Effects of Exercise on Muscle Metabolites and Sarcoplasmic Reticulum Function in Ovariectomized Rats

I. GIGLI [1,2], L. E. BUSSMANN[1]

[1]*Institute of Biology and Experimental Medicine - CONICET,* [2]*Department of Medicine, Faculty of Veterinary Sciences, University of Buenos Aires, Buenos Aires, Argentina*

Summary

The relationships between exercise and metabolites as well as between exercise and sarcoplasmic reticulum function were studied in gastrocnemius muscle of ovariectomized-trained rats. Prolonged moderate-intensity exercise, treadmill up-hill run for 90 min with a 10° incline, decreased the muscle glycogen content. Exercise until exhaustion further lowered the glycogen concentration to 13 % of the control, together with a significant decrease of ATP and glucose-6-phosphate concentrations. Also, Ag^+-induced Ca^{2+} release, measured in whole muscle homogenate, showed a 30 % reduction on exhaustion, while Ca^{2+} uptake was unaffected by this exercise. ATPase activities, of both homogenate and SR vesicles, and Ca^{2+} transport in the latter preparation were not altered on exhaustion. It could be concluded from these results that muscular fatigue in ovariectomized rats after aerobic exercise is caused by the change in energy supply and Ca^{2+} release from the SR, this latter possibly due to metabolites generated by the exercise.

Introduction

It is well known that exercise produces several modifications in skeletal muscle. These changes could lead to muscular fatigue, characterized by a decline in muscular force-generating capacity. In contracting muscle, the need for ATP reflects the requirement of myosin ATPase involved in transducing chemical energy into mechanical output, with that related to ion transport, namely sarcolemmal Na^+ exchange and sarcoplasmic reticulum Ca^{2+} sequestration (Green 1997). Therefore, sustained performance of a muscle depends on its ability to balance the demand for ATP by the contractile elements with the supply of ATP by the mitochondria. After exercise, the concentration of some muscle metabolites, such as lactate, ATP, phosphocreatine (PCr), and glucose-6-phosphate (G6-P) change, and could affect normal muscular function (Allen *et al.* 1995).

Due to the important role of sarcoplasmic reticulum (SR) in the contraction-relaxation cycle, it has been suggested that an alteration in its function could play a role in the development of muscular fatigue (Westerblad *et al.* 1991). However, the effect of exercise on SR activity remains controversial. Although some laboratories have reported a decrease in SR ATPase activity or in calcium uptake (Byrd *et al.* 1989, Belcastro

et al. 1993), others have reported no changes (Fitts *et al.* 1982, Chin and Green 1996).

A possible explanation of those contradictory results on SR function could be ascribed to the different training programs employed, and the duration of the exercise. It is also possible that the different hormonal status of the animals play a role, since the relationship between ovarian cycle and muscle function has been shown in humans (Phillips *et al.* 1996, Sarwar *et al.* 1996) and mice (McGoldrick *et al.* 1998). Furthermore, we have previously demonstrated (Gigli and Bussmann, 2001) a decrease of mitochondrial respiration after exercise in ovariectomized and estrous rats, but not in pseudopregnant rats or ovariectomized rats treated with progesterone. Because sexual steroid hormones could affect skeletal muscles, the present study was undertaken to determine the effect of exercise, without muscle damage, on SR activity, and muscle metabolite changes deprived of sexual steroid hormone influence.

Methods

Chemicals

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

Animals

Ovariectomized Sprague-Dawley female 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 20:00 to 8:00 h). Animals were provided with food and water *ad libitum* until the time of the experiment, the average weight was 237±3.1 g.

The animal procedures were reviewed and approved by the Animal Research Committee from our Institution, which follow the guidelines of the Animal Welfare Act, USDA.

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

On the day of the experiment, the animals were randomly assigned to control (24 h rest) or exercise groups. Animals in the exercise groups run at 21m min^{-1} on a 10° uphill incline for 90 min or until exhaustion.

Animals in the exercise groups were killed by decapitation immediately after the exercise session. Control animals were killed in the same way 24 h following their last training session. Truncal blood was collected and allowed to clot; serum aliquots were collated and stored frozen at –20 °C until assay. Gastrocnemius muscles were rapidly exposed and they were either frozen *in situ* by means of metallic tongs for determination of metabolites or excised and used for obtaining the different tissue fractions.

Muscles 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 an Ultra-Turrax homogenizer (IKA-Labortechnik, Germany). Homogenization was done in the presence of 0.5 mM-PMSF (phenylmethylsulphonyl fluoride), 25 µM-ZPCK (N-CBZ-L-phenylalanine chloromethyl ketone), 25 µM-TLCK (N´-p-tosyl-lysine chloromethyl ketone) and 25 µM-TPCK (L-1-tosylamide-2-phenyl-ethylchloromethyl ketone), as protease inhibitors.

Muscle dry weight was assessed weighing the tissue before and after freeze-drying.

Sarcoplasmic reticulum fractions

Low speed centrifugation steps were carried out in a Sorvall RC5B (DuPont, Wilmington, DE, USA) with a SS 24 rotor. Homogenate was centrifuged at 800x *g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 14 000 x g for 10 min. The supernatant from the 14 000 x g centrifugation was saved, and the pellet was resuspended in buffer A and centrifuged again at 14 000 x g for 10 min. The combined supernatants of the 14 000 x g centrifugations were made in 600 mM KCl and incubated in ice for 30 min. They were then layered onto a 30 % sucrose cushion and sedimented at 145 000 x *g* for 60 min in a Sorvall CombiPlus RC5B (DuPont, Wilmington, DE, USA) with a rotor T-865.1. The pellet was resuspended in buffer A, and used as SR fraction.

ATPase activity

Enzyme activity was measured according to Simonides and van Hardeveld (1990), except that Ca^{2+} ionophore A 23187 (1 µM) was used instead of Triton X-100. The oxidation of NADH was followed in a Gilford Response spectrophotometer (Ciba-Corning Diagnostic Corp, USA) at 37 °C. Assay buffer contained: 20 mM Hepes, 200 mM KCl, 10 mM NaCl, 10 mM NaN_3, 1 mM EGTA, 15 mM $MgCl_2$, 5 µM tetrakis 2-pyridylmethyl- ethylenediamine (TPEN), 10 µM

phosphoenolpyruvate (PEP), 2.96 µM NADH, pyruvate kinase and lactate dehydrogenase 15 U ml^{-1}, pH 7.4. The assay final volume was 0.5 ml. The reaction started with the addition of 5 µl of 500 mM Mg-ATP. Calcium-dependent activity was calculated as the total activity minus the basal activity (without Ca^{2+}). Samples, in this and all other determinations, were analyzed at least in duplicate.

Calcium uptake-release

Ca^{2+} transport was determined in a Jasco FP-777 fluorimeter with the accessory CA-261 for calcium measurement (Jasco Corporation, Tokyo, Japan), using as a probe Fura-2 pentapotasium salt (Molecular Probes, Inc. Eugene, OR, USA) as described (Ruell *et al.* 1995). The reaction medium consisted of 20 mM HEPES, 80 mM KCl, 10 mM NaCl, 10 mM NaN$_3$, 5 mM Na oxalate, 5 µM TPEN, 5 mM Mg-ATP and Fura-2 1µM pH 7.0, in a final volume of 2 ml. Sequestration was started by homogenate or SR addition. The reaction was followed until its decline to a plateau (usually 2.5 min). Adding 20 µl of 14 mM AgNO$_3$, to give a final concentration of 140 µM, started Ca^{2+} release. The reaction was allowed to proceed for about 1.5 min and for calibration purposes EGTA then CaCl$_2$ were added to give a final concentration of 3.5 mM and 5.0 mM, respectively.

Metabolites

Gastrocnemius was ground to a fine powder in a mortar under liquid nitrogen, weighed and extracted with 3 ml perchloric acid 2 N per g. The acid extract was separated from the precipitate and neutralized with NaOH 6 N for 10 min. It was then taken to pH 7.0. Aliquots were analyzed for ATP, PCr, lactate and G6-P by enzymatic reactions linked to each other as described in Kelso *et al.* (1987). The reactions were followed at 340 nm in a spectrophotometer using cells with a total reagent volume of 0.5 ml in buffer 100 mM HEPES, 2 mM MgCl$_2$, 1 mM EGTA, 5 mM AMP, 0.1 mM DTT, pH 7.0.

Muscle glycogen concentration, expressed as glucose units, was determined by an enzyme-coupled assay using a modification of the method of Passonneau and Lauerdale (1974). Glucose 6-phosphate dehydrogenase (G6-Pdase) activity was fluorometrically assayed at pH 9.2, following the reduction of NADP$^+$.

Creatine phosphokinase (CK) and aspartate amino transferase (AST) serum levels were determined spectrophotometrically using Wiener lab kits (Wiener lab, Rosario, Argentina).

Protein determination: was performed by the method of Lowry *et al.* (1951) using bovine serum albumin standard. Samples were digested with 1 N NaOH before assaying.

Statistical analyses

Results are given as means ± S.E.M. for muscle metabolites, G6-Pdase, ATPase activities, and Calcium uptake-release. Differences between the control group and the exercised groups were tested for significant differences using a one-way analysis of variance. Significant values were tested using Student's t test or Mann-Whitney at P<0.05. Due to the fact that CK and AST do not have normal distribution, the medians were tested for significant differences using a Wilcoxon test. The results are expressed as median plus range.

Results

Performance data

The exhaustion groups ran on average a total of 3 hours. Serum creatine phosphokinase (CK) and aspartate amino transferase (AST) activities in untrained ovariectomized rats did not differ from the values in the trained control group (data not shown). CK activity in the running groups were 5909 U l^{-1} (range 3962-8667) in the 90-min group; 4556 U l^{-1} (range 3405-6067) in the exhausted group, and 5385 U l^{-1} (range 3838-7284) in the control group. AST activities were 145 U l^{-1} (range 115-181) in the 90-min group; 125 U l^{-1} (range 113-141) in exhausted group, and 127 U l^{-1} (range 88-153) in the control group. Neither CK nor AST activities in running groups were different from those found in the control group (n=6). Serum lactate levels (n=8), after a significant (P<0.05) increase in the 90-min running group (1.66 mmol. l^{-1}± 0.16 U l^{-1}), declined at exhaustion (1.05 mmol. l^{-1}± 0.25 U l^{-1}) as compared with the control group (1.24 mmol. l^{-1}± 0.22 U l^{-1}I).

Gastrocnemius muscle water content in the experimental groups did not differ from the control values. Gastrocnemius dry weight in the control group was 244 ± 2.0 (n=7) mg g^{-1} wet weight and in the 90-min and exhausted groups, 240 ± 2.3 (n=6) and 241 ± 5 (n=6) mg g^{-1} wet weight, respectively.

Muscle metabolism

Prolonged running reduced the content of muscle glycogen by 60 % in the 90-min group and by 86 % in the exhausted group (Table 1).

Table 1. Effect of exercise on gastrocnemius muscle metabolites.

Group	Glycogen	Lactate	ATP	G6P	PCr
Control	13.7 ± 2.8 (6)	4.55 ± 0.2 (8)	4.61 ± 0.3 (8)	2.33 ± 0.3 (8)	10.39 ± 0.9 (8)
90-min	5.6 ± 0.6 (6)*	4.12 ± 0.3 (8)	4.12 ± 0.3 (8)	1.55 ± 0.2 (8)	11.47 ± 1.1 (8)
Exhausted	1.8 ± 0.2 (6)*	2.21 ± 0.5 (7)*	2.68 ± 0.5 (7)*	0.34 ± 0.5 (7)*	8.26 ± 0.9 (7)

*Metabolite concentration is expressed as μmol . g wet weight^{-1}. Values are mean ± S.E.M. for the number of rats given in parentheses. *P<0.05 vs. control value.*

Muscle lactate, ATP, G6-P and PCr concentrations were determined in the extracts of frozen gastrocnemius (Table I). The metabolites studied did not change in the 90-min group with respect to control group. Lactate, ATP, and G6-P concentrations decreased in the exhausted group with respect to control group (P<0.05). The PCr content did not change in the exhausted group with respect to the control group.

On the other hand, the enzyme G6-Pdase did not exhibit changes in the running groups. The values were as follows: control 156 ± 15.3, 90-min run 152 ± 12.6 and run to exhaustion 151 ± 9.8 μmol NADP$^+$ reduced g^{-1} min^{-1} (n=6).

ATPase activity

In muscle homogenate without added Ca^{2+}, also known as Mg^{2+}-dependent ATPase or basal activity, there were no differences in enzyme activity between control and the exercise groups. Maximal Ca^{2+} dependent activity in the absence of ionophore was achieved at a concentration of 100 μM of free Ca^{2+}. Figure 1 shows the activity of calcium-activated ATPase, with and without
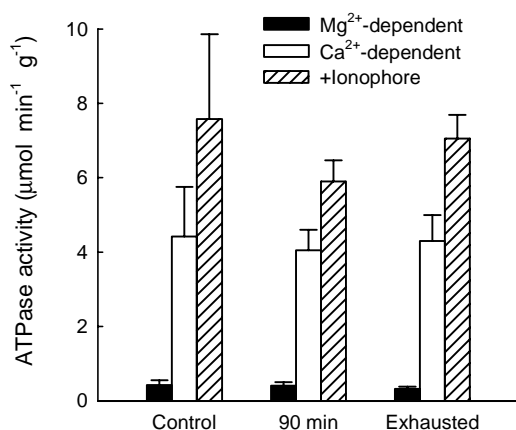

Fig. 1. *Effect of exercise on ATPase activity in rat gastrocnemius homogenates. Closed bars: Mg^{2+}-dependent or basal, open bars: Ca^{2+}-dependent and hatched bars: Ca^{2+}-dependent in the presence of ionophore A 23187 activities, expressed as μmol of ATP hydrolyzed min^{-1} g wet weight^{-1}. Values are mean ± S.E.M.. Number of rats: control n=12; 90-min group n=9; exhausted group n=7.*
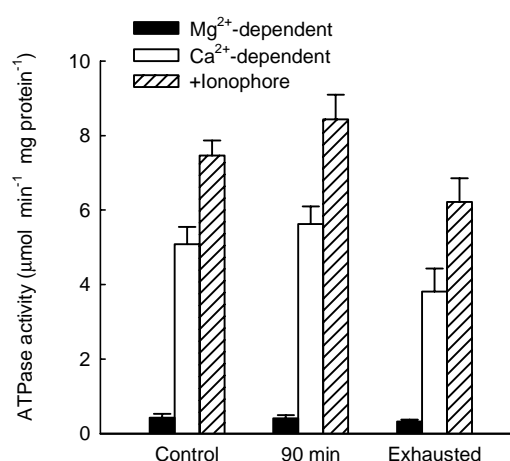

Fig. 2. *Effect of exercise on ATPase activity in rat gastrocnemius sarcoplasmic reticulum. Closed bars: Mg^{2+}-dependent or basal, open bars: Ca^{2+}-dependent and hatched bars: Ca^{2+}-dependent in the presence of ionophore A 23187 activities, expressed as μmol of ATP hydrolyzed min^{-1}mg protein^{-1}. Values are mean ± S.E.M. Number of rats: control n=11; 90-min group n=11; exhausted group n=7.*

ionophore A 23187 in gastrocnemius homogenates. The values, either in the 90-min run or in the exhausted group,

were not significantly different (P>0.05) from those obtained in the control group.

The incubation in the presence of calcium ionophore A 23187 renders the measurement independent of membrane integrity, as it allows the bidirectional flux of the cation. Under this condition, Ca^{2+}-ATPase showed its maximal activity. The increase in the Ca^{2+}-ATPase activity by ionophore addition was similar in the three groups and no significant differences were found among them.

The activity of Ca^{2+}-ATPase in SR fraction of gastrocnemius is depicted in Fig. 2. The SR yield did not differ between animals in the control group and those obtained from other groups (1.03 ± 0.07 mg protein per g of wet weight tissue). Neither the 90-min nor the exhausted groups were found significantly different from the control group. When Ca^{2+} ionophore A 23187 was present, the ATPase activity in both the 90-min and the exhausted groups did not differ from the control values.
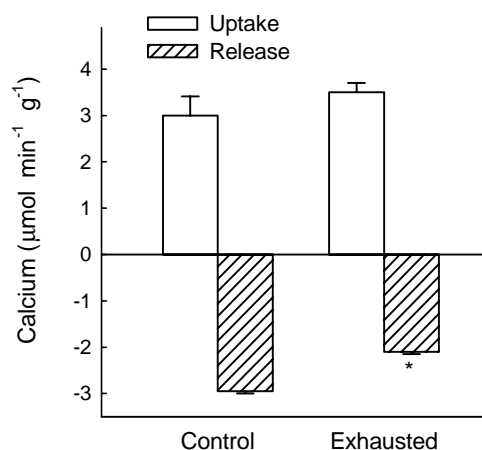
Calcium uptake and release

Ca^{2+} pump activity was measured in gastrocnemius homogenates and the SR. Uptake in whole homogenates in the exhausted group did not differ from the control group (Fig. 3). On the other hand, when Ca^{2+} release was stimulated by the addition of $AgNO_3$, significant differences were found between the exhausted and the control group ($P<0.05$). Release of Ca^{2+} in the 90-min running rats did not show any differences compared to controls (data not shown).

Calcium transport was also measured in sarcoplasmic reticulum from gastrocnemius in exhausted and control groups (Fig. 4). In this case, neither calcium uptake nor its release showed differences compared to the control group.
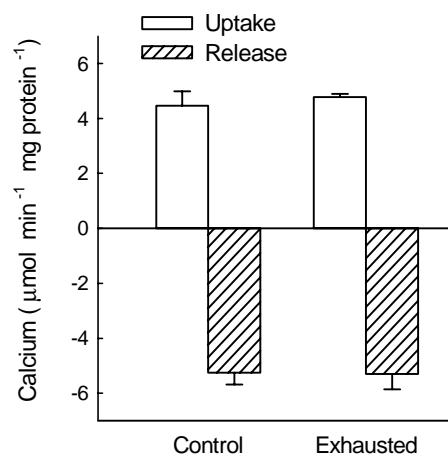

Fig. 3. *Effect of exercise on Ca^{2+}- uptake and release in gastrocnemius homogenates. Open bars: Ca^{2+}-uptake and hatched bars: Ca^{2+}-release as µmol of Ca^{2+} min^{-1} g wet weight^{-1}. Values are means ±S.E.M., n=5 for control and exhausted group. * Significantly lower than control values at P<0.05.*

Fig. 4. *Effect of exercise on Ca^{2+}-uptake and release in gastrocnemius sarcoplasmic reticulum. Open bars: Ca^{2+}-uptake and crosshatched bars: Ca^{2+}-release as µmol of Ca^{2+} min^{-1} mg protein^{-1}. Values are mean ±SE.*

Discussion

Even though the two-week-training program does not enhance the aerobic condition of the rats (Benzi *et al.* 1975), it does reduce muscle injury. The increase of CK and AST in the serum has long been associated with muscle lesion (Garbus *et al.* 1964). In untrained rats, this increment lasted for up to 24 h when considering AST; gender differences were also reported for both enzymes (Shumate *et al.* 1979). Training does reduce muscle injury and prevent the rise of serum CK that follows prolonged exercise (Schwane and Armstrong 1983). Our results concerning CK and AST showed no changes in their serum levels, and hence revealed that no major alteration in muscle integrity was produced by our exercise protocol. We studied the gastrocnemius muscle because it is actively recruited during uphill exercise and represents a composition of the fast muscle fiber types in rats (Armstrong *et al.* 1984). Muscle fatigue could be produced by substrate depletion or by accumulation of metabolic end products (Allen *et al.* 1995). Our results

show a rapid and pronounced decline in glycogen concentration at 90-min and exhaustion, 60 % and 87 % respectively compared with the controls. We did not observe, with our exercise protocol, any increase in muscle lactate concentrations. On the contrary, a decrease was found in the exhausted group. Running to exhaustion elicited a decrease of 85 % in G6-P muscle concentration as compared to the controls. It has been reported that muscle G6-Pdase activity increases after an acute bout of exercise, this being associated with tissue injury (Schwane and Armstrong 1983). However, in our case, enzyme activity did not increase after the exercise with respect to controls, therefore the changes in G6-P concentration are not due to an increase of the G6-Pdase. Since glycogen, and G6P decrease in the exhausted group, it is unlikely that the exercise protocol used produced an increase in fatty acid metabolism. Furthermore, serum acyl derivatives of carnitine measured by electrospray ionization mass spectrometry did not change after exercise (data not shown).

Sustained muscle performance relies on its ability to balance the demand for ATP of the contractile elements with the mitochondria supply. Edwards (1975) reported that the slowing of relaxation during fatigue is related to ATP depletion rather than to the accumulation of lactic acid. After exhaustion by aerobic exercise, we observed a decrease in ATP concentration, with PCr content unchanged or already restored at the time of sampling. Because H^+ is a product of the creatine kinase reaction, the rate of PCr resynthesis is slowed in cells with low pH. In accordance with this, we did not observe changes in PCr concentration possibly due to the resynthesis of this metabolite at the time of sampling, since the exercise was performed under aerobic conditions. In agreement with the results of others (Dosset-Mercer et al.1994, Chin and Green 1996), we did not observe differences in total, basal or Ca^{2+}-activated ATPase activity or in Ca-uptake between control and exercise groups, either in the homogenate or in SR. This discrepancy with previous reports (Byrd et al. 1989, Belcastro et al. 1993) could be due to the methods employed for determining the enzymatic activities or to the exercise procedure. Our observation of a 29 % decrease in Ag^+-induced Ca^{2+} release in whole homogenate is consistent with an earlier report (Favero et al. 1993). It is known that sarcoplasmic reticulum (SR) Ca^{2+} release channel function is modified by ligands that are generated during exercise, and the results indicate that exercise depressed Ca^{2+} release from SR by directly modifying the Ca^{2+} release channel (Favero et al. 1995). The exposure of skinned fibers to H_2O_2 was reported to be capable of causing protein damage and thus to depress force production, partly by inhibiting Ca^{2+} release (Brotto and Nosek 1996). Furthermore, the same action on Ca^{2+} release was attributed to lactate in experiments with in vitro addition of the metabolite (Spangenburg et al. 1998). This latter mechanism, however, does not apply to our results since the exercise protocol, which we used did not increase muscle lactate concentration.

SR isolation implies a series of steps, including incubation in 0.6 M KCl, which would remove most of the ligands not covalently bound, produced by the exercise. This could explain our results and those of other authors (Byrd et al. 1989), concerning the effect of exercise on Ca^{2+} transport in homogenate and its disappearance in the SR.

In conclusion, after 90-min exercise glycogen concentration was decreased and the ATP levels were maintained during the first 90 min of exercise. The exercise until fatigue resulted in diminished ATP synthesis and SR calcium release. We did not observe this decrease in SR fraction probably, due to the KCl incubation, which is a part of the SR isolation method.

Estradiol has been reported to protect muscle against physiological damage (Bar et al. 1988, Koot 1991), and has also been related with an increase of fatty acid oxidation and an inhibition of glucose metabolism during exercise. Ovariectomized rats have been used as a model of postmenopausal bone loss (Kalu 1991), and could be useful model for identifying exercise effects on muscles depleted of sexual steroid hormones. Further studies with normal cycling rats at different times of the estrous cycle would provide deeper insight into the role of sexual steroids in relation to muscle fatigue.

Acknowledgements

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Reprint requests

L. E. Bussmann, Instituto de Biologia y Medicina Experimental-CONICET. Vuelta de Obligado 2490, Buenos Aires (1428), Argentina, Fax: +54 11 4786-2564, e-mail address: bussmann@dna.uba.ar