

Improved cardiac performance after ischemia in aged rats supplemented with vitamin E and α -lipoic acid

JEFF S. COOMBES,¹ SCOTT K. POWERS,¹ KARYN L. HAMILTON,¹ HAYDAR A. DEMIREL,¹
R. ANDREW SHANELY,¹ MURAT A. ZERGEROGLU,¹ CHANDAN K. SEN,^{2,3}
LESTER PACKER,² AND LI LI JI⁴

¹Center for Exercise Science, University of Florida, Gainesville, Florida 32611; ²Department of Molecular and Cell Biology, University of California, Berkeley, California 94720; ³Department of Physiology, University of Kuopio, Kuopio, Finland 70211; and ⁴Department of Kinesiology, University of Wisconsin, Madison, Wisconsin 53706

Received 8 May 2000; accepted in final form 8 August 2000

Coombes, Jeff S., Scott K. Powers, Karyn L. Hamilton, Haydar A. Demirel, R. Andrew Shanely, Murat A. Zergeroglu, Chandan K. Sen, Lester Packer, and Li Li Ji. Improved cardiac performance after ischemia in aged rats supplemented with vitamin E and α -lipoic acid. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R2149–R2155, 2000.—The purpose of these experiments was to examine the effects of dietary antioxidant supplementation with vitamin E (VE) and α -lipoic acid (α -LA) on biochemical and physiological responses to in vivo myocardial ischemia-reperfusion (I-R) in aged rats. Male Fischer-334 rats (18 mo old) were assigned to either 1) a control diet (CON) or 2) a VE and α -LA supplemented diet (ANTIOX). After a 14-wk feeding period, animals in each group underwent an in vivo I-R protocol (25 min of myocardial ischemia and 15 min of reperfusion). During reperfusion, peak arterial pressure was significantly higher ($P < 0.05$) in ANTIOX animals compared with CON diet animals. I-R resulted in a significant increase ($P < 0.05$) in myocardial lipid peroxidation in CON diet animals but not in ANTIOX animals. Compared with ANTIOX animals, heart homogenates from CON animals experienced significantly less ($P < 0.05$) oxidative damage when exposed to five different in vitro radical producing systems. These data indicate that dietary supplementation with VE and α -LA protects the aged rat heart from I-R-induced lipid peroxidation by scavenging numerous reactive oxygen species. Importantly, this protection is associated with improved cardiac performance during reperfusion.

aging; free radicals; lipid peroxidation; myocardial performance

THERE IS MUCH INTEREST in nutritional antioxidant supplementation as a therapeutic preventative strategy against myocardial ischemia-reperfusion (I-R) injury. The interest results from findings that reactive oxygen species (ROS) contribute significantly to myocardial I-R injury (11). ROS, such as superoxide anions, hydroxyl radicals, and peroxy radicals formed during reperfusion, can lead to lipid peroxidation within the

cardiac myocyte, resulting in decreased cardiac performance (3).

It is widely believed that an optimum antioxidant supplement contains more than one nutrient, and recently the combination of two antioxidants, vitamin E (VE) and α -lipoic acid (α -LA), has generated scientific interest. VE is the major lipid peroxidation chain-breaking antioxidant and is located in the lipid phase of the cell. When VE quenches a ROS, a VE radical is formed that must be recycled back to its reduced form to continue to provide antioxidant protection. Importantly, α -LA is capable of recycling VE (26). In addition, α -LA, reduced α -LA (dihydrolipoic acid), and their metabolites also function as antioxidants (26). α -LA has also been reported to be an effective glutathione substitute, capable of increasing cellular glutathione levels and further improving the antioxidant status of the myocardium (14).

The combination of VE and dihydrolipoic acid (DHLA) has been shown to improve rodent cardiac performance in experiments using an in vitro I-R model (15, 16). In these studies, young adult animals were fed a high VE diet (10,000 IU/kg diet), and DHLA was added to the perfusion medium. The myocardial protection from I-R injury was reported to be due to the synergism of both of the two compounds (15), as opposed to their individual antioxidant abilities (15). At present, it is unknown whether this protection is realized in vivo.

Currently, limited information exists regarding the ability of antioxidant supplementation to protect senescent hearts from I-R-induced injury. This is unfortunate, given that, compared with young animals, myocardial I-R in senescent animals results in a greater myocardial injury (21). This observation could be linked to the fact that aging is associated with reduced myocardial antioxidant protection (32). Therefore, these experiments examined the effects of dietary sup-

Address for reprint requests and other correspondence: J. Coombes, School of Human Movement Studies, Connell Bldg., Univ. of Queensland, St. Lucia, QLD 4072, Australia (E-mail: jcoombes@hms.uq.edu.au).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

plementation of antioxidants (VE and α -LA) on I-R-induced myocardial injury in senescent animals. Specifically, we tested the hypothesis that the dietary combination of VE and α -LA would provide protection from I-R damage in aged animals. Furthermore, on the basis of known antioxidant properties of VE and α -LA, we postulated that dietary supplements with these antioxidants would protect the heart against I-R lipid peroxidation induced by superoxide radicals, hydroxyl radicals, hydrogen peroxide, and peroxy radicals.

METHODS

Experimental animals and dietary supplementation. Male Fischer-344 rats (18 mo old) were divided into two dietary groups: 1) a control diet (CON), $n = 25$, and 2) a VE- and α -LA-supplemented diet (ANTIOX), $n = 26$. CON animals were fed the AIN-93M purified diet, which contains 75 IU DL- α -tocopherol acetate/kg diet. ANTIOX animals were fed the AIN-93M purified diet with 10,000 IU DL- α -tocopherol acetate/kg diet and 1.65 g α -lipoic acid/kg diet. Diets were professionally prepared (Harlan Teklad, Madison, WI). Animals were fed the diet for 14 wk. Randomly selected animals from the two dietary groups were exposed to the I-R protocol (CON, $n = 15$; ANTIOX, $n = 14$). The remaining animals served as sham controls by undergoing the same surgical interventions without I-R. These sham controls provided baseline data for the myocardial levels of lipid peroxidation and antioxidant capacity. Furthermore, ventricular tissue from sham controls was used for the heart homogenates that were subjected to the *in vitro* oxidative challenges.

Experimental protocol. The *in vivo* I-R procedure has been explained in detail in previous work from our laboratory (5, 27). Briefly, animals were anesthetized and mechanically ventilated, and the chest was opened by thoracotomy. Coronary occlusion was achieved by a ligature around the left coronary artery; occlusion was maintained for 25 min, followed by a 15-min period of reperfusion. During this time, cardiac performance was monitored by a catheter placed in the ascending aorta. At the completion of the experimental protocol, animals were euthanized with an overdose of pentobarbital sodium, and the heart was removed, washed in an antioxidant buffer (1 mM butylated hydroxytoluene and 100 μ M EDTA in a 50 mM sodium phosphate buffer, pH = 7.4), and rapidly frozen for subsequent biochemical analyses.

Assay of tissue levels of VE. Myocardial levels of VE were determined with HPLC by use of the protocol of Cort et al. (6). Tissue preparation before VE analysis consisted of mechanical homogenization in acetone (Ultra-Turrax T25, IKA Works, Cincinnati, OH), double extraction with petroleum ether, and then reconstitution in isooctane before injection into the HPLC (ABI Analytical Spectroflow 400). Aliquots (20 μ l) of the isooctane extract were injected onto a 250 \times 4-mm, 10- μ m LiChrosorb SI column (Baird and Tatlock, Dagenham, UK).

Assay of tissue levels of α -LA. At physiological pH, the salts of α -LA dissociate and form lipoate. Therefore, determination of α -LA levels in physiological fluids or tissues is specifically a measure of lipoate content. Tissue levels of lipoate were measured according to Sen et al. (31), with slight modification. Frozen left ventricle samples (0.5–0.6 g) were ground to powder in liquid nitrogen with a mortar and pestle and then homogenized on ice in 2 ml of 20% metaphosphoric acid with a Teflon homogenizer. The homogenate was ultrasonicated intermittently for 100 s (10-s periods with 10 s of rest). The sample was extracted in hexane. After hexane evaporation

under nitrogen, the extract was solubilized in a solvent containing 50% 0.2 M monochloroacetic acid, 30% acetonitrile, and 20% methanol. Samples were filtered (0.22 μ m) and frozen at -80°C for HPLC analysis. Samples (0.2 ml) were separated on an Alltima C₁₈ column (250 \times 4.6 mm, 5 μ m; Alltech Associates, Deerfield, IL) by use of a mobile phase consisting of 50% 50 mM sodium biphosphate in water, 30% acetonitrile, and 20% methanol and a flow rate of 1 ml/min. α -LA was detected at a retention time of 9.5 min with a Coulechem II multielectrode electrochemical detector (model 5100A, ESA, Chelmsford, MA). The electrodes were set at the following potentials: electrode 1, +0.45V; electrode 2, +0.85V; and guard cell: +0.90V. Data were collected using a PE Nelson 900 series interface [Perkin Elmer (PE), San Jose, CA] and processed using the PE Nelson Turbochrome 4 (Perkin Elmer) software. Racemate mixture of lipoate, used as a standard, was provided by ASTA Medica (Frankfurt, Germany). Using this method, we obtained a linear ($r^2 = 0.996$) standard curve in the range of 0.2–0.75 nmol of lipoate.

Biochemical assessment of endogenous antioxidant enzymes. To determine whether our dietary treatments altered endogenous antioxidant enzymes, a small sample of the left ventricle from all animals was assayed to measure the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). SOD and GPX activities were determined spectrophotometrically with a modification of the procedures described by Oyanagui (25) and Flohé and Günzler (8), respectively. CAT was assayed using the procedure described by Ji et al. (18).

Lipid peroxidation measurements. To determine the degree of oxidative damage in the heart, left ventricular levels of two by-products of lipid peroxidation were measured. Malonyldialdehyde (MDA) levels were determined spectrophotometrically by use of the thiobarbituric acid-reactive substance (TBARS) method previously described by Mihara and Uchiyama (24), with 1,1,3,3-tetramethoxypropane used as the standard.

Lipid hydroperoxides were quantified using the ferrous oxidation/xenol orange technique reported by Hermes-Lima (17). Cumene hydroperoxide was used as the standard for this assay, and values were expressed as cumene hydroperoxide equivalents (CHE).

***In vitro* measurement of tissue antioxidant capacity.** To evaluate the antioxidant potential of VE and α -LA supplementation, heart homogenates from the sham surgery animals were subjected to five different ROS-generating systems and then analyzed for lipid peroxidation with the TBARS assay described above. Sections of the left ventricle from both ANTIOX and CON animals were homogenized at a concentration of 10:1 in either 0.9% (wt/vol) saline solution (for the aqueous generating systems) or ethanol (for the lipid phase system). Aliquots of the homogenates were incubated at a concentration of 10 mg protein/ml in the presence or absence of an ROS generating system. Five ROS generating systems were used. 1) Superoxide radicals were generated by a hypoxanthine-xanthine oxidase system, according to the method of Fridovich (9). 2) Hydrogen peroxide (100 μ M) was added directly to heart homogenates according to the method of Gonzalez Flecha et al. (12). 3) Hydroxyl radicals were generated in the heart homogenates by adding 0.1 μ M ferrous sulfate (FeSO_4) to heart homogenates. 4) Peroxyl radicals were generated in the aqueous phase of the homogenate by the addition of 9.4 mM AAPH [2,2'-azobis(2-amidinopropane)-dihydrochloride] (19). 5) Peroxyl radicals were generated in lipids in the heart homogenate by thermal decompo-

sition of 5 mM AMVN [2,2'-azobis(2-4 dimethylvaleronitrile)] (19).

After incubation of the heart homogenates in each system, butylated hydroxytoluene (200 mM) and deferoxamine (1 mM) in ice-cold trichloroacetic acid (20% wt/vol) were added to stop the oxidative reaction. TBARS formation was then determined, as described in *Lipid peroxidation measurements*.

Statistical analysis. Biochemical data were subjected to a one-way ANOVA. Performance measures were analyzed using a two-way repeated-measures ANOVA. Scheffé's test was used post hoc, with significance established a priori at $P < 0.05$.

RESULTS

Figure 1 contains the mean (\pm SE) myocardial VE and α -LA concentrations in both CON and ANTIOX diet animals. These data indicate that our feeding protocol resulted in a significant ($P < 0.05$) increase in the myocardial VE levels in the ANTIOX animals compared with CON animals. However, no significant differences ($P > 0.05$) in α -LA levels existed between the two groups.

Cardiac performance was assessed during ischemia and reperfusion by utilizing a fluid-filled catheter placed in the ascending aorta to measure peak systolic pressure. The effects of the different diets on peak arterial pressure and rate-pressure product during the experimental protocol are shown in Fig. 2. Note that no significant differences existed between experimental groups before and during ischemia in either measure. However, at 2, 5, and 15 min of reperfusion, both peak

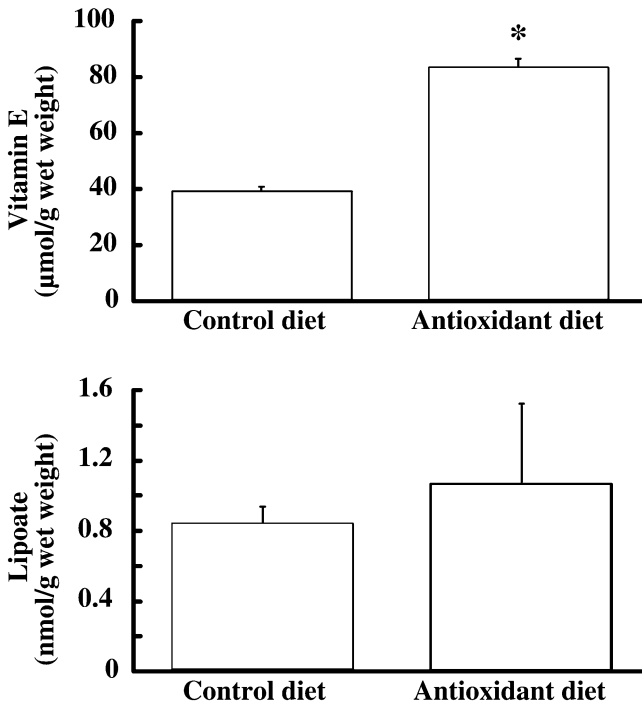


Fig. 1. Comparison of myocardial vitamin E (A) and lipoate concentrations (B) between control (CON) diet and antioxidant (ANTIOX, vitamin E and α -lipoic acid) diet animals. Values are means \pm SE. *Significantly greater than CON diet ($P < 0.05$).

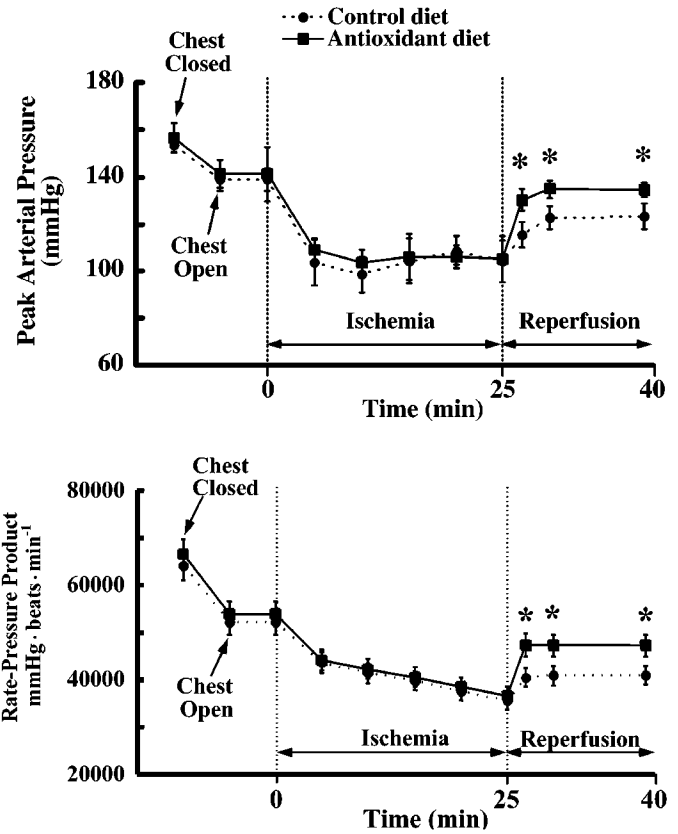


Fig. 2. Effects of CON and ANTIOX diets on peak arterial systolic pressure (A) and the rate-pressure product (B) during ischemia and reperfusion. Values are means \pm SE. *ANTIOX significantly greater than CON ($P < 0.05$).

systolic pressure and rate-pressure product were significantly ($P < 0.05$) higher in the ANTIOX animals compared with CON diet animals.

Two markers of lipid peroxidation were used to determine the effects of the different diets on cardiac damage due to I-R. Figure 3 contains the mean (\pm SE) values for myocardial TBARS and CHE in both experimental groups. First, note that similar results were obtained using the two markers of lipid peroxidation. Also notice that, in the CON diet group, I-R surgery resulted in a significant increase ($P < 0.05$) in both myocardial TBARS and CHE levels compared with sham surgery animals. Finally, a key finding was that antioxidant ANTIOX I-R animals had significantly lower ($P < 0.05$) myocardial TBARS and CHE levels than CON I-R animals.

Table 1 contains mean (\pm SE) activities of GPX, SOD, manganese SOD (MnSOD), copper-zinc superoxide dismutase (Cu-ZnSOD), and CAT. Several observations are noteworthy. First, compared with CON diet animals, myocardial GPX activity in ANTIOX animals was significantly higher ($P < 0.05$) in both the sham and the I-R surgery groups.

Second, SOD activity was greater ($P < 0.05$) in ANTIOX animals that underwent I-R surgery compared with CON diet I-R surgery animals. Furthermore, CON diet I-R surgery animals displayed lower

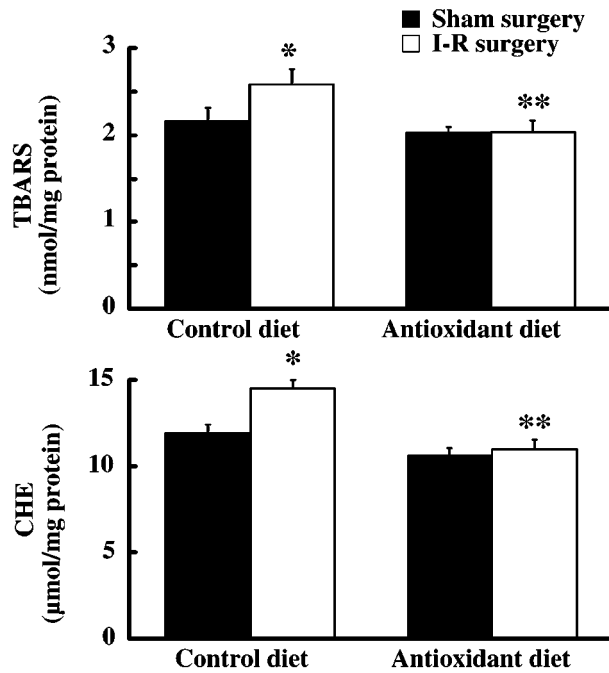


Fig. 3. Lipid peroxidation levels in the myocardium of rats undergoing either ischemia-reperfusion (I-R) or sham surgery and consuming either a CON or an ANTIOX diet. TBARS, thiobarbituric acid-reactive substance; CHE, cumene hydroperoxide equivalents. Values are means \pm SE. *Significantly greater than sham surgery animals ($P < 0.05$); **significantly less than CON diet I-R surgery animals ($P < 0.05$).

($P < 0.05$) total SOD activity compared with CON diet sham surgery animals. Analysis of the different isoforms of SOD indicated that these differences in total enzyme activity were due to changes in the manganese isoform of the enzyme. Finally, dietary supplementation with VE and α -LA did not alter the myocardial activity of CAT in either sham surgery or I-R surgery groups.

Figure 4A indicates that the myocardium from ANTIOX animals was significantly ($P < 0.05$) better protected against lipid peroxidation in all four aqueous radical-generating systems compared with the myocardium from CON diet animals. Similarly, compared with CON diet animals, hearts from antioxidant

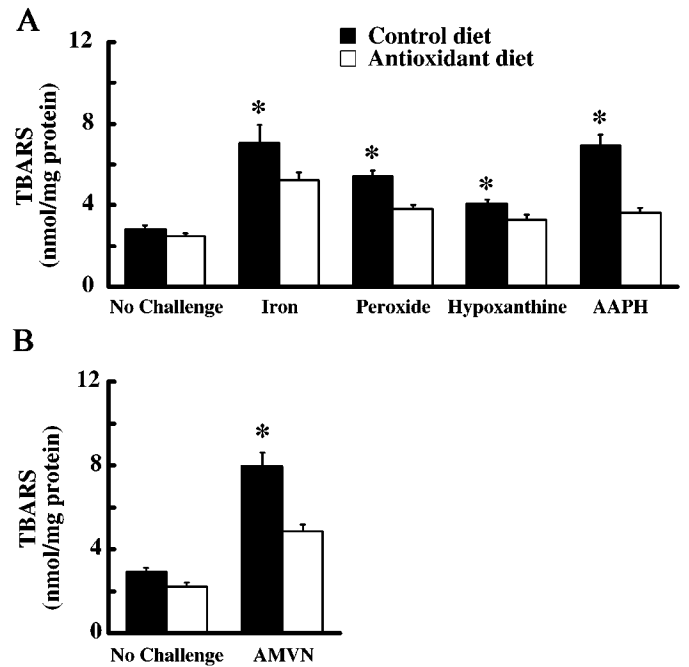


Fig. 4. Lipid peroxidation (TBARS assay) in heart homogenates from either CON or ANTIOX diet animals subjected to 4 in vitro aqueous phase oxidative challenges (A) and 1 lipid phase challenge (B). AAPH, 2,2'-azobis(2-amidinopropane)-dihydrochloride; AMVN, 2,2'-azobis(2,4-dimethylvaleronitrile). Values are means \pm SE. *Significantly greater than ANTIOX diet animals exposed to the same treatment ($P < 0.05$).

ANTIOX animals experienced less ($P < 0.05$) lipid peroxidation when exposed to the lipid phase (AMVN) oxidative challenge (Fig. 4B).

DISCUSSION

Overview of principal findings. This is the first experiment to examine the effects of VE and α -LA supplementation on cardiac performance and biochemistry during in vivo I-R in aged rats. A major finding of this study was that antioxidant supplementation improved cardiac performance during postischemia reperfusion. In addition, our data support the hypothesis that the dietary combination of VE and α -LA reduces myocardial lipid peroxidation resulting from an in vivo I-R

Table 1. Effects of antioxidant supplementation and I-R surgery on endogenous antioxidant enzymes

Antioxidant Enzyme	Control Diet		Antioxidant-Supplemented Diet	
	Sham surgery <i>n</i> = 11	I-R surgery <i>n</i> = 15	Sham surgery <i>n</i> = 10	I-R surgery <i>n</i> = 14
Glutathione peroxidase, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$	0.36 \pm 0.02	0.43 \pm 0.04	0.49 \pm 0.04 [†]	0.58 \pm 0.03 [†]
Total superoxide dismutase, U/mg protein	81.6 \pm 3.5	74.5 \pm 3.5*	78.8 \pm 3.1	80.2 \pm 2.8 [†]
Copper-zinc superoxide dismutase, U/mg protein	36.9 \pm 2.2	42.3 \pm 4.4	36.2 \pm 2.0	36.8 \pm 2.9
Manganese superoxide dismutase, U/mg protein	44.6 \pm 2.9	32.2 \pm 2.2*	42.6 \pm 2.6	43.4 \pm 2.3 [†]
Catalase, U/mg protein	1.50 \pm 0.07	1.68 \pm 0.12	1.57 \pm 0.07	1.64 \pm 0.06

Values are group means \pm SE; *n*, sample size. I-R, ischemia-reperfusion. *Different from sham surgery with same diet ($P < 0.05$); [†]different from control diet group with the same surgical treatment ($P < 0.05$).

insult. Furthermore, *in vitro* experiments demonstrated that this dietary antioxidant combination protected the myocardium against lipid peroxidation induced by five different radical-generating systems. Collectively, these data indicate that dietary supplementation with VE and α -LA protects the senescent heart against lipid peroxidation by scavenging a variety of ROS and that this protection is associated with improved cardiac performance during reperfusion after myocardial ischemia.

Antioxidant supplementation improves cardiac performance during reperfusion. An important observation in this study was that dietary supplementation with VE and α -LA improved cardiac performance during myocardial reperfusion after ischemia. Both peak arterial pressure and the rate-pressure product were significantly higher during reperfusion in ANTIOX animals compared with CON rats. Upon reoxygenation of hypoxic heart tissue, cardiac performance generally remains depressed for several days. The cause of this reperfusion-induced cardiac dysfunction is believed to be due, at least in part, to an increased production of ROS during reoxygenation of the hypoxic tissue (3, 11). ROS have been shown to participate in numerous degenerative cellular processes, such as membrane lipid peroxidation (13). For example, damage to the sarcoplasmic reticulum membrane may result in dissipation of the important transsarcolemmal calcium gradient and an increase in the cytosolic calcium concentration (10). This could result in a sustained contractile activation resulting in hypercontracture, distortion of the myocardial cytoskeleton, and diminished contractile performance (10). The present study suggests that this chain of deteriorating events might be attenuated by preloading the myocardium with nutritional antioxidants that decrease the ROS-induced cellular injury.

Again, this is the first study to demonstrate *in vivo* myocardial protection from I-R injury by use of the combination of VE and α -LA. Our findings are in agreement with previous reports regarding the myocardial benefits of using a combination of VE and α -LA by use of an *in vitro* model (15, 16). In contrast, the current data are contradictory to recent findings from our laboratory in which young female rats were used (5). Using the same antioxidant regimen and I-R protocol as the current experiments, we observed that the antioxidant supplementation resulted in less I-R-induced myocardial lipid peroxidation; nonetheless, this dietary intervention did not improve myocardial contractile performance during reperfusion. At least two possibilities can explain these discrepancies. First, compared with young adult animals, senescent animals have a reduced antioxidant capacity (32). Therefore, it is possible that dietary antioxidant supplementation may be more beneficial in protecting against ROS-mediated cardiac injury in older animals compared with young animals.

A second possibility is that gender or strain differences may exist in the responses of the animals to I-R. The previous study from our lab used female Sprague-Dawley rats, whereas the present study used male

Fischer-344 rats. In this regard, it has been reported that enzymatic antioxidant defenses, specifically SOD and CAT, vary with gender and strain in rats (28). Furthermore, the female gonadotropic hormone estrogen and its metabolites have been shown to have antioxidant capability (33). Therefore, collectively, it appears that age, gender, and strain differences could have contributed to our divergent findings.

Antioxidant supplementation reduces I-R-induced myocardial lipid peroxidation. Lipid peroxidation is one of the most damaging processes that occurs in the myocardium during I-R. The oxidative modification of lipids results in alterations to the fluidity and permeability of membrane peroxidation (13). It has been reported that lipid peroxidation of the sarcoplasmic reticulum, which occurs during I-R, may result in altered calcium handling and subsequent contractile dysfunction (29). In the present study, two measures of lipid peroxidation were used to determine whether dietary supplementation reduced left ventricular lipid damage after I-R. Compared with sham animals, I-R increased myocardial lipid peroxidation in CON diet animals. In contrast, compared with sham, I-R did not increase myocardial MDA and CHE in ANTIOX animals. These findings are in agreement with studies that have reported decreased *in vitro* I-R-induced lipid peroxidation with prefeeding of VE alone (20) or in combination with α -LA (5). However, the current study is the first investigation to support the notion that this dietary antioxidant regimen reduces I-R-induced lipid peroxidation in the senescent heart under *in vivo* physiological conditions.

In vitro oxidative challenges. To determine the mechanism by which dietary supplementation with VE and α -LA provides protection against I-R-induced myocardial lipid peroxidation, *in vitro* experiments were conducted that oxidatively challenged heart homogenates from both CON and ANTIOX animals that were not exposed to I-R. Because of the antioxidant properties of VE and α -LA, we hypothesized that heart homogenates from ANTIOX animals would quench superoxide radicals, hydroxyl radicals, hydrogen peroxide, and peroxy radicals generated *in vitro*. Our data support this postulate. Indeed, after exposure to five ROS generating systems, lower levels of MDA were detected in heart homogenates from ANTIOX animals compared with CON diet animals. Therefore, the finding that the nutritional combination of the two antioxidants results in a myocardium that is protected against all five ROS generating systems supports the notion that combining aqueous and lipid phase antioxidants is therapeutically beneficial (15).

Effects of antioxidant supplementation and I-R on myocardial antioxidant enzymes. The effect of the antioxidant supplementation on activities of myocardial enzymes important in protection against oxidative stress was also investigated. Two interesting observations warrant discussion. First, activity of GPX increased in ANTIOX animals in both the sham and I-R groups. This finding agrees with previous unpublished data from our laboratory and from other investigators

(23, 30). The explanation for the increased GPX activity with VE supplementation could be the increase in cellular selenium concentrations that accompanies VE supplementation (30). Selenium is a cofactor for GPX, and higher cellular levels of VE have been shown to stimulate increased expression of the enzyme.

The second interesting finding was a significantly greater total SOD activity in the myocardium of ANTIOX animals exposed to I-R surgery compared with CON diet I-R surgery animals. Also, myocardial total SOD activity was lower in the CON diet I-R surgery animals compared with CON diet sham surgery animals. These differences in myocardial total SOD activity were due to a higher activity of the manganese isoform. This finding is in agreement with two recent studies reporting that vitamin E feeding upregulated MnSOD protein expression and expression in aortic segments of rats (23) and in rats fed a high-fructose diet (7). The mechanisms to explain these findings are unknown and warrant further investigation.

The decreased activity of the mitochondrial isoform of SOD in aged animals exposed to myocardial I-R may be the result of an accumulation of hydrogen peroxide. Indeed, it has been demonstrated that hydrogen peroxide is a negative allosteric modifier of MnSOD activity (4). In the ANTIOX animals, the presence of additional antioxidants such as α -LA, DHLA, or their metabolites, all of which have been shown to quench hydrogen peroxide, may have protected MnSOD from I-R-induced downregulation.

Limitations of the model. The male Fischer-344 rat was chosen as the experimental subject because 1) the nature of these invasive experiments precludes the use of human subjects, 2) this strain of rat is highly inbred and does not display large interanimal variations in coronary collateral circulation, and 3) the rat is a widely accepted model for the study of dietary antioxidant interventions and an accepted model for aging research (1).

At the completion of the feeding period, our animals were ~21.5 mo old. The experimental rationale for investigating this age group of animals is as follows. Our objective in these experiments was to investigate the effects of antioxidant supplementation on I-R-induced cardiac injury in old animals that were not "old age survivors" (i.e., older than the median life span of this strain). Because the median life span of male F-344 rats ranges from 22 to 29 mo, 21.5-mo-old animals are approaching senescence but are not old age survivors.

The decision to use 10,000 IU of VE and 1.65 g of α -LA per kg diet was based on previous work demonstrating that these dosages of VE (15, 16) and α -LA (26) have provided beneficial results. Also, this dietary dosage of VE results in serum tocopherol levels in the rat of 3 mg/dl, which are similar to values obtained in humans when large doses (600 IU/day) of α -tocopherol are consumed (22).

The surgical procedure used in these experiments has been used successfully in our laboratory (5, 27) and

has been reported to result in both myocardial ischemia and reperfusion (2). However, it is possible that this type of experimental surgery could result in interanimal differences in the magnitude of either ischemia or reperfusion. Nonetheless, we believe that these differences are clinically relevant and better reflect the types of I-R insults that occur in humans.

The decision not to include additional experimental groups that consumed only VE or α -LA was based on the data from Haramaki et al. (16). These investigators reported that it was the combination of the two antioxidants that provided protection and that fewer benefits were observed when they were used individually. Furthermore, in the current experiments, we chose not to include a parallel group of younger animals. This decision was based on previous data from our laboratory indicating that dietary supplementation with VE and α -LA did not improve myocardial performance during I-R (5). Therefore, in the absence of a parallel group of younger animals in our current study, it is not possible to conclude that aging results in a compromised myocardial antioxidant capacity.

Summary and conclusions. These experiments examined the effects of dietary supplementation with VE and α -LA on myocardial physiological and biochemical responses during in vivo I-R in the aged rat. The dietary regimen yielded a significant increase in myocardial VE content after 14 wk of supplementation. Dietary supplementation with these antioxidants improved cardiac performance during reperfusion after ischemia. This improvement in recovery from ischemia appears to be due to the supplemented hearts being protected from a wide range of ROS, resulting in reduced lipid peroxidation. These results indicate that this combination of antioxidant supplements provides protection against myocardial I-R injury in old animals.

Perspectives

This is the first experiment to examine the effects of combining VE and α -LE supplementation on cardiac performance and biochemistry during in vivo myocardial I-R in aged rats. The results confirm previous work using the in vitro model to support the use of this antioxidant combination as a therapeutic defense against I-R oxidative damage. A major new finding of this study was that the antioxidant supplements improved cardiac performance during postischemia-reperfusion. In addition, our data support the hypothesis that the dietary combination of VE and α -LE reduces myocardial lipid peroxidation resulting from an in vivo I-R insult. Furthermore, we performed in vitro experiments indicating that this dietary antioxidant combination protected the myocardium against lipid peroxidation induced by five different radical-generating systems. In summary, this study provides new and important findings relative to the therapeutic role of VE and α -LE in providing protection in the senescent heart during reperfusion after ischemia.

This study was supported by research grants from the American Heart Association-Florida Affiliate (S. K. Powers) and the Finnish Ministry of Education (C. K. Sen).

REFERENCES

- Ames SR. Age, parity, and vitamin A supplementation and the vitamin E requirement of female rats. *Am J Clin Nutr* 27: 1017–1025, 1974.
- Bernier M, Hearse DJ, and Manning AS. Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with anti-free radical interventions and a free radical-generating system in the isolated perfused rat heart. *Circ Res* 58: 331–340, 1986.
- Bolli R, Jeroudi MO, Patel BS, DuBose CM, Lai EK, Roberts R, and McCay PB. Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proc Natl Acad Sci USA* 86: 4695–4699, 1989.
- Bray RC, Cockle SA, Fielden EM, Roberts PB, Rotilio G, and Calabrese L. Reduction and inactivation of superoxide dismutase by hydrogen peroxide. *Biochem J* 139: 43–48, 1974.
- Coombes JS, Powers SK, Demirel H, Jessup J, Vincent HK, Hamilton KL, Naito H, Shanely RA, Sen CK, Packer L, and Ji LL. Effect of combined supplementation with vitamin E and alpha-lipoic acid on myocardial performance during in vivo ischemia-reperfusion. *Acta Physiol Scand* 169: 261–269, 2000.
- Cort WM, Vicente TS, Waysek EH, and Williams BD. Vitamin E content of feedstuffs determined by high-performance liquid chromatographic fluorescence. *J Agric Food Chem* 31: 1330–1333, 1983.
- Faure P, Rossini E, Lafond JL, Richard MJ, Favier A, and Halimi S. Vitamin E improves the free radical defense system potential and insulin sensitivity of rats fed high fructose diets. *J Nutr* 127: 103–107, 1997.
- Flohé L and Günzler WA. Assays of glutathione peroxidase. *Methods Enzymol* 105: 114–121, 1984.
- Fridovich I. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J Biol Chem* 245: 4053–4057, 1970.
- Garcia-Dorado D, Théroux P, Duran JM, Solares J, Alonso J, Sanz E, Munoz R, Elizaga J, Botas J, and Fernandez-Avilés F. Selective inhibition of the contractile apparatus. A new approach to modification of infarct size, infarct composition, and infarct geometry during coronary artery occlusion and reperfusion. *Circulation* 85: 1160–1174, 1992.
- Garlick PB, Davies MJ, Hearse DJ, and Slater TF. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ Res* 61: 757–760, 1987.
- Gonzalez Flecha B, Llesuy S, and Boveris A. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle. *Free Radical Biol Med* 10: 93–100, 1991.
- Halliwell B and Chirico S. Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr* 57: 715S–725S, 1993.
- Han D, Handelman G, Marcocci L, Sen CK, Roy S, Kobuchi H, Tritschler HJ, Flohé L, and Packer L. Lipoic acid increases de novo synthesis of cellular glutathione by improving cystine utilization. *Biofactors* 6: 321–338, 1997.
- Haramaki N, Assadnazari H, Zimmer G, Schepkin V, and Packer L. The influence of vitamin E and dihydrolipoic acid on cardiac energy and glutathione status under hypoxia-reoxygenation. *Biochem Mol Biol Int* 37: 591–597, 1995.
- Haramaki N, Packer L, Assadnazari H, and Zimmer G. Cardiac recovery during postischemic reperfusion is improved by combination of vitamin E with dihydrolipoic acid. *Biochem Biophys Res Commun* 196: 1101–1107, 1993.
- Hermes-Lima M, Willmore WG, and Storey KB. Quantification of lipid peroxidation in tissue extracts based on Fe(III) xylenol orange complex formation. *Free Radical Biol Med* 19: 271–280, 1995.
- Ji LL, Dillon D, and Wu E. Alteration of antioxidant enzymes with aging in rat skeletal muscle and liver. *Am J Physiol Regulatory Integrative Comp Physiol* 258: R918–R923, 1990.
- Kagan VE, Shvedova A, Serbinova E, Khan S, Swanson C, Powell R, and Packer L. Dihydrolipoic acid—a universal antioxidant both in the membrane and in the aqueous phase. Reduction of peroxy, ascorbyl and chromanoxyl radicals. *Biochem Pharmacol* 44: 1637–1649, 1992.
- Klein HH, Pich S, Lindert S, Nebendahl K, Niedmann P, and Kreuzer H. Combined treatment with vitamins E and C in experimental myocardial infarction in pigs. *Am Heart J* 118: 667–673, 1989.
- Lesnefsky EJ, Gallo DS, Ye J, Whittingham TS, and Lust WD. Aging increases ischemia-reperfusion injury in the isolated, buffer-perfused heart. *J Lab Clin Med* 124: 843–851, 1994.
- Machlin LJ and Gabriel E. Kinetics of tissue alpha-tocopherol uptake and depletion following administration of high levels of vitamin E. *Ann NY Acad Sci* 393: 48–60, 1982.
- Mehta J, Li D, and Mehta JL. Vitamins C and E prolong time to arterial thrombosis in rats. *J Nutr* 129: 109–112, 1999.
- Mihara M and Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 86: 271–278, 1978.
- Oyanagui Y. Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal Biochem* 142: 290–296, 1984.
- Packer L, Roy S, and Sen CK. Alpha-lipoic acid: a metabolic antioxidant and potential redox modulator of transcription. *Adv Pharmacol* 38: 79–101, 1997.
- Powers SK, Demirel HA, Vincent HK, Coombes JS, Naito H, Hamilton KL, Shanely RA, and Jessup J. Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *Am J Physiol Regulatory Integrative Comp Physiol* 275: R1468–R1477, 1998.
- Rikans LE, Moore DR, and Snowden CD. Sex-dependent differences in the effects of aging on antioxidant defense mechanisms of rat liver. *Biochim Biophys Acta* 1074: 195–200, 1991.
- Romaschin AD, Wilson GJ, Thomas U, Feitler DA, Tumiati L, and Mickle DA. Subcellular distribution of peroxidized lipids in myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol* 259: H116–H123, 1990.
- Scott DL, Kelleher J, and Losowsky MS. The effect of dietary selenium and vitamin E on glutathione peroxidase and glutathione in the rat. *Biochem Soc Trans* 4: 295–296, 1976.
- Sen CK, Roy S, Khanna S, and Packer L. Determination of oxidized and reduced lipoic acid using high-performance liquid chromatography and coulometric detection. *Methods Enzymol* 299: 239–246, 1999.
- Sohal RS, Sohal BH, and Brunk UT. Relationship between antioxidant defenses and longevity in different mammalian species. *Mech Ageing Dev* 53: 217–227, 1990.
- Tang M, Abplanalp W, Ayres S, and Subbiah MT. Superior and distinct antioxidant effects of selected estrogen metabolites on lipid peroxidation. *Metabolism* 45: 411–414, 1996.