British Journal of Nutrition (2006), **95**, 67–75
DOI: 10.1079/BJN20051602

L-Arginine attenuates xanthine oxidase and myeloperoxidase activities in hearts of rats during exhaustive exercise

Wan-Teng Lin[1,4], Suh-Ching Yang[2], Shiow-Chwen Tsai[3], Chi-Chang Huang[2] and Ning-Yuean Lee[1]*

[1]*Department of Nutrition and Food Sciences, Fu-Jen Catholic University, Taipei, Taiwan, Republic of China*
[2]*School of Nutrition and Health Sciences, Taipei Medical University, Taipei, Taiwan, Republic of China*
[3]*Central Laboratory, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, Republic of China*
[4]*De Lin Institute of Technology, Taipei, Taiwan, Republic of China*

(Received 21 January 2005 – Revised 23 June 2005 – Accepted 12 August 2005)

The present study was to investigate the effects of L-arginine (L-Arg) supplementation on cardiac oxidative stress and the inflammatory response in rats following acute exhaustive exercise on a treadmill. Rats were randomly divided into four groups: sedentary control (SC); SC with L-Arg treatment (SC + Arg); exhaustive exercise (E); exhaustive exercise with L-Arg treatment (E + Arg). Rats in groups SC + Arg and E + Arg received a 2 % L-Arg diet. Rats in groups E and E + Arg performed an exhaustive running test on a treadmill at a final speed of 30 m/min, 10 % grade, at approximately 70–75 % V_{O2max}. The results showed a significant increase in cardiac xanthine oxidase (XO) and myeloperoxidase activities and membrane lipid peroxidation endproduct (malondialdehyde; MDA) levels of exercised rats compared with SC rats. The increased cardiac XO activity and MDA levels in exercised rats were significantly decreased in exercised rats supplemented with L-Arg. Myocardial GSSG content increased whereas the GSH:GSSG ratio was depressed in exercised rats compared with SC rats. Cardiac GSSG levels significantly decreased, whereas total glutathione, GSH and the GSH:GSSG ratio increased in exercised rats supplemented with L-Arg compared with exercised rats. The activities of creatinine kinase (CK) and lactate dehydrogenase (LDH), and lactate, uric acid, and nitrite and nitrate levels in the plasma significantly increased in exercised rats compared with SC rats. The activities of plasma CK and LDH were significantly decreased in L-Arg-supplemented plus exercised rats compared with exercised rats. These findings suggest that L-Arg supplementation reduces the oxidative damage and inflammatory response on the myocardium caused by exhaustive exercise in rats.

L-Arginine: Xanthine oxidase: Myeloperoxidase: Oxidative stress: Exhaustive exercise

The heart tissues are mostly composed of muscles that contain plenty of mitochondria, which need more O_2 compared with other organs. Basically, the heart needs lots of O_2 to work well. Nowadays, O_2 is sometimes apt to be dealt with as a negative factor in oxidative stress instead of as an indispensable factor for life activity. Although increased O_2 flux through the mitochondrial electron transport chain is considered the main source of reactive oxygen species (ROS), other pathways of ROS generation, such as xanthine oxidase (XO) and polymorphoneutrophils, may also be activated during or after strenuous exercise (Ji, 1999).

In previous studies, exhaustive physical exercise has been known to induce free radicals *in vivo* and lead to oxidative damage in several tissues such as muscle, liver, lung, and heart (Kumar *et al.* 1992; Frankiewicz-Jozko *et al.* 1996; Ashton *et al.* 1998; Radak *et al.* 1998). During exhaustive exercise, XO activity was significantly increased in the circulation and tissues (Radak *et al.* 1995; Hellsten *et al.* 1997; Vina *et al.* 2000). XO, a metalloflavoprotein, has a major role for oxygen-derived free radicals in post-ischaemic tissue injury (McCord, 1985). Vina *et al.* (2000) further demonstrated that XO is responsible for free radical production and tissue damage during exhaustive exercise. Recently, it was proposed that XO-derived oxidants are chemotactic to neutrophils (Judge & Dodd, 2004). In human and animal studies, neutrophil infiltration in tissues is associated with strenuous exercise-induced tissue damage (Fielding *et al.* 1993; Belcastro *et al.* 1996). Neutrophils are capable of further generation of free radicals via NADPH oxidase and producing HOCl from H_2O_2 via myeloperioxidase (MPO), a marker for neutrophil infiltration in tissues, in the initiation of exercise-induced muscle damage (Tiidus, 1998). These studies indicate that both XO and MPO are the two main sources of extracellular free radicals during strenuous exercise. These two enzymes are also responsible for exhaustive exercise-induced oxidative stress in several tissues including muscle, liver, and heart.

L-Arginine (L-Arg) is used in current basic and clinical research for its important therapeutic qualities. Most

Abbreviations: AST, aspartate aminotransferase; CAT, catalase; CK, creatinine kinase; E, exhuastive exercise; GPX, glutathione peroxidase; GR, glutathione reductase; L-Arg, L-arginine; LDH, lactate dehydrogenase; MDA, malondialdehyde; MPO, myeloperoxidase; NOx, nitrite and nitrate; ROS, reactive oxygen species; SC, sedentary control; SOD, superoxide dismutase; UA, uric acid; XO, xanthine oxidase.

* **Corresponding author:** Dr Ning-Yuean Lee, fax +886 2 2904 3234, email als2046@mails.fju.edu.tw

pharmacological actions of L-Arg are attributed to NO, which is able to act on a wide range of tissues and is a multipurpose messenger molecule implicated in a wide variety of biological processes (Wu & Morris, 1998). For example, in patients with CVD, intravenous and oral L-Arg administration has been shown to support endothelial function by enhancing vasodilatation and reducing monocyte adhesion (Wang *et al.* 1994). Increasing NO bioactivity through L-Arg supplementation has been shown to reduce blood lactate and NH_3 after maximal or submaximal exercise (Schaefer *et al.* 2002). Recent findings suggest that L-Arg supplementation could significantly enhance the exercised-induced increases of NO production and changes of Fe metabolism (Xiao *et al.* 2003). Several studies have also demonstrated that L-Arg has a protective role against ROS attack. This was possible due to its direct chemical interaction with superoxide anions (O_2^-) *in vitro* (Wascher *et al.* 1997; Lass *et al.* 2002). However, so far as we know, there is no study investigating the effect of L-Arg supplementation on cardiac XO activity, the inflammatory response and antioxidant defence systems during exhaustive exercise. We therefore studied whether dietary L-Arg may protect heart tissue and have beneficial effects on ameliorating exercise-induced oxidative stress and inflammation in the myocardium of rats.

Materials and methods

Thirty-two male Sprague–Dawley rats weighing 280–300 g (8 weeks old) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). All rats were given free access to water and standard pelleted rat food (no. 5001; PMI Nutrition International, Brentwood, MO, USA) and individually housed in a room maintained at 23 ± 2°C with a 12 h light–dark cycle. In order to adapt to the experimental standard diet, rats were fed an AIN-93 diet in the second week. Afterward, rats were randomly divided into four groups: a sedentary control group with AIN-93 diet (SC; *n* 8); sedentary control with AIN-93 containing 2 % L-Arg diet (SC + Arg; *n* 8); exhaustive exercise with AIN-93 diet (E; *n* 8); exhaustive exercise with AIN-93 containing 2 % L-Arg diet (E + Arg; *n* 8). Rats were fed diets for 30 d beginning in the third week. Both diets were identical in energy content (Table 1) (Reeves *et al.* 1993; Lewis & Langkamp-Henken, 2000). The experiment was approved by the Fu-Jen Catholic University Animal Care and Usage Committee and followed the guidelines established by the National Laboratory Animal Breeding and Research Center in Taiwan. It is well known that exercise performance is limited by increase of body temperature during exercise. In order to eliminate this complicating factor, all running tests were conducted in an environmental chamber at 10–12°C to reduce the undue rise in body temperature due to sustained exercise.

Rats in groups E and E + Arg were introduced to treadmill running with 15–20 min exercise bouts at 15–30 m/min for 6 d to accustom them to running by a modification of the method of Ji *et al.* (1991). The treadmill was equipped with an electric shocking grid on the rear barrier to provide the animal with exercise motivation (T510E treadmill device; DR instrument, Taipei, Taiwan). On the day of the exercise test, they were required to run to exhaustion on a six-lane inclined (10°) treadmill at a final speed of 30 m/min, which was approximately 70–75 % of V_{O2max} (Brooks & White, 1978). The measurement of maximal O_2 consumption (100 % V_{O2max}) was considered valid only if the animal ran until it could no longer maintain pace with the treadmill (Somani *et al.* 1995). Therefore, exhaustion was determined as the rat being unable to upright itself when placed on its back (Ji & Mitchell, 1994; de Oliveira *et al.* 2003). To eliminate diurnal effects, the experiments were performed at the same time (09.00 hours to 12.00 hours).

All animals were anaesthetised with ethyl ether and killed immediately after exhaustive exercise. After anaesthesia, body temperature was measured by use of a rectal thermometer. Then, the heparinised blood sample was collected from the abdominal aorta and the heart tissue was carefully removed, rinsed in ice-cold normal saline, blotted dry and stored at −80°C for further analysis.

Heart tissues (100 g/l) were homogenised in ice-cold buffer (sucrose (0·25 mol/l), tri(hydroxymethyl)-aminomethane-HCl (10 mmol/l), and phenylmethylsulfonyl fluoride (0·25 mmol/l); pH 7·4) using a Polytron homogeniser (Kinematica GmbH, Lucerne, Switzerland) with a Teflon pestle and glass tube (Glas-Col, Terre Haute, IN, USA). The homogenates were centrifuged at 10 000 ***g*** for 20 min at 4°C. The supernatant fractions

Table 1. Composition of the diets based on the AIN-93 diet (Reeves *et al.* 1993)*

Ingredient (g/kg)	Group SC	Group SC + Arg	Group E	Group E + Arg
Casein	200	200	200	200
L-Cystine	3	3	3	3
L-Arginine	0	16	0	16
Maize starch	397·5	381	397·5	381
Maltodextrin	132	132	132	132
Sucrose	100	100	100	100
Soyabean oil	70	70	70	70
Cellulose	50	50	50	50
AIN-93 mineral mix	35	35	35	35
AIN-93 vitamin mix	10	10	10	10
Choline bitartrate	2·5	2·5	2·5	2·5
tert-Butylhydroquinone	0·014	0·014	0·014	0·014

SC, sedentary control; Arg, L-arginine; E, exhaustive exercise.

* The components for the diets were purchased from ICN Biochemicals, Inc. (Costa Mesa, CA, USA), with the exception of L-arginine, sucrose and soyabean oil, which were obtained from Sigma-Aldrich-Fluka (St Louis, MO, USA).

For details of diets and procedures, see p. 68.

were transferred to a new Eppendorff tube and used for determination of XO activity, antioxidant enzyme activities and protein levels. The pellets were washed twice in an ice-cold buffer (phosphate buffer; 20 mmol/l; pH 6·0) and sonicated in MPO homogenisation buffer containing 0·5 % hexadecyltrimethylammonium bromide, EDTA (10 mmol/l) and phosphate buffer (50 mmol/l) (pH 6·0) at 25°C for 1 min with an ultrasonic processor (Vibra Cell model VCX-500; Sonics and Materials Inc., Danbury, CT, USA). The homogenates were then centrifuged at 17 000 ***g*** at 4°C for 15 min. Supernatant fractions were aspirated into new tubes and stored on ice until the assay was performed. In addition, all samples were diluted in deionised water for analysis, if necessary. All chemicals used in the present study were purchased from Sigma-Aldrich-Fluka (St Louis, MO, USA) unless stated otherwise.

Plasma parameters

Blood samples were centrifuged at 1400 ***g*** at 4°C for 10 min. The supernatant fractions (plasma) were used for the determination of creatinine kinase (CK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and uric acid (UA) with an automatic analyser (Hitachi 7170; Hitachi, Tokyo, Japan). In addition, nitrate and nitrite (NOx) levels in plasma were quantified by using the Griess reagent to measure nitrite ion concentration (Green *et al.* 1982). NOx concentrations in plasma were measured spectrophotometrically at 550 nm according to a previous study (Schmidt *et al.* 1992).

Assay of xanthine oxidase activity in the heart tissue

XO activity of heart tissue was determined by the method of Westerfeld *et al.* (1959). A diluted sample was added to xanthine (0·1 mmol/l) (dissolved in sodium phosphate buffer, 50 mmol/l; pH 7·5). XO activity was measured at 25°C on a Hitachi U-2000 spectrophotometer at 290 nm for 3 min. One unit of XO activity was defined as 1 μmol urate formation/min at 25°C. XO activity was expressed as U/g protein for specific activity.

Assay of myeloperoxidase activity in the heart tissue

MPO activity of heart tissue was determined as a marker enzyme for measuring neutrophils accumulating in tissue samples, because it is closely correlated with the number of neutrophils present in the tissue (Mullane *et al.* 1985). MPO activity was determined by the method of Schierwagen *et al.* (1990). A portion of diluted sample (50 μl) was added to 1 ml mixed substrate containing H_2O_2 (3 mmol/l) dissolved in 3,3′,3,5′-tetramethylbenzidine (R&D Systems Inc., Minneapolis, MN, USA). MPO activity was measured at 37°C on a Hitachi U-2000 spectrophotometer at 655 nm for 3 min. One unit of MPO activity was defined arbitrarily as the amount of enzyme necessary to catalyse an increase in absorbance of 1·0 at 655 nm/min at 37°C. MPO activity was expressed as U/mg protein for specific activity.

Lipid peroxidation in the heart tissue

The malondialdehyde (MDA) concentration of the heart tissue homogenate was assessed colorimetrically at 586 nm using a commercial kit (Calbiochem 437634; Calbiochem-Novabiochem, La Jolla, CA, USA).

Assay of superoxide dismutase activity in the heart tissue

Superoxide dismutase (SOD) activity of heart tissue was measured with a commercial kit (SD 125; Randox Laboratories, Antrim, UK). A portion of diluted standard or sample (50 μl) was added to 1·7 ml mixed substrate (xanthine (50 μmol/l) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (25 μmol/l)). XO (250 μl; 80 U/l) was added to the mixture and SOD activity was measured by the degree of inhibition of this reaction at 37°C on a Hitachi U-2000 spectrophotometer at 505 nm for 3 min. Activity was expressed as U/mg protein for specific activity.

Assay of catalase activity in the heart tissue

Catalase (CAT) activity of heart tissue was determined at 25°C with a Hitachi U-2000 spectrophotometer UV-VIS spectrophotometer by the method of Beers & Sizer (1952). A diluted sample was added to H_2O_2 (59 mmol/l) (dissolved in potassium phosphate buffer; 50 mmol/l; pH 7·0) and CAT activity was measured at 240 nm for 3 min. One unit of CAT activity was defined as 1 mmol H_2O_2 degraded/min. Activity was expressed as U/mg protein for specific activity.

Assay of glutathione peroxidase activity in the heart tissue

Glutathione peroxidase (GPX) activity of heart tissue was determined with a commercial kit (RS 504; Randox Laboratories, Antrim, UK). A portion of diluted sample (20 μl) was added to 1 ml mixed substrate (glutathione (4 mmol/l), glutathione reductase (GR; 0·5 U/l) and NADPH (0·34 mmol/l) dissolved in phosphate buffer (50 mmol/l); pH 7·2; EDTA, 4·3 mmol/l). Cumene hydroperoxide (40 μl; diluted in deionised water) was added to the mixture and GPX activity was measured at 37°C on a Hitachi U-2000 spectrophotometer at 340 nm for 3 min. One unit of GPX activity was defined as the amount of enzyme that catalysed the reduction of 1 μmol NADPH/min. Activity was expressed as U/mg protein for specific activity.

Assay of glutathione reductase activity in the heart tissue

GR activity of heart tissue was measured with a commercial kit (Calbiochem 359962; Calbiochem-Novabiochem). A portion of diluted sample (200 μl) was added to 400 μl GSSG buffer (2·4 mmol/l; dissolved in potassium phosphate buffer (125 mmol/l); pH 7·5; EDTA, 2·5 mmol/l). NADPH (400 μl; 0·55 mmol/l; dissolved in deionised water) was added to the mixture and GR activity was measured at 340 nm for 5 min on a Hitachi U-2000 spectrophotometer. One unit of GR activity was equal to 1 μmol NADPH oxidised/min. Activity was expressed as mU/g protein for specific activity.

Assay of total and oxidised glutathione in the heart tissue

Total glutathione was determined using a modification of the method of Tietze (1969). Total glutathione was assayed by the addition of 190 μl freshly prepared assay buffer (NADPH (100 μmol/l), 5,5′-dithiobis-(2-nitrobenzoic acid) (5 mmol/l), GR (1 unit/ml), EDTA (1 mmol/l) and phosphate buffer (50 mmol/l); pH 7·2) to 10 μl diluted sample. The change in absorbance was measured after 3 min at 405 nm using a microplate reader (Labsystem Multiskan RC, Helsinki, Finland) and compared with a standard curve, from 0 to 100 μmol/l.

GSSG was determined by the method of Griffith (1980). Diluted samples or standards (70 μl) were derivatised with 1-methyl-2-vinylpyridinium trifluoromethane sulfonate to remove GSH by adding 4 μl 1-methyl-2-vinylpyridinium trifluoromethane sulfonate and 3·2 μl triethanolamine. Samples were left at room temperature for 1 h to allow the reaction to occur. The GSSG that remained was then assayed in the same manner as for total glutathione.

Total protein concentrations in samples

Total protein concentrations of samples were spectrophotometrically estimated according to the method of Lowry *et al.* (1951) using a Bio-Rad DC protein assay kit (catalogue no. 500-0116; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Values are expressed as means with their standard errors. To evaluate differences among the groups studied, two-way ANOVA with Fisher's *post hoc* test was used. SAS software (version 8.2; SAS Institute Inc., Cary, NC, USA) was used to analyse all data. Differences were considered statistically significant when $P<0·05$.

Results

Body weight and endurance time

At the end of 30 d, body weight in the groups SC, SC + Arg, E and E + Arg were 446 (SEM 7), 446 (SEM 10), 447 (SEM 6) and 451 (SEM 8) g, respectively. There were no significant differences in body weight among the four groups. The mean endurance time of treadmill running to exhaustion was 81 (SEM 4) min for group E and 87 (SEM 5) min for group E + Arg, which was not significantly different between the two groups.

Xanthine oxidase activity in the heart tissue

As shown in Fig. 1, XO activity of heart in group E was significantly increased by 87 % compared with that in group SC. However, XO activity was significantly decreased by 44 % in group E + Arg compared with group E ($P<0·05$).

Myeloperoxidase activity in the heart tissue

The degree of neutrophil infiltration in the myocardium is shown in Fig. 2. In comparison with group SC, MPO activity of heart in group E was significantly increased by 18 % ($P<0·05$).

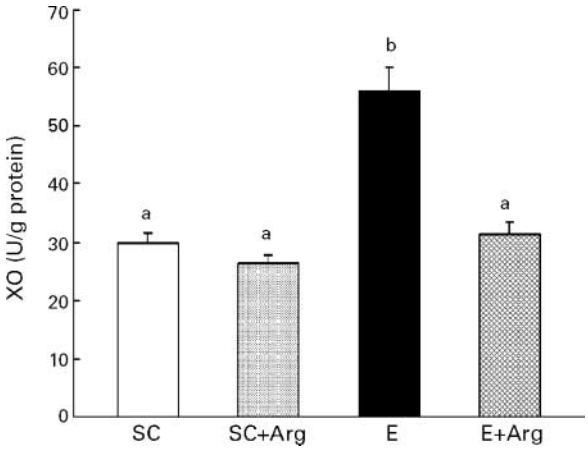

Fig. 1. Effect of L-arginine (Arg) supplementation on xanthine oxidase (XO) activity of heart tissue after exhaustive exercise (E). SC, sedentary control. Values are means for eight rats, with their standard errors represented by vertical bars. Statistical differences among the groups were determined by two-way ANOVA. [a,b]Mean values with unlike letters were significantly different by Fisher's least significant difference test ($P<0·05$). For details of diets and procedures, see p. 68.

Lipid peroxidation in the heart tissue

MDA, a quantitative marker of lipid peroxidation, was measured in the heart tissue as shown in Fig. 3 ($P<0·05$). The content of MDA in group E was significantly elevated by 44 % compared with group SC. When compared with group E, the elevation of MDA content was significantly decreased in group E + Arg ($P<0·05$).

Plasma creatinine kinase, aspartate aminotransferase, lactate dehydrogenase, uric acid, and nitrite and nitrate levels

Plasma CK, AST, LDH, UA, and NOx levels in group E were all significantly elevated by 453, 81, 363, 268 and 93 %, respectively, compared with those in group SC ($P<0·05$) (Table 2). In contrast, the CK and LDH activities of plasma were significantly lowered by 46 and 33 %, respectively, in

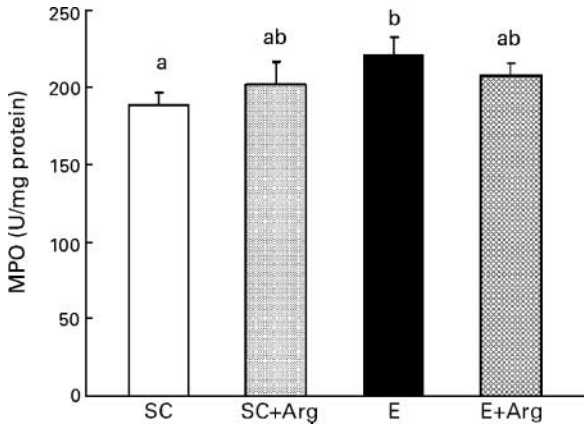

Fig. 2. Effect of L-arginine (Arg) supplementation on myeloperoxidase (MPO) activity of heart tissue after exhaustive exercise (E). SC, sedentary control. Values are means for eight rats, with their standard errors represented by vertical bars. Statistical differences among the groups were determined by two-way ANOVA. [a,b]Mean values with unlike letters were significantly different by Fisher's least significant difference test ($P<0·05$). For details of diets and procedures, see p. 68.

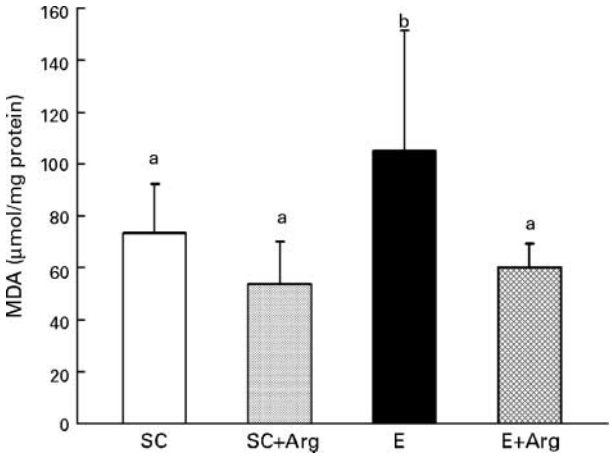

Fig. 3. Effect of L-arginine (Arg) supplementation on malondialdehyde (MDA) activity of heart tissue after exhaustive exercise (E). SC, sedentary control. Values are means for eight rats, with their standard errors represented by vertical bars. Statistical differences among the groups were determined by two-way ANOVA. [a,b]Mean values with unlike letters were significantly different by Fisher's least significant difference test ($P<0·05$). For details of diets and procedures, see p. 68.

group E + Arg compared with group E ($P<0·05$). Elevations of muscle enzymes such as CK, AST, and LDH in plasma are characteristic responses to strenuous exercise and often used as indicators of muscle damage. Especially, changes in activities of AST and LDH have been used previously as indicators of myocardial damage.

Antioxidant enzymes activities in the heart tissue

Table 3 shows the antioxidant enzyme activities. The antioxidant enzyme activities of SOD and CAT in heart tissue were significantly increased by 58 and 62 %, respectively, in group E compared with in group SC ($P<0·05$). In contrast, antioxidant enzyme activities of SOD, CAT, GPX, and GR in group E + Arg were all significantly lower than those in group E ($P<0·05$). It is possible that the higher activity of antioxidant enzymes as a result of exercise might be indicative of a compensatory measure to counteract the possible detrimental effects associated with oxidative stress.

Total and oxidised glutathione in the heart tissue

The levels of total glutathione, GSH and GSSG, and the GSH:GSSG ratio in the heart tissue are shown in Table 4. The levels of total glutathione and GSH showed no difference between groups E and SC. However, the content of GSSG in group E was significantly higher by 63 % compared with that of group SC ($P<0·05$). However, the GSH:GSSG ratio in group E was significantly lower by 55 % than that in group SC ($P<0·05$). When compared with group E, the content of GSSG was significantly lower by 31 % in group E + Arg ($P<0·05$). However, the levels of total glutathione and GSH, and the GSH:GSSG ratio were all significantly increased by 55, 93 and 171 %, respectively, in group E + Arg compared with those in group E ($P<0·05$).

Discussion

Acute physical exercise increases cardiac contractility and heart rate. Strenuous exercise may be associated with a four-fold increase in blood flow through and O_2 consumption by the myocardium. A number of studies have shown free radical-mediated damage during exercise-induced oxidant stress (Ji *et al.* 1991; Ji, 1993; Ji & Mitchell, 1994; Sen *et al.* 1994; Somani *et al.* 1995). The present study assessed the effects of dietary L-Arg supplementation on acute strenuous exercise-induced oxidative damage of myocardium and modulations of cardiac antioxidant enzymes activities. It is the first study to examine the antioxidant potential of L-Arg using an *in vivo* animal exercise model.

The present results demonstrate that in exhaustive exercise with higher anaerobic components, the lactate concentration in plasma of group E (750 (SEM 60) mg/l) was significantly higher than that of group SC (550 (SEM 40) mg/l) ($P<0·05$), but not of group E + Arg (460 (SEM 20) mg/l). This result indicates that the rats are not able to obtain a sufficient amount of O_2 during running; hence, hypoxia metabolism supports a significant part of the exhaustive exercise. This situation is in accordance with some references in the literature (Radak *et al.* 1995; Vina *et al.* 2000). The production of radicals in the heart tissue after acute exhaustive exercise caused some degree of oxidative damage. The importance of this pathway is associated primarily with the consumption of ATP. An increase in the ADP:ATP ratio, followed by AMP

Table 2. Effects of L-arginine (Arg) supplementation on plasma creatinine kinase (CK), lactate dehydrogenase (LDH), uric acid (UA), and nitrite and nitrate (NOx) levels after exhaustive exercise (E)

(Mean values with their standard errors for eight rats)

	Group SC		Group SC + Arg		Group E		Group E + Arg	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CK (U/l)	158[a]	7	231[a]	26	874[b]	223	471[a]	72
AST (U/l)	79[a]	3	90[a]	6	143[b]	16	132[b]	10
LDH (U/l)	169[a]	18	373[a,b]	76	782[c]	146	521[b]	35
UA (mg/l)	9·4[a]	1·0	19·9[b]	2·8	34·6[d]	2·8	28·0[c]	02·1
NOx (μmol/l)	1·38[a]	0·14	1·48[a]	0·20	2·66[b]	0·18	2·69[b]	0·47

SC, sedentary control; AST, aspartate aminotransferase.
[a,b]Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
For details of diets and procedures, see p. 68.

Table 3. Effects of L-arginine (Arg) supplementation on antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR) activities of heart tissue after exhaustive exercise (E)

(Mean values with their standard errors for eight rats)

	Group SC		Group SC + Arg		Group E		Group E + Arg	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
SOD (U/mg protein)	15·41[b]	2·97	6·40[a]	2·10	24·42[c]	4·25	3·69[a]	1·71
CAT (U/mg protein)	39[a]	3	38[a]	4	63[b]	3	45[a]	3
GPX (U/mg protein)	161[a,b]	6	150[a,b]	9	175[b]	11	135[a]	8
GR (mU/g protein)	7·25[a,b]	1·14	7·60[a,b]	0·62	10·01[b]	1·32	6·67[a]	0·94

SC, sedentary control.
[a,b]Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
For details of diets and procedures, see p. 68.

Table 4. Effects of L-arginine (Arg) supplementation on total glutathione, GSH and GSSG concentrations, and GSH:GSSG ratio of heart tissue after exhaustive exercise (E)

(Mean values with their standard errors for eight rats)

	Group SC		Group SC + Arg		Group E		Group E + Arg	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total glutathione (mg/mg protein)	0·047[a]	0·003	0·060[b]	0·006	0·042[a]	0·002	0·065[b]	0·006
GSH (mg/mg protein)	0·039[a,b]	0·003	0·051[b,c]	0·006	0·029[a]	0·002	0·056[c]	0·005
GSSG (mg/mg protein)	0·008[a]	0·001	0·009[a]	0·001	0·013[b]	0·001	0·009[a]	0·001
GSH:GSSG	5·33[b]	0·63	6·19[b]	1·14	2·41[a]	0·36	6·53[b]	0·32

SC, sedentary control.
[a,b]Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
For details of diets and procedures, see p. 68.

and its degradation to hypoxanthine, culminates in UA increasing and O_2^- production by XO (Westing *et al.* 1989; Radak *et al.* 1995; Vina *et al.* 2000). Our data support the findings of the present study in which the XO activity and the UA and MDA concentrations in the heart show similar marked increases immediately following the exhaustive running. This may suggest that the XO-derived free radicals exert some deleterious effect on heart tissue. However, no such increase in XO was observed in the L-Arg-supplemented group in response to exercise. Numerous studies have shown NO, a biologically important molecule, to have many modulatory functions for cells and tissues. NO has been shown to be cardioprotective in ischaemia and reperfusion (Zweier *et al.* 1995; Au *et al.* 2003). Furthermore, NO may exert direct effects on cardiac myocytes (Shiono *et al.* 2002). The inhibition of XO by NO may be mediated through direct binding of NO to the enzyme Fe–S moiety (Hassoun *et al.* 1995). Another study also supported this notion that NO may suppress XO activity (Fukahori *et al.* 1994). Hence, the protective role of NO could probably be due to its property of scavenging free radicals and inhibiting XO; this is in accordance with the present results in the E + Arg group.

MPO has been shown to provide an index of neutrophil infiltration in tissues, and its activity has been reported to be elevated with strenuous running in several tissues of rats, including heart tissue (Belcastro *et al.* 1996). Our data also showed that heart MPO activity in group E was markedly increased after exhaustive exercise. Therefore, it can be concluded that XO-derived oxidants are important in the accumulation of neutrophils after exhaustive exercise. This chemotaxic potential of oxidants from XO is in agreement with previous investigators (Kubes *et al.* 1990, 1991; Nonami, 1997). However, exhaustive exercise-induced cardiac MPO activity was not significantly reduced by L-Arg supplementation.

As described earlier, lipid peroxidation, measured in the form of MDA, significantly increased in the heart tissue of the E group, also supporting the possibility of increased ROS production leading to higher rate of lipid peroxidation (Frankiewicz-Jozko *et al.* 1996; Venditti & Di Meo, 1996). L-Arg supplementation, however, significantly protects heart tissue from ROS-mediated oxidative damage during exhaustive exercise.

Vina *et al.* (2000) have demonstrated that administration of allopurinol, an inhibitor of XO, prevents the increase of the activities of cytosolic enzymes CK and LDH in plasma after exhaustive exercise. Our data also showed that L-Arg supplementation could inhibit the elevation of XO activity and resulted in a significant decrease in plasma levels of CK and LDH in group E + Arg compared with that in group E. This finding was similar to that of a previous report (Vina *et al.* 2000).

Furthermore, Qian *et al.* (2001) and Reid (1998) have suggested that long-term strenuous exercise may stimulate the activity of NO synthase and hence increase NO synthesis, also causing a significant increase in NOx production in plasma. In the present study, the results indicate that in both the E and E + Arg groups there was a significant increase in plasma NOx after exhaustive exercise compared with controls. NO, however, is also a free radical and hence the toxicity of NO (excess production of NO) is markedly enhanced by its reaction with O_2^- from peroxynitrite ($ONOO^-$), which is

extremely cytotoxic (Beckman & Koppenol, 1996). Numerous studies have shown that infusion of $ONOO^-$ into working rat hearts impairs cardiac contractile function by decreasing cardiac efficiency (Liu *et al.* 1997; Schulz *et al.* 1997; Yasmin *et al.* 1997). However, Rubbo *et al.* (1994) reported that NO actually inhibits peroxynitrite-induced lipid peroxidation. The data demonstrated that NO will significantly enhance lipid peroxidation only when rates of NO production approach or are equivalent to rates of O_2^- production by XO. In the present study, we measured the effect of exhaustive exercise at the rate of NOx synthesis in plasma in both exercised groups. Obviously, rats in group E had significant increases in XO activity, MPO activity, MDA levels and GSSG release that will cause oxidative stress in heart tissue. It means that the enhanced production of O_2^- by XO may be possibly as a result of synergy between higher-level NO and O_2^- to form cytotoxic $ONOO^-$ after exhaustive exercise. This is in contrast with the E + Arg group; when NO concentrations are increased, the protective role of NO and/or L-Arg itself may probably be due to the property of scavenging free radicals and possibly inhibiting XO as well as inflammation of neutrophils. Therefore, the present study suggests that the E + Arg group has higher levels of NO to inhibit $ONOO^-$ formation and it is unlikely that $ONOO^-$ may have a role in this process. The present results are in agreement with the findings of Rubbo *et al.* (1994); that NO alone does not induce lipid peroxidation. Thus, we conclude that NO can exacerbate oxidant injury via the production of the potent oxidant $ONOO^-$ or exert a protective role via scavenging O_2^- by termination of free radical-dependent chain-propagation reactions by diverse initiating species.

It is well known that SOD, CAT, GPX and GR are regarded as the first line of the antioxidant enzyme system against ROS generated during exhaustive exercise. The present study showed that these enzymes are increased as a compensatory mechanism in response to the increase of oxidative stress due to exhaustive exercise. It means that the increased XO observed in the present study denotes the increased generation of O_2^-, which will have triggered the activation of SOD by dismutating O_2^- to H_2O_2. This result is supported by previous studies (Ji, 1993; Somani *et al.* 1995; Leeuwenburgh *et al.* 1996). The increased CAT activity levels in heart (162 %) observed in the present study were also presented in Somani *et al.* (1995), who observed CAT activity increase by 358 % in the heart of rats subjected to acute strenuous exercise. Our data show that SOD and CAT activities were significantly lower or were not different in the E + Arg group compared with the control groups. These results indicate that L-Arg supplementation has beneficial effects on attenuating the oxidative stress induced by exhaustive exercise. The increased activity of GPX in heart after acute strenuous exercise has been reported in a previous study; GPX converts H_2O_2 to water and O_2 to cope with abrupt exercise-induced oxidant stress (Somani *et al.* 1995). In addition, we found significantly increased heart GR activity in the E group. However, it is different from the results of a few reports showing no change (Ji, 1993; Leeuwenburgh *et al.* 1996). It may be possible that myocardium shows a marked GR adaptation to GSH depletion and increased GSSG levels in the present study. However, growing evidence (Ji, 1993; Somani *et al.* 1995; Leeuwenburgh *et al.* 1996) suggests that some antioxidant enzymes can be increased in myocardium actively involved in exhaustive exercise. The results of the present study indicate that myocardium of the E group may be more susceptible to oxidative stress than that of the SC group. These disparate results may be due to differences in tissues or organs, exercise intensity, duration and mode of exercise.

GSH is a major non-enzymic antioxidant and has been reported to play an important role in protecting the myocardium from ischaemia and reperfusion-induced oxidative damage (Ji, 2002). Previous studies have shown a decrease in the myocardial GSH of rats after a vigorous swimming exercise and exercise running (Kihlstrom *et al.* 1989; Seward *et al.* 1995; Leichtweis *et al.* 1997). Also, the GSH:GSSG ratio decreases in the myocardium of exhausted rats (Sen *et al.* 1994). Subsequent studies also reported that levels of total glutathione fell in exhaustive-exercise rats (Duarte *et al.* 1993). In the present study, we found that GSSG was significantly increased and that the GSH:GSSG ratio was significantly decreased in the E group, but GSH did not reach statistical significance ($P=0{\cdot}0687$). Also, total glutathione did not change significantly in the E group compared with the SC group. However, L-Arg supplementation significantly increased total glutathione and GSH in both the SC + Arg and E + Arg groups. As a result of the protective effect of L-Arg, GSSG level significantly decreased in comparison with the E group. It means that healthy non-stressed cells maintain notably a high intracellular GSH:GSSG ratio to ensure the availability of GSH, and thereby promote active reduction of H_2O_2 through the GSH redox cycle. The effects of L-Arg supplementation in exhaustively exercised animals on enzymic and non-enzymic antioxidant systems cannot be clearly explained at present. They are probably related to the following two mechanisms. First, L-Arg serves as a precursor of the synthesis of NO that may have an indirect antioxidant role to scavenge O_2^- and inhibit XO activity from exercise-induced stress (Pabla *et al.* 1996). The present results suggest that the protection by L-Arg may, through the generation of NO, reduce ROS formation via the inhibition of XO activity, to prevent cellular damage; but, in the presence of NO synthase inhibitor L-NAME, this protection is abolished (Wascher *et al.* 1997). Second, L-Arg has some protective roles against ROS attack by its direct chemical property interaction with O_2^- (Wascher *et al.* 1997; Lass *et al.* 2002). Hence, it may be possible to maintain normal enzymic and non-enzymic antioxidant systems of the redox cycle after exhaustive exercise. By contrast, non-Arg supplementation of rats significantly increases these antioxidant enzymes and decreases the GSH:GSSG ratio, possibly as a compensatory mechanism to cope with the enhanced production of ROS during exhaustive exercise.

In conclusion, the present results support the mechanism that the increase of XO and MPO activities is the main pathway involved in the production of free radicals during exhaustive exercise. Our data also indicate that the administration of L-Arg could prevent exhaustive exercise-induced production of lipid peroxide levels, elevation of XO activity, and imbalance of antioxidant enzymes activities. Our findings may have important implications in the development of therapeutic strategies aimed at manipulating L-Arg supplementation for NO production and/or L-Arg itself in exercise-induced oxidative stress.

Acknowledgements

This project was supported by the Shin Kong Wu Ho-Su Memorial Hospital of Taiwan, SKH-FJU-92–13.

References

Ashton T, Rowlands CC, Jones E, Young IS, Jackson SK, Davies B & Peters JR (1998) Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. *Eur J Appl Physiol Occup Physiol* **77**, 498–502.

Au A, Louch WE, Ferrier GR & Howlett SE (2003) L-Arginine ameliorates effects of ischemia and reperfusion in isolated cardiac myocytes. *Eur J Pharmacol* **476**, 45–54.

Beckman JS & Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad and ugly. *Am J Physiol* **271**, C1424–C1437.

Beers RF Jr & Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* **195**, 133–140.

Belcastro AN, Arthur GD, Albisser TA & Raj DA (1996) Heart, liver, and skeletal muscle myeloperoxidase activity during exercise. *J Appl Physiol* **80**, 1331–1335.

Brooks GA & White TP (1978) Determination of metabolic and heart rate responses of rats to treadmill exercise. *J Appl Physiol* **45**, 1009–1015.

de Oliveira SL, Diniz DB & Amaya-Farfan J (2003) Carbohydrate-energy restriction may protect the rat brain against oxidative damage and improve physical performance. *Br J Nutr* **89**, 89–96.

Duarte JA, Appell HJ, Carvalho F, Bastos ML & Soares JM (1993) Endothelium-derived oxidative stress may contribute to exercise-induced muscle damage. *Int J Sports Med* **14**, 440–443.

Fielding RA, Manfredi T, Ding W, Fiatarone MA, Evans WJ & Cannon JG (1993) Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in skeletal muscle. *Am J Physiol* **265**, R166–R172.

Frankiewicz-Jozko A, Faff J & Sieradzan-Gabelska B (1996) Changes in concentrations of tissue free radical marker and serum creatine kinase during the post-exercise period in rats. *Eur J Appl Physiol* **74**, 470–474.

Fukahori M, Ichimori K, Ishida H, Nakagawa H & Okino H (1994) Nitric oxide reversibly suppresses xanthine oxidase activity. *Free Radic Res* **21**, 203–212.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS & Tannenbaum SR (1982) Analysis of nitrate, nitrite and [15N]nitrate in biological fluids. *Anal Biochem* **126**, 131–138.

Griffith OW (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* **106**, 207–212.

Hassoun PM, Yu FS, Zulueta JJ, White AC & Lanzillo JJ (1995) Effect of nitric oxide and cell redox status on the regulation of endothelial cell xanthine dehydrogenase. *Am J Physiol* **268**, L809–L817.

Hellsten Y, Frandsen U, Orthenblad N, Sjodin B & Richter EA (1997) Xanthine oxidase in human skeletal muscle following eccentric exercise: a role in inflammation. *J Physiol* **498**, 239–248.

Ji LL (1993) Antioxidant enzyme responds to exercise and aging. *Med Sci Sports Exerc* **25**, 225–231.

Ji LL (1999) Antioxidants and oxidative stress in exercise. *Proc Soc Exp Biol Med* **222**, 283–292.

Ji LL (2002) Exercise-induced modulation of antioxidant defense. *Ann N Y Acad Sci* **959**, 82–92.

Ji LL, Dillon D & Wu E (1991) Myocardial aging: antioxidant enzyme systems and related biochemical properties. *Am J Physiol* **261**, R386–R392.

Ji LL & Mitchell EW (1994) Effects of Adriamycin on heart mitochondrial function in rested and exercised rats. *Biochem Pharmacol* **47**, 877–885.

Judge AR & Dodd SL (2004) Xanthine oxidase and activated neutrophils cause oxidative damage to skeletal muscle after contractile claudication. *Am J Physiol* **286**, H252–H256.

Kihlstrom M, Ojala J & Salminen A (1989) Decreased level of cardiac antioxidants in endurance-trained rats. *Acta Physiol Scand* **135**, 549–554.

Kubes P, Suzuki M & Granger DN (1990) Modulation of PAF-induced leukocyte adherence and increased microvascular permeability. *Am J Physiol* **259**, G859–G864.

Kubes P, Suzuki M & Granger DN (1991) Nitric oxide: an endogenous modulator of leukocyte adhesion. *PNAS* **88**, 4651–4655.

Kumar CT, Reddy VK, Prasad M, Thyagaraju K & Reddanna P (1992) Dietary supplementation of vitamin E protects heart tissue from exercise-induced oxidant stress. *Mol Cell Biochem* **111**, 109–115.

Lass A, Suessenbacher A, Wolkart G, Mayer B & Brunner F (2002) Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. *Mol Pharmacol* **61**, 1081–1088.

Leeuwenburgh C, Leichtweis S, Hollander J, Fiebig R, Groe M & Ji LL (1996) Effect of acute exercise on glutathione deficient heart. *Mol Cell Biochem* **156**, 17–24.

Leichtweis SB, Leeuwenburgh C, Parmelee DJ, Fiebig R & Ji LL (1997) Rigorous swim training impairs mitochondrial function in post-ischaemic rat heart. *Acta Physiol Scand* **160**, 139–148.

Lewis B & Langkamp-Henken B (2000) Arginine enhances in vivo immune responses in young, adult and aged mice. *J Nutr* **130**, 1827–1830.

Liu P, Hock CE, Nagele R & Wong PY (1997) Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. *Am J Physiol* **272**, H2327–H2336.

Lowry OH, Rosebrough NJ, Lewis Farr A & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.

McCord JM (1985) Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* **312**, 159–163.

Mullane KM, Kraemer R & Smith B (1985) Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* **14**, 157–167.

Nonami Y (1997) The role of nitric oxide in cardiac ischemia-reperfusion injury. *Jpn Circ J* **61**, 119–132.

Pabla R, Buda AJ, Flynn DM, Blesse SA, Shin AM, Curtis MJ & Lefer DJ (1996) Nitric oxide attenuates neutrophil-mediated myocardial contractile dysfunction after ischemia and reperfusion. *Circ Res* **78**, 65–72.

Qian ZM, Xiao DS, Ke Y & Liao QK (2001) Increased nitric oxide is one of the causes of changes of iron metabolism in strenuously exercised rats. *Am J Physiol* **280**, R739–R743.

Radak Z, Asano K, Inoue M, Kizaki T, Oh-Ishi S, Suzuki K, Taniguchi N & Ohno H (1995) Superoxide dismutase derivative reduces oxidative damage in skeletal muscle of rats during exhaustive exercise. *J Appl Physiol* **79**, 129–135.

Radak Z, Nakamura A, Nakamoto H, Asano K, Ohno H & Goto S (1998) A period of anaerobic exercise increases the accumulation of reactive carbonyl derivatives in the lungs of rats. *Pflugers Arch* **435**, 439–441.

Reeves PG, Nielsen FH & Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.

Reid MB (1998) Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta Physiol Scand* **162**, 401–409.

Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanaraman B, Barnes S, Kirk M & Freeman BA (1994) Nitric oxide regulation of

superoxide and peroxylnitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J Biol Chem* **269**, 26066–26075.

Schaefer A, Piquard F, Geny B, Doutreleau S, Lampert E, Mettauer B & Lonsdorfer J (2002) L-Arginine reduces exercise-induced increase in plasma lactate and ammonia. *Int J Sports Med* **23**, 403–407.

Schierwagen C, Bylund-Fellenius AC & Lundberg C (1990) Improved method for quantification of tissue PMN accumulation measured by myeloperoxidase activity. *J Pharmacol Methods* **23**, 179–186.

Schmidt HH, Warner TD, Nakane M, Forstermann U & Murad F (1992) Regulation and subcellular location of nitrogen oxide synthases in RAW264.7 macrophages. *Mol Pharmacol* **41**, 615–624.

Schulz R, Dodge KL, Lopaschuk GD & Clanachan AS (1997) Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. *Am J Physiol* **272**, H1212–H1219.

Sen CK, Atalay M & Hanninen O (1994) Exercise-induced oxidative stress: glutathione supplementation and deficiency. *J Appl Physiol* **77**, 2177–2187.

Seward SW, Seiler KS & Starnes JW (1995) Intrinsic myocardial function and oxidative stress after exhaustive exercise. *J Appl Physiol* **79**, 251–255.

Shiono N, Rao V, Weisel RD, Kawasaki M, Li RK, Mickle DAG, Fedak PWM, Tumiati LC, Ko L & Verma S (2002) L-Arginine protects human heart cells from low-volume anoxia and reoxygenation. *Am J Physiol* **282**, H805–H815.

Somani SM, Frank S & Rybak LP (1995) Responses of antioxidant system to acute and trained exercise in rat heart subcellular fractions. *Pharmacol Biochem Behav* **51**, 627–634.

Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* **27**, 502–522.

Tiidus PM (1998) Radical species in inflammation and overtraining. *Can J Physiol Pharmacol* **76**, 533–538.

Venditti P & Di Meo S (1996) Antioxidants, tissue damage, and endurance in trained and untrained young male rats. *Arch Biochem Biophys* **331**, 63–68.

Vina J, Gimeno A, Sastre J, Desco C, Asensi M, Pallardo FV, Cuesta A, Ferrero JA, Terada LS & Repine JE (2000) Mechanism of free radical production in exhaustive exercise in humans and rats; role of xanthine oxidase and protection by allopurinol. *IUBMB Life* **49**, 539–544.

Wang BY, Singer AH, Tsao PS, Drexler H, Kosek J & Cooke JP (1994) Dietary arginine prevents atherogenesis in the coronary artery of the hypercholesterolemic rabbit. *J Am Coll Cardiol* **23**, 452–458.

Wascher TC, Posch K, Wallner S, Hermetter A, Kostner GM & Graier WF (1997) Vascular effects of L-arginine: anything beyond a substrate for the NO-synthase? *Biochem Biophys Res Commun* **234**, 35–38.

Westerfeld WW, Richert DA & Higgins ES (1959) Further studies with xanthine oxidase inhibitors. *J Biol Chem* **234**, 1897–1900.

Westing YH, Ekblom B & Sjodin B (1989) The metabolic relation between hypoxanthine and uric acid in man following maximal short-distance running. *Acta Physiol Scand* **137**, 341–345.

Wu G & Morris SM Jr (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* **336**, 1–17.

Xiao DS, Jiang L, Che LL & Lu L (2003) Nitric oxide and iron metabolism in exercised rat with L-arginine supplementation. *Mol Cell Biochem* **252**, 65–72.

Yasmin W, Strynadka KD & Schulz R (1997) Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc Res* **33**, 422–432.

Zweier JL, Wang P, Samouilov A & Kuppusamy P (1995) Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med* **1**, 804–809.