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THIOCTIC ACID PROTECTS AGAINST ISCHEMIA-REPERFUSION INJURY IN THE ISOLATED PERFUSED LANGENDORFF HEART

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Antioxidant properties of thioctic and dihydrolipoic acid have been demonstrated in membranes and low density lipoproteins (LDL) *in vitro*. *In vivo* studies with dietary supplementation of thioctic acid to rats showed that it can also protect tissues against oxidative damage. Presumably, this action is due to a thioctic acid dihydrolipoic acid (TA/DHLA) coupled antioxidant mechanism, which enhances the activity of other antioxidants (i.e. ascorbate, α-tocopherol) by regenerating them from their radical form.

In the present study, thioctic acid proved to protect against ischemia/reperfusion injury to Langendorff perfused hearts. Hearts isolated from rats fed thioctic acid and subjected to ischemia exhibited better mechanical recovery (left ventricular developed pressure) after reperfusion and lower lactate dehydrogenase leakage. Thioctic acid supplementation also decreased the appearance of fluorescent lipid peroxidation products after ischemia/reperfusion, lowered the rate of 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN) induced lipid peroxidation in heart homogenates, and prevented the loss of α-tocopherol. The total sulfhydryl group content in thioctic acid fed animals was higher and the decrease due to ischemia-reperfusion was not as marked in this group as observed in the control. These results show that dietary supplementation with thioctic acid *in vivo* provides protection against ischemia/reperfusion injury in the Langendorff heart model.

KEY WORDS: Thioctic acid, lipoic acid, dihydrolipoic acid, heart ischemia and reperfusion, lipid peroxidation, α-tocopherol.

INTRODUCTION

Injury of the ischemic heart is believed to be further exacerbated during the re-oxygenation period, a phenomenon called "reperfusion injury". During reperfusion the amount of salvageable myocardium is reduced and necrosis increased due to the destruction of the cardiomyocytes.[1] Calcium overload,[2] mitochondrial dysfunction,[3] arachidonic acid metabolites,[4] and defective myocardial lipid metabolism[5] also contribute to the reperfusion injury. The importance of protecting the reperfused heart has already been recognized clinically. Introduction of new treatments for coronary thrombisis, such as intracoronary artery delivery of thrombolytic medications and precutaneous coronary angioplasty, provide support for this model.

In studies of heart ischemia/reperfusion involving anthracycline (a redox cycling/oxyradical generating drug) or iron (a catalyst of Fenton reaction), it has been shown that reactive oxygen species (ROS) contribute to myocardial injury.[6,7] Direct evidence of radical generation in heart reperfusion has come from spin trapping experiments,

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using spin traps such as 5,5-dimethylpyrroline-N-oxide (DMPO).[8,9] These findings have prompted interest in testing antioxidants and radical scavengers as a means to prevent heart injury during reperfusion.[10]

Thioctic (lipoic) acid is a co-factor for alpha-keto-acid dehydrogenases and is covalently bound as lipoamide to some enzymes in animals.[11] Its reduced form, dihydrolipoic acid (DHLA), is a potent sulfhydryl reductant: redox potential of the TA/DHLA couple is -0.32 V.[12] In aqueous systems, both thioctic acid and dihydrolipoic acid have exhibited antioxidant actions. In particular, dihydrolipoic acid proved to be effective scavenger of superoxide and hydroxyl radicals.[13] Thioctic acid was demonstrated to react with singlet oxygen and oxidizing and reducing radicals.[14,15]

Thioctic acid was not effective in direct elimination of peroxyl radicals, inhibition of lipid peroxidation, and recycling of α-tocopherol in microsomal and lipsomal membranes.[16] In contrast, dihydrolipoic acid was shown to interact directly with water and lipid soluble radicals and enhance the activity of other antioxidants, such as ascorbate, gluthathione, and α-tocopherol.[17–20] In studies with low density lipoproteins (LDL) dihydrolipoic acid, but not thioctic acid, synergistically enhanced ascorbate dependent reduction of α-tocopherol chromanoxyl radicals in LDL.[21]

Thioctic acid has been used in treating diseases in which its levels were found to be low, including liver cirrhosis, diabetes mellitus, atherosclerosis, and polyneuritis. It was suggested that reactive oxygen species (ROS) and free-radical mediated oxidation of lipids and proteins in cell membranes is a common pathway in the etiology of these pathologies.[22]

In an earlier study, we followed the antioxidant activity of thioctic acid by feeding rats a thioctic acid supplemented diet and testing the susceptibility of various tissues to lipid peroxidation.[23] Our results showed that with increasing period of thioctic acid supplementation heart, liver, brain, and skin were progressively more protected against *in vitro* lipid peroxidation induced by an azo-initiator of peroxyl radicals, 2,2′-azobis-(2,4-dimethyl-valeronitrile) (AMVN).

In this research, the protective effects of dietary supplementation of thioctic acid against ischemia/reperfusion injury were investigated in the Langendorff isolated heart model.

MATERIALS AND METHODS

Animals and Perfusion Experiments

Male Sprague-Dawley rats (180–220 g), from Bantin and Kingman (Fremont, California, USA), were housed at 25°C with 12 : 12 hour light–dark cycles with access to diet and water *ad libitum*. Animals were not fasted before heart excision and all perfusion experiments were done within a two day period in a same time window.

The perfusion apparatus constructed essentially as described[24] was temperature regulated at 37°C. This was a retrograde, non-circulating aortic perfusion system (Langendorff, O., 1895) with modified Krebs-bicarbonate buffer (1.2 mM $MgCl_2 \times 6H_2O$, 5.9 mM KCl, 1.7 mM d-Glucose, 25 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 117 mM NaCl, pH 7.4) gassed continuously with 95% O_2 and 5% CO_2.

Before excision of the heart, animals were anesthetized with diethylether and then intravenously injected with 400 units of heparin. Afterward, the aorta was immediately cannulated and the left ventricle perfusion initiated for 10 min (perfusion period). During the perfusion period, a cannula was inserted into the left ventricle through left

atrium and connected to the pressure transducer. Hearts were then subjected to a 40 min period of global ischemia by clamping the aortic cannula. The clamp was reopened to end ischemia and initiate 20 min of reperfusion period. After reperfusion all hearts were frozen and stored in liquid nitrogen. Control hearts were subject to a 60 min period of perfusion without ischemia and stored frozen.

Diet

Thioctic acid supplemented animals had 1.65 g thioctic acid/kg added to the control diet, which was made according to established methods.[25] Animals were fed for a period of 6 weeks.

Left Ventricular Developed Pressure (LVDP)

Developed pressure and heart rate were obtained using a Gould/Statham P23 pressure transducer connected to a Gilson Duograph. LVDP was calculated as the product of developed pressure and heart rate [(mmHg/beat) × (beats/s)]. Percent recovery was calculated by comparing the LVDP before and after ischemia.

Lipid Peroxidation

Heart homogenates were extracted with a solution of methanol and chloroform (1 : 1) and the content of endogenous fluorescent peroxidation products in the lipid phase (such as crosslinked products of dialdehyde with phospholipids) were measured spectrofluorimetrically using a Perkin-Elmer MPF 552 Spectrofluorometer at $\lambda_{excitation} = 365$ nm and $\lambda_{emission} = 425$ nm.[26]

Lipid peroxidation products reacting with thiobarbituric acid (TBARS) were measured spectrophotometrically as described.[27] In this study, the lipid peroxidation was induced in heart homogenates by the azo-initiator, 2,2′-azobis-2,4-dimethylvaleronitrile (AMVN) at 40°C in a medium containing 5 mM AMVN, 1 mg/ml protein in 0.1 M sodium potassium phosphate buffer (pH 7.4).

Carbony Assay

Hearts (150–250 mg pieces) were minced and placed in a 60 mm cell culture plate, containing 3 ml of 0.1 M phosphate buffer (pH 7.4), and a protease inhibitor cocktail with 0.1% digitonin, for 15 min. The resulting fluid was collected and centrifuged at a top speed in a clinical, table-top centrifuge for 5 min to sediment the solid debris. The resulting supernatant was collected and analyzed for carbonyl content by following spectrophotometrically the conversion of 2,4-dinitrophenylhydrazine to 2,4-dinitrophenylhydrazone.[28]

Enzyme Assays

The activity of lipoamide dehydrogenase for reducing thioctic acid to dihydrolipoic acid was assayed spectrophotometrically.[29,30] Essentially, the enzyme activity was determined by measuring the absorbance ($\lambda = 412$ nm) of the reaction product from 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and dihydrolipoic acid. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.4 mM NADH, 0.1 mM NAD,

TABLE I
Effect of dietary supplementation with thioctic acid on recovery of left ventricular pressure (LVDP) in isolated perfused rat hearts after 40 min of global ischemia

	LVDP (mm Hg)	
	Before ischemia	After ischemia and reperfusion
Control	69 ± 12	23 ± 11
Thioctic acid supplemented	71 ± 10*	47 ± 9**

Note. Mean values ± SD are given; $n = 6$. Student's t-test was done to determine the significance of difference in mean between Control and Thioctic Acid Groups.* Before ischemia P (vs. Control) < 0.2. **After ischemia and reperfusion P (vs. Control) < 0.001.

0.1 mM thioctic acid, 0.5 mM DTNB, and 1.65 U/ml lipoamide dehydrogenase from bovine heart (EC 1.8.1.4, # L-2002 from Sigma).

Lactate dehydrogenase (LDH) activity in the heart effluent fractions was measured using a commercial kit (Sigma Diagnostic LDH EC 1.1.1.27 UV optimized test) spectrophotometrically at $\lambda = 340$ nm.

Levels of Antioxidants

Consumption of endogenous α-tocopherol was monitored by an HPLC method using in-line electrochemical and Ultraviolet spectrophotometric detectors. Tocopherol was extracted and measured as previously described.[31] An internal tocopherol standard was included in all samples.

Estimation of Total Sulfhydryl Groups

The amount of protein and non-protein bound reduced thiol groups in heart homogenates were estimated spectrophotometrically with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTN B) according to the procedure described by Sedlak *et al.*[32]

Reagents

Heparin was obtained from the Upjohn Company (Kalamazoo, Michigan, USA) and thioctic acid was a gift from Asta-Medica (Frankfurt, Germany). All other chemicals, enzymes, and reagents were acquired from the Sigma Chemical Company (Saint Louis, Missouri, USA).

RESULTS

Left Ventricular Developed Pressure (LVDP)

The pre-ischemic LVDP in hearts obtained from control diet or thioctic acid diet fed rats (69 ± 12 and 71 ± 10 mmHg, respectively) were not significantly different ($P = 0.18$).

After 40 min of global ischemia and 20 min reperfusion, difference in the mean mechanical recovery of the two diet groups was significant ($P < 0.001$). The mean recovery of thioctic acid summplemented group was 68% (47 ± 9 mmHg) as compared to the 34% (23 ± 11 mmHg) recovery in hearts obtained from animals fed the control diet (Table 1).

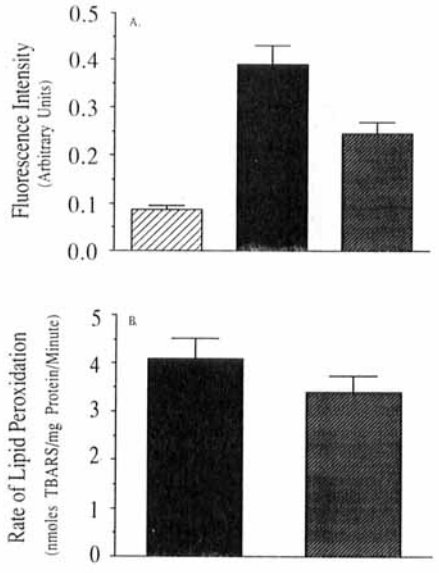

FIGURE 1 Assessment of thioctic acid dietary supplementation on lipid peroxidation in rat hearts homogenates after 10 min perfusion, 40 min ischemia, and 20 min reperfusion period ($n = 6$ each group). ▨ 60 min baseline value, ■ control diet, ▨ thioctic acid. A. Content of endogenous fluorescence lipid peroxidation products. B. Susceptibility of heart homogenates to *in vitro* lipid peroxidation induced by AMVN over 30 min period at 40°C.

Inhibition of Lipid Peroxidation

When hearts were subjected to ischemia and reperfusion, the content of endogenous lipid peroxidation products increased 4.5 fold in hearts obtained from animals on control diet. Thioctic acid supplementation partially prevented accumulation of endogenous lipid peroxidation products since the fluorescence increased only 2.8 fold (Figure 1A).

Supplementation with thioctic acid increased the resistance of the heart homogenates to *in vitro* AMVN-induced lipid peroxidation followed for 30 mins. The rate of lipid peroxidation in hearts obtained from thiotic acid fed animals and subjected to ischemia-reperfusion was 17.5% lower as compared to control heart preparations from animals on control diet (Figure 1B).

Protein Carbonyls Content

No significant difference was observed in the protein carbonyl content in the hearts obtained from either the control or thioctic acid fed animals after the 60 min period of perfusion (Figure 2). In control diet hearts, ischemia and reperfusion resulted in a significant increase in the carbonyl protein content (from 2.0 ± 0.17 to 3.85 ± 0.40 nmole/mg protein). This increase was partially prevented in the hearts obtained from animals fed thioctic acid diet (2.65 ± 0.2 nmolel/mg protein).

Enzyme Assays

Thioctic acid used in our ischemia-reperfusion experiments could be readily and

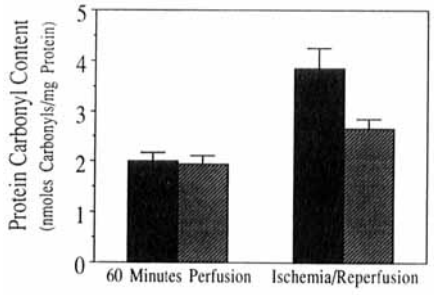

FIGURE 2 Comparison of protein carbonyl content in control and thioctic acid diet group hearts subjected to either 60 min perfusion period or 10 min perfusion, 40 min ischemia, and 20 min reperfusion periods. In each group $n = 6$. ■ control diet, ▨ thioctic acid diet.

quantitatively converted into dihydrolipoic acid by incubation with lipoamide dehydrogenase in the presence of NADH. When 0.1 mM of thioctic acid was incubated with lipoamide dehydrogenase we found that 0.05 mM of dihydrolipoic acid was formed after 20 min incubation as measured by the reaction with DTNB.

During the 10 min perfusion period, only low LDH activity was detected in the effluent (40.4 ± 12.0 IU/L) for the both the control and thioctic acid-supplemented hearts. Immediately after ischemia a substantial increase (4–4.5 fold) in activity was detected in the coronary effluent of hearts from control diet group, whereas, only a 2.3 fold increase was detected in the thioctic acid supplemented group (Figure 3). In either control or thioctic acid diet groups, hearts subjected only to a 60 min period of perfusion did not exhibit elevation in the LDH activity (data not shown).

Consumption of α-Tocopherol

The concentration of endogenous α-tocopherol in control rat heart homogenates was 0.4–0.6 nmole/mg protein. After 6 weeks of dietary supplementation with thioctic acid, its concentration did not change. The data on the consumption of α-tocopherol

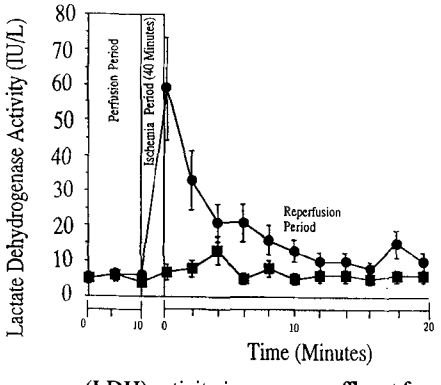

FIGURE 3 Lactate dehydrogenase (LDH) activity in coronary effluent from isolated Langendorff hearts obtained from either control or thioctic acid fed rats and subjected to 10 min perfusion, 40 min ischemia, and 20 min reperfusion periods. Value given are Mean ± SD for $n = 6$ in each group. -●- control diet, -■- thioctic acid diet.

TABLE II
Effect of dietary supplementation with thioctic acid on the content of α-tocopherol and total sulfhydryl groups in isolated Lagendorff perfused rat hearts

	Control	Thioctic acid supplemented
	α-Tocopherol (nmoles/mg protein)	
60 min perfusion	0.40 ± 0.09	0.45 ± 0.08**
10 min perfusion 40 min ischemia 20 min reperfusion	Undetectable	0.15 ± 0.02
	Total Sulfhydryl Groups (nmoles/mgprotein)	
60 min perfusion	21.30 ± 2.3	26.02 ± 3.0*
10 min perfusion 40 min ischemia 20 min reperfusion	12.91 ± 1.6	21.77 ± 2.4

Note: Mean values ± SD are given; $n = 4$. Significance in the difference of means was determined by the Student's t-test. *Difference in mean total sulfhydryl groups after 60 min of perfusion in Control vs. Thioctic Acid supplemented group was significant ($P < 0.006$). **Differences in mean Vitamin E in same experiment was not significant between two groups ($P < 0.5$).

in the course of ischemia-reperfusion are shown in Table II. There was no significant differences in the contents of α-tocopherol after 60 min period of perfusion in heart homogenates obtained from rats fed control or thioctic acid supplemented diet. However, 40 min of ischemia followed by 20 min of reperfusion caused a pronounced decrease in α-tocopherol levels. Only 23–30% of α-tocopherol remained in the thioctic acid supplemented group and less than 4% in normal diet group.

Estimation of Total Sulfhydryl Groups

The total sulfhydryl groups in hearts was 22% higher in thioctic acid group as compared with control diet group. These values were similar in hearts subjected only to 60 min perfusion. Ischemia and reperfusion caused a 40% decrease in the total sulfhydryl in control diet hearts and only 17% in thioctic acid hearts as compared to 60 min perfusion hearts (Table II).

DISCUSSION

In our laboratory we have shown that α-tocopherol can act as a membrane free radical harvesting center and can be enzymatically or non-enzymatically regenerated into its active form. Thiols like dihydrolipoic acid and glutathione synergistically enhance α-tocopherol regeneration and the well known synergistic effects of these physiologically important antioxidants (reductants) with α-tocopherol are mediated by their ability to transfer electrons, necessary for recycling chromanoxyl radicals in membranes, to vitamin C (ascorbic acid).[17] *In vitro* studies with membranes and lipoproteins have demonstrated that dihydrolipoic acid displays greater antioxidant action compared with thioctic acid. Dihydrolipoic acid, but not thioctic acid, scavenges peroxyl radicals and enhances the activity of antioxidants (i.e. ascorbate, α-tocopherol) by regenerating them from their radical form.[17,20,21] Thus knowledge of the biochemistry of the

α-tocopherol cycle led us to investigate whether ischemia reperfusion damage to the heart could be prevented by dietary supplementation of thioctic acid.

Thioctic acid has been shown to be effective against ischemia reperfusion injury. Its administration in the perfusate significantly reduced ventricular fibrillation and prolonged the control sinus rhythm time in isolated hearts subjected to ischemia.[33] It was also efficient in reducing reoxygenation injury in isolated working rat hearts and reperfusion damage in isolated ischemic rat hindlimbs.[34] In our experiments, we subjected rat hearts to 10 min perfusion, 40 min ischemia, and 20 min reperfusion periods using the Langendorff isolated heart model. This particular protocol has proven to provide a reliable means of inducing injury which can be used to test recovery by antioxidant intervention.[7,24] We showed that thioctic acid dietary supplementation for 6 weeks resulted in improved recovery of hearts after ischemia reperfusion. Thioctic acid hearts displayed better mechanical properties (developed pressure) and lower lactate dehydrogenase activity in heart effluent after ischemia period.

However, the mechanisms of these protection are not well understood. Evidence exists to suggest that the observed protection is due to the antioxidant action of thioctic acid.[33,35] The intraperitoneal administration of thioctic acid (100 mg/kg i.p.) reduces the generation of peroxyl radicals in rat intestine after occlusion by ligation.[36] If the reduction of exogenously introduced thioctic acid to dihydrolipoic acid occurs in the organism then dihydrolipoic/thioctic acid couple may act as a protector against oxidative damage. This type of conversion was demonstrated in liver and hepatocytes.[37]

The reduction of thioctic acid to dihydrolipoic acid is catalyzed by lipoamide dehydrogenase. It is known that the activity of lipoamide dehydrogenase exists in myocardial cells.[38,39] Our *in vitro* results showed that thioctic acid, used in these experiments, is readily converted to dihydrolipoic acid by lipoamide dehydrogenase in the presence of reduced pyridine dinucleotides. This suggests that such a conversion may also be feasible *in vivo*. Reduction of thioctic acid to dihydrolipoic acid within cardiomyocytes may therefore increase their resistance to oxidative damage during reperfusion, as demonstrated in this study. We assessed the protection of intracellular targets against oxidation after thioctic acid supplementation. Specifically, we measured the amounts of protein carbonyl content, which isindicative of protein damage and determined lipid damage by measuring the fluorescence peroxidation products (such as aminoiminopropene Schiff base).[40,41] Supplementation with thioctic acid significantly decreased both protein and lipid damage during reperfusion.

These data support the hypothesis that thioctic acid protection against ischemia reperfusion injury is part due to its antioxidant activity. Thioctic acid supplementation also prevented the loss of α-tocopherol after ischemia-reperfusion (α-tocopherol in the hearts subjected to ischemia and reperfusion was significantly higher in thioctic acid fed animals than control group). This higher content of α-tocopherol may be due to the interaction of its radical with ascorbate and glutathione, which subsequently are recycled by dihydrolipoic acid. Furthermore, the sulfhydryl group content in thioctic acid fed animals was higher and the decrease due to ischemia-reperfusion was not as marked in this group as observed in the control. This suggests that thioctic acid may also protect SH groups against oxidation.

Our experiments have shown that thioctic acid is an effective therapeutic agent against heart ischemia reperfusion injury and this protection may be due to its antioxidant action.

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