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Exercise and ovarian steroid hormones
Their effects on mitochondrial respiration

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Abstract

The effect of exercise on mitochondria respiration was studied in gastrocnemius muscle of ovariectomized rats, pseudopregnant rats, and estrous rats. The estrous cycles were followed by vaginal smears. Rats were made pseudopregnant (PSP) by 45 s cervical stimulation with a glass rod on the day of estrous. The treadmill protocol (21m/min, 10 grade uphill) induced a significant decrease in state 3 oxygen consumption (oxidative phosphorylation) in estrous (0.26 ± 0.02 *vs.* 0.49 ± 0.05 μatoms O min^{-1} mg $protein^{-1}$) and ovariectomized rats (0.18 ± 0.03 *vs.* 0.40 ± 0.03 μatoms O min^{-1} mg $protein^{-1}$). In contrast, pseudopregnant and progesterone-treated ovariectomized rats did not decrease state 3 nor state 4 respiratory rates. These results show that the effect of exercise on mitochondria respiration does vary according to the hormonal status.

Keywords: Ovarian hormones; Mitochondria respiration; Exercise; Skeletal muscle; Rat

Introduction

It seems to be clear that exercise produces many modifications to skeletal muscle. Since Gollnick and King [1] have reported damage in muscle mitochondria after exercise, several studies have been focussed on the relationship between mitochondria and exercise. In this regard, opposite results were found. Terjung et al. [2] have found neither ultrastructural nor biochemical changes in muscle mitochondria after exercise. In contrast, McCutcheon et al. [3] have observed mitochondria alteration after exercise in horses.

The integrity of the mitochondria membrane is essential to the oxidative phosphorylation function. Any disruption of this membrane is likely to affect the cellular respiratory capacity.

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Conflicting results were reported in mitochondrial function of rats running until exhaustion. While some authors have reported no changes in mitochondrial respiration [2,4], others reported a decrement in their oxygen consumption after similar exercise [5]. Meanwhile a two week-training protocol does not elicit any change in the oxidative capacity of the gastrocnemius muscle [6], endurance exercise increases mitochondria enzyme levels, such as cytochrome c and citrate synthase, enhancing its oxidative capacity. Thus, mitochondria activity could be affected by exercise according to their oxidative capacity. While Terjung used a variable protocol, which ranged from a 6 to 12 week training period [2], Dohm subjected the animals to a fixed (12 weeks) exercise program length [5]. Therefore, a possible explanation to the diverging results could be the different running protocols used.

Even though several reports show a relationship between ovarian cycle and muscle function, there is no information about mitochondrial respiration during different stages of the ovarian cycle. Chen and Tang [7] showed that inspiratory muscle endurance during the midluteal phase of the menstrual cycle was greater than in the midfollicular phase. Phillips et al. [8] observed maximum voluntary force during the follicular phase and a rapid fall at the time of ovulation. Sarwar et al. [9] reported that the quadriceps muscle was stronger and showed more fatiguability during the ovulatory phase. Maximum isometric force in mice was observed during the luteal phase [10]. Bar et al. [11] suggest that estradiol plays a role in protecting skeletal muscle since ovariectomized female rats present post-exercise damage like males, and estradiol treatment in females, as well as in males, reduce post-exercise damage. On the contrary, Tiidus et al. [12] observed estrogen treatment induced reduction in vitamin C concentration in muscle, liver and heart in male rats, and concluded that estradiol would enhance the susceptibility to the effects of exercise. The objective of this work was to study the effect of exercise on mitochondria respiration in different moments of the ovarian cycle in rats. Consequently, we study the effect of exercise on mitochondria respiration in four different groups of rats: ovariectomized, ovariectomized which received progesterone replacement therapy, 10th day pseudopregnant, and first estrous after pseudopregnancy.

Methods

Chemicals

All chemicals were reagent grade obtained from Sigma Chemical Co., St. Louis, MO, USA unless otherwise indicated.

Animals

Ovariectomized and normal cycling female Sprague-Dawley rats, obtained from the Institute colony, were housed in an environmentally controlled room (22 $\pm$ 2°C) with reversed light-dark cycles in order to allow them to exercise in darkness (lights on from 08:00 pm to 08:00 am). Animals were provided with food and water *ad libitum* until the time of the experiment. The average weight was 233.7$\pm$ 3.1 g and did not differ from ovariectomized, pseudopregnant, estrous or ovariectomized rats that received progesterone treatment.

Daily vaginal smears were taken to establish the timing of the estrous cycle in each cycling rat, and this was continued throughout pseudopregnancy. Rats were made pseudopreg-

nant (PSP) by 45 s cervical stimulation with a glass rod on the day of estrous. The first appearance of leukocytes denoted day 1 of pseudopregnancy.

Progesterone treatment, to those ovariectomized rats who received steroid replacement, was given in the form of implants made from Sylastic tubing (40 mm long × 3.2 mm i.d., Dow-Corning, Midland, MI., USA). Two progesterone filled implants were inserted s.c. in the back of the neck 10 days before the experiment.

Exercise protocol

The animal procedures were reviewed and approved by the Animal Research Committee from our Institution.

Animals were trained to run in a treadmill, starting with the training protocol 10 days before the experiment. Its speed was progressively increased until animals were able to run at 21 m min^{-1} on a 5° uphill incline.

The day of the experiment, the animals of each treatment group were randomly assigned to exercise control group (24 h rest) and exercise group. Animals in the exercise groups run at 21 m min^{-1} on 10° uphill for 90 minutes. The treatment groups were 1) ovariectomized, 2) ovariectomized which received progesterone replacement therapy, 3) 10th day pseudopregnant, and 4) first estrous after pseudopregnancy.

Tissue sampling

Exercise animals were killed by decapitation immediately following the exercise session. Control animals were killed in the same way 24 hrs following their last training session. Troncal blood was collected and allowed to clot. Serum aliquots were collated and stored frozen at −20°C until assay.

Metabolites determination

Gastrocnemius muscles were rapidly exposed and they were frozen *in situ* by means of metallic tongs. Frozen tissue was ground to a fine powder in a mortar under liquid nitrogen, weighed and extracted with 3 ml perchloric acid 2 N per g. Precipitated protein was sedimented by centrifugation. The supernatant was neutralized with KOH and the $KClO_4$ precipitate was sedimented by centrifugation. The supernatant was made 0.6N NaOH, incubated 10 min to inactivate enzymes which survived acid extraction, and then taken to pH 7.0. Lactate concentration was assessed by a fluorometric assay [13]. Muscle glycogen concentration was determined by an enzyme-coupled assay using a modification of the method of Passonneau and Lauerdale [14] and it is expressed as glucose units. Muscle dry weight was assessed by weighing the tissue before and after freeze-drying.

Mitochondrial fraction preparation

Entire gastrocnemius muscle were homogenized in buffer A (20 mM Tris, 300 mM sucrose, 1 mM NaN_3 pH 7.4) with three bursts of 10 s in a Ultra-Turrax homogenizer (IKA-Labortechnik, Germany). Homogenization was done in the presence of 0.5 mM-PMSF (phenylmethylsulphonil fluoride), 25 μM-ZPCK (N-CBZ-L-phenylalanine chloromethyl ketone), 25 μM-TLCK (N′-p-tosyl-lisine chloromethyl ketone) and 25 μM-TPCK (L-1-tosylamide-

2-phenyl-ethylchloromethyl ketone), as protease inhibitors. All centrifugation steps were carried out in a Sorvall RC5B (DuPont Wilmington, DE, USA) with a SS 24 rotor. Homogenate was centrifuged at 800 *g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 14,000 *g* for 10 min. The supernatant from the 14,000 *g* centrifugation was saved; the pellet was resuspended in buffer A and centrifuged again at 14,000 *g* for 10 min. The pellet was resuspended in buffer A and used as a mitochondrial fraction.

Mitochondrial respiration

Oxygen uptake was measured polarographically at 37°C with a Clark-type oxygen electrode mounted in a 0.6 ml volume temperature-controlled chamber (Instech Laboratories, Inc. Horsham, PA, USA). It was assessed immediately after obtaining the mitochondrial fraction. Mitochondria were diluted to a protein concentration of 100 $\mu g\ ml^{-1}$ in incubation medium, which consisted of 250 mM Sucrose, 5 mM $MgCl_2$, 20 mM KCl, 7 mM K_2O_4P, 10 mM HEPES pH 7.0. State 4 (S4) respiration rates were measured with 5 mM glutamate, malate, pyruvate, and succinate, either combined or alone, as substrates. After 5 min, respiration state 3 (S3) was initiated by addition of 175 μM ADP; after oxygen consumption reached a plateau, ADP at the same concentration was added again. For uncoupled respiration, 40 μM 2,4 dinitrophenol (DNP) was added instead of ADP. S3 and S4 respiratory rates, the P/O ratios, and the respiratory control ratio were calculated as described by Chance and Williams [15].

The structural integrity of mitochondria was determined using substrate 2 mM NADH in the presence of ADP. None of the preparations tested from control or run rats were capable of oxidizing NADH.

Spectrophotometric assays of marker enzymes were performed in a Gilford Response spectrophotometer (Ciba-Corning Diagnostic Corp, USA) at 37 °C. Citrate synthase was assessed according to Srere [16], malate dehydrogenase as described by Mullinax at al. [17] and succinate-cytochrome c reductase activity was determined by the method of Tisdale [18].

Radioimmunoassay of progesterone and estradiol were performed as described [19]. Protein determination was performed by the method of Lowry et al. [20] using bovine serum albumin standard. Samples were digested with 1 N NaOH before assaying.

Statistical analyses

Statistical significance was determined by two-way ANOVA followed by a linear contrast. Results are given as mean ± SE. A p value of <0.05 was considered to be statistically significant.

Results

The ovariectomized-trained rats, which run for 90 min, showed an increase in serum lactate level. This increase in lactate concentration was not seen in the gastrocnemius muscle, indicating the moderate intensity of the exercise. Gastrocnemius muscle water content in the experimental groups did not differ from the control one, meanwhile glycogen concentration fell to about 60%, indicative of the intermediate strength of the exercise (Table 1).

The activities of the mitochondrial marker enzymes in the mitochondrial fraction from gastrocnemius muscle of the exercised animals were not different from those found in the

Table 1
Effect of exercise on serum lactate and lactate, glycogen and water content of gastrocnemius muscle in ovariectomized trained rats.

Group	Serum Lactate	Lactate	Glycogen	Dry Weight
Control	1.24 ± 0.22 (8)	4.55 ± 0.25 (8)	13.7 ± 2.8 (6)	244 ± 2.0 (n=7)
Run	1.66 ± 0.16 *(8)	3.54 ± 0.3 (8)	5.6 ± 0.6 *(6)	240 ± 2.3 (n=6)

Serum lactate concentrations are expressed as mmol. l^{-1}, metabolites as μmol. g wet $weight^{-1}$ and dry weight as mg. g wet weight. Values are mean ± SE for the number of rats given in parentheses.

* $P<0.05$ compared to control value.

rested group (Table 2). Demonstrating that the exercise protocol and mitochondria isolation procedure we have used did not produce organelle disruption and leakage of matrix enzymes.

The phosphorylative function of muscle mitochondria showed a different behavior after exercise, depending on the hormonal status of the experimental rats. Oxygen consumption in ovariectomized rats was altered by exercise. In the absence of ADP (S4 conditions), there was no difference between control and 90-min run rats. In contrast, S3 mitochondrial respiration showed a significant decrease after the exercise (Figure 1). State 4 respiration rate was measured with glutamate, malate, pyruvate, and succinate, either combined or alone. Exercise decreased ($p<0.05$) S3 respiration rate when either of the substrates were used (data not shown). Hence, mitochondrial respiration was measured using malate plus glutamate in the other groups. Therefore, the mitochondrial data in this paper will deal with the results obtained from malate plus glutamate. The reduction in oxygen consumption in S3 was not due to either an impairment in translocation of ADP or an inhibition of the F_1-ATPase complex, since the addition of the protonophore DNP did not increase respiration to control levels (Figure 1, insert).

To search for a possible involvement of the steroid hormone in mitochondrial respiration, we used rats made PSP by cervical stimulation, due to the ultra short estrous cycle in the rat. These rats have a luteal phase of 12–14 days and were used in the 10th day of PSP (serum progesterone 80.0 ± 6.4 ng ml^{-1}, n=10) or on the day of the first estrous after PSP (serum estradiol 36.2 ± 4.5 pg ml^{-1}, n=10).

When the experiment was done with rats in the day of estrous after PSP, similar results to those in ovariectomized rats were obtained. That is, a similar S4 in run and control groups and a diminished S3 mitochondrial respiration in the run group compared to that in the control group (Figure 2). However, running in the luteal phase, which means exercising with high levels of progesterone, neither produce modifications in S3 nor S4 oxygen consumption

Table 2
Enzyme activities in mitochondria from gastrocnemius muscles of control and run ovariectomized rats.

	Succinate Dehydrogenase	Malate dehydrogenase	Citrate synthase
Control	0.32 ± 0.02	33.3 ± 7.9	5.46 ± 1.26
Run	0.28 ± 0.06	38.2 ± 6.2	7.28 ± 2.31

Control: animals not exercised the day of experiment, Run: animals exercised for 90 min.

Enzymatic activities are expressed as μmol min^{-1} mg $protein^{-1}$.

Values are mean ± SE (n= 5).

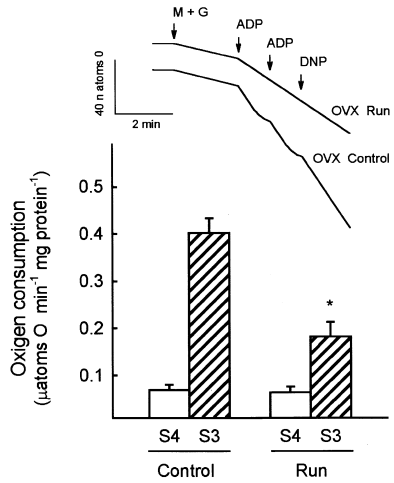

Fig. 1. Oxidative Respiratory State 4 (S4) And Phosphorylative State 3 (S3) In Isolated Mitochondria From Ovariectomized Rats. Control: animals not exercised the day of the experiment, Run: animals exercised for 90 min Oxygen consumption rates expressed as μatoms O min^{-1} mg $protein^{-1}$. Values are mean ± SE for n=7 rats. * Significantly lower than control values at $P<0.05$. Insert depicts a typical registry of oxygen consumption; arrows indicate the moment of additions of malate plus glutamate (M+G, S4), two consecutive of ADP (S3) and 2,4 dinitrophenol (DNP, uncoupled respiration).

(Figure 3). The changes elicited by exercise on S3 in ovariectomized and estrous rats are reflected in the respiratory control ratio (RCR, the rate of oxidation in S3/the rate of oxidation in S4) which was diminished in those groups.

However, phosphorylative respiration remained tightly coupled since the ratio between phosphorylated ADP to oxygen consumed (P/O) were unchanged by exercise in all the stages of the ovarian cycle (Table 3).

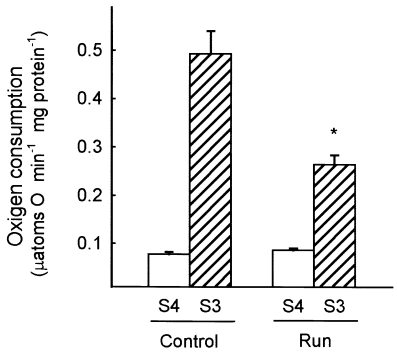

Fig. 2. Oxidative Respiratory State 4 (S4) And Phosphorylative State 3 (S3) In Isolated Mitochondria From Estrous Rats. Control: animals not exercised the day of experiment, Run: animals exercised for 90 min. Estrous indicate the presence of enucleate cornified cells in the vaginal smears after 12 days of luteal phase.Oxygen consumption rates expressed as μatoms O min^{-1} mg $protein^{-1}$. Values are mean ± SE for n=9 rats. * Significantly lower than control values at $P<0.05$.

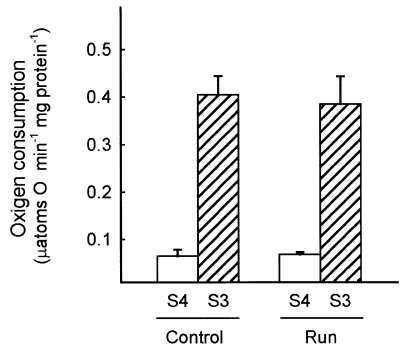

Fig. 3. Oxidative Respiratory State 4 (S4) And Phosphorylative State 3 (S3) In Isolated Mitochondria From PSP Rats. Control: animals not exercised the day of experiment, Run: animals exercised for 90 min. PSP rats were used in the 10th day of luteal phase. Oxygen consumption rates expressed as μatoms O min^{-1} mg $protein^{-1}$. Values are mean ± SE for n=7 rats.

Preincubation of mitochondria from ovariectomized control rats with the post-mitochondrial supernatants from the exercised animals for one hour in an ice bath did not modify S3 respiration rate. Also *in vitro* incubation in the same condition, with progesterone (1 and 0.1 μg ml^{-1}), estradiol (0.1 μg and 0.1 ng ml^{-1}) or both steroids together in the same concentration, did not produce any change in mitochondrial respiration either in run or in control rats (data not shown).

Progesterone administration to ovariectomized rats, by means of Sylastic implants, partially mimics the steroid serum level of PSP rats (41 ± 2.8 ng ml^{-1}, n=8). Under this condition S3 respiration, somehow lower in these animals than in the control of the other groups, was not affected by exercise (Figure 4).

Discussion

The controversial reports on the effects of exercise on mitochondrial respiration [2, 4, 5] could not be attributed to the muscle oxidative capacity. Even though, animals were trained

Table 3
Respiratory parameters of mitochondria from gastrocnemius muscles of control and run rats at different stages of ovarian cycle.

	Ovariectomy		Estrous		PSP	
Group	P/O ratio	RCR	P/O ratio	RCR	P/O ratio	RCR
Control	3.00 ± 0.08	6.6 ± 1.2	2.87 ± 0.08	6.5 ± 0.7	2.95 ± 0.07	6.4 ± 0.7
Run	2.97 ± 0.07	3.2 ± 0.5*	2.90 ± 0.09	3.1 ± 0.3*	2.90 ± 0.08	5.9 ± 1.0

Control: animals not exercised the day of experiment, Run: animals exercised for 90 min.

Estrous indicates the presence of enucleate cornified cells in the vaginal smears after 12 days of luteal phase. PSP rats were in the 10th day of luteal phase. P/O ratio: amount of ATP produced per oxygen consumed. RCR: respiratory control ratio. Data were taken from the experiments of Figures 1, 2 and 3. Values are mean ± SE, n=7 for ovariectomized and PSP rats, n=9 for estrous rats.

* $P<0.05$ compared to control value.

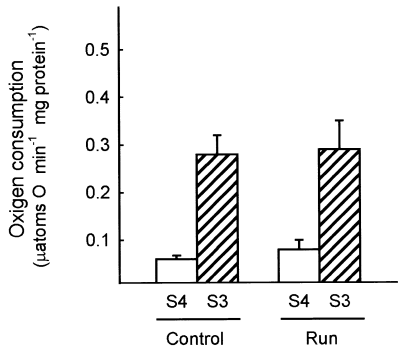

Fig. 4. Oxidative Respiratory State 4 (S4) And Phosphorylative State 3 (S3) In Isolated Mitochondria From Progesterone Treated Ovariectomized Rats. Control: animals not exercised the day of experiment, Run: animals exercised for 90 min Progesterone treatment was done for 10 days by s.c. Sylastic implants. Oxygen consumption rates expressed as μatoms O min^{-1} mg $protein^{-1}$. Values are mean ± SE for n=4 rats.

under different durations of the total length and exercise session [2,4], both protocols exceed the period of time, where a further increase does not enhance the new established mitochondrial oxidative capacity [21]. In our study we used a two week-training protocol, which does not produce an increase in the aerobic condition of the rats [6]. Under this state it was reported that exercised animals showed no mitochondrial respiration levels different from the control [2, 4]. In contrast, we found a decrease in oxygen consumption when the hormonal status was taken into account. Ovariectomized as well as estrous rats showed lowered S3 than those obtained from their respective controls. Conversely, mitochondrial respiration in pseudopregnant and ovariectomized/progesterone treated rats was not affected by exercise.

Starkov et al. [22] reported that male sex hormones and progesterone, when added to the isolated rat liver mitochondria before or after the addition of protonophores, reverse the protonophore-induced uncoupling. Our results with steroids supplemented *in vitro* preclude any direct action of this kind. Incubation with up to 10 times the physiological serum levels of progesterone, estradiol, or both together, neither restore nor diminished S3 respiration of mitochondria in ovariectomized rats and those rats in luteal phase. Conversely, *in vivo* treatment with progesterone has prevented the lowering of S3 respiration.

Our finding that estrous animals have a higher S3 than the pseudopregnant or ovariectomized rats suggest that estradiol could enhance mitochondrial respiration in control animals. Moreover, in control animals, we found a higher S3 phase in pseudopregnants (PSP) than in ovariectomized/progesterone treated rats. The increase of PSP's serum estradiol levels observed at day 10 [23], could account for these results. Since only the ovariectomized and estrous rats had a decreased in S3 after exercise, we suggest that progesterone has a protective effect against the mitochondrial damage caused by exercise. It is unclear whether the possible effect of progesterone on basal (non-exercised) animals is also dependent on estrogens. Phillips et al. [24] proposed that in women, hormone replacement therapy has a protective effect on muscle strength. Accordingly, our results show that female sex hormones have a protective effect on mitochondrial respiration. Specifically, the presence of only one ovarian hormone (progesterone) is not sufficient to reestablish the respiratory conditions of both ova-

riectomized or pseudopregnant-control animals. However, the possible protective effect of progesterone receives support from recent observations. McGoldrik [10] showed that isolated mouse soleus muscle has the highest force generation value during diestrus.

The exercise effect on mitochondrial respiration in ovariectomized and estrous rats is not due to an uncoupled effect since DNP did not increase the respiration to control levels [25]. A likely mechanism of mitochondria alteration may involve the accumulation of free radicals [26]. This could be the initial event leading to the molecular alteration underlying muscle fatigue. The fluctuating hormonal *milieu* in the female rat through the cycle may alter the balance between prooxidants and antioxidants under exercise conditions. The homeodynamic capacity of mitochondrial respiration is reflected by the decrease in S3 during estrous and by the lack of change in S3 during the luteal phase.

Kasapovic et al. [27] reported a decrease in the rat's liver superoxide dismutase activity after ovariectomy and a restoration effect in ovariectomized rats injected with sexual steroid hormones. Moreover, protein modification by lipid peroxidation products was observed during the natural life span of pig corpus luteum [28]. Accordingly, we observed a lowered muscular fatigue incidence in rats in diestrus than those in estrous.

The study of the complicated interaction among hormonal status, exercise, and generation of reactive oxygen species, in different times of the estrous cycle would provide more information about the role of sexual steroids in mitochondrial respiration damage and the propensity to muscular fatigue.

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