L-Arginine supplementation improves antioxidant defenses through L-arginine/nitric oxide pathways in exercised rats

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Shan L, Wang B, Gao G, Cao W, Zhang Y. L-Arginine supplementation improves antioxidant defenses through L-arginine/nitric oxide pathways in exercised rats. J Appl Physiol 115: 1146–1155, 2013. First published August 15, 2013; doi:10.1152/japplphysiol.00225.2013.—L-Arginine (L-Arg) supplementation has been shown to enhance physical exercise capacity and delay onset of fatigue. This work investigated the potential beneficial mechanism(s) of L-Arg supplementation by examining its effect on the cellular oxidative and nitrosative stress pathways in the exercised rats. Forty-eight rats were randomly divided into six groups: sedentary control; sedentary control with L-Arg treatment; endurance training (daily swimming training for 8 wk) control; endurance training with L-Arg treatment; an exhaustive exercise (one time swimming to fatigue) control; and an exhaustive exercise with L-Arg treatment. L-Arg (500 mg/kg body wt) or saline was given to rats by intragastric administration 1 h before the endurance training and the exhaustive swimming test. Expression levels and activities of the L-Arg/nitric oxide (NO) pathway components and parameters of the oxidative stress and antioxidant defense capacity were investigated in L-Arg-treated and control rats. The result show that the L-Arg supplementation completely reversed the exercise-induced activation of NO synthase and superoxide dismutase, increased L-Arg transport capacity, and increased NO and anti-superoxide anion levels. These data demonstrate that L-Arg supplementation effectively reduces the exercise-induced imbalance between oxidative stress and antioxidant defense capacity, and this modulation is likely mediated through the L-Arg/NO pathways. The findings of this study improved our understanding of how L-Arg supplementation prevents elevations of reactive oxygen species and favorably enhances the antioxidant defense capacity during physical exercise.

L-arginine; endurance training; an exhaustive exercise; supplementation effect

RECENTLY, THERE IS AN INCREASING interest in the role of the cellular balance between oxidative stress and antioxidant defense capacity in tissue damage and fatigue resulting from physical exercise. Studies have shown that physical exercise increases the production of reactive oxygen species (ROS), such as superoxide anion (O2·−). ROS have a strong tendency to extract electrons to reach a chemically more stable structure. Therefore, they are capable of eliciting oxidative damage to various cellular components (15, 33). The extent of oxidative damage during physical exercise is determined by the levels of ROS generated and the antioxidant defense capacity (29, 40). When the antioxidant system is not adapted to excessive production of ROS, oxidative stress is initiated. In recent years, a general awareness of the importance of antioxidant system in disease prevention and progression has been developed (9, 11).

However, our understanding of the relationship between antioxidant defense capacity and exercise-induced tissue damage/fatigue is quite limited. This knowledge is important in assessing the necessity and adequacy of antioxidant supplementation in physical exercise.

Both O2·− and nitric oxide (NO) are important mediators of oxidative stress during physical exercise (10). It has been shown that physical exercise induces the potent O2·−-generating enzyme, NADPH oxidase, and the cytokine-induced NO synthase (iNOS) (18). iNOS promotes biosynthesis of NO using L-arginine (L-Arg), NADPH, and oxygen as substrates. NO reacts rapidly with O2·−, producing oxidant peroxynitrite (ONOO−). Studies have shown that iNOS promotes production of both O2·− and NO in L-Arg-depleted cells, leading to ONOO−-mediated cellular injury (32, 38, 39). It has also been reported that iNOS is involved in the production of O2·− at low concentrations of L-Arg during physical exercise, where imbalanced biosynthesis of O2·− and NO would lead to an O2·−/NO-mediated injury (2, 21).

To prevent oxidative stress-induced damage, the organism is well equipped with antioxidant defense systems, including enzymes, such as superoxide dismutase (SOD) and catalase, and nonenzymatic substances, such as reduced glutathione and vitamins (12, 30). Antioxidants can be both synthesized in vivo and absorbed through diet. Much research has focused on the supplementation of nutraceutical agents for reducing oxidative stress. L-Arg is currently used in many basic and clinical research settings due to its well-recognized therapeutic qualities (7). Most of the pharmacological actions of L-Arg have been thought to associate with NO production. It has been well recognized that NO has many physiological functions, including vasodilatation, inhibition of platelet aggregation and neutrophil adhesion, and scavenging of superoxide generated during and after maximal or submaximal exercise (6). Recent findings suggested that L-Arg supplementation can significantly enhance exercise-induced NO production and alter ROS metabolism. Indeed, there is accumulating evidence showing L-Arg has a protective role against oxidative stress, and this action is likely mediated via its interaction with O2·− (3, 20). Nevertheless, the mechanism(s) of L-Arg supplementation’s protective effects remains less understood.

This work investigated the potential beneficial mechanism(s) of L-Arg supplementation by examining its effect on the cellular oxidative and nitrosative stress pathways in the exercised rats. The expression levels and activities of the L-Arg/NO pathway components and parameters of the oxidative stress and antioxidant defense capacity were investigated in L-Arg-treated and control rats. Finally, the L-Arg supplementation efficacy in reducing the exercise-induced imbalance between...
oxidative stress and antioxidant defense through the L-Arg/NO pathway was evaluated.

MATERIALS AND METHODS

Animals and reagents. Adult male Sprague-Dawley rats (6-wk-old), weighing 202 ± 10 g, were used in all experiments. The animals were allowed free access to standard laboratory chow and water in an air-conditioned room with a 12:12-h light-dark cycle. Animals were kept in the animal facility for at least 1 wk before the training program was started. All animal experiments were carried out in compliance with the Animal Management Rules of the Ministry of Health of the People’s Republic of China (document no. 55, 2001) and the Guidelines for the Care and Use of Laboratory Animals of Nanjing Sport Institute, which approved this study.

L-Arg, gastric inhibitory polypeptide, leupeptin, L-cysteine, NADPH, EDTA, and EGTA were purchased from Sigma (St. Louis, MO). L-[3H]Arg (1.5 TBq/mmol) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TRIzol, Taq DNA Polymerase, and cDNA synthesis kit were purchased from Fermentas UAB (Vilnius, Lithuania). All assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and Abcam (Hong Kong, China).

Experimental groups and exercise protocols. Rats were randomly divided into six groups: 1) sedentary control treated with saline (sedentary, n = 8), 2) sedentary control treated with L-Arg (see below, sedimentary + L-Arg, n = 8), 3) endurance training treated with saline (endurance training, n = 8), 4) endurance training treated with L-Arg (endurance training + L-Arg, n = 8), 5) an exhaustive exercise treated with saline (an exhaustive exercise, n = 8), and 6) an exhaustive exercise treated with L-Arg (an exhaustive exercise + L-Arg, n = 8).

The loading of the endurance swimming training used in endurance training and endurance training + L-Arg rats was determined based on the minimum lactate threshold value, which was obtained using a method as described by Bocanegra et al. (4). Briefly, rats were selected randomly from six groups (n = 6) and subjected to a swimming test that was composed of different exercise (3, 6, 9, 12, 15, 18 min) and recovery (3, 6, 9, 12 min) times. The rats were also loaded with a lead sheath attached to their tail, weighing approximately at 0, 1, 2, 3, 4, and 5% of their body weight. During exercise and recovery, blood samples were taken at various times and used for lactate assays. The minimum lactate threshold value was calculated as described by Voltarelli et al. (36).

The swimming exercise protocol used in endurance training and endurance training + L-Arg rats was modified from a previously published procedure (19). Briefly, rats were trained 6 days/wk for 8 wk in a 60 × 90-cm tank filled with 50 cm of water in depth (water temperature: 33 ± 5°C). Rats were loaded with a lead sheath and allowed to swim in group of three or four animals. The swimming duration and loading were progressively increased in 1-wk period, from 20 to 60 min/day and from 1 to 2.6% of body weight, respectively. All training sessions took place during morning between 9:00 and 10:00 AM. Rats in exhaustive exercise and exhaustive exercise + L-Arg groups were subjected to an exhaustive swimming test on the last day of the 8-wk period, and rats were determined to be fatigued when they failed to rise to the surface to breathe within a 5-s period.

L-Arg treatment. Rats in sedentary + L-Arg, endurance training + L-Arg, and exhaustive exercise + L-Arg groups received daily L-Arg treatment (500 mg/kg, 1 h before training) by intragastric administration for 8 wk. Rats in sedentary, endurance training, and exhaustive exercise groups received saline as controls. The exhaustive exercise rats with L-Arg supplementation were treated with L-Arg for 8 wk, and these rats did not exercise during this period of time. The exhaustive exercise rats without L-Arg supplementation were treated with saline for 8 wk, and these rats did not exercise during this period of time. The only exercise these rats did was an exhaustive exercise for 20 min on the last day of the 8-wk period. Before the treatment day, rats were acclimated to a 3-day handling, which included weighing and massaging the stomach area.

Blood and tissue sample collections. At the end of the experiments, rats were euthanized by overdose of anesthetics (via intraperitoneal injection of pentobarbital sodium, 40 mg/kg body wt), and blood and tissue samples were collected for further analysis. Blood samples were collected from abdominal aorta, centrifuged immediately after collection, and stored at −20°C for further analysis. Immediately after the blood was collected, the abdominal aorta tissues were quickly dissected out, frozen in liquid nitrogen, and kept at −80°C for further analysis. The skeletal muscle deep portion of the vastus lateralis muscle was immediately excised from both legs, freeze-clamped between aluminum tongs precooled with liquid nitrogen, and stored at −80°C for later analysis.

Total RNA preparation and RT-PCR analysis. Total RNA of the aortic and muscle tissues was extracted by using the TRI Reagent protocol (27). The RT-PCR was used to amplify a 523-bp iNOS cDNA fragment from rat aortic or muscle tissue RNA. The sequences of the forward (5′-AGTCTCCCTATTCGAGCC-3′) and reverse (5′-TGAGACACGGCTGAACACCTC-3′) primers were chosen based on the published rat smooth muscle iNOS cDNA sequence (37). RT-PCR was also performed to amplify a 510-bp catieonic acid transporter 2 (CAT2) cDNA fragment from the aortic or muscle tissues. The sequences of the forward (5′-CTATGGCGAGGATGGTGTG-3′) and reverse (5′-GGACAGGAAGACGGCAGA-3′) primers were chosen based on the published rat smooth muscle CAT2 cDNA sequence (24).

Western blot analysis. Total proteins were extracted from aortic and muscle tissues, solubilized with 1% SDS, and separated on 8% denaturing SDS polyacrylamide gels (15–25 μg/lane). Proteins were then transferred onto a nitrocellulose membrane by wet electroblotting for 4 h. Blots were blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (20 mmol/l Tris-HCl, 200 mmol/l NaCl, 0.1% Tween 20) before incubation with mouse anti-iNOS monoclonal antibody (Sigma). Incubation with the primary antibody was at a dilution of 1:500 for 1 h at room temperature and, after washing, with the second antibody (horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin antibody) at 1:1,500 for 1 h. Specific proteins were detected by enhanced chemiluminescence. Prestained protein markers were used for molecular mass determinations (37).

Analysis of L-Arg transport capacity in aortic and muscle tissues. L-Arg transport capacity was determined by measuring the intracellular incorporation of L-[3H]Arg (13, 14). Briefly, the transport capacity assays were performed by incubating tissues with 0.5 ml of Krebs-Henseleit buffer (mM/l: 140 NaCl, 5 KCl, 0.9 CaCl₂, 1 MgCl₂, 5.6 g-glucose, and 25 HEPES, pH 7.4) containing L-[3H]Arg (2 μCi/ml) at 37°C for 15 min. Tissues were then washed with ice-cold 10 mmol/l unlabeled L-Arg in PBS and lysed with 200 μl methanolic acid. Total radioactivity of L-[3H]Arg was measured by liquid scintillation counting (mmol·mg⁻¹·min⁻¹). A similar method described above was used to measure the level of L-citrulline (L-Cit).

Analysis of iNOS activity in aortic and muscle tissues. iNOS activity in aortic and muscle tissues was determined by measuring the conversion of L-[3H]Arg to L-[3H]Cit (13, 14). Briefly, tissue samples (5 mg) were fast frozen and ground to powder in liquid nitrogen using a mortar and pestle before sonication in five volumes of the homogenization buffer (50 mmol/l Tris-HCl, pH 7.4, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 0.1% l-mercaptoethanol, 5 g/ml leupeptin, 5 g/ml pepstatin A, 1 mmol/l PMSE, and 5 mmol/l DTT). To analyze iNOS activity, 200 μl of tissue homogenates were incubated in a reaction buffer containing 1 mmol/l L-[3H]Arg (containing 1 mCi [3H]), 1 mmol/l NADPH, 5 mol/l BH₄, 10 mol/l FAD, and 10 mol/l flavin mononucleotide for 30 min at 37°C. The reaction was stopped by adding 50 μl of ice-cold Tris-HCl buffer (pH 5.5) containing 1 mmol/l L-Cit, 1 mmol/l EGTA, and 1 mmol/l EDTA. The reaction substance was applied to 1 ml of Toyopearl SP-550(Na⁺) resin to absorb L-[3H]Arg. The iNOS activity was then quantified by determining the radioactivity of L-[3H]Cit.
radioactivity on liquid scintillation spectroscopy (Wallac/Perkin Elmer, 1,415) and presented as nanomoles of L-[^3H]Cit per minute per milligram protein.

Analysis of serum and muscle tissues parameters. Assay kits for determinations of antioxidant parameters including anti-O$_2^-$ and superoxide scavenging ability, SOD activity, and nitrate (NO$_3^-$)/nitrite (NO$_2^-$) levels were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and assay kit for measurement of nitrotyrosine (3-NT) level was purchased from Abcam (Hong Kong, China). Below is a brief description of each method.

Anti-O$_2^-$ was detected by pyrogallol anti-O$_2^-$ method. The principle of pyrogallol anti-O$_2^-$ method is to use O$_2^+$ scavenger to reduce the absorption peak area of pyrogallol autoxidation product and to use ultraviolet spectrophotometer to measure and indirectly calculate the O$_2^+$ clearance rate for evaluating the antioxidation activity of the test sample.

SOD activity was assayed using a kit which utilizes a highly water soluble tetrazolium salt, 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, that produces a water soluble dye on reduction with O$_2^+$. The rate of inhibition of the activity of xanthine oxidase by SOD was measured and expressed as the enzyme required for a 50% inhibition of xanthine oxidase activity per minute per milligram protein.

As NO is usually rapidly converted to NO$_3^-$ and NO$_2^-$, the total concentration of NO$_3^-/NO_2^-$ is commonly used as a quantitative measure of NO production. NO level was assayed using a NO assay kit. This method provides an accurate, convenient measure of total NO$_3^-/NO_2^-$ in a simple two-step process. The first step converts NO$_3^-$ to NO$_2^-$ utilizing NO$_3^-$ reductase. The second step uses Griess Reagents to convert NO$_2^-$ into a deep purple azo compound. The amount of the azochromophore accurately reflects NO amount in samples.

3-NT was measured using a solid-phase enzyme-linked immunoabsorbent assay, based on the sandwich principle, according to the manufacturer’s recommendations.

Statistical analysis. Data were expressed as means ± SE. Two-way ANOVA with Fisher’s post hoc test (with SAS software, version 8.2, SAS Institute) was used to determine the significance of differences between the groups. Differences were considered statistically significant when $P$ value is less than 0.05 ($P < 0.05$).

RESULTS

Determination of working load used in the endurance swimming training. We performed an anaerobic swimming capacity test to determine the working load used in the endurance swimming training. The blood lactate concentrations from test rats and the curve evaluations showing the minimum lactate threshold are presented in Fig. 1. The blood lactate concentration was 7.01 ± 0.98 mmol/l at 1 min postexercise and was increased to the maximum level of 15.14 ± 0.73 mmol/l at 3 min postexercise. A straight line was ruled from A to B, and the vertical line was drawn between B and Y-axis, where point C represents the minimum lactate threshold loading, corresponding to 2.6% of body weight (Fig. 1). This loading capacity was used in the endurance swimming training.

Effects of L-Arg supplementation on the body weight, heart weight, and swimming time to fatigue. At the end of the experiments, we examined the effects of L-Arg supplementation on body weight, heart weight, and swimming time to fatigue in the exercise rats. As shown in Fig. 2A, rats in endurance training group gained ~12% less body weight than did sedentary group rats (365 ± 3.68 vs. 419 ± 4.95 mg) over the 8-wk of the endurance training. The body weight in endurance training + L-Arg group (422 ± 12.85 mg) increased significantly after 8 wk of L-Arg supplementation, compared to the endurance training group (422 ± 12.85 vs. 365 ± 3.68 mg). However, there was no difference among endurance training + L-Arg (422 ± 12.85 mg), exhaustive exercise (421 ± 6.32 mg), and sedentary groups (419 ± 4.95 mg). The body weights in sedentary + L-Arg group (435 ± 3.82 mg) and exhaustive exercise + L-Arg group (434 ± 5.96 mg) showed a tendency of increase, compared with their respective untreated controls. As illustrated in Fig. 2B and C, the heart weight (Fig. 2B) and heart weight-to-body weight ratio (Fig. 2C) increased by ~20% in endurance training group, whereas there was no difference between other groups.

In addition, the swimming time to fatigue (the one-time exhaustive exercise) in exhaustive exercise + L-Arg rats was significantly increased. The maximum performance in exhaustive exercise + L-Arg group was 20.1 ± 2.08 min, whereas this value in the exhaustive exercise group was 9.01 ± 2.71 min (more than a twofold increase in the time to exhaustion) (Fig. 2D).

Effects of L-Arg supplementation on the expression and function of CAT2 and iNOS in aortic tissues. We first examined whether L-Arg supplementation modulates the expression of the membrane transporter CAT2, which is responsible for L-Arg transport into cells. RT-PCR and analysis using aortic tissues showed that both the endurance training and the exhaustive exercise reduced the mRNA levels significantly, whereas L-Arg supplementation reversed the exercise-induced reductions (Fig. 3, A and C). Of note, the CAT2 mRNA level in endurance training + L-Arg group was about threefold higher than that in the endurance training group and was about onefold higher than that in the sedentary and sedentary + L-Arg groups (Fig. 3, A and C).

To evaluate the effects of L-Arg supplementation on L-Arg transport capacity, we measured L-[^3H]Arg uptake in the aortic tissues. Results shown that the L-Arg transport capacity changed similarly to the alterations of CAT2 mRNA; i.e., exercise reduced L-Arg transport capability, whereas L-Arg supplementation reversed the exercise-induced reductions (Fig. 3, E, F, and H).
The effects of L-Arg supplementation on the expression and activity of iNOS was examined. In contrast to the changes of CAT2, the expression levels of iNOS (at both mRNA and protein levels) were increased following the endurance training, whereas L-Arg supplementation effectively reversed these elevations (Fig. 3, B, C, and D). L-Arg supplementation also reduced the iNOS mRNA level in the exhaustive exercised rats (Fig. 3, B and C).

The iNOS activity was determined by quantifying the conversion of L-[3H]Arg to L-[3H]Cit. The assays showed a similar change pattern in the iNOS activity as that in the iNOS mRNA, i.e., exercise increased iNOS activity, whereas L-Arg supplementation reversed the exercise-induced elevation. The iNOS activity in endurance training group was about one fold higher than that in the sedentary group, and this elevation was completely reversed by L-Arg supplementation (endurance training + L-Arg rats) (Fig. 3G). A similar change level (Fig. 3E) was observed in the exhaustive exercise and exhaustive exercise + L-Arg group. Thus results showed that L-Arg supplementation significantly enhanced L-Arg transport capacity and inhibited the exercise-induced iNOS activation in aortic tissues (Fig. 3, E and G).

**Effects of L-Arg supplementation on the serum levels of NO, anti-\(O_2^-\), SOD, and 3-NT.** To determine the cellular levels of oxidative stress and antioxidant responses, we measured serum levels of NO, anti-\(O_2^-\), 3-NT, and SOD. Results showed that both the endurance training and exhaustive exercise reduced the serum levels of NO and anti-\(O_2^-\) significantly, whereas L-Arg supplementation reversed these elevations (Fig. 4, A and B). The serum levels of NO and anti-\(O_2^-\) in endurance training and exhaustive exercise groups were elevated by ~1-fold and 2.5-fold, respectively, compared with the endurance training group.

In contrast to the changes of NO and anti-\(O_2^-\), the serum 3-NT and SOD level were significantly elevated after the endurance training and the exhaustive exercise. We also observed that L-Arg supplementation effectively reversed these elevations. SOD activity in endurance training and exhaustive exercise groups was elevated by ~60 and 64%, respectively, compared with that in the sedentary group, and L-Arg supple-
mentation completely reversed this elevation (Fig. 4C). Similarly, 3-NT level in endurance training and exhaustive exercise groups was elevated by about two- to threefold, compared with that in the sedentary group, and L-Arg supplementation effectively reduced this elevation (Fig. 4D). Hence, our data showed an exercise-induced overproduction of $O_2^-$ and oxidative stress, manifested as reduced serum levels of NO and anti-$O_2^-$ and increased 3-NT and SOD.

Effects of L-Arg supplementation on the expression and function of CAT2 and iNOS in muscle tissues. In addition to measuring the expression and function of CAT2 in aortic tissues, we also examined its expression and activity in the muscle tissues of L-Arg-treated sedentary, endurance training, and exhaustive exercise rats, and their respective controls. A and B: representative images of RT-PCR gels for detection of CAT2 (A) and iNOS (B) mRNA levels. C, correlating quantifications of CAT2 and iNOS mRNA levels. D: representative images of Western blot detection of iNOS protein expression. E: measurements of L-Arg transport capacity. F: measurements of L-citrulline (L-Cit) level. G: measurements of iNOS activity. H: ratio of L-Arg to L-Cit. Values are means ± SE of each group (n = 8). Two-way ANOVA was used to determine significance of the differences: $P < 0.05$ vs. *sedentary, #sedentary + L-Arg group, $\ddagger$endurance training group, $\ddagger$endurance training + L-Arg group, and * an exhaustive exercise group.

Fig. 3. Effects of L-Arg supplementation on the expression and function of cationic amino acid transporter 2 (CAT2) and inducible nitric oxide synthase (iNOS) in aortic tissues in L-Arg-treated sedentary, endurance training, and exhaustive exercise rats, and their respective controls. A and B: representative images of RT-PCR gels for detection of CAT2 (A) and iNOS (B) mRNA levels. C: correlating quantifications of CAT2 and iNOS mRNA levels. D: representative images of Western blot detection of iNOS protein expression. E: measurements of L-Arg transport capacity. F: measurements of L-citrulline (L-Cit) level. G: measurements of iNOS activity. H: ratio of L-Arg to L-Cit. Values are means ± SE of each group (n = 8). Two-way ANOVA was used to determine significance of the differences: $P < 0.05$ vs. *sedentary, #sedentary + L-Arg group, $\ddagger$endurance training group, $\ddagger$endurance training + L-Arg group, and * an exhaustive exercise group.
muscle tissues. RT-PCR analysis showed that both the endurance training and the exhaustive exercise did not affect the CAT2 mRNA levels; however, L-Arg supplementation significantly increased CAT2 expression in the endurance exercised rats (Fig. 5, A and C). The CAT2 mRNA level in endurance training/L-Arg group was about onefold higher than that in the endurance training group (Fig. 5, A and C). No significant change was observed in the exhaustive exercise rats (both exhaustive exercise and exhaustive exercise/L-Arg groups).

Compared with the alterations of CAT2 mRNA levels, the L-Arg transport capacity was more dramatically affected by exercise and by L-Arg supplementation. Both the endurance training and the exhaustive exercise remarkably reduced L-Arg-transport capability in the muscle tissues, whereas L-Arg supplementation effectively reversed the exercise-induced reductions (Fig. 5, E and H). The transport capacity in the endurance training group was reduced by ~65%, compared with the sedentary group, and L-Arg supplementation increased the transport capacity in the endurance training rats and the exhaustive exercise rats by about fourfold and onefold, respectively (Fig. 5E).

The effects of L-Arg supplementation on the expression and activity of iNOS was examined in muscle tissues. Similar to that observed in the aortic tissues, the iNOS mRNA level was increased following the endurance training, whereas L-Arg supplementation reversed this elevation (Fig. 5, B and C). No significant change was observed in the exhaustive exercise rats (both exhaustive exercise and exhaustive exercise/L-Arg groups).

The activity assay showed a similar change in the iNOS activity as that in the mRNA level, i.e., exercise increased iNOS activity, whereas L-Arg supplementation reversed the exercise-induced elevations (Fig. 5E). The iNOS activity in the endurance training group was about twofold higher than that in the sedentary group. This elevation was completely reversed by L-Arg supplementation (endurance training + L-Arg group) (Fig. 5E). A similar change was observed in the exhaustive exercise and exhaustive exercise + L-Arg groups. Of note, the iNOS activity was significantly decreased in sedentary rats treated with L-Arg (sedentary/L-Arg group), compared with its control (sedentary group) (Fig. 5E).

Effects of L-Arg supplementation on the muscle levels of NO, anti-O₂⁻, SOD, and 3-NT. In addition to evaluating the serum levels of oxidative stress and antioxidant responses, we also measured muscle levels of NO, anti-O₂⁻, SOD, and 3-NT. The muscle NO level was significantly elevated in the endurance training but not the exhaustive exercise rats. The level in
endurance training group was about onefold higher than that in the sedentary group, and L-Arg supplementation significantly increased muscle NO levels in both sedentary and exercise rats (Fig. 6A). The muscle NO level was increased by about threefold in the sedentary/L-Arg and exhaustive exercise/L-Arg groups, compared with their respective controls (sedentary and exhaustive exercise groups) and increased by ~45% in the endurance training + L-Arg group, compared with the endurance training group. Analysis of the muscle anti-O_2^- level showed a similar pattern as that in the muscle NO level, i.e., both the endurance training and the exhaustive exercise reduced muscle anti-O_2^- level, whereas L-Arg supplementation...
tion completely reversed the exercise-induced changes (Fig. 6B). In contrast to the alterations of NO and anti-O$_2^-$ levels, the muscle SOD and 3-NT levels were significantly elevated following the endurance training and exhaustive exercise. However, these elevations were effectively reversed by L-Arg supplementation. SOD activity in the endurance training and exhaustive exercise groups was elevated by about onefold, compared with that in the sedentary group, and L-Arg supplementation effectively reversed these elevations (Fig. 6C). Similarly, 3-NT level in endurance training and exhaustive exercise groups was elevated by about onefold, compared with that in the sedentary group, and L-Arg supplementation effectively reduced these elevations (Fig. 6D). Hence, results demonstrated an exercise-induced overproduction of O$_2^-$ and oxidative stress, manifested as reductions of NO and anti-O$_2^-$ and elevations of SOD and 3-NT. It is likely that the alterations of serum NO, anti-O$_2^-$, SOD, and 3-NT levels were mainly contributed by the changes in the muscle tissues. However, we cannot exclude the possible contributions from other tissues and organs (such as the cardiovascular and central nervous systems) to the exercise-induced elevations in serum NO and anti-O$_2^-$ levels as the functions of these organs/systems during exercise were also increased.

**DISCUSSION**

It has been long recognized that supplementation of L-Arg, the endogenous physiological precursor of NO, improves endothelial vasodilator function of coronary and peripheral arteries (23). More recently, L-Arg supplementation was also shown to reduce moderate-intensity exercise-induced oxidative stress and enhance high-intensity exercise tolerance (1, 5). In this study, we investigated whether L-Arg supplementation attenuates endurance training and an exhaustive exercise-induced oxidative stress using rat models. Results showed that the L-Arg supplementation effectively reversed exercised-induced elevation of 3-NT level and activation of iNOS and SOD and, meanwhile, increased tissue and serum levels of CAT2, NO, and anti-O$_2^-$. These alterations may contribute effectively to the improved balance between oxidative stress and antioxidant defense capacities observed in exercise rats with L-Arg supplementation.
During physical exercise, a sufficient amount of l-Arg transport into cells is critical for maintaining adequate levels of l-Arg such that optimal coupling of l-Arg with iNOS can occur (25, 31). Therefore, factors affecting the transporter’s expression level and function have potential to limit the production of NO. Without ample amount of l-Arg, iNOS will principally utilize O$_2^-$ to form O$_2^-$, which can potentially lead to oxidative stress/damage (15, 17). In this study, we observed that the aortic and muscle tissue levels of l-Arg transport capacity in exercise rats were significantly reduced and iNOS activity was remarkably elevated (Figs. 3 and 5). Furthermore, l-Arg supplementation effectively reversed these alterations. These results demonstrate that the endurance training caused l-Arg depletion and iNOS activation, and l-Arg supplementation effectively enhanced l-Arg transport capacity and inhibited the exercise-induced iNOS activation. As we also detected an elevation of the transporter mRNA, the enhanced transport capacity is likely contributed (at least in part) by the enhanced expression of the transporter protein. Thus our observations suggested a critical role of iNOS in the effects of l-Arg supplementation. However, studies cannot exclude the possible contributions of the other NOS isoforms. The endothelial NOS and neuronal NOS may also have contributed (to a certain extent) to the conversion of l-Arg to l-Cit.

The metabolism of NO is known to involve two different pathways. One is its interaction with O$_2^-$ to form ONOO$,^-$, which is related to l-Arg/NOS/ONOO$/^-$3-NT pathway (35, 39), and the other is associated with the so-called NO bioavailability (i.e., NO involvement in the different biological functions). Since NO is rapidly converted to NO$_3$ and NO$_2$ by l-Arg/NOS/NO$_2$/NO$_3$ pathway, the total concentration of NO$_2$/NO$_3$ is used as a quantitative measure of NO production. It has recently been reported that dietary l-Arg supplementation increases plasma NO$_2$/NO$_3$ (NO$_2$/NO$_3$) levels and improves exercise efficiency and exercise tolerance in rats (22, 26). In this work, results showed that the plasma and muscle levels of NO$_2$/NO$_3$ in the endurance training and exhaustive exercise groups were significantly reduced, and 3-NT levels were increased in exercised rats, and supplementation of l-Arg significantly reversed these alterations. These findings confirm that supplementation of l-Arg can effectively increase NO bioavailability. It is worth mentioning that l-Arg supplementation increased NO level in sedentary rats (as it did in exercised rats). The reason for this elevation is unclear at this time, but presumably it might be associated with inhibition of iNOS in these rats. It has also been shown that SOD preserves bioavailable NO by preventing the reaction of NO with excess amount of O$_2^-$ (8). To assess the mechanism(s) by which overproduction of O$_2^-$ (resulting from l-Arg deficiency) leads to oxidative stress, studies examined serum and muscle levels of SOD and anti-O$_2^-$ in the exercised rats. Results showed that the levels of SOD activity were significantly increased, and l-Arg supplementation completely reversed these elevations. As SOD is known to be induced by O$_2^-$, its increase in the exercise rats indicates an increased production of O$_2^-$. Indeed, the serum and muscle levels of anti-O$_2^-$ were drastically reduced by acute exercise, and exercise training and l-Arg supplementation effectively reversed these alterations (Figs. 4 and 6). Thus the protective effect of l-Arg supplementation likely resulted from a decreased generation of O$_2^-$. Interestingly, these exercised rats with l-Arg supplementation maintained their body weight, while a significant loss of body weight was observed in the rats without l-Arg treatment. Taken together, these results demonstrate that l-Arg supplementation significantly reduces oxidative stress and enhances the antioxidative capabilities in exercised rats.

l-Arg has been shown to have a protective role against oxidative stress via its association with O$_2^-$ (25). In this study, results demonstrate a protective role of l-Arg supplementation in the endurance exercised and the exhaustive exercise rats. The protective effects of l-Arg supplementation might be associated with the following two different, but related, mechanisms. First, as a precursor to the biosynthesis of NO, l-Arg may act as an indirect antioxidant by supplying endogenous l-Arg, inhibiting iNOS activity, and scavenging O$_2^-$ (34, 41). Second, as iNOS can mediate the production of a significant amount of O$_2^-$ at low levels of l-Arg during exercise (16, 28), the capacity of iNOS in contributing to O$_2^-$ production could be greatly diminished by l-Arg supplementation. It is likely that iNOS and SOD were overactivated, excess amount of O$_2^-$ was produced, and more ONOO$^-$ was generated in exercise rats without l-Arg supplementation. In contrast, the serum and tissue NO levels were increased effectively in exercise rats with l-Arg supplementation, and such elevations might be beneficial in scavenging free radicals and inhibiting iNOS activation. Thus data suggest that the exercise rats with l-Arg supplementation have higher levels of l-Arg to inhibit iNOS activity and promote NO bioavailability.

Studies showed that the l-Arg supplementation effectively reversed endurance training-induced body weight loss (Fig. 2). The body weight of the endurance training rats was reduced by $\sim$12%, compared with sedentary rats. This may reflect an energy imbalance in exercised rats. The rapid and efficient fuel utilization during exercise might be associated with an energy imbalance. In contrast, body weight in endurance training + l-Arg group was only reduced by $\sim$1%, compared with sedentary rats, suggesting that supplementation of l-Arg effectively corrected energy imbalance and maintained energy homeostasis in exercised rats. As an ergogenic aid, l-Arg has been shown to enhance both aerobic- and resistance exercise-associated muscle strength and recovery (3). In addition, l-Arg has been shown to play a role in ornithine and urea metabolism, which may benefit energy balance (2). Thus we presume that the energy imbalance resulting from endurance training is likely remedied by supplementation of l-Arg.

In summary, this study showed that the endurance training and exhaustive exercise cause the cellular oxidative stress and antioxidant responses in rats, and l-Arg supplementation effectively inhibits iNOS and SOD activation, promotes NO bioavailability, and enhances the antioxidant defense capacities.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: L.S. and Y.Z. conception and design of research; L.S. and G.G. performed experiments; L.S. and B.W. analyzed data; L.S. and W.C. interpreted results of experiments; L.S. prepared figures; L.S. drafted manu-

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