

Biochemical Adaptations in Muscle

EFFECTS OF EXERCISE ON MITOCHONDRIAL OXYGEN UPTAKE AND RESPIRATORY ENZYME ACTIVITY IN SKELETAL MUSCLE*

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SUMMARY

The capacity of the mitochondrial fraction from gastrocnemius muscle to oxidize pyruvate doubled in rats subjected to a strenuous program of treadmill running. Succinate dehydrogenase, reduced diphosphopyridine nucleotide dehydrogenase, DPNH cytochrome *c* reductase, succinate oxidase, and cytochrome oxidase activities, expressed per g of muscle, increased approximately 2-fold in hind limb muscles in response to the training. The concentration of cytochrome *c* was also increased 2-fold, suggesting that the rise in respiratory enzyme activity was due to an increase in enzyme protein. The total protein content of the mitochondrial fraction increased approximately 60%. These changes in the concentration of cytochrome *c* and total mitochondrial protein are of special interest because they suggest that exercise could serve as a useful tool for studying the biosynthesis of mitochondrial proteins.

Mild exercise, such as that used in previous studies, was found to have no effect on the level of succinate dehydrogenase in muscle, suggesting that the failure of earlier studies to show an increase in respiratory enzyme activity resulted from the use of an insufficient exercise stimulus.

Mitochondria from muscles of the exercised animals exhibited a high level of respiratory control and tightly coupled oxidative phosphorylation. Thus, the increase in electron transport capacity was associated with a concomitant rise in the capacity to produce adenosine triphosphate. This adaptation may partially account for the increase in aerobic work capacity that occurs with regularly performed, prolonged exercise.

Comparative studies have shown that a good correlation exists between the ability of a muscle to perform prolonged exercise and its content of respiratory enzymes (1, 2). Paul and Sperling (1) found the breast muscle of the nonflying chicken to have low

α -ketoglutarate and succinate Q_{O_2} values and to be poor in mitochondria. In contrast, the breast muscles of mallards and pigeons, which spend long periods in active flight, are rich in mitochondria and have Q_{O_2} values which are approximately 10 times as high as those found in the chicken (1). Similarly, Lawrie (2) has reported that the levels of cytochrome oxidase, succinate oxidase, and succinate dehydrogenase activity in psoas muscle of the sedentary laboratory rabbit are approximately one-third to one-half as high as those found in the active wild hare, and only one-fourth to one-sixth as high as the values obtained on horse psoas.

Little is known regarding the factors which control the biogenesis of mitochondria and the biosynthesis of specific mitochondrial constituents. It does appear clear, however, from studies on the effects of thyroxine feeding, that mitochondria in striated muscle and various other tissues are capable of undergoing adaptive changes in both composition and number (3-6). Lee and Lardy (3) found that feeding desiccated thyroid to rats for 10 days increases the specific activity of mitochondrial α -glycerophosphate dehydrogenase 2- to 3-fold in heart muscle and about 20-fold in liver. The activities of a number of other mitochondrial enzymes, including those of the respiratory chain, also increase significantly in response to thyroxine but to a lesser extent (3-5), while still others appear to be unaffected by hyperthyroidism (3). Electron microscopic studies have shown that thyroxine administration can also increase the number and alter the morphology of mitochondria in skeletal muscle (6).

In view of the evidence, obtained from the above studies with thyroxine, that mitochondria are capable of undergoing adaptive changes, it appeared possible that the differences between the respiratory enzyme levels of active and inactive muscles might be due not only to genetic differences but also to an adaptive process. To test this possibility, controlled studies on the chronic effects of exercise are necessary. Surprisingly few such studies have been reported. Hearn and Wainio (7), in a study on rats subjected to 30 min of swimming daily for 5 to 8 weeks, found no increase in the level of succinate dehydrogenase activity in skeletal muscle. With the use of the same exercise program, Gould and Rawlinson (8) observed no change in the levels of malate dehydrogenase and ATPase activity in rat skeletal muscle. These negative results might reflect the relative mildness of the exercise stress used, since 30 min of swimming is well within the exercise capacity of the untrained rat. It has been observed in

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our laboratory that untrained rats can swim for over 6 hours if the water temperature is kept near 32°.

It appeared of interest, therefore, to investigate the chronic effects of more vigorous and prolonged exercise on skeletal muscle. The data reported in the present paper show that a strenuous program of running increases mitochondrial capacity for oxidative phosphorylation and that this results, at least in part, from an increased ability to carry out terminal electron transport.

EXPERIMENTAL PROCEDURE

Animal Care and Exercise Program—Male rats of a Wistar strain (specific pathogen-free CFN rats), aged 6 weeks were obtained from Carworth Farms, placed in individual cages, and maintained on a diet of Purina laboratory chow and water. They were divided into four groups.

An exercising group was trained to run on a motor-driven treadmill similar to the one described by Kimeldorf (9). It consists of a wide, endless belt riding on metal rollers. A Lucite box, partitioned into individual compartments 30 cm long by 10 cm wide, is suspended over the belt, providing a limited area for each animal to run in. Motivation is provided by a shock grid at the rear of the compartments. Animals learn to avoid being shocked by keeping pace with the belt movement. The treadmill was set at an 8° incline. The animals were exercised 5 days per week. They initially ran for 10 min at 22 m per min, twice daily, 4 hours apart. The work load was progressively increased over 12 weeks. At the end of this period the animals were running continuously for 120 min at 31 m per min, with 12 sprints at 42 m per min, each lasting 30 sec, interspersed at 10-min intervals through the workout. Animals were maintained at the final work level until they were sacrificed; this period varied from 1 to 6 weeks. The exercising group was provided with food and water *ad libitum*.

An exercising control group ran on the treadmill for 10 min per day, 5 days per week. Running speed was increased to 31 m per min over 6 weeks and then maintained at this level. The purpose of this program was to maintain running skill, and familiarity with the procedure, while keeping the exercise stimulus relatively minimal. Food intake was adjusted to maintain their body weights in the same range as that for the exercising groups.

Sedentary control rats were divided into a paired weight group, in which the food intake was adjusted so as to maintain the body weights approximately the same as those of the exercising rats, and a free eating group which was provided with food *ad libitum*. These animals were not subjected to treadmill running.

Preparation of Homogenates and Mitochondria—Animals were not exercised for 24 hours prior to killing. They were killed by decapitation, after an overnight fast, and exsanguinated. Muscles were rapidly dissected out, chilled in ice-cold Ringer's solution, blotted, weighed, chopped into a fine mince with scissors, and homogenized in a glass Potter-Elvehjem homogenizer. Because of the abundant connective tissue, it was found necessary to use loosely fitting tubes, and pestles with cutting teeth on the bottom. The tubes were immersed in ice water during the procedure. After gross breaking up of the tissue, homogenization was completed with 10 complete passes of the tube. The homogenates contained 1 g of muscle per 10 ml. Homogenates for assays of succinate dehydrogenase, succinate oxidase, and cytochrome oxidase activities were prepared in 0.01 M potassium phosphate buffer, pH 7.4.

Preliminary studies confirmed the finding of Ernster, Ikko, and Luft (10), and of Hedman (11) that sucrose media are unsatisfactory for the isolation of skeletal muscle mitochondria. Mitochondrial yield, in terms of respiratory and enzymatic activity, from muscles homogenized in 0.175 M KCl was approximately twice as great as from paired muscles homogenized in sucrose. Muscle mitochondria were, therefore, prepared from homogenates made in 0.175 M KCl. To isolate mitochondria for spectrophotometric assays of enzyme activity, the homogenates were first centrifuged for 10 min at $700 \times g$. The supernatant fluid was decanted, and the sediment was resuspended in 0.175 M KCl and centrifuged for 10 min at $700 \times g$. The $700 \times g$ supernatant suspensions were combined, centrifuged again at $700 \times g$, decanted, and centrifuged at $14,000 \times g$ for 20 min. The mitochondrial pellet was washed once by resuspension in 0.175 M KCl and recentrifuged at $14,000 \times g$. The mitochondria from 1 g of muscle were finally suspended in 2 ml of 0.01 M potassium phosphate buffer, pH 7.4, and frozen and thawed twice prior to measurement of DPNH dehydrogenase and DPNH cytochrome *c* reductase activities.

Mitochondria for O_2 uptake studies were isolated from the final $700 \times g$ supernatant fluid by centrifugation at $6000 \times g$ for 20 min. The mitochondria were suspended in 0.25 M sucrose and were used immediately.

Assay Methods—Oxygen uptake was measured in a Gilson differential respirometer, at 30°, with air as the gas phase. The center wells of the Warburg flasks contained 0.2 ml of 15% KOH and a wick of pleated filter paper.

Mitochondrial respiration was measured in triplicate. Each flask contained, in a final volume of 2 ml, 6.5 mM $MgCl_2$, 30 mM KCl, 62.5 mM sucrose, 40 mM potassium phosphate buffer, 10 mM potassium pyruvate, 1.0 mM sodium malate, 25 mM glucose, 2.5 mM ATP, 2 mg of hexokinase, and mitochondria from 0.5 g of muscle. The pH of the mixture was 7.2. After 10 min of thermoequilibration, O_2 uptake was measured for 10 min in the absence of phosphate acceptor. The ATP, glucose, and hexokinase were then tipped in from the side arm. At this point, the first flask was chilled and the reaction was stopped with 5% trichloroacetic acid. O_2 uptake was measured for two more 10-min periods in the remaining flasks; the reaction was then terminated by the addition of 5% trichloroacetic acid. The mixtures were filtered, and the P_i present was measured. Phosphate esterified was measured by P_i disappearance during the 20-min period of respiration in the presence of the P_i -trapping system. Respiratory control ratios were calculated by dividing the rate of O_2 uptake in the presence of phosphate acceptor by the rate in the absence of phosphate acceptor.

Succinate oxidase and cytochrome oxidase activities were measured manometrically as described by Potter (12).

O_2 uptakes are expressed as microliters of dry O_2 under standard conditions.

Spectrophotometric assays were performed in a Beckman model DU spectrophotometer with a thermostated cell compartment in 1-ml cuvettes of 1-cm light path, at 30°. Readings were taken at 15- or 30-sec intervals. Initial reaction rates were determined from a segment of the linear portion of the change in absorbance and corrected for the rates of any nonenzymatic activity. Enzymatic activities are reported as micromoles of substrate oxidized per min.

DPNH dehydrogenase activity was assayed by the method of Minakami, Ringler, and Singer (13). The reduction of potas-

TABLE I

Oxygen consumption by mitochondrial fraction from gastrocnemius muscles of rats in exercising and sedentary groups

The methods used for isolating mitochondria and determining O₂ consumption, respiratory control, and P:O ratio are described under "Experimental Procedure." The substrate used was pyruvate plus malate. There were eight animals in each group. The values shown are means \pm the standard errors of the mean. Oxygen uptake is expressed as microliters of O₂ utilized per hour by the mitochondria from 1 g of fresh muscle.

Group	Oxygen uptake	Respiratory control index	P:O ratio
	$\mu\text{l O}_2/\text{hr/g}$		
Sedentary.....	506 \pm 53	14.7 \pm 2.6	2.7 \pm 0.2
Exercising.....	1022 \pm 118 ^a	16.1 \pm 2.2	2.6 \pm 0.1

^a Exercising versus sedentary, $p < 0.01$.

sium ferri cyanide, at different concentrations, was measured at 420 m μ and the results were extrapolated to V_{max} with respect to oxidant. The reaction mixture contained 40 mM potassium phosphate buffer, pH 7.4, 0.15 mM DPNH, and 0.2, 0.4, or 0.6 mM K₃Fe(CN)₆. The reaction was started by the addition of 0.02 ml of mitochondrial suspension.

DPNH cytochrome *c* reductase activity was measured by the method of Nason and Vasington (14). The reduction of cytochrome *c* was followed at 550 m μ .

Succinate dehydrogenase activity was measured by the method of Bonner (15). Potassium ferri cyanide reduction was followed at 420 m μ .

The cytochrome *c* content of muscle was determined as described by Rosenthal and Drabkin (16).

Inorganic phosphate was measured by the method of Fiske and SubbaRow (17).

Protein was determined by the biuret method (18). Mitochondria were prepared for protein measurement by the procedure of Cleland and Slater (19).

Materials—DPNH, cytochrome *c* (type II), ATP, and hexokinase (type III) were obtained from Sigma.

RESULTS

Group Differences in Exercise Capacity, Body Weight, and Gastrocnemius Muscle Weight—To determine whether or not the running program had produced an adaptive increase in exercise capacity, the animals were subjected to an "all-out" exercise test, in which they ran to exhaustion at a treadmill speed of 31 m per min. After 12 weeks of training the average all-out run time for the exercising group was 186 \pm 18 min, compared to 29 \pm 3 min for the exercising control group ($p < 0.001$). The exercising group gained weight considerably more slowly than the free eating sedentary control group. The average final body weight of the exercising group was 353 \pm 17 g compared to 491 \pm 21 g for the free eating sedentary controls ($p < 0.01$). The final weights of the paired weight sedentary control group averaged 348 \pm 9 g, while the exercising controls, in which the food intake was also restricted, weighed 367 \pm 7 g. The weights of the animal's leg muscles did not appear to be directly affected by the endurance exercise, but roughly paralleled their body weights. This is illustrated by the weights of the gastrocnemius muscles which averaged 2.10 \pm 0.06 g for the exercising group; 2.08 \pm 0.04 g for the paired weight sedentary controls; and 2.62 \pm 0.12 g for the free eating sedentary controls.

Despite these differences in weight, no differences were found between the paired weight and free eating sedentary controls in the specific activities of the respiratory enzyme systems measured in muscle. Therefore, in the following sections, the results obtained on animals from these two groups have been combined and are referred to jointly under the headings "sedentary group" or "sedentary controls."

Respiratory Activity and Oxidative Phosphorylation in Mitochondrial Fraction from Muscle—The respiratory activity of the mitochondrial fraction from gastrocnemius muscles was measured with pyruvate plus malate as the substrate. During uncontrolled respiration (*i.e.* in the presence of nonlimiting amounts of P_i and ADP) the mitochondrial fraction of muscles from the exercising group utilized approximately twice as much O₂ as that from the sedentary controls (Table I). As also shown in Table I, respiratory control and P:O ratios were not significantly different for the two groups. It appears from these results that the exercise program brought about a major increase in the ability of the involved muscles to oxidize pyruvate. The high level of respiratory control, together with P:O ratios in the same range as those of the controls, indicates a concomitant increase in the capacity to form ATP.

Cytochrome Oxidase and Succinate Oxidase Activities—Whole homogenates of gastrocnemius and soleus muscles were assayed for cytochrome oxidase and succinate oxidase activities. As shown in Table II, the activity of both of these enzyme systems was significantly greater in the exercising group ($p < 0.001$). These results, obtained with whole homogenates and, therefore, not subject to errors due to possible differences in the percentage yield of mitochondria, provide further evidence that endurance exercise increases the respiratory capacity of muscle. For the sake of consistency, the O₂ uptakes in Table II are given per g of fresh muscle. However, as the water content of muscle from the two groups was not different, the findings are the same when O₂ uptakes are expressed, in the conventional manner, as microliters of O₂ utilized per hour per mg of dry muscle. The water content of fresh muscles averaged 0.78 \pm 0.02 ml per g for the sedentary group and 0.78 \pm 0.01 ml per g for the exercising group.

Succinate Dehydrogenase—In view of the finding of Hearn and Wainio (7) that 30 min of daily swimming for 6 weeks did not

TABLE II

Cytochrome oxidase and succinate oxidase activities in muscles of rats in exercising and sedentary groups

Cytochrome oxidase and succinate oxidase activities were measured, as described under "Experimental Procedure," in whole homogenates of gastrocnemius and soleus muscles. Each value represents the mean \pm the standard error of the mean for muscles from nine rats. Activity is expressed as microliters of O₂ utilized per min per g of fresh muscle.

Group	Cytochrome oxidase	Succinate oxidase
	$\mu\text{l O}_2/\text{min/g}$	
Gastrocnemius		
Sedentary.....	305 \pm 15	73 \pm 5
Exercising.....	551 \pm 31 ^a	117 \pm 8 ^a
Soleus		
Sedentary.....	427 \pm 16	95 \pm 10
Exercising.....	691 \pm 52 ^a	160 \pm 8 ^a

^a Exercising versus sedentary, $p < 0.001$.

increase the level of succinate dehydrogenase in the skeletal muscles of rats, it is of interest that, in the present study, a highly significant increase in the activity of this enzyme occurred in the exercising animals. Homogenates of gastrocnemius muscles from nine sedentary controls oxidized an average of 8.3 ± 0.7 μ moles of succinate per min per g of fresh muscle, compared to 15.1 ± 1.4 μ moles per min per g for nine animals in the exercising group ($p < 0.001$). In contrast, a value of 7.7 ± 0.9 μ moles per min per g was obtained on three rats made to swim 30 min per day, 5 days per week, for 6 weeks; this value is not significantly different from that for the sedentary controls, and confirms the observation of Hearn and Wainio (7).

DPNH Dehydrogenase and DPNH Cytochrome *c* Reductase Activities—From the findings on muscles of the exercising animals described in the preceding two sections, it appeared clear that an increase had occurred in the activity of the components of the respiratory chain involved in the oxidation of succinate. As DPN is responsible for linking the oxidation of the majority of other metabolites to O_2 , it seemed important to also investigate the effect of the exercise program on DPNH dehydrogenase activity. As shown in Column 2 of Table III, the DPNH dehydrogenase activity of the mitochondrial fraction from gastrocnemius muscles of the exercising group was significantly higher than that of the sedentary group ($p < 0.001$). The protein content of the mitochondrial fraction was also significantly higher in the exercising group ($p < 0.001$), as shown in Table III, Column 3. As a result, the values of DPNH dehydrogenase activity for the two groups are not significantly different when expressed in terms of mitochondrial protein (Table III, Column 4).

DPNH cytochrome *c* reductase activity was also significantly greater in the mitochondrial fraction of the exercising group, with an average value, for seven gastrocnemius muscles, of 0.60 ± 0.09 μ mole of DPNH oxidized per min by the mitochondria from 1 g of muscle, as compared to 0.25 ± 0.05 μ mole per min per g of muscle for seven sedentary controls ($p < 0.01$).

Cytochrome *c*—In addition to measuring the activity or “functional capacity” of the respiratory chain in the skeletal muscles of exercising and sedentary animals, it appeared of interest to

obtain direct information regarding the concentration of some of its components. As the cytochromes and flavoproteins of the respiratory assembly appear to occur in simple molar ratios to each other (20), it appeared practicable to use changes in the concentration of one component as an indicator, representative of the response of the entire respiratory chain. Cytochrome *c* was chosen for this purpose because, compared to the other cytochromes, it is relatively easy to extract from muscle. The average concentration of cytochrome *c* in the gluteus muscles of 10 exercising rats was 6.46 ± 0.58 μ moles per g of muscle; this was significantly higher than the value of 3.47 ± 0.18 μ moles per g obtained on 10 sedentary controls ($p < 0.001$). This increase in the cytochrome *c* content of muscles of the exercising rats favors the interpretation that the rise in mitochondrial electron transport activity, which was of the same order of magnitude, is the result of an increase in the concentration of the enzyme systems which make up the respiratory chain.

DISCUSSION

A 2-fold increase in the capacity to oxidize pyruvate occurred in the muscles of rats subjected to a program of strenuous exercise. Concomitantly, the activities of the enzymes of the mitochondrial electron transport chain approximately doubled in the muscles of the exercised rats. The concentration of cytochrome *c* also increased 2-fold, providing evidence that the rise in respiratory enzyme activity is due to a net increase in enzyme protein. This is supported by the finding of a general increase in mitochondrial protein.

The finding that mitochondria obtained from the muscles of the exercised animals exhibited a high level of respiratory control and tightly coupled oxidative phosphorylation indicates that the increase in mitochondrial electron transport capacity is associated with a rise in the capacity to produce ATP. In contrast, mitochondria from tissues of hyperthyroid animals, which also show an increase in respiratory enzyme activity (4, 5) are characterized by partial uncoupling of oxidative phosphorylation and loss of respiratory control (21). Furthermore, on electron microscopic examination, the mitochondria in the skeletal muscles of the exercised animals showed no evidence of swelling or other gross alteration in structure¹ such as have been reported in the tissues of hyperthyroid animals (6, 22).

In contrast to the program of treadmill running, 30 min of daily swimming, which represents a mild work load for the rat, has no effect on the level of succinate dehydrogenase in rat gastrocnemius muscle. This confirms the report of Hearn and Wainio (7), and indicates that a greater exercise stress is needed to bring about an adaptive increase in the enzymes of the mitochondrial electron transport chain. In the present study, the exercise load was progressively increased over a 12-week period to try to achieve a near maximal increase in aerobic work capacity. We now have preliminary data which indicate that similar results can be obtained in as short a time as 3 weeks if the exercise periods are sufficiently prolonged.

The intracellular concentrations of numerous substances, including pyruvate, lactate, P_i , ADP, and AMP increase in muscle during exercise (cf. References 23 and 24). Whether or not one of these acts as an inducer of the biosynthesis of the enzymes involved in mitochondrial electron transport is not known. However, it appears clear from the present data that either an increase in the number of mitochondria, an increase in

TABLE III

Effect of exercise program on DPNH dehydrogenase activity and on mitochondrial protein concentration

DPNH dehydrogenase activity was measured in the mitochondrial fraction of gastrocnemius muscles as described under “Experimental Procedure.” The concentration of ferricyanide was varied, and the results were extrapolated to V_{max} with respect to oxidant. Each value represents the mean \pm the standard error of the mean for muscles from 10 rats. Enzymatic activity is expressed as micromoles of DPNH oxidized per min by the mitochondria from 1 g of fresh muscle (Column 2) and as micromoles of DPNH oxidized per min per mg of mitochondrial protein (Column 4).

1. Group	2. DPNH dehydrogenase	3. Mitochondrial protein	4. DPNH dehydrogenase
	μ moles/min/g muscle	mg/g muscle	μ moles/min/mg protein
Sedentary.....	5.6 ± 0.6	2.97 ± 0.20	1.96 ± 0.30
Exercising....	11.8 ± 1.5^a	4.67 ± 0.30^a	2.54 ± 0.26^b

^a Exercising versus sedentary, $p < 0.001$.

^b Exercising versus sedentary, $p > 0.1$.

¹ J. R. Williamson and J. O. Holloszy, unpublished observations.

the size of the mitochondria, or an alteration in the composition of the mitochondria must have occurred. Studies in progress on the chronic effects of exercise on the levels of activity of the citric acid cycle enzymes, and on the number and size distribution of mitochondria in muscle may help to determine which of these possibilities is correct. Regardless of which alternative obtains, the finding that exercise can induce an increase in the concentrations of specific mitochondrial enzymes and in total mitochondrial protein in muscle may provide a useful tool for studying the biogenesis of mammalian mitochondria, or the biosynthesis of specific mitochondrial constituents, or both.

The increase in the capacity of muscle to oxidize pyruvate and form ATP has interesting implications relative to the ability of muscle to perform aerobic work. It is well known that physically trained individuals, as compared to untrained, are characterized by the ability to attain a higher maximum rate of O_2 consumption during strenuous exercise (25, 26) and to maintain lower levels of blood lactate during moderate exercise (27, 28). It has been suggested that cardiovascular adaptations, resulting in the delivery of more blood and O_2 to the working muscles, are responsible for these effects of training (27, 29). However, it is not possible to explain on this basis the finding that trained individuals have lower blood lactate concentrations than untrained even when the exercise load is light (27, 28), as it has been shown recently that cardiac output and, therefore, also peripheral blood flow, is actually lower at a given level of submaximal exercise in the trained than in the untrained state (30). Trained muscles appear to compensate for this decrease in blood flow and meet their O_2 requirements during work by increased extraction of O_2 from the blood, as evidenced by a greater arteriovenous O_2 difference (30). It appears likely, therefore, that during moderate exercise the rate of aerobic metabolism of pyruvate in the muscles of sedentary individuals is limited not by the supply of oxygen but by the capacity of the mitochondria for pyruvate oxidation. The increase in respiratory activity induced in muscle by physical training could play a major role in decreasing lactate production during submaximal exercise. Lower concentrations of P_i and ADP in the cytoplasm brought about by the increase in the rate of oxidative phosphorylation would be expected to result in a slower rate of glycolysis (24, 31-34). This, in turn, would decrease the rate of reduction of DPN in the cytoplasm. A decrease in extramitochondrial DPNH concentration, together with a lower concentration of pyruvate second to the slowing of glycolysis and the increase in the capacity for pyruvate oxidation, would reduce the rate of lactate production. There is evidence that a rise in the concentration of lactate in muscle may play an important role in the development of muscle fatigue during exercise (35). Thus, a reduction in the rate of lactate production by the above mechanisms could contribute to the increase in the capacity for prolonged submaximal physical activity that is associated with regularly performed exercise.

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