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Changes of transferrin-free iron uptake by bone marrow erythroblasts in strenuously exercised rats

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The effects of strenuous exercise on transferrin-free iron (Fe II) uptake by bone marrow erythroblasts in rats were investigated. Female Sprague-Dawley rats were randomly assigned to one of six groups, three of which underwent 3, 6, or 12 months of strenuous exercise (swimming 2 hr/day, 5 days/week) or their corresponding three control groups. At the end of experiments, bone marrow erythroblasts were isolated for Fe II uptake assay in vitro. It was found that the amounts of iron uptake into cytosole and stroma of the cells of rats in the groups undergoing 3 and 12 months of exercise did not differ from those in their corresponding sedentary groups. In addition, analysis of nonspecific and specific Fe II uptake by cytosole and stroma did not display any significant difference between the exercise and corresponding sedentary groups. However, the amount of Fe II uptake into cytosole and stroma was significantly increased in rats that exercised for 6 months compared with the corresponding controls. Nonspecific iron uptake into stroma was significantly higher in the exercise group than in the sedentary group (0.120 ± 0.018 vs. 0.049 ± 0.006 pM/10^6 cells, $P < 0.01$). The V_{max} of the specific iron uptake into stroma was significantly higher (0.326 ± 0.024 vs. 0.238 ± 0.037 pM/10^6 cells, $P < 0.05$) and the K_m of iron uptake into cytosol lower (0.08 ± 0.01 vs. 0.21 ± 0.03 μM, $P < 0.001$) in the exercise groups than in the control groups. These results indicate that 6 months of strenuous exercise could significantly increase Fe II uptake by the cells, probably by affecting the number and/or affinity of the putative iron carrier and the permeability to iron of cell membrane. The increased ability of cell-free iron accumulation in exercise might be a self-protective mechanism for body cells from the free iron-induced free radical reaction in addition to providing more iron for cell heme synthesis. (J. Nutr. Biochem. 11:367–373, 2000)

Keywords: transferrin-free iron; bone marrow erythroblasts; strenuous exercise; carrier-mediated iron uptake

Introduction

Normal iron status in athletes is especially important because of the central role of this mineral in oxygen transport and the synthesis of hemoglobin, myoglobin, and certain enzymes essential to energy production. On the other hand, exercise can affect iron status of athletes. During the past decades, a considerable amount of research has been devoted to investigating the effects of exercise on body iron status in both humans and animals.[1–9] However, most studies have addressed this issue by measuring plasma indices of iron status and tissue iron levels. Little is known about the effects of strenuous exercise on cellular iron accumulation.

It is generally believed that transferrin (Tf) and transferrin receptor (TfR)-mediated iron uptake (or Tf- and TfR-dependent iron transport) is the major route or the normal source for cellular iron accumulation under physiologic conditions. The number of TfRs on the cellular membrane is an important factor in determining the amount of iron acquired by a cell in vivo.[10–13] A number of studies, however, show that non-Tf-bound iron can also enter the cells by a process independent of Tf and TfR. Several groups have reported evidence for the existence of a Tf- and TfR-independent iron transport system on the membrane of mammalian cells such as intestinal mucosal cells,[14–16] liver

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cells,[17,18] reticulocytes,[19–23] and some cultured cells.[24–26] The mechanism involved in this transport system is not yet completely documented but evidence supports the possibility that an iron carrier-mediated process is involved in the non-Tf-bound iron transport across the cell membrane.[11] It also has been suggested that the most important physiologic function of this system is likely to participate in the process of Tf-bound iron uptake, playing a key role in translocation of iron from endosome to cytosol after iron is released from Tf within the endosome. The existence of this system, which is the same as that of the Tf- and TfR-dependent system, has the same important physiologic role in the determination of cellular iron.[13]

In a previous study,[27] we investigated the effect of strenuous exercise (3 months of swimming) on TfR expression and transferrin-bound iron (Tf-Fe) uptake in bone marrow erythroblasts in rats to address further the issue of exercise and iron status at the cellular level. It was found that both the TfR number on the cell surface and Tf-Fe uptake in bone marrow erythroblasts were significantly increased in the strenuously exercised rats. Similar results were observed in exercised rats that swam for 6 or 12 months in a subsequent investigation.[28] However, it is unknown whether the strenuous exercise can affect non-Tf-bound iron (or Tf-free iron) uptake by the cells. Therefore, the experiments in this study were designed to investigate the effects of strenuous exercise on Tf-free iron uptake by bone marrow erythroblasts in rats.

Materials and methods

Animals and exercise protocol

The use of animals for this study was approved by the Department of Health of the Hong Kong Government and the Animal Ethics Committee of the Hong Kong Polytechnic University. Female Sprague-Dawley rats, aged 2 months, 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 a relative humidity of 60 to 65% and a 12-hr dark-light period. After being kept under the standard laboratory conditions for 1 week, the animals were randomly assigned to one group undergoing 3 (n = 8), 6 (n = 11), or 12 months (n = 8) of strenuous exercise or to a corresponding control group (n = 7, 8, and 7, respectively). Laboratory rodent diets for rats (PMI Nutrition International, the Richmond Standard, St. Louis, MO USA) and distilled water were provided ad libitum throughout the experimental period. Swimming was performed according to the procedure described previously.[27] Briefly, the rats in exercised groups swam in groups of three in a glass swimming basin (45 cm width × 80 cm length × 80 height) filled with tap water to a depth of 50 cm so that the rats could not rest by supporting their body with their tails. The water temperature was maintained at 35 ± 1°C. The rats swam 5 days per week. The daily training lasted for 30 min for the first week and 60 min for the second week. The 2-week swimming period was considered to be a training period[29,30] so that increased exercise could be tolerated later. After the training period, 120 min of exercise per day were given, lasting for different periods (3, 6, or 12 months). After swimming, rats were placed in a dry tank and warmed with overhead lamps. The rats in the corresponding control groups remained sedentary in their cages and received approximately the same amount of handling as the exercised animals throughout the entire experiment.

Bone marrow mononuclear cells

A method described previously[27] was used for cell preparation. In brief, animals were unfed for 24 hours following the last exercise regimen of the 3-, 6-, or 12-month period. The bone marrow cells were then isolated and suspended in ice-cold saline buffered with 20 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethansfulfonic acid] (HEPES), pH 7.4, at 4°C. The crude cell suspension was passed through two layers of nylon mesh.[31] The mononuclear cells were obtained by Histopaque (Sigma Chemical Co., St. Louis, MO USA) density gradient sedimentation. The cells were then washed three times with ice-cold Hanks' balanced salt solution (HBSS) buffered with 20 mM HEPES, pH 7.4, containing 2% bovine serum albumin. Subsequently the cells were suspended in the same buffer to a final concentration of 1×10^8 cells/mL. The cell suspension was kept at 4°C for no longer than 3 hr before use. To determine the percentage of erythroblast in cell suspensions, cell counts were carried out on dried cell smears stained with May-Grunwald-Giemsa stains.[32]

Measurement of Tf-free iron uptake

$Iron^{59}$ ($FeCl_3$, 5 μCi/μg) was purchased from Radiochemical Center (Amersham, Buckinghamshire, England). Radioactive iron solution was prepared according to Morgan.[19] Briefly, $^{59}FeCl_3$ was mixed with $^{56}FeSO_4$ (in 0.1 M HCl) in a molar ratio of 1:10, followed by a 50-fold molar excess of 2-mercaptoethanol and 0.27 M sucrose to give an iron concentration of 62.5 μM. No attempt was made to eliminate oxygen from the solutions. However, as described previously[19,22,23] the iron in this solution prepared with this method should be in the ferrous state because it reacted immediately and completely with 2,2′-bipyridine and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine with the production of the colored products characteristic of their reaction with ferrous iron. Thus, the iron in the above solution will be called Fe II in this article. The solution was used within 2 hr after preparation.

The following procedure was used to determine Tf-free iron uptake by the bone marrow erythroblasts in all experiments, except where indicated. The washed bone marrow cells in 0.27 M sucrose (1×10^6 cells) were added to the incubation medium, which consisted of 0.27 M sucrose buffered to pH 6.5 with 4 mM piperazine diethanesulfonic acid (PIPES). After being prewarmed in a shaking water bath for 10 min at 37°C, the radioactive Fe II solution was added. The total incubation volume was 0.50 mL, with an iron concentration of 2 μM. The incubation was then performed at 37°C in a shaking water bath for the desired period of time, after which the cells were centrifuged at 1,000 × g for 10 min at 4°C and washed three times with ice-cold saline. During the third wash, the cells were transferred to fresh test tubes. The cells were hemolyzed with 20 mM HEPES, pH 7.4, and centrifuged at 10,000 × g for 20 min at 4°C to separate cytosolic and stromal fractions of the cells. These two fractions were counted for radioactive iron separately. In certain experiments, the effect of different concentrations of iron on cellular Tf-free iron uptake was determined. The same procedure was used except that the Fe II solution was added in amounts required giving the desired final iron concentrations (0–4 μM).

Analytical methods

Cell counts were made using a hemocytometer. Radioactivity was measured in a three-channel γ-counter (Packard 5003 COBRA Q, Packard Instrument Co., Meriden, CT USA). The data were expressed as means ± SE. The statistical calculation was performed using the Student's t-test.

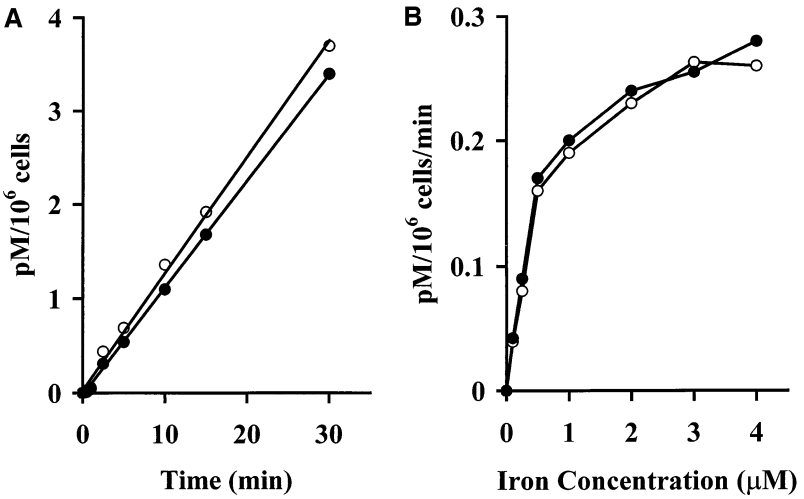

Figure 1 (A) Time course of Fe II uptake by cytosolic (○) and stromal (●) fractions of rat bone marrow cells. (B) Effect of varying the Fe II concentrations in the incubation medium on the rate of iron uptake into cytosol and stromal fractions of the cells. The data are the means of duplicate determinations.

Results

Time course and effect of iron concentration

Preliminary experiments showed that the uptake of Fe II by bone marrow cells was optimal when incubation was performed at pH 6.5 in isotonic sucrose solution. Therefore, these conditions were used to determine the time course of Fe II uptake by the cells in this study. The results indicated that the Fe II uptake into both the cytosolic and the stromal fractions increased with the time in a linear manner for at least 30 min (*Figure 1A*). This linear relationship was found in cells derived from both the exercised and control rats. Because iron uptake was linear with respect to time, the rate of iron uptake was determined by taking a single incubation time point and calculating the rate of iron uptake in terms of picomolars of iron per 10^6 cells per minute. The 30-min incubation period was used to determine the effect of different concentrations of iron on Tf-free iron uptake. The rate of Fe II uptake into the cytosolic and stromal fractions of the cells showed evidence of saturation as the iron concentration in incubation medium was raised to approximately 1 μM, but it continued to increase in a linear manner with further increase in concentration (*Figure 1B*). Hence, there was evidence of both a saturable or specific and a nonsaturable or nonspecific uptake processes. These results were similar to the previous observations in both rabbit and rat reticulocytes by Morgan.[19] The specific process was analyzed by the Eadie-Hofstee method to determine the maximum rate of iron uptake (V_{max}) and the Michaelis constant (K_m) after subtracting the nonspecific part of the uptake, as determined by the slope of the linear portion of the uptake curve. The mean K_m values were 0.170 ± 0.033 and 0.133 ± 0.023 μM for uptake into the cytosolic and stromal fractions, respectively. The corresponding V_{max} values were 0.267 ± 0.028 and 0.263 ± 0.040 pM/10^6 cells/min.

Effects of strenuous exercise on Tf-free iron uptake by bone marrow cells

3 months of exercise. After 3 months of experiment, bone marrow cells were isolated from the exercised and sedentary rats. The cells were used to determine the effect of exercise on cellular Fe II uptake using the procedure described in Materials and methods. It was found that incorporation of iron into both cytosol and stroma increased with the increase of iron concentrations in the exercise and the sedentary groups. At all concentration points, no significant differences in the rates of iron incorporated into cytosol or stroma were found between the exercise and the sedentary groups (*Figure 2A and 2B*). The rates of nonspecific uptake by cytosol were 0.057 ± 0.012 and 0.053 ± 0.010 pM/10^6 cells/min in the exercise and the sedentary groups, respectively ($P > 0.05$; *Figure 3*). The corresponding stromal values were 0.063 ± 0.013 and 0.047 ± 0.008 pM/10^6 cells/min ($P > 0.05$). In addition, no significant differences in V_{max} and K_m values of the cytosolic and stromal fractions were found between the exercise and sedentary groups (*Table 1*). These results indicate that 3 months of strenuous exercise did not affect Tf-free iron uptake by bone marrow cells in rats.

6 months of exercise. The amounts of iron uptake into both cytosol and stroma of bone marrow cells was significantly higher in the rats exercised for 6 months than in the corresponding control rats (*Figure 2C and 2D*). The rate of nonspecific uptake into the stroma was 0.120 ± 0.018 pM/10^6 cells/min in the exercise group, which was significantly higher than that of the corresponding control group (0.039 ± 0.006 pM/10^6 cells/min; $P < 0.01$). The corresponding values in cytosol did not differ between the exercise and sedentary groups ($P > 0.05$; *Figure 3*). After subtracting the nonspecific uptake, the specific uptake of iron into two fractions was calculated. The K_m value in the cytosolic fraction was significantly lower in the exercised rats (0.08 ± 0.01 μM) than in the control animals (0.21 ± 0.03 μM; $P < 0.01$), but this difference was not found in the K_m values of the stromal fraction ($P > 0.05$). In contrast, the V_{max} value in the stromal fraction, but not in the cytosolic fraction ($P > 0.05$), was found to be significantly higher in the exercised group (0.326 ± 0.024 pM/10^6 cells/min) than in the corresponding control group (0.238 ± 0.037 pM/10^6 cells/min; $P < 0.05$). The above results show

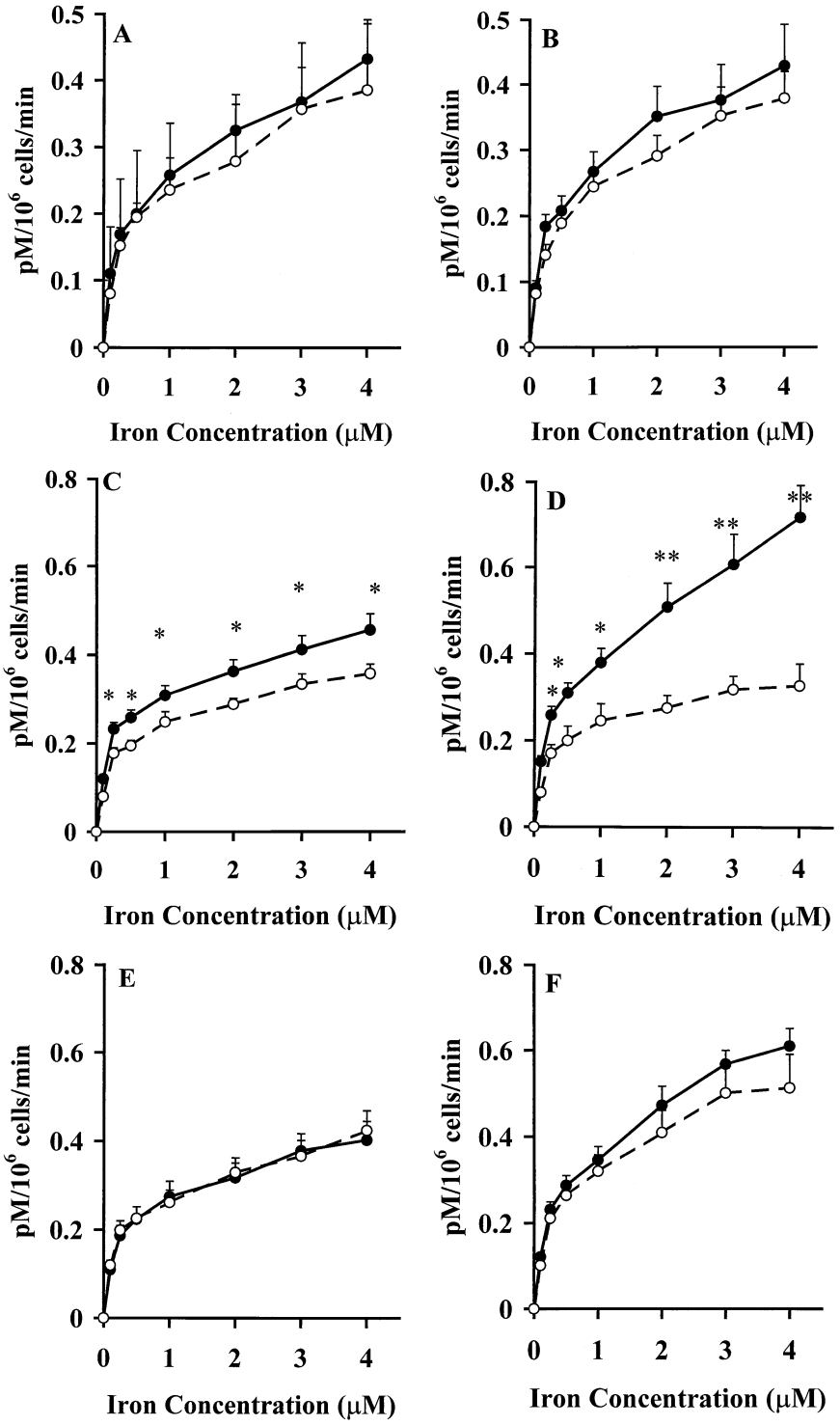

Figure 2 Fe II uptake into cytosolic (A,C,E: 3, 6, and 12 months, respectively) and stromal (B,D,F: 3, 6, and 12 months, respectively) fractions in exercised (●) and sedentary (○) rats. The values are means ± SE. *$P < 0.05$; **$P < 0.01$; **$P < 0.001$ versus their corresponding control groups.

that the Fe II uptake by bone marrow cells was enhanced by 6 months of strenuous exercise.

12 months of exercise. Fe II uptake into cytosol and stroma of the cells was also determined over 12 months in exercise and corresponding control groups. No significant difference in the amount of Fe II uptake by the cells was found between the exercise and sedentary groups (*Figure 2E and 2F*). Further analysis showed that the nonspecific uptake of iron into cytosolic and stromal fractions in the exercise group did not differ from that in the sedentary group (*Figure 3*). However, a significantly higher K_m in the specific iron

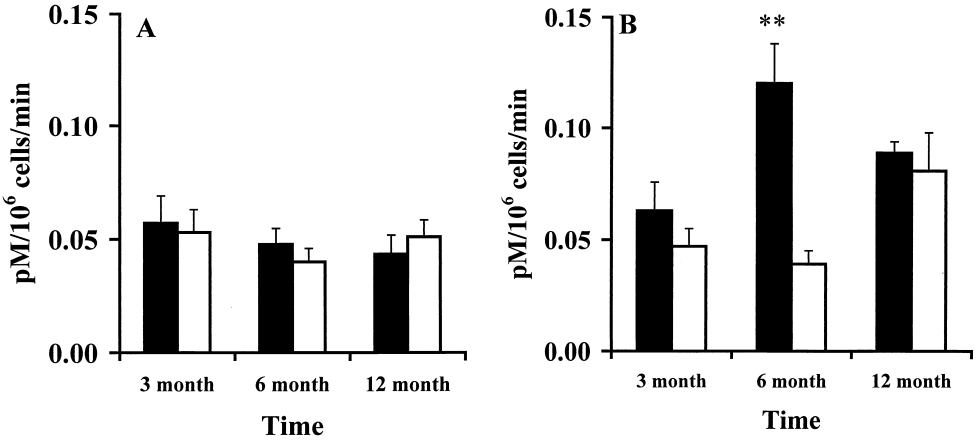

Figure 3 The nonspecific iron uptake into (A) stromal and (B) cytosolic fractions of the bone marrow cells in the exercise (filled columns) and sedentary (open columns) groups. The values are means ± SE. $^{**}P < 0.01$ versus corresponding control.

uptake into stroma (0.25 ± 0.04 μM) was found in the exercise group than in the control group (0.13 ± 0.02 μM; $P < 0.05$). The K_m value of the cytosol in the exercise group (0.12 ± 0.03 μM) was not significantly different from that in the sedentary group (0.17 ± 0.03 μM; $P = 0.07$). Iron uptake into the stroma in the exercise group had a relatively greater V_{max} (0.405 ± 0.047 pM/10^6 cells/min) than did that in the control group (0.290 ± 0.032 pM/10^6 cells/min), but statistical difference did not reach a significant level ($P = 0.07$; *Table 1*).

Discussion

The present study demonstrates that non-Tf-bound iron uptake into the cytosol and stroma of cells could be significantly affected by 6 months of exercise. The increased ability of cells in Tf-free iron uptake might be important for the cells to acquire enough iron for the enhanced heme synthesis during long-term exercise, although its physiologic importance remains to be investigated. The uptake of Tf-free iron showed evidence of both a saturable or specific process and a nonsaturable or nonspecific process. The changes in K_m and V_{max} in the exercised rats suggest that the increased iron uptake into cells might be associated with the changes in the number and/or affinity of the putative iron carrier or transporter on the membrane of cells. The significantly increased nonspecific iron uptake might imply that the exercise could lead to the changes in structure and/or functions of plasma membrane and therefore affect membrane iron transport. The duration and intensity of exercise might be two major factors in determining the changes of ability of cell accumulation of Tf-free iron. The Tf-free iron uptake by the cells was not significantly increased in rats exercised for 3 and 12 months. The former was most likely due to the relatively shorter duration of exercise. The latter might suggest that long-term and regular exercise could result in the formation of self-adaptive mechanisms. However, the precise causes resulting in the difference in the effects of duration on Tf-free iron uptake by the cells must be addressed.

On the other hand, the increased ability of the cells to acquire Tf-free iron, which was found in the rats that swam for 6 months, suggests that a given period of exercise could lead to a more effective removal of "free" iron from extracellular fluid or plasma by body cells. This exercise-induced change in Tf-free iron uptake by cells might be a

Table 1 K_m and V_{max} values for specific Fe II uptake into cytosol and stroma in exercised and sedentary rats

Time (months)	Groups	K_m (μM)		V_{max} (pM/10^6 cells/min)	
		Cytosol	Stroma	Cytosol	Stroma
3	Exercise ($n = 8$)	0.19 ± 0.03	0.15 ± 0.04	0.259 ± 0.026	0.290 ± 0.044
	Sedendary ($n = 7$)	0.18 ± 0.04	0.12 ± 0.03	0.244 ± 0.033	0.261 ± 0.051
6	Exercise ($n = 11$)	0.08 ± 0.01[b]	0.12 ± 0.03	0.295 ± 0.022	0.326 ± 0.024[a]
	Sedentary ($n = 8$)	0.21 ± 0.03	0.15 ± 0.02	0.288 ± 0.023	0.238 ± 0.037
12	Exercise ($n = 8$)	0.17 ± 0.03	0.25 ± 0.04[a]	0.278 ± 0.030	0.405 ± 0.047
	Sedentary ($n = 7$)	0.12 ± 0.03	0.13 ± 0.02	0.270 ± 0.030	0.290 ± 0.032

The values are means ± SE.
[a] $P < 0.05$.
[b] $P < 0.001$ versus their corresponding sedentary groups.

self-protective response to exercise for the body cells from the toxic effects of the "free" iron-induced free radical reaction. It has been well documented that mobilization of iron from storage locations such as liver and spleen and erythropoiesis and erythrocyte turnover are increased in exercise. Several research groups have provided evidence that exercise can increase the level of loosely bound iron and transit iron in tissues.[8,33–35] It is possible that the formation of "free" iron in local tissue may be relatively higher and the free radical reaction induced by "free" iron can hence be subsequently enhanced. However, exercise also increases the ability of cells to accumulate "free" iron. It will decrease the amount of free iron in local tissues and hence protect cells from toxic damage induced by "free" radical reaction. The balance between formation of "free" iron and the ability of "free" iron accumulation by cells might be important for normal cellular functions in strenuous exercise.

As mentioned in Introduction, Tf-bound iron uptake is a major route of cellular iron accumulation under physiologic conditions. In a previous study,[27] it was found that 3 months of strenuous exercise in rats could induce a significant increase in TfR expression, Tf endocytosis, and consequently Tf-Fe uptake in bone marrow erythroblasts. In addition, longer periods of strenuous exercise (6 or 12 months) had similar results.[28] However, in this study, the significant increase in Fe II uptake by the cells was found in rats that swam only for 6 months, not for 3 or 12 months. The amount of Fe II uptake by the cells was not increased with the increase in the expression of TfR and Tf-bound iron uptake. It indicated that the effects of strenuous exercise on Tf-bound and Tf-free iron uptake by the cells were different. It might imply that Tf-bound and Tf-free iron uptake by the cells was mediated by different membrane transporters or mechanisms. This result does not favor our previous suggestion that the TfR might function as a membrane iron carrier[13,23] to mediate Fe II transport across cellular membrane. Therefore, it is reasonable to believe that Fe II uptake by bone marrow cells found in this study is independent of Tf and TfR. Although it is possible that Fe II used in the present study is bound by the residual Tf present in the cells and taken up by the Tf-Fe uptake mechanism, this is not supported by the fact that the pH used in this study is 6.5. It has been demonstrated that at this pH, Tf binds iron very poorly and very little Tf can be internalized by the cells.[19,36] The saturable or specific component of Fe II uptake into the cells suggested the existence of a membrane iron carrier protein-mediated process similar to that found previously in reticulocytes of rats and rabbits.[19,21–23]

The mechanism of the increased Fe II uptake by the cells or expression of the putative iron carrier on the membrane induced by strenuous exercise is unknown. The expression of TfR or Tf-Fe uptake by most types of cells is regulated by intracellular iron content at the post-transcriptional level. If intracellular iron levels decrease, the expression of TfR increases and hence cells acquire more Tf-Fe. However, in contrast to TfR regulation, some observations have not found that the non-Tf-bound iron uptake is responsive to the change in iron concentration within the cells.[24,25] Therefore, further investigation will be required to elucidate the causes responsible for the increased expression of the putative iron carrier protein during exercise. In addition, why and how the nonsaturable iron uptake into cytosol and stroma is increased in exercise should be addressed. The understanding of these two issues is critical for fully elucidating the effect of exercise on iron metabolism and the role of the changed membrane iron transport in exercise.

In conclusion, our findings indicate that a given period of strenuous exercise was able to significantly affect the amounts of Fe II uptake by bone marrow cells. The relevant mechanism is unknown; however, it is probably due to the changes in the putative iron carrier number, the affinity of this carrier with iron, and/or the permeability of cell membrane. This increased ability of cells to accumulate "free" iron, induced by strenuous exercise, might play an important role in providing more iron for the increased heme synthesis as well as in protecting the cells from the "free" iron-induced free radical reaction during exercise.

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