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Effects of dietary calcium restriction and acute exercise on the antioxidant enzyme system and oxidative stress in rat diaphragm

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Itoh, Masayuki, Shuji Oh-ishi, Hidekazu Hatao, Christiaan Leeuwenburgh, Colin Selman, Hideki Ohno, Takako Kizaki, Hiroyuki Nakamura, and Takeshi Matsuoka. Effects of dietary calcium restriction and acute exercise on the antioxidant enzyme system and oxidative stress in rat diaphragm. *Am J Physiol Regul Integr Comp Physiol* 287: R33–R38, 2004. First published February 5, 2004; 10.1152/ajpregu.00598.2003.—Calcium deficiency is considered to increase intracellular calcium level; thus the aim of the current study was to elucidate whether dietary calcium restriction enhanced exercise-induced oxidative stress in rat diaphragm. Twenty male Wistar rats were randomly assigned to either a control group or a group subjected to 1 mo of calcium restriction. In addition, each group was subsequently subdivided into rested or acutely exercised group. Dietary calcium restriction significantly ($P < 0.05$) upregulated the activities of manganese-superoxide dismutase (Mn-SOD), copper-zinc-superoxide dismutase (Cu-Zn-SOD), and glutathione peroxidase (Gpx) but not catalase. Acute exercise, in addition to calcium restriction, decreased both SOD isoenzymes in the diaphragm of calcium-restricted rats ($P < 0.05$). On the other hand, calcium restriction resulted in increased Gpx mRNA expression ($P < 0.05$). In control rats, acute exercise significantly ($P < 0.05$) increased the expressions of both SOD mRNAs, whereas in the calcium-restricted rats, it increased that of Mn-SOD mRNA ($P < 0.05$) but decreased that of Gpx mRNA ($P < 0.05$). Furthermore, reactive carbonyl derivative, a marker of protein oxidation, was significantly greater in the calcium-restricted rats than in the control rats after acute exercise ($P < 0.05$). The results suggest that antioxidant enzymes in rat diaphragm were upregulated in response to an increased oxidative stress by dietary calcium restriction but that upregulation is not enough to cope with exercise-induced further increase of oxidative stress.

superoxide dismutase; glutathione peroxidase; catalase; reactive carbonyl derivative

CALCIUM IS AN INORGANIC SUBSTANCE, which in many terrestrial organisms, including humans, may become deficient with advancing age, and this negative calcium balance appears to be associated with several conditions, including osteoporosis (11). It is also known that calcium deficiency increases intracellular calcium and blunts the calcium gradient between intracellular and extracellular spaces through the sodium/calcium exchange system (10). Intracellular calcium overload appears to increase oxidative stress; for example, phospholipase A_2 (PLA$_2$), a modulator of free radical generation in contracting muscle, is calcium dependent (24). In addition, oxidative stress also seems to result in an increase of intracellular free calcium; for example, Andrade et al. (2) have indicated that hydrogen peroxide causes an increase in intracellular calcium in skeletal muscle fibers, which is prevented by a reducing agent. Therefore, oxidative stress may disturb cellular calcium homeostasis in cells (29), consequently affecting gene expression (34) and protein synthesis (5). Thus, because a change in the concentration of intracellular calcium is a key regulator of a wide variety of cellular functions (7, 29), any rise in intracellular calcium concentration is expected to play a critical role in diaphragmatic dysfunction, especially for patients with airflow obstruction such as chronic obstructive pulmonary disease.

Physical exercise has been widely employed as a useful tool for comprehensive pulmonary rehabilitation as well as preventive measures against aging and/or osteoporosis (25, 33). However, paradoxically, physical exercise is also well known to markedly increase the blood flow and the oxygen uptake of active muscles, probably leading to an increased generation of reactive oxygen species (9, 28), which may result in oxidative damage to active muscles (9, 30). The diaphragm is a unique skeletal muscle that works continuously, and it is actively involved during exercise. Therefore, the diaphragm is potentially exposed to the increased production of reactive oxygen species and any resulting oxidative stress during exercise. Indeed, several studies have suggested that reactive oxygen species have the ability to cause the dysfunction of diaphragm (32, 36). Change in antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (Gpx), and catalase (Cat) should provide indirect evidence of the exposure to reactive oxygen species and oxidative stress.

Many studies suggest that a failure of muscle calcium homeostasis is a key step in exercise-induced muscle damage (3, 17, 20), although few have examined the effect of calcium deficiency on exercise-induced oxidative stress (including antioxidant enzymes system) (22, 27). The aim of the current study was to elucidate the synergism of dietary calcium restriction and acute exercise on the antioxidant enzyme system of rat

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diaphragm, measuring various antioxidant enzyme activities and determining their mRNA expressions, using real-time RT-PCR (12).

MATERIALS AND METHODS

Animals, Diet, and Exercise

A total of 20 male Wistar rats, weighing 60–80 g, at 4 wk of age, were obtained from Japan SLC (Shizuoka). Rats were held five per cage at 24°C under artificial lighting (12:12-h light-dark cycle; with lights on from 0700 to 1900). The animals were cared for in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan, based upon the Helsinki Declaration, 1964. After rats had been fed ad libitum with a control diet (1.04% calcium, Oriental MF, Oriental Yeast, Tokyo) for 8 wk, they were divided randomly into two groups. Rats in control group ($n = 10$) were fed a control diet for one more month, and rats in calcium-restricted group were fed a diet with a significant reduction in calcium (<0.01% calcium, Japan Crea, Tokyo) for 1 mo ($n = 10$). Subsequently, both groups were subdivided into two groups: individuals killed at rest (control/rested rats; calcium-restricted/rested rats) and killed immediately after an acute bout of exercise (control/exercised rats; calcium-restricted/exercised rats) ($n = 5$, in each group). During the last week of the experiment period, the exercise rats were acclimated to running on a rodent treadmill (15 m/min, 10 min/day, every 3 days, and 3 times in total). Acute exercise was performed on a treadmill with a speed of 20–22 m/min, 0% grade for 60 min. Electric shocks were used sparingly to motivate rats to run.

Tissue Preparation

Rats were anesthetized with sevoflurane, and blood was drawn into a heparinized syringe from the inferior vena cava. After rats had been killed by decapitation, the costal diaphragms were excised immediately, weighed, and frozen in liquid nitrogen. The muscle tissues were then stored at −80°C for biochemical analysis. The femur bones were also removed and cleaned of soft tissue. Subsequently, a portion of costal diaphragm tissues was minced in an ice-cold medium containing 0.25 M sucrose, 10 mM 2-amino-2-hydroxymethyl-1,3-propanediol, and 0.1 mM EDTA (pH 7.4, wt/vol 1:9), and homogenized on ice in brief bursts by a Polytron homogenizer (Kinematika, Lucerne, Switzerland). The homogenate was centrifuged at 750 *g* (4°C) for 20 min, and the supernatant was used for various assays. Protein content was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using BSA as a standard.

Bone Mineral Estimation

In regard to the femur, total surface area (total projected area of tissue including bone and marrow) and bone mineral content were measured using a dual-energy X-ray absorptiometry (Dexa) (DCS-600, Aloka, Tokyo). Bone mineral density was calculated as (bone mineral content)/area.

Antioxidant Enzyme Activities

Total SOD (EC 1.15.1.1) activity was determined using the method of Crapo et al. (8). One unit of SOD activity was defined as the amount required to inhibit the rate of reduction of cytochrome *c* by 50%. Mn-SOD was distinguished from Cu-Zn-SOD according to the cyanide procedure, in which the reaction mixture contains KCN at 1 mM final concentration. Gpx (EC 1.11.1.9) activity was assayed spectrophotometrically according to Tappel (39). Cat (EC 1.11.1.6) activity was measured by the method of Aebi (1).

mRNA Expressions of Antioxidant Enzymes

Total RNA extraction. Total RNA was isolated from 100 mg of diaphragm tissue using Trizol reagent (Life Technologies, Eggenstein,

Germany). RNA was resuspended in 50 μl of RNase-free water, and the quantity and purity of the total RNA were assessed spectrophotometrically.

Primers. Primers for each antioxidant enzyme mRNA expression were designed as follows: GAPDH forward primer, GGC AAG TTC AAC GGC ACA, and reverse primer, CGC CAG TAG ACT CCA CGA CA; Cu-Zn-SOD forward primer, GAT TAA CTG AAG GCG AGC AT, and reverse primer, CCG CCA TGT TTC TTA GAG T; Mn-SOD forward primer, GCT GGA GCC GCA CAT TAA C, and reverse primer, CAG CGC CTC GTG GTA CTT CT; Gpx forward primer, TGC AAT CAG TTC GGA CAT CA, and reverse primer, ACC ATT CAC CTC GCA CTT C; Cat forward primer, TTG AGC CCA GCC CGC ACA AG, and reverse primer, CAC GCG AGC ACG GTA GGG AC.

Real-time RT-PCR assay. Real-time RT-PCR was carried out with the iCycler iQ (Bio-Rad, Tokyo). According to the manufacturer's instructions, a master-mix of the following reaction components was prepared to the indicated concentration: 23 μl RNase-free water, 0.25 μl forward primer (0.5 μM), 0.25 μl reverse primer (0.5 μM), 25 μl 2 × QuantiTect SYBR Green RT-PCR Master Mix (QIAGEN), 0.5 μl QuantiTect RT Mix (QIAGEN). Forty-nine microliters of master-mix was filled in each well of the 96-well microplate, and 1 μl, containing 1 ng of total RNA from each sample, was added as PCR template. RNA standards were also added to give final concentrations of 0.04–25 ng of total RNA per RT-PCR reaction mixture. The following real-time RT-PCR protocol was used; reverse transcription (50°C for 30 min), PCR initial activation step (95°C for 15 min), amplification and quantification program (repeat 40 times; 94°C for 15 s, 58°C for 30 s, 72°C for 45 s with a fluorescence measurement), melting curve program (50–90°C with heating rate of 0.5°C/min and continuous fluorescence measurement). A standard curve was generated using the fluorescent data from the serial dilution of rat diaphragm total RNA. All samples were run in duplicate. Quantities of Mn-SOD, Cu-Zn-SOD, and Gpx in each sample were normalized to the corresponding input total RNA based on GAPDH quantitation. Values were expressed as the ratio of Mn-SOD, Cu-Zn-SOD, Gpx, or Cat mRNA expressions to GAPDH mRNA expressions.

The cycle number at which a significant increase in the fluorescence signal is first detected is defined as the threshold cycle. With the use of serial dilutions of the total RNA, the standard curve was generated on the basis of the linear relationship existing between the threshold cycle and the logarithm of the starting total RNA concentration. Melting curve analysis demonstrated that each of the primer pairs amplified a single predominant product with a distinct melting temperature. Melting temperature was used to identify specific products in subsequent analysis. As an additional control of specificity, the length of PCR products was confirmed by agarose gel electrophoresis.

Determination of Reactive Carbonyl Derivative

Reactive carbonyl derivative was determined by the 2,4-dinitrophenyl hydrazine (DNPH) method of Nakamura and Goto (23). Briefly, proteins in two equal portions of supernatant were precipitated with 10% TCA. The precipitates were treated with either 2 N HCl alone (control) or 2 N HCl containing 10 mM DNPH at 15°C for 1 h. After the reaction, the mixture was centrifuged, the precipitates were washed with an ethanol-ethyl acetate (1:1) mixture three times, and the final precipitates were dissolved in 8 M urea. The absorbance was measured at 360 nm, and the carbonyl content was obtained as nanomoles per milligram protein using a molar extinction coefficient of $22,000^{-1}$. Samples processed similarly but without DNPH treatment were used as controls.

Statistical Analysis

Data are expressed as means ± SE. The Mann-Whitney *U*-test was applied to the data, and statistical significance level was set at $P < 0.05$, using StatView (version 5.0, SAS Institute).

Table 1. *Effect of dietary calcium restriction on body weight, diaphragm weight and protein content, and bone mineral content*

Experimental Subgroup	Body Weight, g	Diaphragm		Bone Mineral	
		Weight, mg/100 g body wt	Protein content, mg/g wet wt	Bone mineral content, mg/bone	Bone mineral density, mg/cm^2
Control					
Rested	323.1±5	309.4±8.7	33.0±4.9	287.4±2.8	138.2±2.5
Exercised	321.5±6.8	327.6±14.5	33.9±1.0		
Calcium restricted					
Rested	335.2±5.2	293.5±14.1	29.8±2.3	266.4±3.1*	127.2±1.4*
Exercised	335.0±8.2	305.1±10.5	34.3±3.0		

Data are expressed as means ± SE; $n = 5$ in each group. *$P < 0.05$ vs. control (rested).

RESULTS

Body Weight, Diaphragm Weight and Protein Content, and Bone Mineral Estimation

There was no significant difference in body weight, diaphragm weight, and protein content between groups (Table 1). As was expected, both bone mineral content and bone mineral density of rat right femur were significantly reduced after 1-mo dietary calcium restriction (Table 1), implying that dietary calcium restriction used in the current study was sufficient to cause bone resorption in the rat femur. Therefore, the calcium-restricted rats could be regarded to be in a condition of calcium deficiency.

Antioxidant Enzyme Activities in the Rat Diaphragm

As shown in Fig. 1, both of Mn-SOD and Cu-Zn-SOD activities significantly increased after calcium restriction. Acute exercise decreased both of these activities only in the calcium-restricted group but had no significant change in the control group. With respect to Gpx, the activity was also upregulated by calcium restriction (Fig. 2*A*). Acute exercise increased the Gpx activity in the control rats, but it induced no further increase in the calcium-restricted rats. There was no significant difference in Cat activity between any of the indicated groups (Fig. 2*B*).

mRNA Expressions of Mn-SOD, Cu-Zn-SOD, Gpx, and Cat

Dietary calcium restriction did not alter either Mn-SOD or Cu-Zn-SOD mRNA expression (Fig. 3). In the control group, acute exercise increased the mRNA expressions of both SOD isoenzymes, whereas in the calcium-restricted group, only the expression of Mn-SOD mRNA was significantly increased by acute exercise. As shown in Fig. 4*A*, the expression of Gpx mRNA was markedly increased by calcium restriction, but its increased expression was significantly lowered after acute exercise. As for Cat, neither dietary calcium restriction nor acute exercise statistically affected the mRNA expression (Fig. 4*B*).

Reactive Carbonyl Derivative

Dietary calcium restriction had no significant effect on the level of reactive carbonyl derivative, as a marker of protein oxidation, in rat diaphragm. After acute exercise, however, reactive carbonyl derivative was significantly higher in the calcium-restricted rats than in the control rats, as shown in Fig. 5.

DISCUSSION

The current data demonstrate that dietary calcium restriction increased the activities of certain antioxidant enzymes (Mn-SOD, Cu-Zn-SOD, and Gpx) in rat diaphragm but not of Cat. This suggests the presence of an adaptive phenomenon to dietary calcium restriction, probably implying that dietary calcium restriction imposes oxidative stress in rat diaphragm. Indeed, the level of reactive carbonyl derivative, as a marker of protein oxidation, in the calcium-restricted rats was significantly higher than in the control rats after acute exercise. Thus these findings indicate the possibility that calcium restriction induces oxidative stress and strenuous exercise enhances calcium restriction-induced oxidative stress.

SOD, Gpx, and Cat are regarded as the first line of the antioxidant defense system against reactive oxygen species
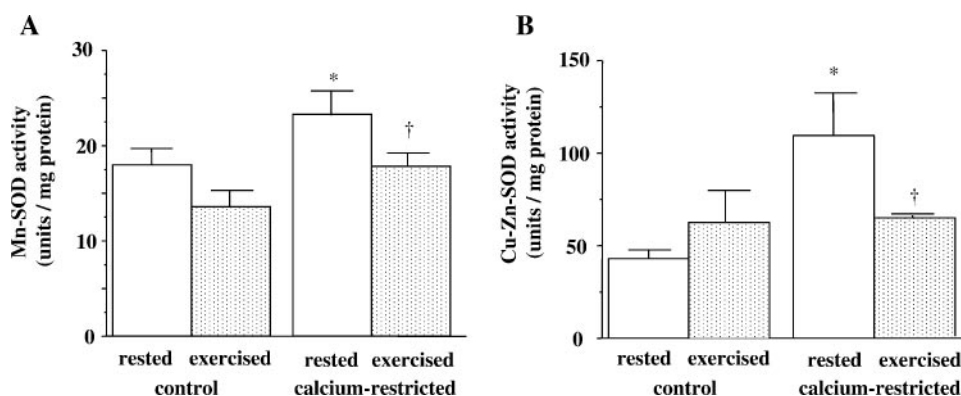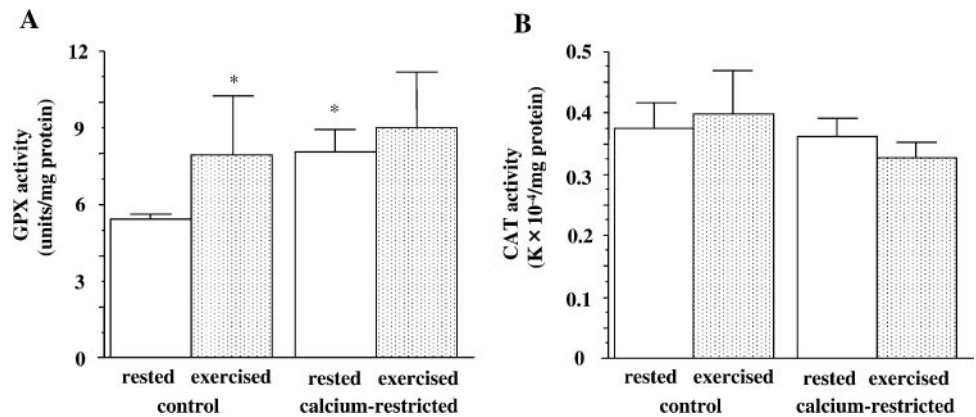

Fig. 1. Activities of Mn-superoxide dismutase (Mn-SOD) (*A*) and Cu-Zn- superoxide dismutase (Cu-Zn-SOD) (*B*) in rat diaphragm. Values are means ± SE; $n = 5$ in each group.*$P < 0.05$ vs. control (rested); †$P < 0.05$ vs. calcium restricted (rested).

Fig. 2. Activities of glutathione peroxidase (Gpx) (*A*) and catalase (Cat) (*B*) in rat diaphragm. Values are means $\pm$ SE; $n = 5$ in each group.*$P < 0.05$ vs. control (rested).

generated in vivo during oxidative stress. There have been few reports on a relationship between calcium deficiency and antioxidant enzymes (22, 27). Oh-ishi et al. (27) revealed that dietary calcium restriction upregulated both Mn-SOD and Cu-Zn-SOD in rat soleus muscles, as in the diaphragm in the current study, while Miyazaki et al. (22) reported a decrease in Mn-SOD activity, but no change in Cu-Zn-SOD activity, in heart from the calcium-restricted rats. However, exhaustive exercise had no effect on either SOD isoenzyme activity in the soleus muscles and the heart of calcium-restricted rats (22, 27). Many previous studies have shown that acute exercise increased the activities of total SOD and/or SOD isoenzymes in several tissues such as skeletal muscles, diaphragm, liver, heart, and red blood cell, or did not alter activities, but rarely decreased those activities (38). Although there is a paucity of data concerning the effect of acute exercise on antioxidant enzymes system of diaphragm (6, 18, 26), it seems that, under a physiological condition, acute exercise does not alter either of SOD isoenzyme activities in rat costal diaphragm. Our results, however, showed that acute exercise decreased the activities of both SOD isoenzymes in costal diaphragm from the calcium-restricted rats (but not from the control rats).

Previous works have shown that reactive oxygen species may inactivate antioxidant enzymes in vitro (4, 35). Because strenuous physical exercise is known to result in an increased production of reactive oxygen species in skeletal muscle (9, 28), the results of this study suggest that diaphragm of the calcium-deficient rats may be more susceptible to oxidative

stress than that of the control rats. It might be possible that both Mn-SOD and Cu-Zn-SOD were subjected to similar inactivation in the calcium-deficient rats during exercise.

With regard to Gpx, many of previous studies have shown significant elevation of Gpx activity in skeletal muscle after acute exercise (15, 16, 19, 26). In the current study, dietary calcium restriction increased Gpx activity as well as SOD isoenzyme activities in rat diaphragm. However, acute exercise did not show further increase in Gpx activity of the calcium-restricted rats, although it increased Gpx activity in the control rats. Thus this result might suggest that dietary calcium restriction caused a maximum induction of Gpx activity, and, consequently, acute exercise did not offer its additional increase in the calcium-restricted rats. According to Oh-ishi et al.'s work (27), 1-mo dietary calcium restriction had no overt effect on Gpx activity in soleus muscles, and acute exercise did not change its activity in 1-mo calcium-restricted rats. Miyazaki et al. (22) also have shown that neither 1-mo dietary calcium restriction nor acute exercise had any overt effect on Gpx activity of rat heart. The differences among the current result and previous studies might be due, in part, to the differences of tissue used and the intensity of exercise. With respect to Cat, most studies, including the current study, have reported no change in skeletal muscle Cat activity after acute bout of exercise (14, 21).

Dietary calcium restriction did not change either of the mRNA expressions of both SOD isoenzymes, although it increased the activities of both SOD isoenzymes. Thus this

Fig. 3. Mn-SOD mRNA expression (*A*) and Cu-Zn-SOD mRNA expression (*B*) in rat diaphragm. Values are means $\pm$ SE; $n = 5$ in each group. Values were expressed as the ratio of Mn-SOD or Cu-Zn-SOD to GAPDH mRNA expressions. *$P < 0.05$ vs. control (rested); †$P < 0.05$ vs. calcium restricted (rested).
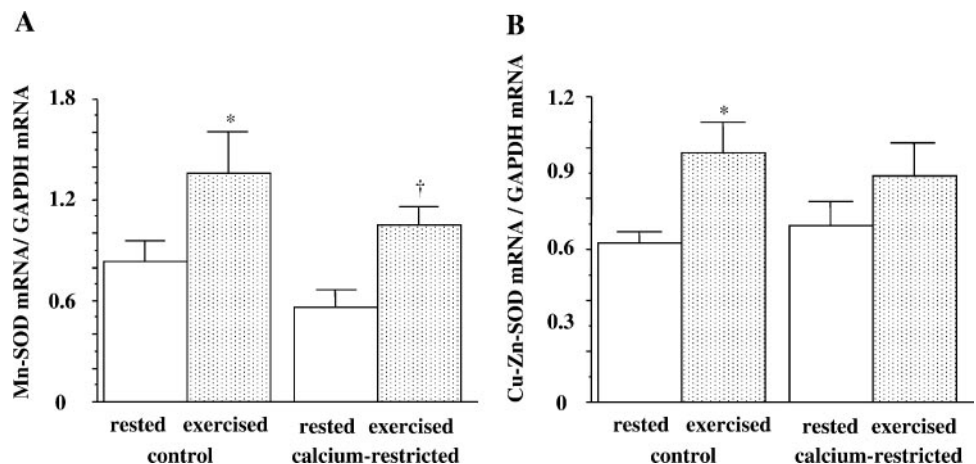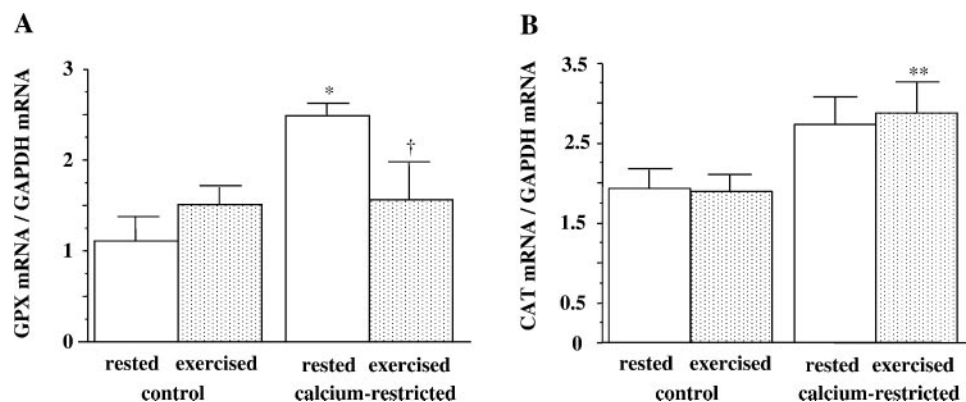
Fig. 4. Gpx mRNA expression (*A*) and Cat mRNA expression (*B*) in rat diaphragm. Values are means ± SE; $n = 4$–5 in each group. Values were expressed as the ratio of Gpx or Cat to GAPDH mRNA expressions. *$P < 0.05$ vs. control (rested); †$P < 0.05$ vs. calcium restricted (rested); **$P < 0.05$ vs. control (exercised).

finding may suggest that those activities in the calcium-restricted rats could be controlled by a translational process and/or posttranslational process but not by a transcriptional process. On the other hand, acute exercise increased the mRNA expressions of both SOD isoenzymes in the control rats and only Mn-SOD mRNA expression in the calcium-restricted rats. As for Mn-SOD in the calcium-restricted group, its activity was decreased by acute exercise, while its mRNA expression was increased by acute exercise. As we mentioned earlier, there is a possibility that Mn-SOD was inactivated by ROS. In addition, we should state the possibility that the upregulation of Mn-SOD mRNA by acute exercise might be an adaptation phenomenon to compensate for the decreased Mn-SOD activity.

Unlike our current results, Oh-ishi et al.'s previous work (27) has shown that dietary calcium restriction increased the mRNA expressions of both SOD isoenzymes in rat soleus muscle, and acute exercise enhanced their expressions in a state of severe calcium deficiency. This might imply that the mRNA expressions of SOD isoenzymes could be influenced by the extent and duration of calcium deficiency. Recently, Hollander et al. (13) have reported that acute exercise may stimulate Mn-SOD mRNA transcription in deep vastus lateralis due to the increased binding of transcription factors such as NF-κB and activator protein-1 but not Cu-Zn-SOD mRNA transcription. Our results, however, seem to suggest that acute exercise may stimulate the transcriptions of not only Mn-SOD mRNA

but also Cu-Zn-SOD mRNA. Therefore, further investigation will be needed for the precise mechanism.

In the current study, Gpx mRNA expression increased in response to dietary calcium restriction, being parallel to the increase of its activity. Therefore, this finding might indicate that its activity in the calcium-restricted rats could be mainly controlled by a transcriptional process. On the other hand, acute exercise decreased its mRNA expression only in the calcium-restricted rats, unlike SOD isoenzyme mRNAs. This result may also imply that diaphragm of calcium-deficient rats could be subject to more severe oxidative stress during physical exercise and that a susceptibility of mRNA to oxidative stress differs in each mRNA.

Recently, reactive carbonyl derivative has been often measured as an index of tissue damage by exercise-induced oxidative stress (31). Our findings have shown that, after acute exercise, the level of reactive carbonyl derivative was greater in the calcium-restricted rats than in the control rats, possibly implying that acute exercise can increase oxidatively modified proteins under a condition of calcium deficiency. Calcium deficiency is considered to increase intracellular calcium level, and the increased level of intracellular calcium is able to induce more superoxide generation (24, 29). Therefore, acute exercise is readily expected to enhance oxidative stress induced by dietary calcium restriction.

People who have airflow obstruction such as chronic obstructive lung disease often need a pulmonary rehabilitation, and many of them are elderly people. Furthermore, quite recently, airflow obstruction has been shown to be an important risk factor for osteoporosis (37). Therefore, they might be in a status of calcium deficiency. Therefore, we should pay attention to the exercise intensity at the beginning of rehabilitation because there is a possibility that a single bout of exercise can cause free radical-induced diaphragmatic damage in calcium-deficient subjects, probably leading to clinically relevant forms of respiratory muscle dysfunction or fatigue.

In summary, the results suggest that dietary calcium restriction increases certain antioxidant enzymes in rat diaphragm in response to increased oxidative stress but that the increased level of antioxidant enzyme system is not enough to cope with exercise-induced oxidative stress.

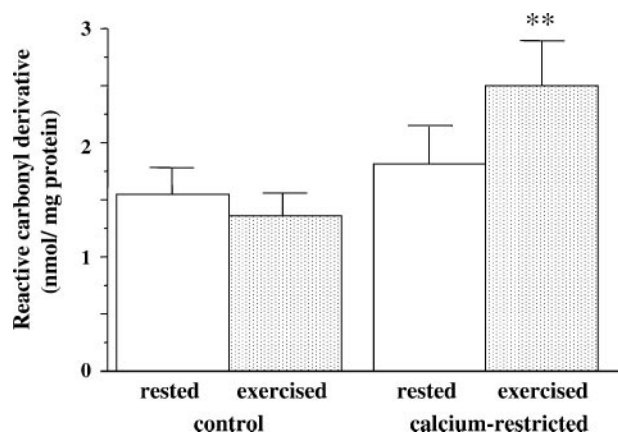Fig. 5. Reactive carbonyl derivative in rat diaphragm. Values are means ± SE; $n = 5$ in each group.**$P < 0.05$ vs. control (exercised).

ACKNOWLEDGMENTS

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