Alterations by Norepinephrine of Cardiac Sympathetic Nerve Terminal Function and Myocardial β-Adrenergic Receptor Sensitivity in the Ferret Normalization by Antioxidant Vitamins

Chang-seng Liang, MD, PhD; Naomi Kenmotsu Rounds, MD; Erdan Dong, MD, PhD; Suzanne Y. Stevens, PhD; Junya Shite, MD; Fuzhong Qin, MD

Background—Chronic excessive norepinephrine (NE) causes cardiac sympathetic nerve terminal abnormalities, myocardial β-adrenergic receptor downregulation, and β-adrenergic subsensitivity. The present study was carried out to determine whether these changes could be prevented by antioxidants.

Methods and Results—Ferrets were administered either NE (1.33 mg/d) or vehicle by use of subcutaneous pellets for 4 weeks. Animals were simultaneously assigned to receive either antioxidant vitamins (β-carotene, ascorbic acid, and α-tocopherol) or placebo pellets. NE increased plasma NE 4- to 5-fold but had no effect on heart rate, heart weight, arterial pressure, or left ventricular systolic function. However, myocardial NE uptake activity and NE uptake-1 site density were reduced, as well as cardiac neuronal NE, tyrosine hydroxylase, and neuropeptide Y. In addition, there was a decrease in myocardial β-adrenergic receptor density with a selective decrease of the β1-receptor subtype, reduction of the high-affinity site for isoproterenol, decreased basal adenyl cyclase activity, and the adenyl cyclase responses to isoproterenol, Gpp(NH) p, and forskolin. All of these changes were prevented by antioxidant vitamins. The effects of NE on myocardial β-adrenergic receptor density, NE uptake-1 carrier site density, and neuronal NE were also prevented by superoxide dismutase or Trolox C.

Conclusions—The toxic effects of NE on the sympathetic nerve terminals are mediated via the formation of NE-derived oxygen free radicals. Preservation of the neuronal NE reuptake mechanism is functionally important, because the antioxidants also prevented myocardial β-adrenergic receptor downregulation and postreceptor abnormalities. Thus, antioxidant therapy may be beneficial in heart failure, in which cardiac NE release is increased. (Circulation. 2000;102:96-103.)

Key Words: norepinephrine ■ antioxidants ■ receptors, adrenergic, beta ■ heart failure

Congestive heart failure is associated with excessive sympathetic stimulation and increased circulating and interstitial cardiac norepinephrine (NE) concentrations. The increase in cardiac interstitial NE is probably caused by heightened cardiac sympathetic nerve activity and reduced neuronal uptake of NE. A decrease of cardiac neuronal uptake of NE also has been shown to occur after exogenous administration of NE. Because the changes of neuronal uptake are associated with reductions of cardiac catecholaminergic and tyrosine hydroxylase profiles, we suspect that NE exerts a direct toxic effect on cardiac sympathetic nerves. A direct demonstration of NE-induced sympathetic denervation has been shown in the lateral saphenous veins in dogs. It has been speculated that the sympathetic nerve endings probably are damaged by NE-derived free radicals. The toxic metabolites of NE probably are formed outside the neurons and taken up into the nerve terminals by the desipramine-sensitive NE transporter. Such oxidative products as adrenochrome have been shown to induce coronary spasm, arrhythmias, myocardial necrosis and ultrastructural damage, and ventricular dysfunction. In addition, tetrahydroisoquinolone compounds formed by condensation of epinephrine with acetaldehyde have been shown to cause chemical sympathectomy.

The purpose of this study was to further investigate whether oxidative products of NE were involved in cardiac...
sympathetic nerve terminal abnormalities by administration of antioxidant vitamins or superoxide dismutase (SOD). The integrity of the sympathetic nerve terminals was assessed by measuring myocardial tissue NE uptake activity, NE uptake-1 carrier site density, and the contents of 3 sympathetic neuronal markers: catecholamines, tyrosine hydroxylase, and neuropeptide Y. Furthermore, to determine the functional significance of the sympathetic nerve terminal changes, we measured myocardial β-adrenergic receptor density and post-receptor adenylyl cyclase function. Chronic NE has been shown to reduce myocardial β-adrenergic receptor density and uncouple the β-receptors. We speculate that improvement of myocardial NE uptake by the antioxidants would reduce cardiac interstitial NE concentration and prevent the agonist-induced downregulation of myocardial β-adrenergic receptors and β-adrenergic subsensitivity.

Methods

Study Protocols and Animal Preparations
Adult male ferrets weighing 1.4 to 2.1 kg were chosen for 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 for the Care and Use of Laboratory Animals. The study comprises 2 phases. In phase 1, we aimed to determine whether vitamins A, C, and E prevented the NE-induced abnormal...
ities on the sympathetic nerve terminals or myocardial \( \beta \)-adrenergic receptors. In phase 2, we investigated whether the effects of vitamins A, C, and E were mediated via inhibition of oxidative stress by administering polyethylene-conjugated recombinant human SOD (PEG-rhSOD; OXIS Health Products, Inc) and measurements of tissue reduced to oxidized glutathione ratio, a measure of total oxidative stress. Furthermore, because vitamin E could be an active ingredient of the vitamin mixture, we administered the vitamin E derivative 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox C; Sigma-Aldrich Co) to animals treated with either NE or vehicle.

To implant pellets, we anesthetized the animals with intramuscular ketamine (37.5 mg/kg) and xylazine (2 mg/kg). Under a sterile technique, each animal received 2 sets of sustained-release pellets (Innovative Research of America) placed subcutaneously at the nape of the neck. The first set of pellets contained either NE (40 mg) or vehicle. The amount of NE was calculated to deliver 1.33 mg/d for 30 days. In phase 1, the second set of pellets was either 3 antioxidant vitamin or 3 placebo pellets. The vitamin pellets contained either 10 mg \( \beta \)-carotene, 100 mg ascorbic acid, or 100 mg \( \alpha \)-tocopherol, each calculated to be released over a 30-day period. The dose and duration of NE administration were chosen from pilot experiments. The doses of antioxidant vitamins used in the study are 3 to 10 times the current recommended dietary allowances for humans and are within the human therapeutic ranges based on body weight.

In phase 2, ferrets underwent the same surgical procedure as in phase 1 and were randomly divided into 5 groups according to the NE and drug treatment: (1) vehicle and placebo pellets, (2) NE and placebo pellets, (3) NE and PEG-rhSOD (20 mg, 3100 U of SOD activity/mg), (4) vehicle and Trolox C (100 mg), and (5) NE and Trolox C. PEG-rhSOD is a recombinant human copper-zinc SOD coupled to polyethyleneglycol (1 to 5 strands) with average molecular weights of 5000 to 1 000 000. The high molecular weights confer long half-lives. Half-time for serum clearance after injection in mice or rats is \( \approx \) 36 hours and after injection in dogs is \( \approx \) 5 days.

Trolox C is a water-soluble vitamin E derivative devoid of the phytol side chain. The doses of PEG-rhSOD and Trolox C were chosen empirically on the basis of information available from acute or short-term studies.

Hemodynamic Measurements

Hemodynamic studies were performed in intact animals after anesthesia with intramuscular ketamine and xylazine 4 weeks after pellet implantation. The carotid arteries were cannulated for measuring aortic and left ventricular (LV) pressures with a Spectramed P23XL pressure transducer (Spectramed, Inc) and a 2F transducer-tipped model SPR-407 Millar catheter (Millar Instruments, Inc), respectively. The heart rate, mean aortic pressure, LV pressure, and the first derivative of LV pressure by electronic differentiation were recorded on a Brush model 480 multichannel recorder (Gould, Inc, Instruments Systems Division).

Resting hemodynamic measurements were obtained in animals \( \approx \) 1 hour after the placement of the Millar catheter. An arterial sample was obtained for measuring plasma NE with the radioenzymatic Cat-A-Kit assay system (Amersham). Hemodynamic measurements were obtained in triplicate and the averages used for the statistical analysis. Isoproterenol (0.4 \( \mu \)g/kg) was then administered as a single intravenous bolus, and peak LV dp/dt was obtained as a measure of myocardial \( \beta \)-adrenergic responsiveness.
Myocardial NE Uptake Activity and Noradrenergic Neurotransmitter Contents

After the hemodynamic studies, the animals were killed with sodium pentobarbital (>100 mg/kg). The heart was removed and rinsed in an ice-cold oxygenated normal saline. Muscle slices from a designated region of the LV free wall below the atrioventricular groove were taken for measuring tissue NE uptake activity6,7 and neuronal catecholaminergic histofluorescence using the sucrose–potassium phosphate–glyoxylic acid condensation method.6

In addition, in phase 1, fresh LV free wall tissue blocks were immersion-fixed for 5 to 7 days in a 1.8% paraformaldehyde and 7.5% picric acid solution (pH 7.4) at 4°C for immunocytochemical staining for tyrosine hydroxylase or neuropeptide Y. The tissue blocks were prepared as described previously6 and incubated with either an affinity-purified polyclonal rabbit anti-tyrosine hydroxylase antibody (1:250 dilution in 1% Triton X-100 buffer; Chemicon International, Inc) or an anti–neuropeptide Y antibody (1:4000 in 0.4% Triton-X 100 buffer; Incstar Corp).

All tissue block sections for NE histofluorescence and immunocytochemistry were photographed at ×30 magnification with 35-mm slide film. The neurotransmitter profiles were morphometrically analyzed and quantified as the number of profiles counted in a 0.00885-mm³ field. The results of 7 fields were averaged for each ventricle.

Ventricular Membrane Preparation for NE Uptake-1 Carrier Site Receptor, β-Adrenergic Receptors, and Adenylyl Cyclase Activity

Crude muscle membrane fractions were prepared by homogenization for measuring myocardial NE uptake-1 carrier site density by specific binding of [3 H]nisoxetine (New England Nuclear; specific activity 85 Ci/mmol),8 β-adrenergic receptor density by specific binding of [125 I]iodocyanopindolol (ICYP, New England Nuclear; specific activity 2200 Ci/mmol),28 β-adrenergic receptor subtypes identified by analysis of displacement curves with the highly β1-selective antagonist CGP20712A (CIBA-Geigy Pharmaceuticals),28 β2-adrenergic receptor agonist binding by isoproterenol competitive inhibition,15,29 and basal and stimulated adenylyl cyclase activity.30 The adenylyl cyclase activity was stimulated by isoproterenol (0.1 mmol/L in the presence of 0.1 mmol/L GTP), guanylylimidodiphosphate [Gpp(NH)p] (0.1 mmol/L), or forskolin (1 mmol/L). Pilot studies showed that isoproterenol, Gpp(NH)p, and forskolin produced dose-dependent increases of myocardial adenylyl cyclase activity in ferrets. The agonists produced a plateau adenylyl cyclase activity at the concentrations used in the study. The samples were assayed for cAMP levels by the competitive protein-binding technique using the cAMP assay system (Amersham Life Science). The protein content was determined by use of bichinchoninic protein assay reagents (Pierce) with BSA as a standard.

Myocardial Glutathione Contents

In phase 2, ventricular myocardium was taken for measuring reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations with 5,5-dithiobis-2-nitrobenzoic acid (Sigma-Aldrich Co) as a substrate in a glutathione reductase–coupled enzymatic assay.31 The rate of formation of 2-nitro-5-thiobenzoic acid, which is proportional to the amount of GSH, was measured at a wavelength of 412 nm on a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer. The ratio of reduced to oxidized glutathione ([GSH]/[GSSG]) was calculated.

The individuals responsible for biochemical, receptor, and adrenergic neuronal marker profile measurements had no knowledge of the group identity of the animals at the time of study.
results showed significant differences between treatments (NE versus vehicle), intervention (antioxidants versus placebo), and interaction between treatment and intervention. A value of $P<0.05$ was considered statistically significant.

**Results**

**Phase 1: Studies With Vitamins A, C, and E**

The ferrets were divided into 4 groups according to the pellets they received: (1) a vehicle and 3 placebo pellets ($n=12$); (2) a vehicle and 3 antioxidant vitamin pellets ($n=12$); (3) an NE pellet and 3 placebo pellets ($n=14$); and (4) an NE and 3 antioxidant vitamin pellets ($n=14$). Table 1 shows body weight, right ventricular and LV weights, heart rate, mean arterial pressure, and LV $dp/dt$. ANOVA showed no differences in any of the parameters among the groups except for a slight but significant difference in body weight between 2 groups of animals.

Isoproterenol administration increased heart rate and LV $dp/dt$ (Figure 1). However, the magnitude of changes was significantly reduced in animals treated with NE compared with vehicle. Antioxidant vitamins had no effects on the inotropic and chronotropic responses to isoproterenol in the vehicle-treated animals but enhanced the responses in NE-treated animals to levels similar to those seen in the vehicle-treated group.

Figure 2 shows that plasma NE was markedly elevated in ferrets receiving subcutaneous NE and that its levels were not affected by antioxidant vitamin treatment. In addition, NE administration reduced myocardial tissue NE uptake activity and NE uptake-1 carrier site density. These changes were attenuated by antioxidant vitamins. The dissociation constant of the nisoxetine binding was affected by neither NE nor antioxidants. The dissociation constant for ICYP among the groups. The curves were fitted to a 2-site model for measuring the fractions of high- and low-affinity receptor populations. The fraction of high-affinity $\beta$-adrenergic receptors was reduced by NE (Figure 7). Antioxidant treatment had no effects on the distribution of the high- and low-affinity sites in the vehicle-treated animals but prevented the reduction of fraction of high-affinity $\beta$-adrenergic receptors in NE-treated ferrets. There were no significant differences in the dissociation constant for ICYP among the groups.

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Figure 3 shows the representative cardiac catecholaminergic histofluorescence, tyrosine hydroxylase, and neuropeptide Y immunoreactive profiles in 3 groups of animals. ANOVA showed significant differences between treatments (NE versus vehicle), between interventions (antioxidant vitamins versus placebo), and in the treatment-intervention interaction. Figure 4 shows that the sympathetic nerve terminal marker profiles were markedly reduced in the NE-treated ferrets. Antioxidant vitamin treatment did not affect the parameters in the vehicle group but prevented the reductions of the parameters in the NE-treated ferrets.

NE administration reduced myocardial total $\beta$-adrenergic receptor density (Figure 5). This was secondary to a specific reduction of $\beta_2$-subtype receptors; $\beta_2$-subtype receptor density was unaffected by NE. The figure also shows that antioxidant vitamin treatment, which had no effects in the vehicle-treated animals, prevented the decreases of total $\beta$-adrenergic receptor density and $\beta_2$-subtype density in NE-treated ferrets. There were no significant differences in the dissociation constant for ICYP among the groups.

Figure 6 shows representative isoproterenol competition curves in the vehicle, NE, and NE plus antioxidant vitamin groups. The curves were fitted to a 2-site model for measuring the fractions of high- and low-affinity receptor populations. The fraction of high-affinity $\beta$-adrenergic receptors was reduced by NE (Figure 7). Antioxidant treatment had no effects on the distribution of the high- and low-affinity sites in the vehicle-treated animals but prevented the reduction of fraction of high-affinity $\beta$-adrenergic receptors in NE-treated ferrets. There were no significant differences in the dissociation constants of high-affinity and low-affinity sites for isoproterenol among the groups.

**TABLE 2. LV Adenylyl Cyclase Activity**

<table>
<thead>
<tr>
<th>Adenylyl Cyclase Activity</th>
<th>Vehicle</th>
<th>Antioxidants</th>
<th>Vehicle</th>
<th>Antioxidants</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Basal activity, pmol · mg$^{-1}$ · min$^{-1}$</td>
<td>6.5±0.9</td>
<td>6.5±0.7</td>
<td>3.9±0.6*</td>
<td>6.1±0.8†</td>
</tr>
<tr>
<td>$\Delta$ Stimulated activity, pmol · mg$^{-1}$ · min$^{-1}$</td>
<td>8.5±1.7</td>
<td>7.4±0.8</td>
<td>4.1±0.7*</td>
<td>8.2±0.8†</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>39±11</td>
<td>32±6</td>
<td>15±2*</td>
<td>24.3±3†</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>168±15</td>
<td>175±18</td>
<td>105±9*</td>
<td>151±16†</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

* $P<0.05$ vs vehicle placebo group.
† $P<0.05$ vs NE placebo group.
Table 2 shows that basal adenylyl cyclase activity was reduced by NE. In addition, the increases of adenylyl cyclase activity produced by isoproterenol, Gpp(NH)p, and forskolin were reduced by NE. Antioxidant vitamin treatment did not affect basal and stimulated adenylyl cyclase activity in vehicle-treated animals, but it attenuated the reduction of basal and stimulated adenylyl cyclase activity in NE-treated ferrets. ANOVA showed a significant interaction between the treatment and intervention for each of these parameters.

Phase 2: Studies With PEG-rhSOD and Trolox C

Table 3 shows that NE administration had no effects on body weight, heart weight, heart rate, mean aortic pressure, or LV dP/dt in any of the groups, nor did PEG-rhSOD or Trolox C change any of the parameters.

Figure 8 shows that subcutaneous NE increased GSSG and decreased the GSH/GSSG ratio in cardiac tissue. GSH concentration did not differ among the groups. The changes of GSSG and GSH/GSSG ratio were abolished by administration of PEG-rhSOD or Trolox C. PEG-rhSOD and Trolox C also increased the LV dP/dt response to isoproterenol and β-adrenergic receptor density (Figure 9) and the reductions of myocardial NE uptake-1 carrier site density and catecholaminergic histofluorescence profiles produced by NE administration in ferrets (Figure 10). Trolox C alone had no effects in the vehicle-treated ferrets.

Discussion

NE administration produced abnormalities in the cardiac sympathetic nerve terminals and the myocardial β-adrenergic receptor–coupled G-protein–adenylyl cyclase signal transduction system in ferrets. These changes probably are mediated via oxidative products from the metabolism of NE, because the presynaptic and postsynaptic abnormalities of the cardiac sympathetic nervous system were prevented by co-administration of vitamins A, C, and E (phase 1) or by PEG-rhSOD or Trolox C (phase 2).

We showed that NE administration decreased cardiac neuronal NE histofluorescence, tissue NE uptake activity, NE uptake-1 carrier site density, and immunostained profiles of tyrosine hydroxylase and neuropeptide Y in ferrets. The findings suggest that the defects of the

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Placebo</th>
<th>NE</th>
<th>Trolox C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>1.72±0.06</td>
<td>1.69±0.07</td>
<td>1.60±0.03</td>
<td>1.76±0.07</td>
</tr>
<tr>
<td>LV weight, g</td>
<td>3.9±0.1</td>
<td>4.3±0.1</td>
<td>4.4±0.1</td>
<td>4.2±0.1</td>
</tr>
<tr>
<td>RV weight, g</td>
<td>1.5±0.1</td>
<td>1.5±0.1</td>
<td>1.5±0.1</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>135±6</td>
<td>148±7</td>
<td>150±8</td>
<td>148±5</td>
</tr>
<tr>
<td>Aortic pressure, mm Hg</td>
<td>108±8</td>
<td>113±4</td>
<td>111±6</td>
<td>96±6</td>
</tr>
<tr>
<td>LV dP/dt, mm Hg/s</td>
<td>3300±220</td>
<td>3696±529</td>
<td>3665±529</td>
<td>3241±246</td>
</tr>
</tbody>
</table>

RV indicates right ventricular. Values are mean±SEM. ANOVA showed no statistically significant differences among the groups.

Figure 8. LV GSSG concentration and GSH/GSSG ratio in vehicle- and NE-treated animals with and without PEG-rhSOD or Trolox C. Bars denote SEM. *P<0.05 vs vehicle group. †P<0.05 vs NE group.

Figure 9. Changes of LV dP/dt in response to isoproterenol administration and myocardial total β-adrenergic receptor density in vehicle- and NE-treated animals with and without PEG-rhSOD or Trolox C. See legend to Figure 8.
sympathetic nerve terminals are present both on the neurolemmal NE transport system and within the nerve terminals where NE, tyrosine hydroxylase, and neuropeptide Y are stored. Because neuropeptide Y is not taken up by the nerve endings after corelease with NE,32 the decrease of neuropeptide Y cannot be explained by the impaired NE transporter site.

Earlier studies suggested that catecholamine-induced myocardial necrosis was produced by adrenochrome,11,12 a major oxidative metabolite of epinephrine. However, the amount of adrenochrome present in the tissue probably is too low to produce significant biological effects.33 More recently, direct measurements of increased hydroxyl free radical generation by nonenzymatic auto-oxidation of NE have been made in the heart after NE administration34 and cardiac sympathetic nerve stimulation.35 The increase in cardiac tissue GSSG and decrease of the GSH/GSSG ratio by NE in the present study are consistent with oxidative stress. Our results also showed that the NE-mediated toxicity on the sympathetic nerve terminals was prevented by PEG-rhSOD. Similarly, NE cardiotoxicity in isolated hearts was completely abolished by SOD,36 suggesting that the NE toxicity is mediated by free oxygen radicals produced by NE.

β-Carotene and α-tocopherol are lipid-soluble. They exert antioxidant functions and prevent lipid peroxidation in biological lipid phases, such as cell membranes or low-density lipoprotein by quenching oxygen free radicals.37 In this process, α-tocopherol is oxidized to an inactive α-tocopheroxyl radical. Ascorbic acid is a water-soluble antioxidant. It potentiates the effects of α-tocopherol by regenerating α-tocopherol from its radical at the interface between cell membrane or lipoprotein and water.38 Results of phase 2 of the study indicate that Trolox C is effective in preventing the toxic effects of NE on the sympathetic nerve terminals and myocardial β-adrenergic receptors. Trolox C contains only the 6-chromane head structure of α-tocopherol, which is believed to be responsible for the antioxidant activities.39 Furthermore, given the similar positive effects of a specific antioxidant agent such as PEG-rhSOD in our study, we can state with certainty that the beneficial effects observed in phase 1 experiments resulted at least in part from the antioxidant properties of vitamin E.

In summary, our findings indicate that the toxic effects of NE on the sympathetic nerve terminals may be prevented by antioxidant vitamins and that α-tocopherol may be considered for use in heart failure, in which cardiac NE release is increased, to preserve the integrity of presynaptic NE uptake, thus reducing the rise of interstitial NE and agonist-induced β-adrenergic receptor downregulation.

Acknowledgments
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