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Original Contribution

VITAMIN E AND OXIDATIVE STRESS IN THE HEART OF THE CARDIOMYOPATHIC SYRIAN HAMSTER

REN-KE LI, MICHAEL J. SOLE, DONALD A. G. MICKLE, JOSHUA SCHIMMER, and DAVID GOLDSTEIN
The Centre For Cardiovascular Research, The Toronto Hospital, University of Toronto, Toronto, Canada

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Abstract—Myocardial deterioration is relentlessly progressive in almost all patients who develop overt symptoms. Many dilated cardiomyopathies are associated with a marked increase in cardiac sympathetic tone which may be toxic to myocytes. Microvascular spasm, leading to diffuse, focal reperfusion injury, also appears to be an important mechanism of cardiomyocyte loss in many models of dilated cardiomyopathy. Free radicals may mediate both catecholamine-induced damage and reperfusion injury. We hypothesized that myocardial antioxidant reserve may be significantly reduced in dilated cardiomyopathy and that α-tocopheryl acetate may be of benefit. The enzymes superoxide dismutase, catalase and glutathione peroxidase were measured in the myocardial tissue of control and cardiomyopathic hamsters in early (25–50 days) and late (275–320 days) stages of the cardiomyopathy. In another study, myocardial glutathione peroxidase activity and protein oxidation was measured in control and late stage cardiomyopathic hamsters receiving α-tocopheryl (70 mg/kg/day) or vehicle for 1 month. There were no significant differences in glutathione peroxidase activity between control and cardiomyopathic hamsters in the early stage of the cardiomyopathy. Superoxide dismutase and catalase activities did not change with aging; however, glutathione peroxidase decreased over 30% , α-tocopherol was reduced by approximately 50% and protein oxidation increased more than 2-fold in the hearts of late stage cardiomyopathic hamsters. α-Tocopheryl acetate administration restored α-tocopherol levels, glutathione peroxidase activity and protein oxidation to normal. We conclude that the decompensating heart has significantly limited antioxidant reserve and that this reserve is sensitive to the intake of antioxidant supplements. © 1998 Elsevier Science Inc.

Keywords—Cardiomyopathy, Vitamin E, Heart failure, Alpha-tocopherol, Oxidative stress, Free radical, Glutathione peroxidase

INTRODUCTION

In spite of recent advances in the therapy of heart failure, it is clear that myocardial decompensation is relentlessly progressive in almost all patients who develop overt symptoms. There is growing recognition of the importance of reactive oxygen intermediates in the pathogenesis of heart failure. In a guinea pig model of heart failure, induced by aortic stenosis, a decrease in superoxide dismutase and glutathione peroxidase activities was associated with increased lipid peroxidation[1]; α-tocopherol therapy was of considerable benefit to both structure and function.[2] Hill and Singal[3] reported an antioxidant deficit and an increase in oxidative stress in the failing hearts of rats following myocardial infarction; that is α-tocopherol and the antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase were decreased whereas lipid peroxidation was increased. Finally, an increase in myocardial oxidative stress has also been reported in canine mitral insufficiency, a paradigm of volume overload-induced left ventricular dysfunction. α-Tocopherol treatment both increased antioxidant reserve and improved cardiac contractility.[4] All of these models represent forms of acute surgically induced cardiac disease. There have

Address correspondence to: Dr. Michael J. Sole, The Toronto Hospital, EN 13-208, 200 Elizabeth Street, Toronto, Ontario, Canada M5G 2C4.

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been no studies of oxidative stress in natural forms of chronic heart disease such as dilated cardiomyopathy.

The cardiomyopathic Syrian hamster is a unique, reproducible, genetically transmitted (autosomal recessive), animal model of cardiac hypertrophy, dilatation and failure.[5] Hamsters younger than 40–50 days of age display little gross or light microscopic evidence of heart disease; foci of cardiac necrosis, which calcify, first appear between 40 and 70 days. The heart gradually hypertrophies then dilates and the animals ultimately die of congestive failure or sudden death at 9 to 11 months of age (approximately 40% of the life expectancy of a normal hamster).[5] Free radicals are known to be increased at the onset (40–90 days) of hamster heart disease[6,7]; however, there has been no evaluation of oxidative stress and its possible importance or prevention during the stages of cardiac decompensation and failure.

In this study we examine oxidative stress, cardiac antioxidant reserve and the effects of supplemental α-tocopheryl acetate in the cardiomyopathic hamster. We show that myocardial protein oxidation is increased and that cardiac antioxidant defences consisting of α-tocopherol stores and glutathione peroxidase activity are significantly decreased during the late stages of the cardiomyopathy. All of these alterations may be restored by the administration of α-tocopheryl acetate.

MATERIALS AND METHODS

1. EXPERIMENTAL PROTOCOLS

Young (25–30 day old) and adult (300 day-old) male cardiomyopathic Syrian hamsters (CHF 147, Canadian Hybrid Farms, Nova Scotia) and disease-free golden hamsters (CHF GS, Canadian Hybrid Farms) were used in these experiments.

Young and old cardiomyopathic and normal hamster hearts were harvested for analysis of their antioxidant enzyme activities and α-tocopherol levels. Normal and cardiomyopathic adult hamsters were separated into two groups: α-tocopherol and control. The α-tocopherol hamsters were injected subcutaneously daily for 4 weeks with all-rac-α-tocopheryl acetate (70 mg/kg) dissolved in carrier (20 μl ethanol in saline); the control animals were treated identically but with an equal volume of carrier alone. The hamsters were sacrificed by decapitation and the hearts were isolated and immediately frozen in liquid nitrogen for glutathione peroxidase, α-tocopherol and protein oxidation determinations.

The protocols were approved by The Toronto Hospital Animal Experimentation Committee.

2. MEASUREMENT OF ANTIOXIDANT ACTIVITIES

Superoxide dismutase: copper/zinc-superoxide dismutase was extracted and analysed at 415 nm as described by L'Abbe and Fisher[8] using a Cobas-Fara analyser (Hoffmann-La Roche Inc., Nutley, N.J.). Frozen myocardium (0.2 gram) was homogenized in 0.5 ml of 0.05M potassium phosphate buffer containing 0.1mM ethylenediamine tetraacetic acid (EDTA, pH 7.8). The enzyme was extracted by the addition of 0.35 ml of chloroform/ethanol (3:5) solution. After centrifugation for 5 min at 12,000 *g*, the supernatant was carefully collected and used for enzyme assay.

Catalase

Catalase was analysed by the method of Cohen *et al.*[9] Frozen myocardium (0.2 gram) was homogenized in 0.5 ml of ice cold potassium phosphate buffer (0.05M, pH 7.0). The homogenate was centrifuged at 12,000 *g* for 12 min. The supernatant was collected and 0.01 ml of absolute ethanol was added and incubated at 4°C for 30 min before 10% Triton X-100 was added to a final concentration of 1%. The samples were then used to determine catalase activity.

Glutathione peroxidase

Glutathione peroxidase was extracted and assessed using the method reported by Doroshow *et al.*[10] Frozen myocardium (0.2 gram) was homogenized in 0.5 ml of a sucrose-EDTA solution (0.25M sucrose, 0.001M EDTA). The homogenate was centrifuged at 12,000 *g* for 60 min at 4°CC and the supernatant was assayed on a Cobas-Fara analyser at 340 nm.

3. MEASUREMENT OF MYOCARDIAL α-TOCOPHEROL

The method of Ingold *et al.*[11] was used to measure α-tocopherol levels in hamster myocardial tissue. The sample in 1 ml of water containing an internal standard (2,2,5,7,8-pentamethylhydroxychroman) was homogenized, transferred into a methanol-rinsed glass tube, and incubated on ice for 2 min. After the addition of 2 ml ethanol and 3 ml heptane to the homogenate and vortex mixing, the samples were centrifuged at 560 *g* for 10 min at 4°C to produce a biphasic mixture. The aqueous phase was recovered for protein analysis. The organic phase containing α-tocopherol was dried under nitrogen and the residue reconstituted in 100 μl of hexane. A 25 μl sample was injected into a Waters HPLC with a Supelco LC-NH_2 25 cm $\times$ 4.6 mm i.d. column (Supelco Inc., Bellefonte, PA) and resolved with hexane:isopropanol (97:3, v:v). Standard solutions of α-

tocopherol (Sigma Chemical Co. catalogue # T 3251) containing internal standard were used to generate a standard curve for the calculation of the α-tocopherol levels.

4. MEASUREMENT OF MYOCARDIAL OXIDIZED PROTEIN

Myocardial specimens (0.4 gram) were homogenized in 0.05M Hepes buffer (137.0 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4 and 0.6 mM Mg SO_4, pH 7.4) in the presence of protease inhibitors (0.5 μg/ml leupeptin, 0.7 μl/ml pepstatin, 40 μl/ml phenymethylsulfonic acid and 0.5 μl/ml aprotinin) and EDTA to a final concentration of 1.1 mM. One hundred μl of 20% streptomycin (in 50 mM Hepes buffer, pH 7.2) was added to the homogenate and the sample was incubated on ice for 15 min. The homogenate was then centrifuged at 11,000 *g* for 10 min and the supernatant was collected for the determination of the carbonyl content and measurement of protein concentration.

Each sample was divided into equal aliquots and an equal volume of trichloroacetic acid (20% TCA) was added into each fraction. To one of these fractions was added 500 μl of derivatization solution (10 mM 2,4-dinitrophenyhydrazine (DNPH) in 6 M guanidine hydrochloride-0.5 M potassium phosphate, pH 2.5) and to the other was added 500 μl of the same solution without DNPH (as a control). The samples were incubated at room temperature for 30 min with vortexing every 5 min. After the addition of 500 μl of 20% TCA and mixing for 5 min, the samples were centrifuged for 10 min at 11,000 *g* and the pellets were washed three times with 1 ml ethanol-ethyl acetate (1:1). The protein pellets were dissolved in 0.8 ml of 6 M guanidine solution and the absorbance of the solution was read at 370 nm. The oxidized protein concentration was calculated based on the μmoles of DNPH bound to protein (the reference absorbance of 22,000 M^{-1} cM^{-1} for aliphatic hydrazones was used).

The protein concentration was measured by Bio-Rad protein assay using a Cobas-Fara analyser and calculated based on a protein standard curve (0.50, 0.75, 1.00, 1.25, 1.50 mg/ml BSA in Hepes buffer).

5. DATA ANALYSIS

All results were expressed as mean ± 1 SD. One-way analysis of variance was used to assess differences among groups of superoxide dismutase, catalase, glutathione peroxidase and α-tocopherol. If the F ratio was significant from the ANOVA, a Duncan's multiple-range *t*-test was employed to specify differences between the groups.

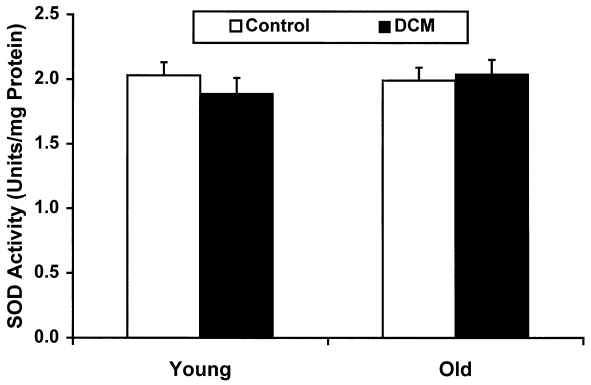

Fig. 1. Myocardial superoxide dismutase (SOD) activities of young (25–30 days old) and adult (300 days old), control (clear bar) and cardiomyopathic (dark bar) hamsters. Results are expressed as mean ± I SD. $n = 7$/group.

RESULTS

Superoxide dismutase (1.99 ± 0.10, 2.04 ± 0.11 U/mg protein, $n = 7, 7$) and catalase (10 ± 0.8, 10 ± 1.0 U/L) activities of the hearts of young and old cardiomyopathic hamsters were similar to those in normal hamsters (2.03± 0.10, 1.89 ± 0.12 U/mg protein, $n = 7, 7$; 10 ± 0.8, 10 ± 1.1 U/mg protein, $n = 7, 7$), respectively (Figs. 1 and 2). The disease and the age of the animals did not affect these activities. Although there was no difference in glutathione peroxidase activity between young control and cardiomyopathic hamsters (108 ± 4.2, 112 ± 3.2 U/mg protein ($n = 7,7$)), myocardial glutathione peroxidase activity in adult dilated cardiomyopathic hamsters (78 ± 2.4 U/mg protein, $n = 7$) was significantly lower ($p < 0.01$) than in control animals (102 ± 1.6 U/mg protein, $n = 7$) (Fig. 3). The disease, but not the age of the animals, affected myocardial glutathione peroxidase activity.

Adult cardiomyopathic animals exhibited a reduc-

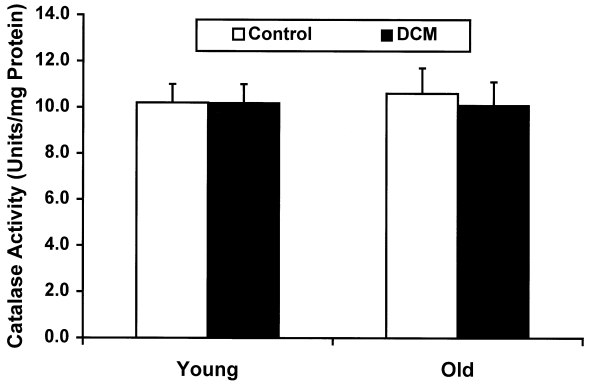

Fig. 2. Myocardial catalase activities of young (25-30 days old) and adult (300 days old) control (clear bar), and cardiomyopathic (dark bar) hamsters. Results are expressed as mean ± I SD. $n = 7$/group.

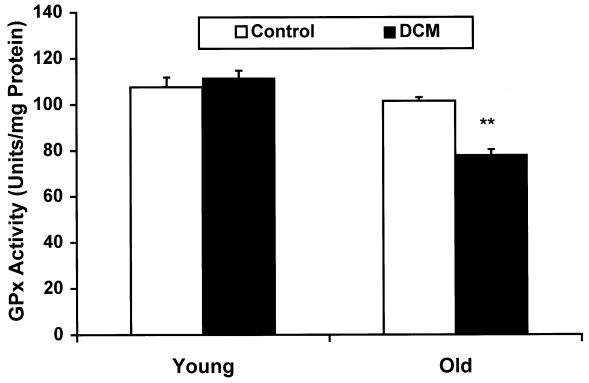

Fig. 3. Myocardial glutathione peroxidase (GPx) activities of young (25-30 days old) and adult (300 days old), control (clear bar) and cardiomyopathic (dark bar) hamsters. Results are expressed as mean ± I SD. $n = 7$/group. ** $p < 0.01$.

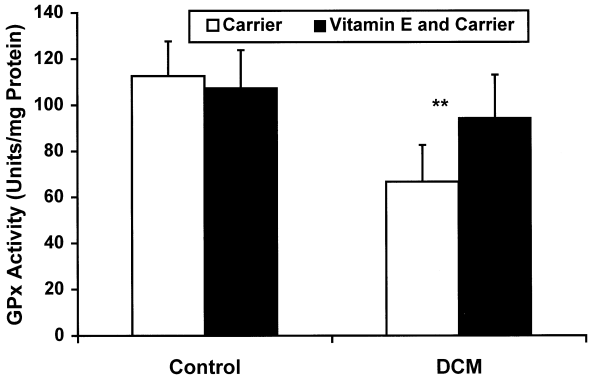

Fig. 5. Myocardial glutathione peroxidase in adult control and cardiomyopathic (DCM) adult hamsters injected subcutaneously daily for 4 weeks with α-tocopheryl acetate (70mg/kg) dissolved in carrier (dark bar) or with carrier alone (clear bar). $n = 6$/group. ** $p < 0.01$.

tion ($p < 0.01$) in myocardial α-tocopherol levels (0.35 ± 0.15 nmole/mg protein, $n = 6$) relative to control myocardial levels (0.55 +/ 0.12 nmoles/mg protein, $n = 6$)(Fig. 4). The administration of α-tocopheryl acetate to the dilated cardiomyopathic hamsters increased ($p < 0.05$) the myocardial level of α-tocopherol to 0.48 ± 0.08 nmoles/mg protein ($n = 6$) which was not significantly different from control levels. α-Tocopheryl acetate administration did not change the myocardial glutathione peroxidase activities of the control animals (Fig. 5). The myocardial glutathione peroxidase activities of the α-tocopherol-supplemented and nonsupplemented control animals were 113 ± 16 ($n = 6$) and 107 ± 17 U/mg protein, ($n = 6$), respectively. α-Tocopheryl acetate increased ($p < 0.01$) myocardial glutathione peroxidase activities from 66 ± 16 U/mg protein ($n = 6$) to 94 ± 19 U/mg protein ($n = 6$) in the cardiomyopathic animals. The glutathione peroxidase activities of the α-tocopheryl acetate-supplemented animals were not significantly different from the activities of the control animals.

The level of oxidized protein in the adult cardiomyopathic myocardium (1.5 ± 0.37 μmoles/mg protein, $n = 6$) was significantly higher ($p < 0.01$) than that of control hearts (0.75 ± 0.31 μmoles/mg protein, $n = 6$)(Fig. 6). α-Tocopheryl acetate restored cardiomyopathic myocardial protein oxidation levels to 0.91 ± 0.30 μmoles/mg protein ($n = 6$). This level was not significantly different from the myocardial oxidized protein levels in the α-tocopheryl acetate-treated (0.72 ± 0.5 μmoles/mg protein, $n = 6$) and the non-α-tocopheryl acetate-treated (0.75 ± 0.31 μmoles/mg protein, $n = 6$) control animals.

DISCUSSION

There is growing recognition of the importance of reactive oxygen intermediates in the pathogenesis of

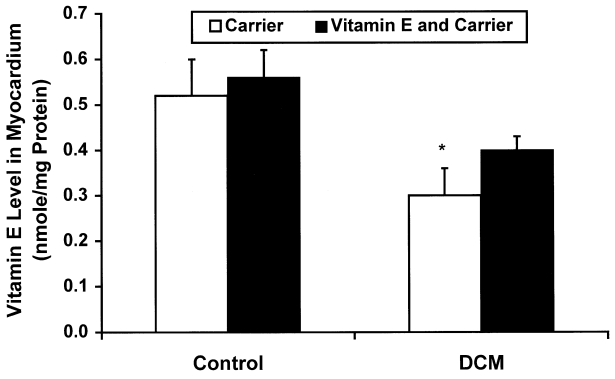

Fig. 4. Myocardial α-tocopherol levels in control and cardiomyopathic (DCM) adult hamsters injected subcutaneously daily for 4 weeks with α-tocopheryl acetate (70mg/kg) dissolved in carrier (dark bar) or with carrier alone (clear bar). $n = 6$/group. * $p < 0.05$.

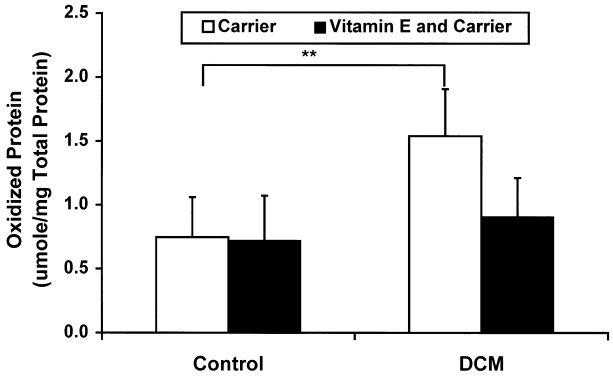

Fig. 6. Myocardial levels of oxidized proteins as measured by protein carbonyl content in control and cardiomyopathic (DCM) adult hamsters injected subcutaneously daily for 4 weeks with α-tocopheryl acetate (70mg/kg) dissolved in carrier (dark bar) or with carrier alone (clear bar). $n = 6$/group. ** $p < 0.01$.

atherosclerosis and myocardial ischemic injury. The present study adds to recent evidence that they also contribute to the deterioration of the failing myocardium.

Congestive heart failure in the hamster, as in humans, is associated with an increase in both circulating catecholamines and cardiac sympathetic tone.[12] Catecholamines are well-established causes of oxidative stress and cardiomyopathy.[13] Myocardial damage may occur through the excessive stimulation of intracellular metabolism and myocyte calcium overload,[14] auto-oxidation of catecholamines to toxic adrenochromes,[15,16] or through the production of hydrogen peroxide as a normal byproduct of tyrosine hydroxylation. α-Tocopherol deficiency exacerbates catecholamine (isoprenaline) cardiomyopathy whereas pretreatment with this vitamin is protective.

Oxygen free radicals are a product of reperfusion injury.[17] Catecholamines contribute to cardiac microvascular spasm which leads to diffuse, focal reperfusion injury and myocyte loss.[12] Factor[18] and ourselves[19] have shown this to be a critical pathway in the pathogenesis of hamster cardiomyopathy and of other paradigms of dilated cardiomyopathy such as hypertensive-diabetic cardiomyopathy, viral and Chagasic myocarditis, and following acute brain injury. Microvascular spasm in the acute head injury model occurs coincident with a massive elevation in plasma catecholamines[20] and conversely, hamster cardiomyopathy or the cardiomyopathy following murine viral myocarditis may be prevented by the administration of an α1-adrenergic antagonist[21] or the peripheral ganglionic blocker, chlorisondamine.[22]

A variety of other mechanisms such as cytokine stimulation,[23] nitric oxide generation[24] and mitochondrial DNA mutations,[25] although undoubtedly important contributors to myocardial free radical generation in heart failure, have not yet been explored in the hamster model.

The antioxidant status of adult cardiomyopathic hamsters was abnormal. Although superoxide dismutase and catalase activities were unaffected in the adult cardiomyopathic hamster myocardium, glutathione peroxidase activity and α-tocopherol levels were decreased. The major myocardial defense against oxyradical membrane injury is α-tocopherol and glutathione peroxidase[26,27]. α-Tocopherol reduces primary lipid peroxyl radicals to lipid peroxides. The α-tocopheroxyl radical is relatively stable and is readily reduced by ascorbic acid to regenerate α-tocopherol[26,27]. The resultant lipid peroxides are reduced by glutathione peroxidase and the formation of lipid alkoxyl or peroxyl radicals is prevented. Hydroperoxide reduction by glutathione peroxidase suppresses hydroperoxide-derived lipid peroxidation and prevents α-tocopherol oxidation. Glutathione peroxidase is more susceptible to oxyradical inactivation compared to superoxide dismutase and catalase.[28] Lipid peroxyl radical scavenging by α-tocopherol prevents radical-mediated glutathione peroxidase inactivation. In addition to its free radical chain-breaking function, α-tocopherol appears to have a posttranscriptional stabilization effect on glutathione peroxidase mRNA,[29] but no effect on either superoxide dismutase or catalase activities. Thus, the decrease in myocardial glutathione peroxidase in the adult cardiomyopathic hamsters can be explained by decreased α-tocopherol levels.

The unsaturated phospholipids of the cardiomyocyte subcellular organelles are susceptible to oxyradical injury. The sarcolemma may be the most susceptible organelle being the most exposed to extracellular oxyradicals with ischemia and reperfusion injury.[31] Lipid peroxides can form lipid alkoxyl or peroxyl radicals which are capable of abstracting hydrogen from adjacent polyunsaturated lipid molecules to propagate the lipid peroxidation reaction. The alkoxyl and peroxyl radicals can also damage proteins. Although all amino-acid side-chains are susceptible to free radical injury, arginine, lysine, proline, histidine, cystine, methionine, tryptophan, tyrosine and phenylalanine are more susceptible.[31] Free radicals can even attack the α-carbon atom of the peptide bond. Oxidative modification of the proteins makes them more susceptible to degradation by proteases.[31–33] Secondary and tertiary structural changes also occur. With oxyradical-derived injury of the cardiomyocyte organelles, membrane permeability characteristics and the capacity to maintain transmembrane ionic gradients are compromised. Myocardial structure and function is ultimately disrupted.

The change in antioxidant status in the cardiomyopathic hamster was associated with increased levels of oxidized proteins as measured by protein carbonyl content. Oxidative cleavage of the peptide backbone and oxidation of the side chains of glutamyl, aspartyl, lysyl, arginyl, prolyl and theonyl generates carbonyl derivatives[31] which were measured. The increase in carbonyl content was reversed with an increase in myocardial α-tocopherol levels and is consistent with the hypothesis that membrane lipid peroxidation products oxidize nearby proteins. Since the physiological mechanism of removing oxidized protein is rapid and through proteolysis, the increase in oxidized proteins suggests that the oxyradical injury is severe and affects not only the membrane associated proteins but also proteolytic enzymes and protein synthesis during the later stages of the cardiomyopathy.

Oxidative stress may contribute to the pathogenesis

of heart failure in several ways. Firstly, oxygen free radicals can profoundly affect calcium handling by the myocyte producing significant and irreversible myocardial dysfunction.[34,35] In addition, oxygen free radical products of neurohormones and related substances such as catecholamines (adrenochromes)[16] are directly toxic to cells. Finally, reactive oxygen species appear to be important for the induction of apoptosis[36] and hence engender the inexorable myocyte loss which culminates in myocardial failure. These observations suggest that antioxidant supplements, such as α-tocopherol may be of benefit in heart failure.

CONCLUSIONS

We found that myocardial protein oxidation, a marker of oxidative stress, was markedly increased during the late stages of hamster cardiomyopathy. Conversely, myocardial antioxidant defences, glutathione peroxidase activity and α-tocopherol stores, were significantly reduced. α-Tocopherol administration was able to completely restore these abnormalities. A generalization of these data suggest that congestive heart failure may significantly encroach on myocardial antioxidant reserve; as this reserve is sensitive to the intake of antioxidants such as α-tocopherol, such supplements should be further evaluated as part of the therapeutic strategy for heart failure.

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