Antioxidant vitamins prevent cardiomyocyte apoptosis produced by norepinephrine infusion in ferrets

Fuzhong Qin, Naomi K. Rounds, Weike Mao, Keisuke Kawai, Chang-seng Liang*

Department of Medicine, University of Rochester Medical Center, Cardiology Unit, Box 679, 601 Elmwood Avenue, Rochester, NY 14642, USA

Received 7 August 2000; accepted 18 April 2001

Abstract

Background: Norepinephrine (NE) induces apoptosis in cultured neonatal rat myocytes. To determine whether this change occurred in intact animals after chronic subhypertensive doses of NE, and whether the effect was mediated via oxidative stress produced by NE, we measured myocyte apoptosis and apoptotic gene proteins in ferrets receiving chronic NE with and without antioxidant vitamin treatment.

Methods: Ferrets were administered either subcutaneous NE or vehicle and simultaneously assigned to receive antioxidant vitamins (β-carotene, ascorbic acid and α-tocopherol) or vehicle for 4 weeks. Resting hemodynamics and plasma NE were measured at 4 weeks. Animals were then sacrificed for measuring cardiac myocyte size by electron microscopy, and oxidative stress by reduced to oxidized glutathione (GSH/GSSG) ratio and mitochondrial DNA 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG). Cardiomyocyte apoptosis was detected by both terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay and monoclonal antibody to single-stranded DNA (Mab) staining. Western blot analysis was used to measure the expression of the antiapoptotic protein Bcl-2 and apoptotic protein Bax.

Results: NE administration produced a 4-fold increase in plasma NE, but had no effect on resting heart rate, heart weight, arterial pressure, left ventricular systolic function or cardiac cell size. NE infusion decreased tissue GSH/GSSG ratio, and increased mtDNA 8-oxo-dG, and TUNEL- and Mab-positive apoptotic cells. These changes were associated with a 27% decrease in Bcl-2 protein, a 42% increase in Bax and a 57% reduction in the ratio of Bcl-2/Bax. All of the changes were prevented by co-administration of antioxidant vitamins. Conclusion: NE administration at a dose which produced no significant increase in blood pressure or myocyte hypertrophy caused cardiomyocyte apoptosis in intact animals. This effect was associated with an increase in oxidative stress, up-regulation of Bax protein and down-regulation of Bcl-2 protein. Antioxidant vitamins prevented the changes produced by NE. The findings suggest that NE-induced myocyte apoptosis is mediated by oxidative stress, and that antioxidant vitamins may be beneficial in heart failure in which cardiac NE release is increased. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Adrenergic (ant)agonists; Apoptosis; Free radicals; Heart failure

1. Introduction

Cardiac sympathetic stimulation occurs early in congestive heart failure [1]. Over a period of time as in a transgenic animal model overexpressing nerve growth factor in the heart [2,3], sympathetic stimulation of the heart may lead to cardiac hypertrophy, β-adrenergic substrain sensitivity, myocardial ischemia and ventricular dysfunction. Myocardial hemorrhage, necrosis and heart failure also have been shown to occur in animals after large doses of catecholamines [4,5]. These effects of catecholamines on the heart are thought to be a result of oxygen supply and demand mismatch, causing tissue ischemia and necrosis. However, at levels like those achieved in heart failure, NE does not produce prominent myocardial necrosis. Instead, NE may cause progressive cardiac muscle damage by programmed cell death (apoptosis) in cultured rat cardiomyocytes [6–8] via the formation of reactive oxygen species [9], but whether these changes occur in animals after chronic exposure to NE at clinically relevant concentrations is not known. Our laboratories have shown previously that chronic infusion of NE at doses achieving...
myocardial interstitial NE levels comparable to those observed in heart failure produces β-adrenoceptor down-regulation, β-adrenergic subsensitivity, and reduction of noradrenergic nerve terminal profiles [10]. In a recent study [11], we reported that the decrease of cardiac noradrenergic nerve terminal profiles produced by chronic NE was associated with an increase in tissue oxidized glutathione (GSSG) and a decrease in the ratio of reduced to oxidized glutathione (GSH/GSSG) in animals. The study further showed that the increase in oxidative stress by NE is pathophysiologically important because both the decrease in GSH/GSSG and reduction of noradrenergic nerve terminal function were prevented by co-administration of antioxidant vitamins (β-carotene, ascorbic acid, and α-tocopherol) or the free radical scavenger superoxide dismutase [11].

The purpose of the present study was to determine if chronic administration of NE produced cardiomyocyte apoptosis in intact animals, and whether the changes could be prevented by antioxidant vitamins as was the cardiac adrenergic nerve terminal function. The antioxidant effect of the vitamins was evident by the prevention of increase of oxidized glutathione and mitochondrial DNA (mtDNA) 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), a stable marker of oxidative DNA damage by reactive oxygen species [12]. Apoptosis was measured by two independent methods. The standard method using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) detects double-stranded DNA breakage [13], a finding occurring late in apoptosis, whereas the second method utilizing monoclonal antibody against single-stranded DNA (Mab) labeling is more sensitive and detects early apoptotic cells [13,14]. In addition, as apoptotic oncoproteins (Bcl-2 and Bax) [15] are implicated in the regulation of apoptosis in response to oxidative stress [16–18], we proposed to measure the protein expression of the apoptosis-related genes, and to determine whether these changes were prevented by antioxidant vitamins.

2. Methods

2.1. Animal model and experimental protocol

Adult ferrets weighing 1.5–2.1 kg were used in this study. The study was approved by the University of Rochester Committee on Animal Resources and conformed to the guiding principles approved by the Council of the American Physiological Society and the National Institutes of Health guide on the humane care and use of laboratory animals.

The animals were anesthetized with intramuscular ketamine (37.5 mg/kg) and xylazine (2 mg/kg) for placement of sustained release pellets (Innovative Research of America, Sarasota, FL) under a sterile technique. Each animal received two sets of pellets placed in individual subcutaneous pockets at the nape of neck. The first set of pellets was either NE (40 mg) or vehicle. The amount of NE was calculated to deliver at 1.33 mg/day for 30 days. The second set of pellets contained either antioxidant vitamin or three placebo pellets. The vitamin pellets contained 10 mg β-carotene, 100 mg ascorbic acid and 100 mg α-tocopherol, each calculated to be released over a 30-day period.

The study comprises two phases. In Phase I, we determined the effects of NE and antioxidant vitamins on cardiomyocyte apoptosis. Samples were taken from our prior experiments [11] in which the ferrets were divided randomly to four groups: (1) a vehicle and three placebo pellets (vehicle); (2) a vehicle and three vitamin pellets (antioxidants); (3) a NE pellet and three placebo pellets (NE+placebo); and (4) a NE pellet and three vitamin pellets (NE+antioxidants). Additional experiments were performed in Phase II to examine the effects of NE and antioxidant vitamins on cardiomyocyte size and tissue oxidative stress. Ferrets underwent the same procedures as in Phase I, and were divided into three experimental groups: (1) Vehicle; (2) NE, and NE+antioxidants. Oxidative stress was assessed by measuring myocardial GSH/GSSG ratio and mtDNA 8-oxo-dG. We also measured myocardial concentrations of α-tocopherol in Phase II animals.

2.2. Echocardiographic and hemodynamic measurements

Hemodynamic and echocardiographic studies were performed 4 weeks after pellet implantation as described in our recent study [11]. Arterial samples were obtained for measuring plasma NE using the radioenzymatic Cat-A-Kit assay system (Amersham, Arlington Heights, IL) [19]. A Toshiba model SSH-60 echocardiographic system (Toshiba America Medical System, Tustin, CA) was used to measure left ventricular end-diastolic dimension (EDD) and end-systolic dimension (ESD), using a 5-MHz transducer. Left ventricular fractional shortening was calculated as [(EDD−ESD)×100]/EDD.

After the hemodynamic study, the animal was given a lethal dose (>100 mg/kg) of intravenous sodium pentobarbital in Phase I. In Phase II, animals were killed with 10% KCl to arrest the heart in diastole for the morphometric studies. In both phases of the study, the heart was quickly removed after sacrifice, and the right and left ventricles were separated from the septum and rinsed in ice-cold oxygenated normal saline. Transmural myocardial samples from left ventricle were frozen in liquid nitrogen for the TUNEL assay, detection of early apoptosis by Mab and Western blot analyses for apoptosis-related gene products (Phase I). In Phase II, fresh myocardial samples were prepared for electron microscopy and glutathione content. The rest was frozen in liquid nitrogen for measuring mtDNA 8-oxo-dG and α-tocopherol.
2.3. TUNEL assay

The frozen sections were fixed in 4% methanol-free formaldehyde in phosphate-buffered saline (PBS). The sections were stained using the Apoptosis Detection System (Promega, Madison, WI), according to the manufacturer's instructions. Briefly, the sections were incubated with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP. For negative control, deoxynucleotidyl transferase was omitted. For positive control, the sections were first treated with 10 unit/ml of DNase I to induce apoptosis. To identify myocytes, sections were incubated with mouse monoclonal α-sarcomeric actin antibody (Sigma–Aldrich, St. Louis, MO) and TRITC-conjugated secondary anti-mouse IgG. Finally, to identify nonapoptotic and apoptotic myocyte nuclei, sections were stained with propidium iodide (Sigma–Aldrich). The samples were analyzed under a fluorescence microscope. Four sections randomly picked from each of four pieces were analyzed per animal. Myocyte nuclei were determined by random counting of 10 fields per section. The number of TUNEL-positive myocyte nuclei was scored per 10,000 cardiomyocytes.

2.4. Detection of apoptosis by monoclonal antibody to single-stranded DNA

Frozen tissue sections were fixed in 85% methanol in PBS. The sections were incubated with mouse anti-single-stranded DNA monoclonal antibody (Chemicon International Inc, Temecula, CA). The sections were incubated with biotin-conjugated anti-mouse IgM (Vector Laboratory, Burlingame, CA) and avidin and biotinylated horse-radish peroxidase macromolecular complex (Vector Laboratory), and stained with 3-amin-9-ethylcarbazole (Vector Laboratory) and hematoxylin (Vector Laboratory). Mab was omitted for negative control. For the positive control, the sections were first incubated with proteinase K (20 mg/ml) to produce apoptotic changes [20]. The samples were examined under light microscopy. The number of apoptotic nuclei was determined as described above.

2.5. Western blot for apoptotic and antiapoptotic proteins

Protein was extracted from the frozen myocardial tissue. Aliquots containing 40 μg of protein were loaded onto 12% SDS–polyacrylamide gel for electrophoresis. Equal loading of myocardial protein was confirmed by Coomassie blue staining and anti-β-actin antibody (Sigma–Aldrich) [21]. Mouse anti-Bax monoclonal antibody and mouse anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used for the detection of Bax and Bal-2. The Phototope-HRP Western Blot Detection Kit (New England Biolab, Beverly, MA) was used to visualize the bands. The autoradiograms were scanned by a GS-700 Imaging Densitometer and the bands were quantified using Quantity One Program (Bio-Rad Laboratories, Hercules, CA).

2.6. Electron microscopic morphometry

Fresh samples were taken from the subendocardial region of the anterior wall of the left ventricle and immediately fixed in 2% glutaraldehyde and 4% paraformaldehyde in PBS. Specimens were post-fixed in 1.0% osmium tetroxide and embedded in Epon epoxy resin. Blocks were sectioned and stained with toluidine blue for light microscopic evaluation. Ultrathin sections were stained with uranyl acetate and lead citrate for examination on a Hitachi 7100 transmission electron microscope. Images of longitudinal and cross-sectional fibers were digitized from an original electron microscopic magnification of 1000 and enlarged onto the computer video monitor with Flashpoint VGA 3.4 software (Integral Technologies, Inc, Indianapolis, IN), resulting in a final magnification of 8000. In each animal, 20 myocytes were measured for myocyte diameter and cross-sectional area, using Image Pro Plus software (Media Cybernetics, L. P., Silver Spring, MD). The cell size was measured by an individual who had no knowledge of the experiments or drug assignment.

2.7. Myocardial tissue glutathione and mtDNA 8-oxo-dG contents

Fresh ventricular myocardium was homogenized in three volumes of 1% picric acid, and the supernatant collected for measuring glutathione using a glutathione reductase-coupled enzymatic assay [22], on a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer (Perkin Elmer, Norwalk, CT). Total glutathione was calculated from a standard curve of purified glutathione, and GSSG was measured by masking GSH with 2-vinyl pyridine in the conversion reaction from 5,5-dithiobis-2-nitrobenzoic acid to 2-nitro-5-thiobenzoic acid. The ratio of GSH to GSSG was calculated.

To measure mtDNA 8-oxo-dG, we isolated mitochondria from the ventricular tissue [23], and extracted mtDNA using a QIAamp Blood kit per manufacturer’s instructions. MtDNA 8-oxo-dG was measured using HPLC and electrochemical detection [24]. Briefly, mtDNA was digested into deoxynucleosides by nuclease P1 and alkaline phosphatase. The deoxynucleoside mixture was filtered through a 0.22-μm Nylon filter and injected into a YMC Basic™S 3 μ 4.6×150 mm column (Waters Corporation, Milford, MA) in a BAS 480 HPLC system (Bioanalytical Systems, Inc., West Lafayette, IN), with a mobile phase of 5% methanol in 100 mM lithium acetate buffer (pH 5.2). Electrochemical detection of 8-oxo-dG and 2′-deoxyguanosine (dG) was performed on a Model 5200A Coulochem II detector equipped with a Model 5011 analytical cell and Model 5021 guard cell (ESA, Inc., Chelmsford, MA). The potential settings on the dual
coulometric detector were +400 mV for detector 1 and +800 mV for detector 2. Purified 8-oxo-dG (ESA Inc.) and dG (Sigma–Aldrich) were used for calibration.

2.8. Myocardial content of α-tocopherol

Muscle samples from the left ventricular free wall were homogenized in an extraction solution containing 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 5 mM MgCl₂, 0.1 mM EDTA and 200 mM SDS, and centrifuged at 1500 g for 5 min. The supernatant was treated with a mixture of ethanol and hexane. Tissue α-tocopherol was extracted and redissolved in methanol [25]. The amount of α-tocopherol was determined using the BAS 480 HPLC and ESA Coulochem II electrochemical detector. A reverse phase 5 μ. S100A 4.6×250 mm C18 column (Whatman, Inc., Clifton, NJ) was used, with a mobile phase of methanol, ethanol, and 2-propanol in a 13.4-mM lithium perchlorate buffer.

2.9. Statistical analysis

Results were presented as means±S.E. The data were analyzed with the RS/1 Research System (Bolt, Beranek and Newman Software Products, Cambridge, MA) and SYSTAT programs (SPSS, Inc., Chicago, IL). Standard parametric analysis of variance and multiple range tests were used to determine the statistical significance of differences among the groups or between two means. However, in experiments with ≤6 samples in each group, nonparametric Kruskal–Wallis analysis of variance and Mann–Whitney statistics were used instead. Values of P<0.05 was considered statistically significant.

3. Results

3.1. Phase I study

3.1.1. Resting hemodynamics

Animals received NE administration showed no overt symptoms or signs of cardiac failure. Table 1 shows body weight, left ventricular weight, heart rate, mean arterial pressure, and left ventricular dp/dt. There were no significant differences in any of the parameters among the groups. Plasma NE content was increased 4–5-fold in ferrets receiving NE compared to vehicle. Antioxidant vitamins had no effect on plasma NE concentration.

3.1.2. Apoptosis of cardiomyocytes by TUNEL assay

Fig. 1 shows the TUNEL staining in myocardial tissue. Specificity of the technique to detect DNA fragmentation was documented by positive labeling of nuclei after exposure of the tissue to DNase I (Fig. 1A, B). DNA fragmentation was not detected when terminal deoxynucleotidyl transferase was omitted in the enzymatic reaction (data not shown). Identification of apoptotic cardiomyocytes was confirmed by double labeling of cells in which the fragmented DNA and α-sarcomeric actin were co-localized in the same cell. TUNEL staining was rare in the interstitial cells, endothelial cells and the smooth muscle cells of the intramyocardial blood vessels. NE administration significantly increased the number of TUNEL-positive nuclei compared to the Vehicle group. Antioxidant vitamins had no effects in the Vehicle group, but prevented the NE-induced myocyte apoptosis in the NE-treated animals (Fig. 1C–H and Fig. 2A).

3.1.3. Apoptosis of cardiomyocyte by Mab to single-stranded DNA

Specificity of the immunohistochemical staining of single-stranded DNA by Mab in apoptotic cardiomyocytes was documented in tissue sections after exposure to proteinase K (Fig. 3A). No staining was detected when Mab was omitted (Fig. 3B). The cells positively labeled with the single stranded DNA Mab showed characteristic elongated and striated features of cardiomyocytes, which were easily distinguishable from non-myocytes under a light microscope at a high magnification (Fig. 4). The number of Mab-positive cells was greater than that detected by TUNEL staining in the same tissue (Fig. 2). NE administration caused an increase in Mab-positive nuclei

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Placebo</th>
<th>Antioxidants</th>
<th>NE Placebo</th>
<th>Antioxidants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of experiments</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>1.78±0.09</td>
<td>2.01±0.07</td>
<td>1.68±0.07</td>
<td>1.90±005</td>
</tr>
<tr>
<td>LV weight (g)</td>
<td>3.8±0.2</td>
<td>3.8±0.1</td>
<td>4.0±0.2</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>181±6</td>
<td>185±5</td>
<td>177±4</td>
<td>186±7</td>
</tr>
<tr>
<td>Mean aortic pressure (mmHg)</td>
<td>118±5</td>
<td>118±5</td>
<td>107±4</td>
<td>120±5</td>
</tr>
<tr>
<td>LV dp/dt (mmHg/s)</td>
<td>3007±109</td>
<td>2867±229</td>
<td>2939±166</td>
<td>3255±324</td>
</tr>
<tr>
<td>Plasma [NE] (ng/ml)</td>
<td>0.31±0.06</td>
<td>0.28±0.08</td>
<td>1.44±0.34*</td>
<td>1.23±0.27*</td>
</tr>
</tbody>
</table>

LV, left ventricular; NE, norepinephrine.

Values are means±S.E.

*P<0.05, vs. the Vehicle groups, as determined by Kruskal–Wallis analysis of variance and Mann–Whitney statistics.
Fig. 1. Demonstration of myocyte apoptosis in ferrets treated with NE. Apoptotic nuclei (arrow) are shown by green fluorescence in the left panels. The localization of nuclei is documented by propidium iodide staining (arrowhead) and peripheral distribution of α-sarcomeric actin antibody labeling of myocyte cytoplasm is also illustrated by red fluorescence in the right panels. [(A–H): magnification ×350]. See text for explanation.
3.2. Phase II study

3.2.1. Cardiac function and myocardial tissue α-tocopherol content

Table 2 summarizes body weight, left ventricular weight, heart rate, mean aortic pressure, left ventricular fractional shortening and myocardial α-tocopherol concentrations in the three experimental groups. NE administration did not cause significant changes in body weight, left ventricular weight, heart rate, aortic blood pressure, or left ventricular fractional shortening. Administration of antioxidant vitamins increased myocardial content of α-tocopherol, but had no effect on heart rate, blood pressure, left ventricular weight or left ventricular fractional shortening in the NE-treated animals.

3.2.2. Cardiomyocyte size

Electron microscopic examinations indicate that NE administration produced no structural changes of the myocytes or evidence of myocardial necrosis (data not shown). Nor did NE or antioxidant vitamins change cardiomyocyte diameter and cross-sectional area (Fig. 9).

3.2.3. Myocardial GSH/GSSG ratio and mtDNA 8-oxo-dG content

NE administration increased myocardial GSSG and mtDNA 8-oxo-dG concentrations. The values were normalized to GSH and mtDNA dG, respectively. Fig. 10 showed that NE increased oxidative stress in the heart as evidenced by the decrease in the GSH/GSSG ratio and increase in mtDNA 8-oxo-dG/dG ratio. The cardiac oxidative stress produced by NE was reduced by antioxidant vitamins which returned cardiac GSH/GSSG ratio and mtDNA 8-oxo-dG/dG ratio to the control values.

4. Discussion

In the present study, we showed that chronic NE administration induced cardiomyocyte apoptosis in ferrets. NE administration also decreased the expression of Bcl-2 protein and increased Bax protein. As a result, the ratio of Bcl-2 to Bax was markedly reduced. These changes were associated with increases in tissue GSSG and mtDNA 8-oxo-dG and prevented by administration of antioxidant vitamins. The results suggest that NE-induced myocyte apoptosis may be mediated by NE-exerted oxidative stress.

4.1. Antioxidant vitamins

Three commonly used antioxidant vitamins were chosen for the study. α-Tocopherol and β-carotene are lipid soluble, and are capable of inhibiting lipid peroxidation of the cell membrane. α-Tocopherol reacts with a variety of oxygen free radicals, including single oxygen, lipid peroxide products, and superoxide radical, to form a relatively
innocuous tocopherol radical, whereas β-carotene is an efficient ‘quencher’ of single oxygen [26]. Ascorbic acid is water soluble. It reacts with superoxide, hydroxyl radical, single oxygen and hydrochlorous acid [27], and potentiates the effects of α-tocopherol by regenerating α-tocopherol from its radical [28].

The doses of antioxidant vitamins we chose for the study are 3–10 times the current recommended dietary allowances for humans, and are within the human therapeutic ranges based on body weight [29]. The doses administered were sufficient to increase cardiac α-tocopherol by 70%, and abolish the NE-induced oxidative stress, as measured by tissue [GSH]/[GSSG] ratio and mtDNA 8-oxo-dG/dG ratio. The findings suggest that the doses of vitamins chosen for the study are clinically relevant and sufficient to produce desired antioxidant effects.

4.2. Detection of apoptosis by two different methods: Mab to single-stranded DNA and TUNEL labeling

The standard TUNEL labeling [30] has been widely regarded as a cellular apoptotic marker [31,32]. Intense
specific for apoptosis [13,20]. Mab-positive cells are present in early apoptotic cells [13,14,20], and absent in necrotic tissues. Compared to the TUNEL staining, significantly more Mab-positive myocytes were detected in our present study. Other studies have also shown that TUNEL staining underestimates the incidence of apoptosis [13,35]. Thus, the detection of single-stranded DNA by Mab is not only a more specific but also a more sensitive method than the TUNEL assay.

4.3. NE and apoptosis

NE induces myocyte apoptosis in cultured adult rat ventricular myocytes [6]. This effect is shared by isoproterenol [7], forskolin and 8-bromo-cAMP, and can be inhibited by either propranolol [6], a nonspecific β-receptor blocker, or atenolol [37], a β₁-selective adrenergic blocker. In contrast, β₂-receptor antagonists had no effect on the NE-induced myocyte apoptosis [37]. Furthermore, the catecholamine-induced apoptosis is attenuated by an inhibitor of protein kinase A [6,38]. The findings suggest that NE produces cardiac apoptosis via a β₁-adrenergic receptor-mediated cAMP-protein kinase dependent pathway which probably involves phosphorylation of L-type Ca²⁺ channels in the heart. Increase in intracellular free Ca²⁺ has been shown to affect mitochondrial membrane permeability and induce apoptosis [39].

Our present study extends the prior observations in cultured myocytes to intact animals. The plasma NE levels achieved in the animals after NE administration were similar to those occurred in rabbits after pacing induced cardiomyopathy [40], and patients with severe heart failure (NYHA Class III and IV) and the worst prognosis [41]. This dose of NE also has been shown to increase the cardiac interstitial NE concentration to levels comparable to those seen in pacing-induced cardiomyopathy [10]. However, the dose of NE we administered was not large enough to produce major changes in heart rate, blood pressure and myocardial systolic performance. Although the use of anesthesia could have affected the hemodynamic responses in our experiments, our findings are consistent with prior studies in conscious dogs that NE infusion does not produce hemodynamic effects unless plasma NE far exceeds 1 ng/ml [42]. Arterial pressure also did not increase significantly in dogs even when the plasma NE concentrations increased to as high as 4.3 ng/ml [10,43]. Furthermore, our present study shows that chronic NE produced no obvious damage on cardiac ultrastructure. Nor did it increase heart weight or cardiomyocyte size.

4.4. NE and oxidative stress

Catecholamines are known to produce oxidative metabolites in the heart. Adrenochrome [44] is a major oxidative metabolite of epinephrine. However, the amount of adrenochrome present in the tissue probably is too low to produce...
significant biological effects [5]. More recently, direct measurement of increased hydroxyl free radical generation by nonenzymatic auto-oxidation of NE has been observed in the heart after NE administration [45] and cardiac sympathetic nerve stimulation [46]. Our present study provides further evidence that NE produced oxidative stress, as judged by the reduction of cardiac GSH/GSSG ratio and increase in the mtDNA 8-oxo-dG/dG ratio, and that oxidative stress was effectively reduced by antioxidant vitamins.

4.5. Oxidative stress and apoptosis

Evidence has accumulated that oxidative stress induces programmed cell death. Direct exposure of cultured rat cardiomyocytes to H$_2$O$_2$ and O$_2^-$ has been shown to induce myocyte apoptosis [9,16,47]. Myocyte apoptosis also occurs after mechanical stretch via increased production of reactive oxygen species; and this change was reduced by a free radical scavenging nitric oxide donor [48]. Oxygen free radicals also have been implicated as a mediator for myocyte apoptosis induced by daunorubicin [49] and cytokine toxicity [50]. In addition, antioxidants such as probucol and pyrrolidine dithiocarbamate have been shown to reduce myocyte apoptosis in rat after a large myocardial infarction [51].

4.6. Signal pathway of NE-induced apoptosis

The Bcl-2 family contains several members of oncoproteins that can inhibit or promote apoptosis [52]. The net effect in tissue is probably determined by the relative expression of the proapoptotic Bax and the antiapoptotic Bcl-2 [53]. Bcl-2 protein is localized in the mitochondria, endoplasmic reticula, and nuclear membranes where most of the oxygen-free radicals are generated and where the free radicals exert their apoptotic effects. Bcl-2 probably acts to prevent apoptosis by scavenging oxygen derived free radicals inside the cells [17]. Bcl-2 gene products have been shown to prevent programmed cell death of ventricular myocytes [54].

In our present study, NE decreased Bcl-2 protein expression and increased Bax expression, leading to a marked reduction of the Bcl-2 to Bax ratio in favor of cell apoptosis. A decrease in the Bcl-2 to Bax ratio by NE also has been reported in cultured myocytes [37]. However, our study is the first to demonstrate that antioxidant vitamins reversed the ratio of Bcl-2 to Bax produced by NE. The changes were associated with reduction of TUNEL- and Mab-positive cardiomyocyte nuclei. Similarly, ascorbic acid and α-tocopherol have been shown to prevent lipopolysaccharide-induced apoptosis by modulation of Bcl-2 and Bax proteins [18]. Likewise, carvedilol, a β-receptor blocker with potent antioxidant properties, prevents epinephrine-induced apoptosis in human coronary artery endothelial cells [55]. The results suggest that free radicals are involved in the regulation of Bcl-2 and Bax protein expression.

The exact mechanism by which NE produces apoptosis is not known. NE may exert this effect via stress-activated protein kinase (SAPK)/c-Jun NH$_2$-terminal kinase (JNK) [56]. Studies have shown a close structural and functional correlation between Bcl-2 and JNK in neuronal apoptosis [57, and that overexpression of Bcl-2 suppresses JNK activity in PC12 cells [58]. The SAPK/JNK pathway is also activated by auto-oxidized dopamine induced apop-
Fig. 8. A representative blot was shown of Bax in the vehicle- and NE-treated animals with and without antioxidant vitamins. Uniform protein loading was evidenced by the β-actin Western blot.

tosis in cultured non-neuronal and neuronal cells [59,60]. This change was associated with a decrease in the Bcl-2 to Bax ratio [60]. There is evidence that activation of SAPK/JNK system occurs in myocyte apoptosis [61], and after β-adrenergic receptor stimulation [62], but the direct proof that NE produces myocyte apoptosis by activation of the SAPK/JNK system is lacking. Other apoptogenic pathways such as mitochondrial cytochrome C, and a number of caspases [16,35,53,63] may play a role as well.

4.7. Significance in chronic heart failure

Evidence has now accumulated that apoptosis may be an important mode of cell death in heart failure [35]. This change may be induced by sympathetic stimulation of the heart that is known to occur in heart failure. Plasma NE correlates with the severity of the left ventricular dysfunction [64] and the mortality [41]. Other factors in heart failure may also contribute to oxidative stress. We recently extended the antioxidant vitamin therapy to pacing-induced

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Placebo</th>
<th>NE Placebo</th>
<th>NE Antioxidants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of experiments</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>1.50±0.03</td>
<td>1.47±0.02</td>
<td>1.48±0.05</td>
</tr>
<tr>
<td>LV weight (g)</td>
<td>3.4±0.1</td>
<td>3.4±0.2</td>
<td>3.6±0.2</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>156±8</td>
<td>131±3</td>
<td>158±7</td>
</tr>
<tr>
<td>Mean aortic pressure (mmHg)</td>
<td>98±8</td>
<td>94±6</td>
<td>99±7</td>
</tr>
<tr>
<td>LV fractional shortening (%)</td>
<td>29±2</td>
<td>27±2</td>
<td>30±2</td>
</tr>
<tr>
<td>Myocardial α-tocopherol (μg/g)</td>
<td>3.7±0.7</td>
<td>4.2±0.4</td>
<td>7.0±0.7*†</td>
</tr>
</tbody>
</table>

LV, left ventricular.
* Values are means±S.E.
*P<0.05, vs. the Vehicle Placebo group, and †P<0.05, vs. the NE Placebo group, as determined by analysis of variance and multiple range test.
cardiomyopathy, and found that as in the present study antioxidant vitamins prevented the oxidative mtDNA damage produced by rapid cardiac pacing [65]. Antioxidant vitamins also reduced the cardiac systolic dysfunction in pacing-induced cardiomyopathy [66]. These findings suggest that antioxidant vitamins may exert an important therapeutic effect in the treatment of human congestive heart failure. The results support the concept that antioxidants (e.g., vitamin E) protect against a variety of oxidative stress and reduce the risk of heart disease [67], although the benefit of antioxidants in cardiovascular diseases remains divergent [68] and is a subject of a recent critical review [69].

4.8. Conclusion

NE administration produces oxidative stress in the heart and induces cardiomyocyte apoptosis. These changes are associated with up-regulation of Bax protein and down-regulation of Bcl-2 protein. Our results showed that concomitant administration of antioxidant vitamins reduced oxidative stress produced by NE, and prevented the NE-induced myocyte apoptosis, probably through the regulation of Bcl-2 and Bax proteins. Additional studies are needed to determine if antioxidant vitamins, when given after an insult has been established, will halt or reverse the damage produced by NE. Our findings further suggest that antioxidant therapy may be beneficial in congestive heart failure in which cardiac NE release is increased.

Acknowledgements

The study was supported in part by US Public Health Service grant HL-07229, American Heart Association grants, and Paul N. Yu Fellowship. The authors thank Amy Mohan, Janice Gerloff, and Robin Stuart Buttles for their expert technical assistance.

References


