34.2 ± 2.0) groups ($P < .05$), although the difference between E_2 and S (35.7 ± 3.0 μmol/min/g wet muscle) groups was no longer significant ($P = .24$). There were no differences noted among groups in β-HADH/CS (an index of the capacity for β oxidation relative to total oxidative capacity) or the ratio of PFK/β-HADH (an index of the capacity for glycolysis *v* β-oxidation).

A significant group effect ($P < .05$) was found for GS fractional velocity P (39.7% ± 6.2%), S (61.9% ± 8.8%), and E_2 (65.8% ± 8.4%) (Fig 1). Using the log-transformed values, P was significantly lower ($P < .05$) than both S and E_2 rats. Statistical adjustment for changes in fat mass did not affect group differences in GS fractional velocity, with P rats (34.9% ± 7.6%) remaining lower ($P < .05$) than both S (75.6% ± 11.5%) and E_2 rats (60.3% ± 7.8%). Additionally, differences in GS activity were not due to variation among groups in circulating fat substrates because FFA was significantly higher ($P < .05$) in E (0.586 ± 0.037) rats than both P (0.409 ± 0.041) and S (0.334 ± 0.051 mmol/L). Plasma glucose was significantly higher ($P < .05$) in P (10.0 ± 1.0) rats than both E (7.5 ± 0.3) and S (7.9 ± 0.3 mmol/L) groups.

DISCUSSION

This study investigated the effects of estradiol on enzymatic pathways of substrate oxidation and storage in skeletal muscle in female rats. The majority of previous studies addressing this question had not controlled for the effect of estradiol on food intake.[1-5] Thus, it has been difficult to differentiate between the effects of estradiol and OVX-induced hyperphagia on skeletal muscle enzyme activity. Using a pair-feeding design, we were able to examine the effects of estradiol on specific skeletal muscle enzymes independent of changes in food intake. Our results suggest that estradiol may affect the regulation of enzymes involved in nonoxidative glucose disposal (GS) and oxidative metabolism (CS) in skeletal muscle.

Table 2. Skeletal Muscle Enzyme Activities (μmol/min/g wet muscle)

Enzyme Measurement	S	P	E_2
GSI + D_{subsat}	0.7 ± 0.4	0.6 ± 0.3	1.1 ± 0.5
GSI + D_{sat}	1.2 ± 0.7	2.0 ± 0.9	2.0 ± 1.0
PFK	0.43 ± 0.07	0.51 ± 0.1	0.45 ± 0.07
CS	33.9 ± 1.4	34.9 ± 2.0	41.4 ± 2.0*
β-HADH	4.4 ± 0.3	4.8 ± 0.3	4.7 ± 0.2

NOTE. Values are mean ± SE.

*$P < .05$ greater than values for P and S.

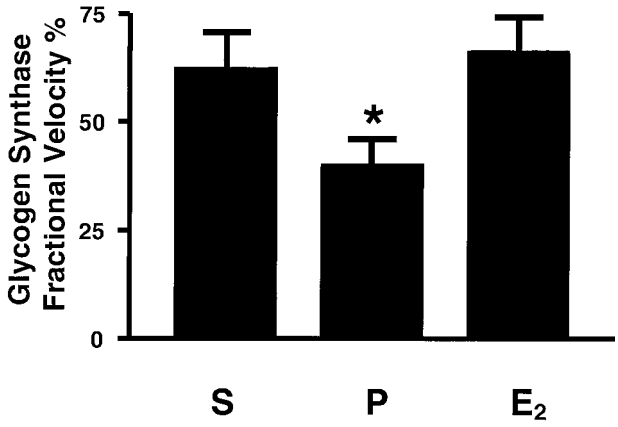

Fig 1. GS fractional velocity in skeletal muscle of sham-operated (S), ovariectomized, and placebo-replaced (P) and ovariectomized and estradiol-replaced (E_2) female rats (bars represent data after statistical control for changes in fat mass). *$P < .05$) E_2 and S greater than P.

Fractional velocity is defined as the ratio of GS activity at a physiologic glucose-6-phosphate concentration to the total activity at a saturated glucose-6-phosphate concentrations and is reflective of in vivo rates of glycogen synthesis.[8,19,20] OVX decreased GS fractional velocity, and this effect was reversed with estradiol treatment (Fig 1). This suggests that estradiol affects the regulation of glycogen synthase activity. Our finding is in accord with prior studies[3,5] that found decreased incorporation of glucose into glycogen in ovariectomized rats and mice. In addition, Rincon et al[4] reported decreased protein expression of GS in ovariectomized rats. Collectively, these prior studies suggest that OVX-induced reduction in GS content may explain the reduced glycogen synthase activity and glycogen formation observed during OVX. Despite these similar results, however, previous studies did not control for food intake. Therefore, it was unclear whether these effects of estradiol on GS were due to the hormone, per se, or hyperphagia. To our knowledge, this is the first study to demonstrate an effect of estradiol availability on skeletal muscle GS fractional velocity independent of changes in food intake.

Despite our pair-feeding paradigm, there were differential changes in body composition among groups. Specifically, S rats gained fat, whereas P and E_2 groups lost fat. This potentially could be explained by an effect of ovarian hormones on energy expenditure, because food intake was kept constant. It has been suggested that ovarian hormones affect energy balance by altering energy expenditure.[21] In the present study, however, we did not measure energy expenditure. Irrespective of the mechanism, we must consider the potential effects of differences in body composition on skeletal muscle enzyme activities. For example, adiposity may partially regulate GS activity.[22] Thus, variations in GS among groups may be due, in part, to differences in the change in fat mass. For several reasons, however, we do not believe this to be the case. First, differences among groups in GS fractional velocity do not parallel differences in the change in fat mass. That is, we would expect GS fractional velocity to be increased in the groups that lost body fat, P and E_2 rats, compared with S rats. Second, after statistical adjustment for changes in fat mass, differences in GS fractional velocity persisted. Finally, group differences in GS activity were not related to plasma free fatty acid levels, an adipose tissue metabolite thought to mediate the effect of adiposity on insulin sensitivity.[23] Specifically, variation in free fatty acid concentrations among groups did not explain the pattern of differences in glycogen synthase. In fact, free fatty acid concentrations were significantly higher ($P < .05$) in E rats compared with both P and S groups (see Results). Thus, despite the elevated free fatty acid level in E rats, GS fractional velocity was similar to S rats. Taken together with our pair-feeding paradigm, we believe that the effects of estradiol deficiency and replacement on the regulation of GS activity were independent of changes in both food intake and adiposity.

CS activity was greater in E_2 rats compared with P and S groups. Unlike the pattern observed with GS, however, CS activity was not decreased in P relative to S rats. Thus, estradiol availability does not appear to regulate CS activity. Instead, the possibility exists that our experimental conditions produced the differences noted in CS activity. The constant release of estradiol from the pellets may have different effects on CS activity compared with the pulsatile release of estradiol in vivo in the S group. Indeed, previous studies have shown that constant-release progesterone replacement after OVX has differing effects on blood volume and pressure despite achieving plasma concentrations similar to normal cycling levels.[24] Additionally, it is plausible that estradiol levels were higher in the E_2 group compared with both P and S groups, although plasma estradiol was not measured. Thus, although the availability of estradiol, per se, did not modulate CS, our results suggest that either the concentrations or cyclicity of release of estradiol may partially regulate CS activity.

There was no effect of estradiol treatment on PFK or β-HADH activity. These results are similar to those observed by Campbell and Febbraio,[10] who found no difference in β-HADH activity among sham-operated controls, OVX + placebo, and OVX + estradiol rats in skeletal muscle (white gastrocnemius) of similar fiber type (mostly type IIB fibers[10]) to the muscle used in the present study. In contrast, Campbell and Febbraio[10] did find an effect of estradiol in the red gastrocnemius muscle (primarily type I fibers[10]) suggesting that the effects of estradiol on fat metabolism may be muscle or fiber type-specific. In addition, we found no differences among groups in the ratio of β-HADH/CS (an index of the capacity for β-oxidation relative to total oxidative capacity) or PFK/β-HADH (an index of the capacity for glycolysis *v* β-oxidation). Thus, it does not appear that OVX or estradiol treatment specifically alters the enzymatic capacity for the oxidative metabolism of glucose or fatty acids in fast-twitch skeletal muscle. We should note, however, that recent findings suggest that estradiol may affect fat oxidation by regulating the entry of fatty acids into the mitochondria via carnitine palmitoyltransferase in both fast- and slow-twitch skeletal muscle.[10]

In conclusion, our findings suggest that estradiol may affect the regulation of GS and CS activity in female rats. Our pair-feeding design allowed us to examine the effects of estradiol on skeletal muscle enzyme activities without the confounding effects of changes in food intake. In addition, the effects of estradiol on muscle enzymes are likely independent of changes

in adiposity. Taken together, our results suggest that estradiol may partially regulate the enzymatic capacity of skeletal muscle for nonoxidative glucose disposal (GS) and overall oxidative metabolism (CS).

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