Increased nitric oxide is one of the causes of changes of iron metabolism in strenuously exercised rats

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Qian, Zhong Ming, De Sheng Xiao, Ya Ke, and Qin Kue Liao. Increased nitric oxide is one of the causes of changes of iron metabolism in strenuously exercised rats. Am J Physiol Regulatory Integrative Comp Physiol 280: R739–R743, 2001.—This study was carried out to investigate the possible role of increased nitric oxide (NO) production in the development of the low iron status in strenuously exercised rats. Female Sprague-Dawley rats were randomly assigned to four groups: sedentary (S1), sedentary + nitro-L-arginine methyl ester (L-NAME; S2), exercise (E1), and exercise + L-NAME (E2). Animals in the E1 and E2 groups swam for 2 h/day for 3 mo. L-NAME in the drinking water (1 mg/ml) was administrated to rats in the S2 and E2 groups for the same period. At the end of third month, hematological indexes and nitrite and nitrate (NOx) contents in the plasma and non-heme iron and NOx levels in the liver, spleen, and bone marrow cells were measured. Three months of exercise induced a significant increase in NOx content and a decrease in iron level both in plasma and tissues. Treatment with L-NAME, an inhibitor of NO synthase (NOS), led to a significant decrease in NOx and an increase in iron level both in plasma and tissues in the exercised rats. The E2 group had a significantly lower NOx content as well as a higher iron level both in plasma and tissues than the E1 group. However, the iron contents in the plasma and tissues of the E2 group were still significantly lower than those found in S1. No difference was found in NOx levels between E2 and S1. These findings showed that exercise was associated with elevation in NOx and reduction in iron in plasma and the tissues. Treatment with L-NAME was able to completely inhibit the effect of exercise on NOx as well as partly recover the decreased iron contents in plasma and tissues resulting from exercise. This suggests that the increased production of NO might be one of the causes of the lower iron status in exercised rats.

Iron is the trace mineral that plays a central role in oxygen transport and the synthesis of hemoglobin, myoglobin, and some important enzymes fundamental to energy production. This mineral has therefore been studied extensively with respect to exercise. In animal studies, an overall trend has suggested depletion of iron stores or suboptimal iron status as an impact of exercise (10, 18, 22, 24). However, the mechanism by which exercise produces this change in iron status is unknown, although some possible causes have been proposed. These include increased iron losses in urine (15), sweat (3), and/or feces (23), reduced intestinal iron absorption (9), hemolysis (7, 26), redistribution of iron stores (24), and a higher turnover of hemoglobin and erythrocytes (11, 17, 25).

In a previous study (20), we found that strenuously exercised rats (swimming 2 h/day for 3 mo) had a significant increase in transferrin-receptor expression and transferrin-bound iron accumulation in bone marrow erythroblasts. Also, plasma iron concentrations and non-heme iron contents in the liver, spleen, and some other tissues were lower in the exercised rats than in the sedentary animals. It was therefore suggested that the increased iron uptake by the bone marrow cells and probably some other cells, such as the muscle cells, might be one of the causes of the decreased plasma and tissue iron levels in the exercised rats. Furthermore, our preliminary data showed that strenuous exercise also induced a significant increase in plasma nitrite and nitrate (NOx). The correlative analysis demonstrated that there was a negative correlation of NOx and iron levels in plasma in the exercised rats. On the basis of these results plus the recent findings on interaction of nitric oxide (NO) with iron metabolism (12, 21), we proposed that increased NO production might be one of the reasons for the changes of iron metabolism found in the exercised rats.

In the present study, we investigated the changes in tissue NOx concentrations and non-heme iron contents as well as the effects of NO synthase (NOS) inhibitor [nitro-L-arginine methyl ester (L-NAME)] on these two indexes in exercised rats. The results indicate that in addition to the increased plasma NOx, exercise can lead to an increase in tissue NOx levels with a significant decrease in tissue iron contents. L-NAME inhibited the effect of exercise on NOx levels and iron contents in tissue and plasma. The data obtained provided further evidence to support the view that the increased production of NO might be one of the causes responsible for the low or suboptimal iron status and other
changes in iron metabolism induced by strenuous exercise.

METHODS

Animals and exercise protocol. The Department of Health of the Hong Kong Government and the Animal Ethics Committee of the Hong Kong Polytechnic University approved the use of animals for this study. Female Sprague-Dawley rats (aged 2 mo), supplied by the Animal House of The Hong Kong Polytechnic University, were housed in pairs in stainless steel rust-free cages at 21 ± 2°C with relative humidity of 60–65% and alternating 12-h periods of light and dark. After being kept under standard laboratory conditions for 1 wk, the animals were randomly assigned to one of the following four groups: sedentary (S1; n = 7); sedentary + L-NAME (S2; n = 7); exercise (E1; n = 6); exercise + L-NAME (E2; n = 8).

Swimming exercise was followed by a modification of the methods of Ruckman and Sherman (22) and Prasad and Pratt (18). Rats in exercise groups (E1 and E2) swam in groups of two or three in a glass swimming basin (45 × 80 × 80 cm width/length/height) filled with tap water to a depth of 50 cm. The water temperature was maintained at 35 ± 1°C. The rats swam 5 days/wk. The daily training lasted for 30 min in the first week and 1 h in the second week. The 2-wk swimming period was considered as a training period (22) so that increased exercise could be tolerated later. After the training period, 2 h of exercise were given per day (9:00–11:00 AM), lasting for 3 mo. L-NAME (Calbiochem) in the drinking water (1 mg/ml) was freshly prepared (daily) and orally administered to rats in the S2 and E2 groups for 3 mo. The inclusion of L-NAME in the drinking water did not alter the water intake of these animals. The consistency of exercise intensity between the rats treated with and without L-NAME was strictly controlled by observing the whole swimming process. Our choice of the word “exercised” in the present study was based on the experimental protocols and animal model used in a number of studies (10, 18, 22). However, it should be pointed out that the term “trained” is indeed a better word to describe the experimental groups than the word exercised, because bouts were repeated daily over a period of time. The rats in the control groups (S1 and S2) remained sedentary in their cages and received approximately the same amount of handling as the exercised animals throughout the entire experiment. At the end of the 3-mo experiment, the animals were not fed for 24 h after the last exercise regimen and then heparinized and killed by 40 mg/kg ip of pentobarital sodium.

Sampling of blood and tissue. Fasting blood samples were collected from heart after death and aliquots were taken immediately for hematological indexes and NO determination. The liver, spleen, kidney, heart, brain, lung, and adrenal gland were removed, weighed, and stored in a freezing chamber below −70°C. The blood in the liver and spleen was washed out by a perfusion of 0.9% saline (iron free) before the samples were stored. Subsequent determination of non-heme iron and NO concentrations in the liver and spleen were performed. The bone marrow cells were isolated from both femora and tibiae by rapidly splitting scraped bones according to the method described previously (20). The NOx contents and non-heme iron concentrations in the cells were measured as well.

Analytical methods. Hb concentration was determined by the cyanmethemoglobin method. Hematocrit (Hct) was measured using the microhematocrit centrifuge (5). Plasma iron and total iron-binding capacity were determined using commercial kits (Sigma, St Louis, MO). Non-heme iron concentrations of tissues and cells were measured according to the method described by Kaldor (13). Protein contents were determined with a commercial kit (Sigma). NOx levels in the plasma and tissues were measured with Griess reagent according to the manufacturer’s instructions (Calbiochem). A number of recent studies has demonstrated that NOx determination by Griess reaction is useful for the evaluation of NO production (2, 16, 19). These studies also showed that L-NAME administration does not significantly interfere with NOx measurement by Griess reaction. The data were evaluated using a two-way ANOVA and Tukey’s post hoc tests. The results were expressed as means ± SE. The statistical calculation was performed using SPSS software for Windows (version 9.0). A probability value of P < 0.05 was taken to be statistically significant. There were no significant interactions between exercise and L-NAME for any variables.

RESULTS

Effect of strenuous exercise and L-NAME treatment on the body and organ weights, hematological indexes, and plasma NOx contents. The average body and organ weights in the strenuously exercised and sedentary rats treated with and without L-NAME were measured at the end of the 3-mo experimental period. The results are presented in Table 1. It was found that swimming led to a significant increase in heart weight. The average heart weights in the E1 and E2 groups were significantly higher than their corresponding values in the sedentary animals. However, no significant differences were found in the weights of the bodies and other organs between the S1 and the E1 groups. Also, L-NAME treatment did not affect the average body and other organ weights, no differences being found in these measurements between S1 and S2 or E1 and E2.

The blood Hct, Hb, and total iron-binding capacity of the S1 and the E1 groups were not significantly different (Table 2). L-NAME treatment did not lead to a significant change in these measurements, and no differences were found between S1 and S2 or E1 and E2. However, the results showed that the E1 group had

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<th>Table 1. Body and organ weights in the strenuous exercised and sedentary rats treated with and without L-NAME</th>
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<td>Sedentary Groups</td>
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<td>Body</td>
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Values are means ± SE (g). S1, sedentary control group; S2, sedentary + L-N-nitro-L-arginine (L-NAME); E1, exercise control group; E2, exercise + L-NAME group. *P < 0.05; †P < 0.01 vs. S1 group.
significantly lower plasma iron (PI) levels and transferrin saturation (TS) than the S1 animals (both \( P < 0.001 \)). Treatment with \( \text{L-NAME} \) led to a significant increase in PI and TS in the E2 group compared with the E1 group (both \( P < 0.05 \)) but not in the sedentary rats. However, the PI and TS in the E2 group were still significantly lower than those in the S1 group (\( P < 0.05 \) and 0.001, respectively). In addition, plasma NOx levels (Table 2) were significantly increased in the E1 group compared with the S1 group (\( P < 0.001 \)). The E2 group had significantly lower NOx levels than the exercised rats treated without \( \text{L-NAME} \) (\( P < 0.01 \)). However, this value in the E2 group was still significantly higher than that of the S1 rats (\( P < 0.05 \)).

**NOx contents in the liver, spleen, and bone marrow cell.** The results showed that strenuous exercise significantly enhanced NOx concentrations in the liver, spleen, and bone marrow (Fig. 1). After the 3 mo of the experiment, the mean NOx contents in the liver, spleen, and bone marrow cells in the E1 group were significantly higher than their corresponding values in the S1 group (\( P < 0.05, 0.001, \) and 0.001, respectively). This indicates that strenuous exercise can result in an increase in NOx contents in the liver, spleen, and bone marrow cells. The treatment with \( \text{L-NAME} \) significantly affected NOx contents in the liver, spleen, and bone marrow cells in the exercised rats but not in the sedentary rats (Fig. 1). Although the mean NOx contents in the liver, spleen, and bone marrow cells in the S2 group were lower than in the S1 group, a significant difference was not reached (\( P = 0.075, 0.096, \) and 0.099, respectively). However, the NOx contents in the liver, spleen, and bone marrow cells in the E2 group were significantly lower than in the E1 group (\( P < 0.05, 0.01, \) and 0.01, respectively). No differences were found in the NOx contents of the liver, spleen, and bone marrow cells between groups E2 and S1.

**Effect of exercise and \text{L-NAME} treatment on non-heme iron concentrations in the liver, spleen, and bone marrow cell.** A significant decrease in the mean non-heme iron levels of the liver, spleen, and bone marrow cells was found in the E1 compared with the S1 (all \( P \) values <0.001; Fig. 2). Treatment of \( \text{L-NAME} \) in the S2 group led to a significant increase in non-heme iron contents in the liver (\( P < 0.05 \)) and spleen (\( P < 0.05 \)) but not in the bone marrow cells (\( P = 0.15 \)) compared with the S1 group. Whereas the E2 group had a significantly higher level of non-heme iron in the liver, spleen, and bone marrow cells than the E1 group (\( P < 0.01, 0.01, \) and 0.05, respectively), it was noted that these values were still significantly lower than those in the S1 group (\( P < 0.05, 0.01, \) and 0.05, respectively). This indicates that \( \text{L-NAME} \) treatment can recover significantly, but only in part, the decreased iron levels in the liver, spleen, and bone marrow cells resulting from strenuous exercise.

### Table 2. Hematological indexes and plasma NOx levels of the strenuous exercised and sedentary rats treated with and without \text{L-NAME} inhibitor (\text{L-NAME})

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<th>Exercise Groups</th>
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<td></td>
<td>( \text{S1} ): control</td>
<td>( \text{E1} ): control</td>
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<td></td>
<td>( n = 7 )</td>
<td>( n = 6 )</td>
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<tr>
<td>Hb, g/dl</td>
<td>15.9 ± 0.7</td>
<td>14.7 ± 1.0</td>
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<tr>
<td>Hct, %</td>
<td>40.5 ± 1.0</td>
<td>39.8 ± 1.3</td>
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<tr>
<td>PI, µg/ml</td>
<td>2.07 ± 0.13</td>
<td>1.06 ± 0.07</td>
</tr>
<tr>
<td>TS, %</td>
<td>52.8 ± 1.6</td>
<td>26.1 ± 1.7*</td>
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<tr>
<td>TIBC, µg/ml</td>
<td>3.91 ± 0.18</td>
<td>4.04 ± 0.15</td>
</tr>
<tr>
<td>NOx, µM</td>
<td>21.5 ± 1.43</td>
<td>42.08 ± 3.47*</td>
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Values are means ± SE. Hct, hematocrit; PI, plasma iron; TIBC, total iron-binding capacity; TS, transferrin saturation (% PI/TIBC). NO, nitric oxide synthase; NOx, nitrate and nitrite. \(*P < 0.001, \dagger P < 0.05 \) vs. \( \text{S1}; \ddagger P < 0.01, \S P < 0.05 \) vs. \( \text{E1} \).

**DISCUSSION**

These experiments confirm our earlier observation (20) that strenuous exercise leads to a significant decrease in plasma iron levels as well as in tissue non-heme iron contents without the marked changes in Hb. In addition to the increase in plasma NOx concentration, a significantly higher level of NOx in tissues such as the liver, spleen, and bone marrow cells was ob-

![Fig. 1. Effects of nitro-L-arginine methyl ester (L-NAME) treatment on the concentrations of nitrate and nitrite (NOx) in the liver (A), spleen (B), and bone marrow cells (C) in strenuous exercised and sedentary rats. The results are expressed as means ± SE. *\( P < 0.05 \) and ***\( P < 0.001 \) vs. the sedentary control (S1). +\( P < 0.05 \) and ++\( P < 0.01 \) vs. the exercise group (E1).](http://ajpregu.physiology.org/10.1152/ajpregu.00820.2011)
served in the exercised rats in this study. Results also showed that exercised rats treated with L-NAME, an inhibitor of NOS, have a significantly lower level of NOx in tissues and cells in the exercised rats than in the S1 group. L-NAME treatment led to a partial recovery of non-heme iron levels in tissues and cells in the exercised rats. The difference of effects of L-NAME on NOx and non-heme iron in tissues in exercised rats implies that there might be other factors in addition to the increased NO production involved in the development of the low iron status induced by strenuous exercise. Further studies are needed to clarify these factors. In addition, the treatment of L-NAME would be expected to have many effects in addition to its direct effect on iron metabolism. It has been shown in rats that ingestion of L-NAME for 2 wk leads to significant reductions in insulin release in response to a glucose load and sodium and potassium imbalances (1). It is unknown at present whether these effects induced by L-NAME ingestion are associated with the partial recovery of non-heme iron levels in tissues and cells found in exercised rats treated by L-NAME.

The results obtained from this study show that exercise did not result in a significant change in Hb concentration in the blood. Because of the possible existence of the expanded blood volume induced by exercise (6, 8), the actual amount of Hb in the blood of exercised rats is probably higher than in sedentary animals. This unchanged or increased Hb in the blood is probably due to the increased transferrin-bound iron uptake, therefore increasing the rate of Hb synthesis and speed of Hb release from the cells. The increased speed of Hb release might be the reason why intracellular iron in bone marrow erythroblasts is lower in exercised rats than in sedentary rats, although exercised rats have a significantly higher number of transferrin-receptor and transferrin-bound iron uptake in bone marrow cells (20). In addition to NO, the intracellular low iron is without a doubt involved in the increased expression of transferrin receptor that leads to lower PI. It is likely that the increased NO and the decreased intracellular iron both are related to the increased transferrin-receptor expression, although it is unknown which one is the cause and which the consequence.

In conclusion, the findings obtained from the present study, in addition to our previous data and new advances in the studies of the interaction of NO and iron metabolism, support the viewpoint that increased NO production might be one of the causes of the changes in iron metabolism found in strenuously exercised rats.

**Perspectives**

Although iron has been studied extensively with respect to exercise, the mechanism by which exercise produces low or suboptimal iron status is unknown. To our knowledge, the present study is the first report on the effect of strenuous exercise on NO production and the possible role of NO in the changes of iron metabolism in strenuously exercised rats. On the basis of this study and our previous data (20), it is proposed for the first time that increased NO production might be one of the causes of the low-iron status in the strenuously exercised rats. The recent findings on the interaction of
NO and iron metabolism reported by others (12, 21) have shown that NO plays a role in the regulation of iron metabolism. NO, in addition to intracellular iron levels, can regulate the expression of transferrin receptor and ferritin, two important proteins in the determination of iron at the cellular level, by interacting with iron regulatory protein (IRP; or iron regulatory factor) (4, 12, 14, 27), thus affecting the number of transferrin receptors and ferritin. NO itself has been reported to be able to cause the removal of iron from ferritin stores (21), thereby reducing the tissue and cell iron levels. The increased NO induced by exercise might lead to an increased binding of IRP to the iron responsive elements of mRNAs both of the transferrin receptor and ferritin, then specifically repress the biosynthesis of cellular iron storage protein ferritin and stimulate the expression of cellular iron accumulation protein transferrin receptor. It results in an increased transferrin-receptor number on the cellular membrane and transferrin-bound iron uptake by bone marrow cells (20), the decreased plasma iron level and tissue non-heme iron contents, and the transfer of iron from storage location to some important cells in iron utilization. To confirm this possibility, further study is needed to investigate whether strenuous exercise can lead to significantly increased IRP activity (or decreased activity of cytosolic aconitase) in the liver, spleen, and bone marrow cells and to identify the effect of l-NAME on IRP activity in strenuously exercised rats. Such studies will provide direct evidence for the involvement of NO and IRP in the changes of iron status in exercise.

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