Cardiac sympathetic neuroprotective effect of desipramine in tachycardia-induced cardiomyopathy

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Liang, Chang-seng, Weike Mao, Chikao Iwai, Shuji Fukuoka, and Suzanne Y. Stevens. Cardiac sympathetic neuroprotective effect of desipramine in tachycardia-induced cardiomyopathy. *Am J Physiol Heart Circ Physiol* 290: H995–H1003, 2006. First published October 7, 2005; doi:10.1152/ajpheart.00569.2005.—Cardiac sympathetic transmitter stores are reduced in the failing heart. In this study, we proposed to investigate whether the reduction of cardiac sympathetic neurotransmitters was associated with increased interstitial norepinephrine (NE) and reactive oxygen species in congestive heart failure (CHF), using a microdialysis technique and salicylate to detect ·OH generation. Rabbits with and without rapid ventricular pacing (340 beats/min) were randomized to receive desipramine (10 mg/day) or placebo for 8 wk. Rapid pacing produced left ventricular dilation and systolic dysfunction. The failing myocardium also showed reduced tissue contents of NE and tyrosine hydroxylase protein and activity. In contrast, myocardial interstitial NE was increased in CHF (0.89 ± 0.11 ng/ml) compared with the sham-operated animals (0.26 ± 0.03 ng/ml). In addition, cardiac oxidative stress was increased in CHF animals as measured by myocardial interstitial ·OH radical, tissue oxidized glutathione, and oxidized mitochondrial DNA. Desipramine treatment produced significant NE uptake inhibition as evidence by an exaggerated pressor response and a greater increase of myocardial interstitial NE in response to intravenous NE infusion but no significant effects on cardiac function or hemodynamics in sham-operated or CHF animals. However, desipramine treatment attenuated the reductions of tissue NE and tyrosine hydroxylase protein and activity in CHF. Desipramine also prevented the reduction of tyrosine hydroxylase produced by NE in PC12 cells. Thus the reduction of cardiac sympathetic neurotransmitters is related to the increased interstitial NE and tissue oxidative stress in CHF. Also, normal neuronal uptake of NE is required for NE or its oxidized metabolites to exert their neurotoxic effects.

oxidative stress; microdialysis; tyrosine hydroxylase; norepinephrine; ceramide.

DEPLETION OF CARDIAC NOREPINEPHRINE (NE) is one of the important salient features in human congestive heart failure (CHF) (7, 9). Prior studies (5, 7, 9, 16, 17) have shown that cardiac NE is depleted in CHF because of increased preferential cardiac release of NE, reduced neuronal reuptake of NE, and impaired NE synthesis. Tyrosine hydroxylase, a rate-limiting enzyme in NE biosynthesis (37), is reduced in heart failure (16, 53), which may account, at least in part, for the reduced production of NE. Recently, we observed in several animal models of experimental CHF (23, 30, 39, 41) that the sympathetic nerve profiles as measured by NE histofluorescence and tyrosine hydroxylase immunocytochemistry are reduced in CHF, but

there is no significant reduction of protein gene product 9.5 (PGP9.5) (41), a panneuronal marker (20, 29, 57). Thus the anatomic integrity of the cardiac sympathetic nerves probably is intact in CHF, and the changes of sympathetic neurotransmitters within the nerve endings are caused by functional abnormalities that are potentially reversible with either effective therapy or removal of primary insult that causes heart failure as discontinuation of cardiac pacing in tachycardia-induced cardiomyopathy (30).

Our laboratories have further shown that the reductions of cardiac sympathetic transmitters, like those in CHF, can also be induced by exogenous administration of NE (40, 41) and inhibited by desipramine, a NE uptake inhibitor (39, 41). The findings suggest that the cardiac sympathetic nerve terminal dysfunction is probably caused by increased interstitial NE in CHF, and the neuronal damaging effect of NE involves the uptake of NE or its oxidative metabolites into the sympathetic nerve endings. However, direct in vivo demonstration of myocardial NE uptake inhibition by desipramine is lacking. In the present study, we employed rapid ventricular pacing to produce left ventricular dilation and failure in rabbits and measured the myocardial interstitial NE concentrations directly using a microdialysis technique (31, 35, 61). We measured the sympathetic neuronal markers NE and tyrosine hydroxylase. Furthermore, desipramine has been shown recently to reduce mitochondrial reactive oxygen species and cell death by inhibiting membrane sphingomyelinase and ceramide production (11, 25, 42). Ceramide is the second messenger in the sphingomyelin-signaling pathway (21). It is highly expressed in the heart and has been shown to play an important role in mediating the cell apoptosis and cardiac dysfunction produced by TNF-α and oxygen free radicals in cardiomyocytes (59). Desipramine has also been shown to reduce ceramide increase, infarct size, and myocyte apoptosis during prolonged ischemia in both isolated perfused hearts (14) and intact rabbits (3). Because reduction of oxidative stress by antioxidants has also been shown to attenuate the neuronal damaging effects of NE (40), we performed additional experiments to determine whether the effects of desipramine on myocardial NE and tyrosine hydroxylase were associated with reductions of interstitial oxygen free radicals and tissue oxidative stress. Cardiac microdialysis of salicylic acid was used to measure myocardial interstitial 2,3-hydroxybenzoic acid (2,3-DHBA), a sensitive and specific marker for hydroxyl radical (45, 50, 54). In addition, myocardial contents of GSH-to-GSSG ratio and mitochondrial DNA (mtDNA) 8-oxo-7,8-dihydro-2 deoxyguanosine

(8-oxo-dG) were measured. Finally, to determine whether desipramine exerts a direct effect on sympathetic cells, we administered desipramine to the cultured rat pheochromocytoma PC12 cells treated with various doses of NE. We also measured ceramide by immunocytochemistry in PC12 cells treated with NE and desipramine. Our results indicate that desipramine attenuates the reduction of tyrosine hydroxylase in both the failing myocardium and the NE-treated PC12 cells and that the neuroprotective effects are related to the inhibitory action of desipramine on NE uptake without any effect on oxidant stress or ceramide production.

METHODS

Animal model. This 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's *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 85-23, Revised 1996).

Adult healthy New Zealand White rabbits (2.8 to 3.6 kg, 4–6 mo of age) were chosen for production of experimental heart failure with the use of a modified rapid cardiac pacing technique as described previously (30, 58). Briefly, under general isoflurane anesthesia, subxyphoid thoractomy and pericardiotomy were performed for placement of two shielded pacing leads (model TPW50; Ethicon, Somerville, NJ) onto the left ventricular apex and the left pectoral muscle, respectively. One week after surgery, rabbits were randomly assigned to receive either pacing (CHF animals, 340 beats/min, model 8086 Prevail implantable programmable pacemaker modified for rapid pacing, Medotronic, Minneapolis, MN) or no cardiac pacing (sham-operated animals). The animals developed progressive heart failure beginning 1–2 wk after the start of rapid pacing.

Experimental design. CHF and sham-operated animals were randomized to receive desipramine or placebo. The drug was delivered at a rate of 10 mg/day for 8 wk with the use of subcutaneous pellets (Innovative Research of America, Sarasota, FL). Animals in a lightly anesthetized state with the use of ketamine (20 mg/kg) and midazolam (0.6 mg/kg) were examined weekly by echocardiograms. At *week 8*, cardiac pacing was discontinued, and the animal was anesthetized for the measurement of resting hemodynamics and cardiac interstitial NE and 2,3-DHBA with the use of the microdialysis technique. Afterwards, the animal was euthanized with intravenous pentobarbital sodium (>100 mg/kg), and the heart was removed, weighed, and rinsed in ice-cold oxygenated normal saline. The left and right ventricles were separated from the septum and weighed. Transmural muscle blocks from left ventricles were processed immediately or stored in liquid nitrogen for later analysis for cardiac sympathetic neurotransmitters and for total cellular and mitochondrial oxidative stress indexes.

Echocardiographic and hemodynamic measurements. Two-dimensional and M-mode echocardiograms were obtained by using a 5-MHz transducer on a Toshiba model SSH-60A sonographic system (Toshiba America Medical System, Tustin, CA). Maximal left ventricular end-diastolic dimension (EDD) and end-systolic dimension (ESD) were measured and used to calculate left ventricular fractional shortening (FS) with the following equation: $FS = [(EDD - ESD)/EDD] \times 100$.

For hemodynamic studies, animals were anesthetized with ketamine (35 mg/kg) and midazolam (0.8 mg/kg). A 20-gauge, fluid-filled catheter (Insyte; Desert Medical; Becton, Dickson and Company; Sandy, UT) was inserted into the left carotid artery and connected to a Statham P23 XL pressure transducer (Spectramed, Oxnard) with an amplifier for measuring aortic pressure, while a 2.2 Fr SPR-249 micromanometer-tipped catheter (Millar Instruments, Houston, TX) was advanced into the left ventricle via the right carotid

artery for measuring the left ventricular pressure. Electrocardiograms, aortic pressure, and the first derivative of left ventricular pressure (dP/d*t*) were recorded on a multichannel recorder (Brush model 480, Gould, Instrument Systems Division, Cleveland, OH) and an IOX data acquisition and analysis system (EMKA Technologies, Falls Church, VA). At least 1 h was allowed to elapse after catheterization before the resting hemodynamic data were taken in triplicate over a 15- to 20-min steady state. The measurements taken from five consecutive beats during the expiratory cycles were averaged for statistical analysis.

Myocardial microdialysis for 2,3-DHBA and NE determination. After completion of resting hemodynamic measurements, rabbits were artificially ventilated with a constant-volume respirator using room air mixed with oxygen. Heart rate, arterial pressure, and ECG were monitored and recorded continuously. The chest was opened through the fifth or sixth rib on the left side. A small incision was made in the pericardium with a fine guiding needle, and a CMA/20 microdialysis probe (10-mm membrane length; CMA Microdialysis, North Chelmsford, MA) was implanted at the midlevel of left ventricular free wall. The dialysis probe was perfused with Ringer solution containing (in mM) 147 NaCl, 2.3 CaCl$_2$, and 4 KCl (pH 7.4) with the use of a CMA/102 microdialysis pump. The outlet side of the probe was connected to a microcentrifuge tube on ice. A 120-min equilibration period was allowed to elapse after probe implantation before the dialysate of 5 μl/min was collected every 4 min into a 5 μl ice-cold solution containing 0.1% Na$_2$EDTA in 0.1 M HClO$_4$ to prevent amine oxidation. Sodium salicylate (1 mM) was added to the microdialysis perfusion to detect the generation of ·OH as reflected by the formation of 2,3-DHBA in the myocardium (46, 47, 50). After baseline measurements were completed, NE was infused intravenously at 2 mmol·kg^{-1}·min^{-1} for 20 min, and dialysate collections were continued for another 25 min after the discontinuation of NE for immediate assays of NE and 2,3-DHBA with the use of a Bioanalytical System 480 HPLC system (BAS, West Lafayette, IN).

The HPLC was equipped with a BAS model 480 pump, a Unijet microbore column (5 μm, 100×1 mm), and a LC/4C electrochemical detector with a glassy carbon electrode. Detector potential was +0.70 V with an Ag/AgCl reference electrode. The mobile phase consisted of 0.15% 1-heptansulfonic acid, 0.01% Na$_2$EDTA, 0.3% triethylamine, and 4% acetonitrile (pH 2.8 with phosphoric acid). An aliquot of dialysate was injected into a 10-μl sample loop. A standard solution of 2,3-DHBA and NE was also injected for calibration.

Cardiac sympathetic nerve terminal neurotransmitters. Left ventricular muscle samples were taken to measure NE with the use of both HPLC and sucrose-potassium phosphate-glyoxylic acid-induced (SPG) histofluorescence (15, 23). For the HPLC assay, muscle samples were minced and suspended in 0.4 N perchloric acid with 5 mM reduced glutathione (pH 7.4), homogenized with a Brinkman Polytron PCU-2 homogenizer, and centrifuged at 500 *g*. The supernatant was then injected into the BAS HPLC with a LC/4C electrochemical detector. A MP-1 catecholamine mobile phase (Bioanalytical Systems) was used. To measure SPG histofluorescence, tissue blocks were sectioned at a thickness of 16 μm and dipped in SPG solution (23, 39). Tissue sections were viewed under epifluorescent illumination by using a Nikon fluorescence microscope (Nikon Instruments, Melville, NY) and photographed onto 35-mm slides with ×30 magnification. The number of catecholaminergic profiles were counted morphometrically in a 0.221 mm^2 (0.003536 mm^3) field. Six fields were counted for each animal, and the results were averaged.

To measure tissue tyrosine hydroxylase, left ventricular muscle blocks were homogenized in 1× lysis buffer (Cell Signaling Technology, Beverly, MA) containing 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM Na$_2$EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 μg/ml leupeptin, and 1 mM PMSF) for 30 min on ice. After centrifugation at 12,000 *g* for 15 min was completed, the supernatant was used for Western blot analysis. Protein content was determined by using a

bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were loaded onto SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (PerkinElmer, Boston, MA) electrically. Blots were then probed with either anti-tyrosine hydroxylase antibody (1:1,000; Cell Signaling Technology) or anti-PGP9.5 antibody (1:1,000; Biogenesis, Kingston, NH). After exposure to horseradish peroxidase-conjugated secondary antibody for 1 h, blots were visualized by using a chemiluminescence detection kit (Cell Signaling Technology). The autoradiograms were scanned on a Microtek model 6800 scanner (Microtek, Carson, CA), and the optical density of bands was determined by using a NIH 1.6 gel image program. The optical density readings of samples were normalized to a control sample in an arbitrary densitometry unit.

Myocardial tyrosine hydroxylase activity assay. Tyrosine hydroxylase activity was measured by the conversion of [^{3}H]tyrosine to dihydroxyphenylalanine and ^3H_2O by using a modification (52) of the procedure of Reinhard et al. (56). Briefly, heart tissue was homogenized in five volumes of $1\times$ lysis buffer (Cell Signaling Technology), containing protease inhibitors and phosphatase inhibitors (see *Cardiac synaptic nerve terminal neurotransmitters*) at 4°C, and centrifuged at 14,000 g. The supernatant was then added to an equal amount of $2\times$ assay buffer to reach the final concentration of 150 mM Tris maleate, 50 μM L-[3,5-^{3}H]tyrosine (51.5 Ci/mM, PerkinElmer), 5 mM ascorbate, 0.45 mg/ml catalase, and 0.5 mM 6-MPH$_4$ at pH 6.8. After 10 min at 37°C, the reaction was stopped on ice. With the use of this reaction, 1 mol of ^3H_2O is generated for each mole of ^{3}H-tyrosine that is converted to dihydroxyphenylalanine by tyrosine hydroxylase. The tyrosine hydroxylase assay is linear over the reaction time for at least 30 min. Released ^3H_2O was separated from unreacted ^{3}H-tyrosine by mixing the reaction samples with 7.5% charcoal in 1.0 M HCl and assayed from the supernatant by liquid scintillation counting (TriCarb 2400 TR liquid scintillation counter, Packard Instrument, Meriden, CT). Tyrosine hydroxylase activity was expressed as picomoles of ^3H_2O formed per milligram of protein in 10 min.

Myocardial glutathione measurement. A Bioxytech GSH/GSSG-412 test kit (OXIS International, Portland, OR) was used to measure GSH and GSSG. Fresh left ventricular myocardium was homogenized in three volumes of 1% picric acid, and the supernatant was collected for measuring with or without 33 mM 1-methyl-2-vinyl-pyridinium trifluoromethanesulfonate (M$_2$VP), a scavenger of reduced GSH. For GSH estimation, 5 μl of supernatant was mixed with 355 μl of assay buffer containing 100 mM NaPO$_4$ and 5 mM EDTA, pH 7.5. For GSSG estimation, 10 μl of homogenate with M$_2$VP was mixed with GSSG buffer containing 100 mM NaPO$_4$ and 5 mM EDTA, pH 10.05. The samples were mixed with 1.262 mM 5,5-dithiobis-(2-nitrobenzoic acid) and 15 units/ml GSH reductase. The mixture was incubated at room temperature for 5 min, and the absorbance was recorded at 412 nm for 3 min after the addition of 3.8 μmol NADPH by using a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA).

Myocardial mtDNA 8-oxo-dG measurement. Left ventricular muscle samples were prepared for isolation of mitochondria (4, 58). The mtDNA was extracted by using QIAamp blood kit (Qiagen, Valencia, CA) per the manufacturer's instructions and digested into deoxynucleosides by nuclease P1 and alkaline phosphatase. The deoxynucleoside mixture was filtrated through a 0.22-μm nylon filter and injected into a YMC Basic S, 3 μ 4.6×150 nm column (Waters, Milford, MA) in the BAS 480 HPLC with a mobile phase of 5% methanol in 100 mM lithium acetate buffer (pH 5.2). Detection of 8-oxo-dG and 2′-deoxyguanosine (dG) was performed on a model 5200A Coulochem II electrochemical detector equipped with a model 5011 analytical cell and model 5021 guard cell (Environmental Science Associate, Chelmsford, MA), and dG (Sigma-Aldrich, St. Louis, MO) was used for calibration.

PC12 cell studies. Liang's laboratory (44) has shown in previous studies that NE reduced NE uptake and NE uptake-1 carrier protein in a dose-dependent manner in PC12 cells without causing significant cell death. Cell death occurred only when the NE concentration

exceeded 100 μM (43, 44). In this study, we investigated the effects of NE, as well as the influence of desipramine and antioxidant enzymes on the effects of NE, on the tyrosine hydroxylase content in PC12 cells. PC12 cells were plated in a 60-mm dish for Western blot analysis, or, at a density of 10^5 cells per well, in a 12-well plate for NE uptake assay and grown for 24 h at 37°C. The medium was then switched to 2% serum RPMI 1640 overnight before exposing the cells to 1, 10, and 100 μM of NE in the presence or absence of 1 μM desipramine for 24 h. PC12 cells were also exposed to NE in the presence and absence of superoxide dismutase (20 μg/ml) and catalase (20 μg/ml) for 24 h. All cell samples were then solubilized in $1\times$ lysis buffer and prepared for Western blot analysis as described above for the heart muscle samples. Equal loading of the protein was confirmed by reblotting the polyvinylidene difluoride membrane with anti-β-actin (1:1,000; Abcam, Cambridge, MA). Finally, to determine whether desipramine reduced the lethal effect of NE on PC12 cells, we measured cell viability of the PC12 cells by 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (44).

NE uptake activity in PC12 cells after NE and desipramine treatment was measured by specific uptake of [^{3}H]NE in an incubation medium containing 35.7 nM l-[7-^{3}H(N)]NE (13.8 Ci/mmol; New England Nuclear, Boston, MA) at 37°C for 15 min (44). After the wash was completed, cells were solubilized and counted for radioactivity in the TriCarb 2400 TR liquid scintillation counter. Nonspecific accumulation of radioactivity was determined by parallel incubation of PC12 cells in the presence of 10 μM nisoxetine in the incubation medium.

Immunocytochemistry of ceramide in PC12 cells. Ceramide was measured in PC12 cells with an anti-ceramide antibody (12), by using an immunocytochemistry protocol for avidin-biotin complex (ABC) detection by Cell Signaling Technology. To study the effects of NE and desipramine on ceramide generation, PC12 cells were treated with 100 μM NE, 1 μM desipramine, or 100 μM NE plus 1 μM desipramine for 24 h and compared with those cells without NE or desipramine treatment. After treatment was completed, PC12 cells were washed by $1\times$ TBS and immersed in 4% paraformaldehyde for 10 min at 4°C. The cells were then permeabilized on coverslips with 0.2% Triton X-100, blocked with a 5.5% goat serum in TBS-Triton buffer, and incubated with a primary anti-ceramide mouse IgM antibody (1:100) in 3% bovine serum albumin in TBS overnight at 4°C. The cells were then treated with a biotinylated anti-mouse IgM secondary antibody and prepared for ABC detection using hydrogen peroxide and Vectastain ABC kits (Vector, Burlingame, CA).

Statistical analysis. Results are presented as means ± SE. Experimental data were managed and analyzed by the RS/I Research System (Bolt, Beraneck and Newman Software Products, Cambridge, MA). The statistical significance of differences among the different experimental groups was analyzed by ANOVA. Student's *t*-test with Bonferroni correction was used to determine the statistical significance of difference between two individual groups. For serial measurements, two-grouping (CHF and drug treatment), repeated-measures ANOVA (SYSTAT version 11, SYSTAT Software, Point Richmond, CA) was used to determine the significance of influence of CHF and desipramine treatment. The significance of changes over a period of time in the same animals was determined by pairwise Bonferroni *t*-tests. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Cardiac function and resting hemodynamics. Rapid ventricular pacing caused a progressive increase in left ventricular EDD and decline in left ventricular FS over 8 wk of pacing (Fig. 1). Desipramine treatment alone had no effects in the cardiac function in sham-operated animals. However, in animals receiving rapid cardiac pacing, desipramine appeared to
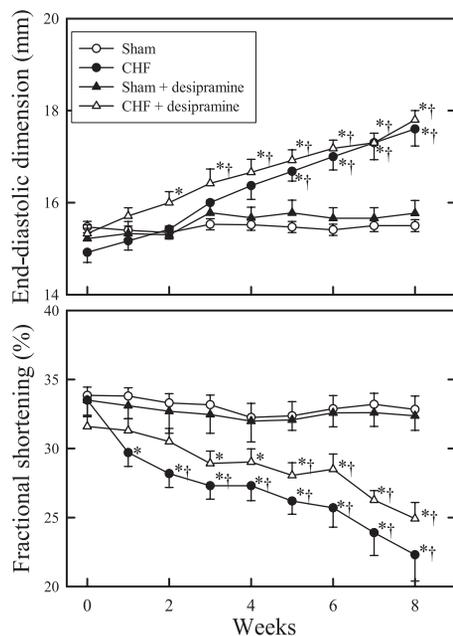
Fig. 1. Effects of desipramine on left ventricular end-diastolic dimension (*top*) and fractional shortening (*bottom*) in rabbits with sham-operated and rapid ventricular pacing. Bars denote ± SE; $n = 9$–17 animals. Two-grouping, repeated-measures ANOVA revealed that rapid ventricular pacing increased left ventricular end-diastolic pressure ($F = 26.256$, degree of freedom $= 1, 42$, $P < 0.001$) and reduced left ventricular fractional shortening ($F = 30.197$, degree of freedom $= 1, 42$, $P < 0.001$). However, no significant effect was produced by desipramine treatment on either left ventricular end-diastolic dimension ($F = 0.727$, degree of freedom $= 1, 42$, $P = 0.399$) or left ventricular fractional shortening ($F = 0.708$, degree of freedom $= 1, 42$, $P = 0.405$). There was also no significant interaction between pacing and desipramine treatment for either left ventricular end-diastolic dimension ($F = 0.235$, degree of freedom $= 1, 42$, $P = 0.630$) or left ventricular fractional shortening ($F = 1.696$, degree of freedom $= 1, 42$, $P = 0.200$). *$P < 0.05$, compared with respective baseline in each group using all pairwise multiple comparison Bonferroni *t*-tests; †$P < 0.05$, compared with the corresponding value (in time) in the sham-operated placebo group.

hasten the dilation of the left ventricle, but the net increase of left ventricular EDD at the end of 8 wk did not differ significantly between the CHF animals with and without desipramine (2.3 ± 0.3 vs. 2.7 ± 0.3 mm). Desipramine also produced no statistically significant differences in left ventricular FS in CHF animals.

Table 1 shows body weight, heart weight, resting heart rate, mean aortic blood pressure, and left ventricular end-diastolic pressure and dP/d*t* in the sham-operated and CHF animals. Cardiac dysfunction was evidenced by the increased left ventricular end-diastolic pressure and reduced left ventricular dP/d*t*. Body weight, left ventricular weight, heart rate, and aortic blood pressure did not differ between the CHF and sham-operated groups. Desipramine produced no changes in any of the hemodynamic parameters in either the sham-operated or the CHF animals.

Microdialysis measurements of myocardial interstitial NE and responses to NE infusion. Figure 2 illustrates the changes of interstitial NE concentration during 20 min of NE infusion and 25 min of recovery. The baseline interstitial NE (in ng/ml) taken within 5 min immediately before NE infusion differed significantly among the four groups of animals ($F = 23.79$, degree of freedom $= 3, 20$, $P < 0.001$). When compared with baseline value in the sham-operated placebo animals ($0.26 \pm$

Table 1. *Hemodynamics and organ weights in sham-operated and CHF animals*

	Sham		CHF	
Variable	Placebo	Desipramine	Placebo	Desipramine
n	14	9	9	9
Body weight, kg	3.42±0.05	3.41±0.11	3.24±0.05	3.09±0.04
LV weight, g	4.06±0.26	3.93±0.22	3.69±0.18	3.46±0.16
Heart rate, beats/min	241±18	201±21	223±20	202±21
MABP, mmHg	88±8	84±9	83±8	69±7
LV EDP, mmHg	7±1	5±1	21±3*	15±3*
LV dP/d*t*, mmHg/s	3938±441	3286±434	2471±436*	2188±423*

Values are means ± SE; $n =$ number of animals. CHF, congestive heart failure; MABP, mean aortic blood pressure; EDP, end-diastolic pressure; LV dP/d*t*, first derivative of left ventricular (LV) pressure. Data were analyzed by ANOVA, and statistical significance of difference between two means was determined by Bonferroni simultaneous 95% confidence intervals for all comparisons. *$P < 0.05$, compared with sham-operated placebo group.

0.03 ng/ml), interstitial NE was increased in animals treated with desipramine (0.74 ± 0.09 ng/ml), CHF (0.89 ± 0.11 ng/ml), and CHF plus desipramine (1.28 ± 0.12 ng/ml). NE infusion produced significant increases in interstitial NE in all experimental animals, but compared with the other groups, the increase of interstitial NE during NE infusion was relatively small in the sham-operated placebo group. The peak interstitial NE (obtained between 12 and 20 min of NE infusion) was greatest in the desipramine-treated CHF animals (4.21 ± 0.76 ng/ml), compared with the sham-operated placebo (0.76 ± 0.09 ng/ml), sham-operated desipramine (2.80 ± 0.27 ng/ml), and CHF placebo (3.13 ± 0.63 ng/ml) groups. Interstitial NE returned to baseline quickly in the sham-operated placebo animals (0.29 ± 0.04 ng/ml), but the values in the other three groups remained elevated above baseline 12 min after the end of NE infusion and were higher than that in the sham-operated placebo group at 20–25 min after end of NE infusion. NE infusion also increased mean aortic blood pressure in all animals. The net increase of mean aortic blood pressure during
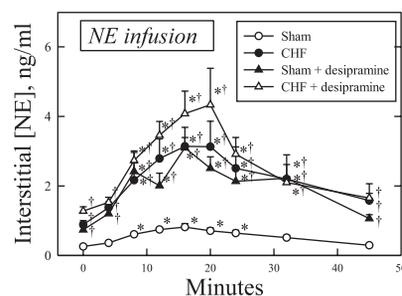

Fig. 2. Effects of desipramine on left ventricular interstitial norepinephrine (NE) concentrations before, during, and after NE infusion (2 mmol·kg^{-1}·min^{-1}) in sham-operated and CHF animals. Bars denote ± SE; $n = 5$–7 animals. Two-grouping, repeated-measures ANOVA revealed that NE infusion increased interstitial NE concentrations in all animals ($F = 24.203$, degree of freedom $= 5, 100$, $P < 0.001$) and that response of interstitial NE concentrations during NE infusion differed significantly between the CHF and sham-operated animals ($F = 32.17$, degree of freedom $= 1, 20$, $P < 0.001$) and between the placebo and desipramine treatment ($F = 21.986$, degree of freedom $= 1, 20$, $P < 0.001$). There was, however, no statistical significance of group and treatment interaction ($F = 3.116$, degree of freedom $= 1, 20$, $P = 0.093$). *$P < 0.05$, compared with respective baseline in each group using all pairwise multiple comparison Bonferroni *t*-tests; †$P < 0.05$, compared with the corresponding value (in time) in sham-operated placebo group.

NE infusion, however, was smaller in CHF animals (23 ± 5 mmHg) compared with sham-operated animals (43 ± 5 mmHg). In addition, consistent with inhibition of NE uptake, desipramine treatment enhanced the pressor response to NE in the CHF animals (37 ± 6 mmHg).

Effect of desipramine on myocardial interstitial 2,3-DHBA. Figure 3 shows the changes of interstitial 2,3-DHBA at rest and during NE infusion in sham-operated and CHF animals. Interstitial 2,3-DHBA was increased sixfold at baseline in CHF animals compared with the sham-operated animals. NE infusion increased 2,3-DHBA in both groups of animals. These changes in sham-operated animals were further exaggerated by desipramine administration.

Myocardial tissue glutathione and mtDNA 8-oxo-dG. Rapid cardiac pacing reduced myocardial GSH and increased GSSG. As a result, the GSH-to-GSSG ratio decreased markedly in the failing myocardium (Fig. 4). It also increased mtDNA 8-oxo-dG. Because there was no change in mtDNA dG, the mtDNA 8-oxo-dG-to-dG ratio increased more than twofold in animals with CHF. The figure also showed desipramine treatment caused a slight increase of 8-oxo-dG-to-2′-dG ratio in sham-operated animals but had no effect on 8-oxo-dG-to-dG ratio in CHF animals.

Effects of desipramine on tissue NE and tyrosine hydroxylase. Table 2 shows the changes in tissue NE, SPG histofluorescence, and tyrosine hydroxylase in sham-operated and CHF animals. As shown before, myocardial NE and tyrosine hydroxylase protein were reduced in CHF animals. Similar to the Western blot analysis, tyrosine hydroxylase activity was reduced by 50–60% in CHF animals. Furthermore, because PGP9.5 did not differ between the groups, the ratio of tyrosine hydroxylase to PGP9.5 was reduced to a similar extent as tyrosine hydroxylase in CHF. The administration of desipramine reduced tissue NE and SPG histofluorescence in sham-operated animals but had no significant effect in tyrosine hydroxylase protein or activity. In contrast to the sham-operated animals, desipramine increased SPG histofluorescence profiles and tyrosine hydroxylase protein and activity in CHF animals.

Effects of desipramine in PC12 cells. Figure 5 shows that NE reduced tyrosine hydroxylase in a dose-dependent fashion
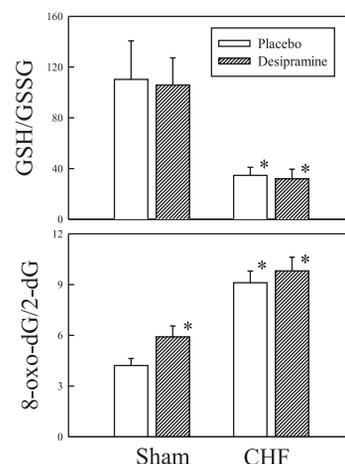
Fig. 4. Effects of desipramine on left ventricular cellular GSH-to-GSSG (*top*) and mitochondrial DNA 8-oxo-dG-7,8-dihydro-2 deoxyguanosine (8-oxo-dG)-to-2′-deoxyguanosine (dG; *bottom*) ratios in sham-operated and CHF rabbits. Bars denote ± SE; $n = 8$–12 animals. $*P < 0.05$, compared with sham-operated placebo group.

in PC12 cells. A 34% reduction of tyrosine hydroxylase was seen with 100 μM NE. We also found that the reduction of tyrosine hydroxylase was abolished by 1 μM desipramine and superoxide dismutase plus catalase (Fig. 5). This dose of desipramine was chosen from our preliminary experiments showing an 88% reduction of NE uptake activity in PC12 cells (39 ± 4 vs. 313 ± 9 fmol·mg^{-1}·min^{-1}, $t = 27.4$, degree of freedom = 10, $P < 0.001$). This dose of desipramine was also sufficient to reduce cell death, as measured by MTT, produced by 500 μM NE in PC12 cells from 51 ± 6% to 29 ± 3% ($P < 0.01$).

The effects of desipramine and NE on ceramide content in PC12 cells are shown in Fig. 6, which shows that desipramine (1 μM) treatment resulted in a marked reduction of ceramide. In contrast, NE, at a dose that effectively reduced tyrosine hydroxylase by 34% (Fig. 5), produced no significant effect on ceramide in either control or desipramine-treated PC12 cells.

DISCUSSION

Our present experiments extend our prior studies on the reduction of cardiac sympathetic neurotransmitters in the failing heart. Our present study is the first to provide direct evidence that the reduction of cardiac sympathetic neurotransmitters in CHF is associated with increases in interstitial NE and concomitant production of ·OH with the use of the cardiac microdialysis technique. The findings suggest that the changes in neuronal transmitters are probably causally related to the increases in interstitial NE or its oxidative products, which is consistent with our prior findings that exogenous NE infusion reduced cardiac sympathetic neuronal profiles (40, 41). Direct demonstration of the effect of NE on neuronal tyrosine hydroxylase was also provided in our present study using PC12 cells.

Our present study also shows that the effect of NE on the cardiac sympathetic nerve endings requires an intact NE uptake mechanism. The administration of desipramine attenuated the reduction of tyrosine hydroxylase protein and enzyme activity in CHF. However, desipramine had a comparatively smaller effect on the restoration of tissue NE, probably because of the increased cardiac release of NE in CHF and the inhibition of
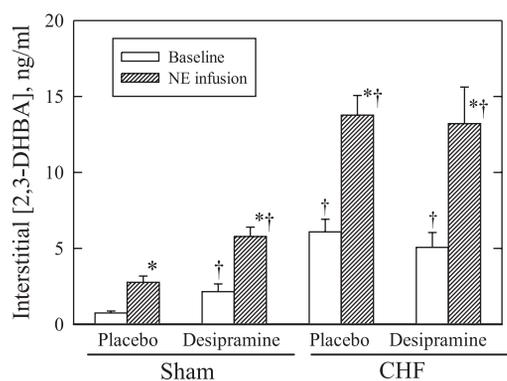

Fig. 3. Effects of desipramine on myocardial interstitial 2,3-hydroxybenzoic acid (2,3-DHBA) concentration in sham-operated and CHF animals. Interstitial 2,3-DHBA was increased in CHF animals and after desipramine administration. Bars denote ± SE; $n = 8$ animals. $*P < 0.05$, compared with baseline value of same group of animals; $†P < 0.05$, compared with corresponding value of sham-operated placebo group.

Table 2. *Effects of desipramine on cardiac sympathetic neurotransmitters in sham-operated and CHF animals*

Variable	Sham		CHF	
	Placebo	Desipramine	Placebo	Desipramine
Norepinephrine				
Tissue [NE], μg/g	2.54±0.23	1.51±0.16*	1.28±0.15*	1.65±0.28*
SPG, profiles/field	480±27	390±18*	146±18*	214±20*†
Tyrosine hydroxylase, arbitrary units				
Tyrosine hydroxylase	1.00±0.07	0.86±0.08	0.50±0.05*	0.80±0.06†
Tyrosine hydroxylase-to-PGP9.5 ratio	1.15±0.04	1.11±0.10	0.73±0.04*	1.03±0.04†
Tyrosine hydroxylase activity, pmol $^3H_2O \cdot mg^{-1} \cdot 10\ min^{-1}$	19.5±3.3	17.3±2.3	7.8±1.7*	17.1±2.8†

Values are means ± SE; $n = 6$–12 animals. NE, norepinephrine; SPG, sucrose-potassium phosphate-glyoxylic acid; PGP9.5, protein gene product 9.5. Data were analyzed by 2-way repeated-measures ANOVA, and statistical significance of difference between two means was determined by Bonferroni simultaneous 95% confidence intervals for all comparisons. *$P < 0.05$, compared with sham-operated placebo group; †$P < 0.05$, compared with CHF placebo group.

NE uptake by desipramine. Inhibition of NE uptake in PC12 cells by desipramine also abolished the reduction of tyrosine hydroxylase produced by NE. Desipramine also reduced the lethal effects of high-dose NE in PC12 cells. Similar effects were produced by superoxide dismutase and catalase in PC12 cells (43). However, desipramine did not affect the changes of either cardiac GSH-to-GSSG or mtDNA 8-oxo-dG-to-dG ratios in the CHF animals. In fact, desipramine actually increased myocardial ·OH as measured by interstitial 2,3-DHBA in the sham-operated animals. Thus the effects of desipramine on the preservation of sympathetic neuronal transmitters are independent of tissue oxidative stress. In addition, the neuroprotective effects of desipramine and superoxide dismutase are additive on sympathetic nerves (1). Thus the loss of noradrenergic transmitters in the failing myocardium is most likely caused by oxidation metabolites of NE that exert effects on the sympathetic nerve endings via the desipramine-sensitive NE uptake site.

Desipramine is a classical NE uptake inhibitor. It has been shown to reduce the NE uptake activity in the ventricular
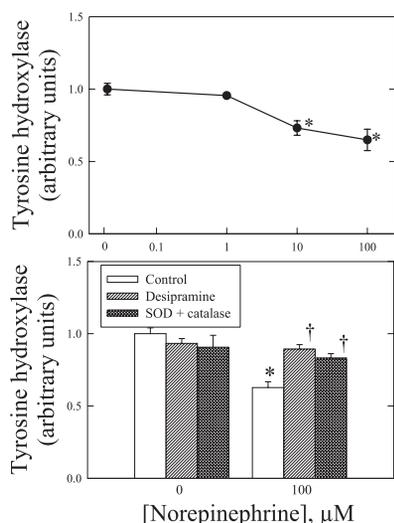

Fig. 5. Effects of NE on tyrosine hydroxylase in PC12 cells. *Top*: NE decreased tyrosine hydroxylase in PC12 cells. *Bottom*: decrease of PC12 tyrosine hydroxylase was abolished by desipramine (1 μM) and reduced by SOD plus catalase. Equal loading of protein for Western blot analysis was confirmed by reblotting membrane with anti-β-actin antibody. Bars denote ± SE; $n = 6$ animals. *$P < 0.05$, compared with control without NE (0 concentration); †$P < 0.05$, compared with corresponding values without desipramine or SOD plus catalase.

muscle preparation ex vivo (39, 40). At the dose administered, desipramine was well tolerated and produced no untoward clinical or hemodynamic effects in our present study. The dose was sufficient to potentiate the pressor response to exogenous administration of NE and to exaggerate the increase in myocardial interstitial NE during NE infusion. The prolonged elevation of myocardial interstitial NE after the discontinuation of NE infusion in the desipramine-treated animals was also consistent with the pharmacological inhibition of NE uptake by desipramine in the heart. In a prior study of open-chest anesthetized dogs, Levy and Blattberg (38) reported that desipramine potentiated and prolonged the chronotropic, pressor, and inotropic responses of the animals to exogenous NE. Desipramine also inhibited the extraction of exogenous NE in the heart, which is consistent with the greater and more prolonged elevations of interstitial NE after desipramine treatment in our study. Our microdialysis study also showed that NE infusion produced a much greater increase in myocardial interstitial NE in CHF placebo animals than in the sham-operated placebo group. These findings are consistent with the reduced myocardial NE uptake activity known to exist in the failing heart. However, unlike the exaggeration of interstitial NE, the magnitude of pressor response to NE infusion was smaller in CHF animals compared with the sham-operated control. The latter phenomenon, which has been described previously (39, 41), probably was related to the reduced responsiveness of vascular postjunctional adrenoceptors to NE that is known to exist in heart failure (19).

The microdialysis method has been used to demonstrate the cardiac release of NE after coronary ischemia-repurfusion (36, 47, 50), NE administration (48), and cardiac sympathetic nerve stimulation (46). The earlier studies have also shown that cardiac sympathetic stimulation could raise interstitial NE to a level high enough to generate oxygen free radicals. In these studies, salicylate was administered to the dialysis perfusate and reacts with the interstitial hydroxyl free radical to form ·OH adducts, such as 2,3-DHBA, which was detected in the dialysate and used as an index of interstitial ·OH radicals (45). A positive correlation has been shown between the release of NE and the formation of ·OH in the heart (49). Likewise, our present study demonstrates a temporal correlation between the amounts of interstitial NE and 2,3-DHBA in the heart during NE infusion.

Our present study demonstrates that CHF is associated with increased myocardial interstitial ·OH. An increase in cardiac ·OH has been described by Ide et al. (26) in dogs with
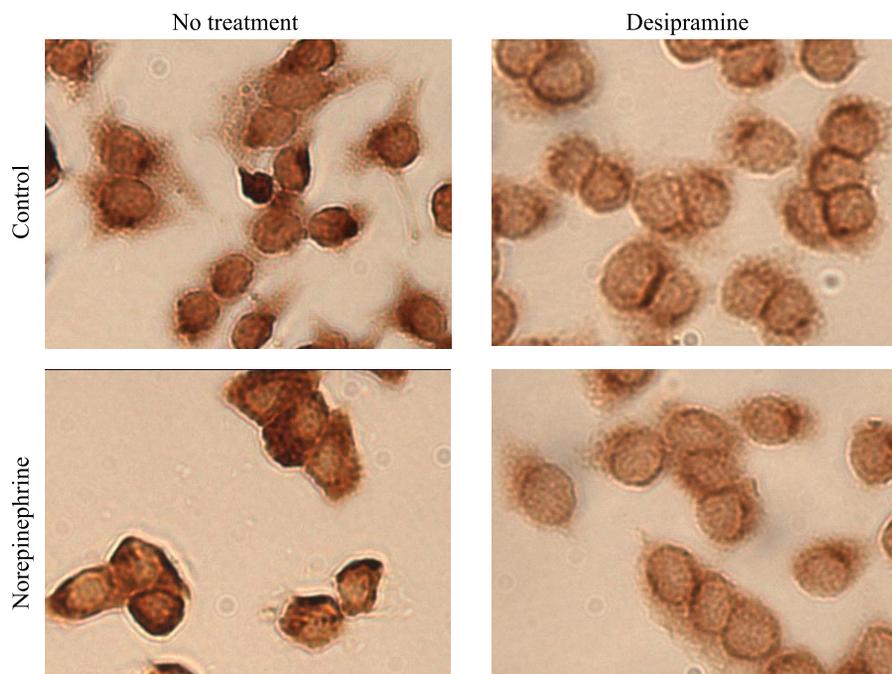
No treatment　　　　　　　Desipramine

Fig. 6. Effects of NE (*bottom*, *left*), desipramine (*top*, *right*), and NE plus desipramine (*bottom*, *right*) on ceramide content in PC12 cells. Immunocytochemical staining revealed that desipramine (1 μM) treatment reduced ceramide content, but NE (100 μM) had no effect on ceramide in either control (*top*, *left*) or desipramine-treated PC12 cells.

pacing-induced heart failure with the use of electron resonance spectroscopy. In the latter study, the authors also found that the increase of ·OH in freeze-clamp failing heart myocardium was abolished by desferrioxamine (an iron chelater), Tiron (a non-enzymatic O_2^-· scavenger), and catalase. The findings suggest that O_2^-· is produced and converted into H_2O_2 and further to ·OH via the iron-catalyzed Harber-Weiss reaction and Fenton reaction in the failing heart. Ide et al. (27) have also shown that O_2^-· is probably produced in the failing myocardium by a functional block of electron transport at mitochondrial electron transport complex I. In addition, O_2^-· may react with nitric oxide, which is increased in the failing myocardium as a result of increased expression and activity of the inducible nitric oxide synthase (60), to form another strong oxidant mediator peroxynitrite (24); the latter also has been shown to cause cardiac and vascular endothelial dysfunction (8, 22, 51, 55). The functional importance of the reactive oxygen and nitrogen species in heart failure is a topic of intense current research and beyond the scope of our present investigation.

Desipramine at the dose employed produced no significant hemodynamic effects in our studies but increased myocardial interstitial NE in sham-operated and CHF animals. Desipramine treatment has also been shown to impair NE clearance and increased plasma NE in patients with CHF (10, 38). The hemodynamic measurements in our studies, however, should be interpreted with caution because of the use of anesthesia that could have modified animals' responses. In addition, desipramine has been shown to have a sympathoinhibitory effect via stimulation of presynaptic α_2-receptors (18). Levy and Blattberg (38) reported that acute administration of desipramine diminished the release of NE from the cardiac nerve endings after stimulation of the left ansa subclavia. On the other hand, after prolonged exposure, desipramine desensitized the central and peripheral α_2-adrenoceptors, causing a decrease of sympathoinhibition and an increase of NE release (13). Thus the effects of desipramine on hemodynamics and plasma NE may

vary depending on the experimental condition and the duration of its administration. The higher interstitial NE in the desipramine-treated CHF animals could have exerted a greater inotropic effect and accounted for the greater average left ventricular FS compared with the CHF placebo animals. However, the differences between the two groups were not statistically significant. Furthermore, because desipramine promoted earlier dilation of the left ventricle (Fig. 1), NE uptake inhibitors are ultimately detrimental in the failing heart despite the potential beneficial effects on the sympathetic nerve endings and FS.

Immunocytochemistry demonstrated that desipramine reduced endogenous ceramide in PC12 cells, indicating that, at the dose employed, desipramine inhibited sphingomyelinase activity and ceramide production. On the other hand, the administration of 100 μM NE did not increase ceramide. The findings suggest that at doses ≤100 μM, the increases in reactive oxygen species and cellular oxidative stress produced by NE (44) are not caused by the formation of ceramide. This is consistent with our results in intact animals that desipramine did not reduce cardiac interstitial ·OH radicals or myocardial oxidative stress in CHF animals. However, a possibility that the apoptotic effects of higher doses of NE are mediated via an action of ceramide exists and warrants further evaluation.

Our present study provides no molecular mechanism to explain the reduction of tyrosine hydroxylase produced by the oxidative metabolites of NE, but several plausible mechanisms have been postulated. Kuhn et al. (34) reported that tyrosine hydroxylase activity was reduced by various catechol-quinones by converting tyrosine hydroxylase to a redox-cycling quinoprotein. This mechanism of action is probably not important in the reduction of tyrosine hydroxylase produced by NE in our study, because unlike our present study, the catechol-quinone-mediated inactivation of tyrosine hydroxylase, which is sensitive to reducing agents, is not inhibited by antioxidant enzymes such as superoxide dismutase or catalase (34). Tyrosine hydroxylase has also been shown to be inhibited by a mechanism

involving peroxynitrite-induced tyrosine nitration (2, 28) or sulfhydryl oxidation of the enzyme (6, 33). Borges et al. (6) showed that peroxynitrite-induced inactivation of tyrosine hydroxylase is associated with dithiothreitol-reversible S-glutathionylation of the enzyme. Studies (32) have also shown that the peroxynitrite-induced inhibition of tyrosine hydroxylase can be reversed by reduced forms of nicotinamide nucleotides. These studies suggest that the reduction of tyrosine hydroxylase during oxidative stress is caused primarily by S-glutathionylation, whereas tyrosine nitration of the enzyme occurs relatively late when extensive depletion of endogenous catecholamines have occurred (32). In a recent study, we observed that NE reduced NE uptake transporter receptor in PC12 cells by endoplasmic reticulum stress (43). Because endoplasmic reticulum stress may also affect biosynthesis of tyrosine hydroxylase, studies are needed to explore the relationships between the increased endoplasmic reticulum stress produced by NE and tyrosine hydroxylase biosynthesis.

In summary, our present study showed that the reductions of cardiac sympathetic neurotransmitters in CHF are associated with elevated interstitial NE and ·OH. Desipramine treatment blocked NE uptake and increased interstitial NE but attenuated the reduction of myocardial tyrosine hydroxylase in CHF. These results suggest the neurotoxic effect of NE or its oxidative metabolites depend on the normal NE reuptake mechanism of the sympathetic nerve endings. However, despite the potential neuroprotective of desipramine, the medication probably has limited therapeutic value because it increased myocardial interstitial NE and could potentially exaggerate the progressive cardiac remodeling in CHF.

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