Alterations in cardiac adrenergic terminal function and β-adrenoceptor density in pacing-induced heart failure

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EVIDENCE HAS ACCUMULATED that sympathetic activation in heart failure plays an important role in the progression of cardiac dysfunction. Recent studies in humans with congestive heart failure secondary to left ventricular systolic dysfunction have shown that administration of β-receptor blockers not only increases the left ventricular ejection fraction but also reduces cardiac mortality and morbidity (5, 27, 31). An increase in cardiac release of norepinephrine (NE) has been observed in early mild heart failure before any obvious generalized activation of the sympathetic nervous system (10). There is also a preferential increase in sympathetic activity of the heart compared with other organs in severe heart failure (10, 13). The marked preferential increase in the release of cardiac NE probably contributes to the eventual depletion of NE in the failing heart (9). The heart is also thought to be functionally denervated, with reduction of tyrosine hydroxylase activity to account for the loss of NE (8, 44).

We recently reported that unlike tyrosine hydroxylase, the neurolemmal marker protein gene product 9.5 (PGP 9.5) content is not reduced in the failing myocardium (44), suggesting that the sympathetic nerves are structurally intact and may recover in function with therapy. We have further shown that the functional alterations in the sympathetic nerve terminals in heart failure include loss of NE and tyrosine hydroxylase, decrease of NE uptake activity, and reduction of NE uptake-1 carrier site density (15). Because neuronal reuptake of NE is the major mechanism for removal of NE from the presynaptic cleft and termination of action of NE on the myocardial β-adrenoceptors, this decrease in myocardial NE uptake activity is expected to increase interstitial NE and decrease myocardial β-adrenoceptor density. Indeed, there is a strong inverse relationship between myocardial NE uptake activity and β-adrenoceptor density in animals with heart failure (15, 23).

To further determine whether the changes in adrenergic neuronal NE uptake play a role in the pathophysiology of myocardial β-adrenoceptor downregulation, we carried out the present study to examine the temporal associations of cardiac sympathetic nerve terminal function and the β-adrenoceptor-coupled adenyl cyclase signal transduction system during the development of and recovery from pacing-induced heart failure in rabbits.

METHODS

Animal preparations. Adult healthy New Zealand White rabbits (2.6–3.8 kg, 4–6 mo old) were chosen. Animals were prepared for experimental heart failure by a modified technique of Spinale et al. (40). 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 (DHHS publication National Institutes of Health 85–23, Revised 1985, Office of Science and Health Reports). Animals were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (3 mg/kg) followed by supplemental...
doses if necessary. The animals were intubated and ventilated with respirators (Harvard Apparatus, South Natick, MA). Through a sterile skin incision, subxyphoid thoracotomy and pericardiotomy were performed. A shielded pacing lead (TPWS50, Ethicon, Somerville, NJ) was then sutured onto the apical region of the left ventricular free wall and brought out through the diaphragm and abdominal wall. A second pacing lead was sutured onto the left pectoral muscle. These pacing leads were routed subcutaneously and exteriorized to the interscapular region, and the wound was closed. Artificial ventilation was discontinued, and the tracheal tube was removed after spontaneous breathing was assured.

One week later, the pacing leads were connected to a model 8086 Preval HVRP programmable pacemaker (Medtronic, Minneapolis, MN). Animals were assigned randomly to receive rapid ventricular pacing at a rate of 360 beats/min for varying durations. A control group of animals underwent identical surgery but received no cardiac pacing. They were euthanized 9 wk after the thoracotomy, corresponding in time to animals after 8 wk of cardiac pacing. The pacemaker and pacing leads were stored in a pocket of a custom-made rabbit jacket.

Experimental protocol. To study the time course of changes during the progression of and recovery from heart failure, the animals were euthanized for study after 2, 4, or 8 wk of rapid cardiac pacing or 1, 2, or 4 wk after cessation of 8 wk of pacing. An electrocardiogram was recorded once a week during the pacing protocol to confirm proper cardiac pacing. A group of animals without rapid pacing was included as control. There were six to eight animals in each group. Echocardiograms were taken once a week during rapid pacing and at 3 days, 1 wk, 2 wk, and 4 wk after cessation of pacing. At the end of the specified time periods, the animals were anesthetized with intramuscular ketamine (28 mg/kg) and midazolam (0.8 mg/kg) for measuring resting hemodynamics and plasma NE. The animals were then given a lethal dose (>100 mg/kg) of intravenous pentobarbital sodium. The hearts were removed and weighed. Each ventricular free wall was separated from the septum and rinsed in ice-cold oxygenated normal saline. The left ventricular weight includes both the septum and the left ventricular free wall. Fresh left ventricular muscle blocks were taken for measurement of NE uptake activity. Other muscle blocks were either rapidly frozen and fixed or stored in liquid nitrogen for measurements of left ventricular noradrenergic nerve terminal profiles, NE uptake-1 carrier site density, β-adrenoceptor density, and adenyl cyclase activity.

Hemodynamic and echocardiographic measurements. Cardiac pacing was discontinued before the final hemodynamic study. Two-dimensional and M-mode echocardiographic studies were performed in a left lateral decubitus position, using a scanning system heart (SSH) sonographic system (Toshiba Medical Systems, Tustin, CA). A 5-MHz transducer was used to measure the maximum left ventricular end-diastolic (EDD, measured in mm) and end-systolic dimensions (ESD, mm). Left ventricular fractional shortening (FS, %) was calculated as [(EDD − ESD) × 100]/EDD.

Animals were then prepared for hemodynamic measurements. A 20-gauge Insize intravenous catheter (Deseret Medical, Becton Dickinson, Sandy, UT) was introduced into the left carotid artery and connected to a pressure transducer (model P23 XL, Spectramed, Oxnard, CA) and an eight-channel Brush recorder (model 480, Gould, Instruments System Division, Cleveland, OH) for measuring arterial pressure and heart rate. A 2-Fr micromanometer-tipped catheter (Millar Instruments, Houston, TX) was introduced into the left ventricle via the right carotid artery and connected to the Brush recorder for measuring left ventricular pressure and its first derivative (dP/dt) using an electronic differentiator. Resting hemodynamic measurements were made in triplicate at 5-min intervals at least 1 h after insertion of the Millar catheter. Averages of the triplicate measurements were used for statistical analysis. An arterial blood sample was taken for measuring plasma NE using the radioenzymatic assay (32).

Myocardial NE uptake activity. Myocardial NE uptake activity was measured in quadruplicate by incubating fresh tissue slices at 37°C for 15 min in 50 nmol/l 1-[3H]NE (13.8 Ci/mmol; New England Nuclear, Boston, MA). Specific 3H-uptake activity, defined as the difference in radioactivity between tissue slices incubated in a [3H]NE-containing solution at 37°C and those at 4°C, is considered to represent NE uptake activity (23).

Myocardial NE uptake-1 carrier site density. Myocardial NE uptake-1 carrier site density was measured by using the radioligand binding technique with [3H]nisoxetine (New England Nuclear) (41). Approximately 80 µg of membrane protein, suspended in 50 mM Tris·HCl buffer (pH 7.4) containing 300 mM NaCl and 5 mM KCI, was incubated in triplicate with 3 nM [3H]nisoxetine and eight concentrations of nonradioactive nisoxetine (0.3125–100 µM) at room temperature for 90 min in a final volume of 0.25 ml. For determination of nonspecific binding, 10 µM nisoxetine was added. The reaction was terminated by addition of ice-cold Tris buffer and filtered immediately through Whatman GF/B filters (Whatman Chemical Separation, Clifton, NJ) on a Brandel cell harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD). The membranes were rapidly washed, dried, and counted for 3H radioactivity by liquid scintillation spectrometry (Tri-Carb 460 CD, Packard Instrument, Downers Grove, IL). The number of receptor binding sites and the dissociation constant were calculated using the EBDA computer software program (Elsevier Science Publisher, Cambridge, UK) (26).

Anatomic studies of ventricular sympathetic nerves. Fresh left ventricular tissue blocks were rapidly frozen and prepared for glyoxal acid-induced histofluorescence for catecholamines and immunochemistry for tyrosine hydroxylase (15). Histofluorescence specific for catecholamines was performed using a modification (1) of the sucrose-potassium-phosphate-glyoxal acid condensation method of de la Torre (6). A sheep anti-tyrosine hydroxylase primary antibody was used for the immunocytotoxic visualization of tyrosine hydroxylase. Sections for NE histofluorescence were photographed at ×30 magnification, whereas slides for tyrosine hydroxylase immunocytochemistry were photographed at ×20 magnification onto 35-mm slides. The number of stained catecholamine profiles was counted in a 0.221-mm2 (0.003536-mm2) field. The number of immunostained tyrosine hydroxylase profiles was counted in a 0.00885-mm2 field. The results of six fields were averaged for each ventricle.

Myocardial β-adrenoceptor density. Myocardial β-adrenoceptor density was measured by using the radioligand binding technique with [125I]iodocyanopindolol (I CYP, 2,200 Ci/mmol; New England Nuclear) (20). Tissue protein was determined using a biinchoninic acid protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard. Approximately 20 µg of membrane protein, suspended in 50 mM Tris·HCl buffer (pH 7.4) containing 120 mM NaCl and 5 mM KCI, was incubated in triplicate with eight concentrations of [125I]ICYP (5–250 pM) at 37°C for 60 min in a final volume of 0.25 ml. Nonspecific binding was determined by parallel incubation of samples containing 100 µM propranolol. The technical details of the assay were similar to those described above for the NE uptake-1 carrier site density.
Table 1. Heart rate, mean aortic pressure, and plasma NE during and after rapid cardiac pacing

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Rapid Cardiac Pacing</th>
<th>Cessation of Pacing</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>week 2</td>
<td>week 4</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>272 ± 14</td>
<td>243 ± 6</td>
<td>242 ± 13</td>
</tr>
<tr>
<td>Mean aortic pressure, mmHg</td>
<td>99 ± 5</td>
<td>93 ± 3</td>
<td>96 ± 6</td>
</tr>
<tr>
<td>Plasma [NE], ng/ml</td>
<td>0.07 ± 0.02</td>
<td>0.56 ± 0.16*</td>
<td>0.46 ± 0.12*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–8. [NE], norepinephrine concentration. *P < 0.05, compared with control. †P < 0.05, compared with week 8 of pacing.
2 and 3). The NE uptake-1 carrier site density did not return to control until 2–4 wk after cessation of pacing.

Cardiac sympathetic nerve terminal profiles. Figure 5 shows representative NE histofluorescence profiles at different stages of rapid cardiac pacing and 1 and 4 wk after cessation of pacing. In addition, we have summarized the group data of NE histofluorescence and immunostained tyrosine hydroxylase profiles in Fig. 6. Figure 5 shows that neither catecholaminergic histofluorescence profiles nor immunostained tyrosine hydroxylase profiles decreased immediately after commencement of rapid ventricular pacing. No significant changes occurred in animals after 2 wk of pacing. However, both neurotransmitter profiles decreased significantly by 4–8 wk of rapid cardiac pacing. Figure 5 also shows that both neuronal NE and tyrosine hydroxylase profiles returned to control within 1 wk after cessation of rapid pacing and remained unchanged thereafter.

Myocardial β-adrenoceptor function. Figure 7 shows that left ventricular β-adrenoceptor density did not change from the control values 2 wk after the start of rapid cardiac pacing but decreased significantly at 4 and 8 wk of rapid pacing. Myocardial β-adrenoceptor density also did not change immediately after discontinuation of rapid ventricular pacing. It remained reduced below control values at 2 wk and did not return to control until 4 wk after cessation of rapid pacing.

In contrast, the production of cAMP by isoproterenol, Gpp(NH)p, and forskolin was reduced soon after the start of rapid cardiac pacing. The adenylyl cyclase activity was reduced at 2 wk of pacing (Fig. 8). It

Fig. 2. Changes in LV end-diastolic dimension and fractional shortening produced by rapid ventricular pacing and after cessation of rapid pacing (solid circles). Open squares show results of control group without rapid pacing. Analysis of variance for repeated measures was used to determine statistical significance of changes in pacing groups. Numbers of measurements were 42, 37, 42, 35, 34, 27, 29, 24, 21, 16, 18, 12, and 5, at baseline (week 0), weeks 1, 2, 3, 4, 5, 6, 7, and 8 of pacing; and 3 days, 1 wk, 2 wk, and 4 wk after cessation of pacing, respectively. Bars denote SE. *Values that differed from baseline at \( P < 0.05 \). †Values that differed from week 8 of pacing at \( P < 0.05 \).

Fig. 3. Changes in LV first derivative of pressure (dP/dt) and end-diastolic pressure produced by rapid cardiac pacing and cessation of rapid pacing. Bars denote SE. *Values that differed from control at \( P < 0.05 \). †Values that differed from week 8 of pacing at \( P < 0.05 \).

Fig. 4. Changes in LV norepinephrine (NE) uptake activity and NE uptake-1 carrier site density produced by rapid cardiac pacing and cessation of rapid pacing. Bars denote SE. *Values that differed from control at \( P < 0.05 \). †Values that differed from week 8 of pacing at \( P < 0.05 \).
remained depressed throughout the rapid pacing. Adenylyl cyclase activation improved after cessation of rapid pacing. The recovery appeared to be faster than that of myocardial β-adrenoceptor density. Significant improvements already occurred within 1 wk after cessation of pacing. The responsiveness of adenylyl cyclase to isoproterenol, Gpp(NH)p, and forskolin returned to baseline within 1–2 wk after discontinuation of rapid pacing.

**DISCUSSION**

Rapid ventricular pacing has been used extensively to study the hemodynamics, neurohormonal alterations, pathogenesis, and efficacy of therapeutic interventions in heart failure (39, 40). Studies have shown that hemodynamic changes occur as early as 24 h after rapid pacing (18), with continuing deterioration of ventricular function (29), myocardial muscle depression (12), and eventual development of clinical heart failure after 3–4 wk of pacing (18). Acute rapid ventricular pacing also causes an increase in plasma NE and reductions of fraction of myocardial β-receptor binding agonist with high affinity as well as adenylyl cyclase activity within 24 h (18), but changes in myocardial β-receptor density, regulatory guanosine nucleotide binding proteins (G proteins), and myocardial NE do not occur until 3–4 wk after cessation of rapid cardiac pacing (18, 25). Furthermore, it has been demonstrated that left ventricular systolic function and myocardial β-adrenoceptor density return to normal within 4 wk after discontinuation of pacing (22, 28).
Our present study is the first longitudinal investigation linking the hemodynamic changes produced by rapid cardiac pacing to the myocardial β-adrenoceptor-coupled adenylyl cyclase system and sympathetic nerve terminal abnormalities in a time-dependent fashion. The study not only confirmed the hemodynamic and myocardial β-adrenoceptor changes mentioned previously in pacing-induced cardiomyopathy but also showed that as in dogs with heart failure (15, 23), sympathetic nerve terminal function was abnormal in the rabbits with pacing-induced heart failure. Left ventricular NE uptake activity and NE uptake-1 carrier site density decreased significantly within 2 wk after the start of rapid cardiac pacing, followed by the reductions of NE histofluorescence and tyrosine hydroxylase profiles and myocardial β-receptor density at 4–8 wk of rapid pacing. The findings suggest that the reduction of the cardiac NE uptake function is an earlier alteration in the process of cardiac sympathetic nerve terminal abnormalities in heart failure. Decreases in NE uptake activity and NE uptake sites have also been shown to occur in the failing human heart (2, 9, 19). There is a positive correlation between cardiac NE uptake and left ventricular ejection fraction (19).

Fig. 6. Changes in LV NE histofluorescence profiles and immunostained tyrosine hydroxylase profiles produced by rapid cardiac pacing and cessation of rapid pacing. Bars denote SE. *Values that differed from control at P < 0.05. †Values that differed from week 8 of pacing at P < 0.05.

Fig. 7. Changes in LV β-adrenoceptor density produced by rapid cardiac pacing and cessation of rapid pacing. Bars denote SE. *Values that differed from control at P < 0.05. †Values that differed from week 8 of pacing at P < 0.05.

droxylase profiles and myocardial β-receptor density at 4–8 wk of rapid pacing. The findings suggest that the reduction of the cardiac NE uptake function is an earlier alteration in the process of cardiac sympathetic nerve terminal abnormalities in heart failure. Decreases in NE uptake activity and NE uptake sites have also been shown to occur in the failing human heart (2, 9, 19). There is a positive correlation between cardiac NE uptake and left ventricular ejection fraction (19).

Studies have linked the reduction of cardiac neuronal uptake of NE to the increased release of NE from the heart and depletion of NE in the failing heart (19). The sympathetic nervous system is activated early after initiation of rapid ventricular pacing (16, 18). This early activation of cardiac sympathetic activity could potentially increase the cardiac synaptic NE concentration to a level sufficient to cause downregulation of NE uptake-1 carrier sites and decreased NE uptake activity within 2 wk. Direct support of a pivotal role of NE on cardiac sympathetic nerve function was provided using an exogenous NE infusion (15). The dose of NE used
was subhypertensive but was sufficient to increase cardiac interstitial NE to a level similar to that found in heart failure (7, 21). This dose of NE was sufficient to decrease NE uptake activity and reduce neuronal profiles of NE histofluorescence and tyrosine hydroxylase (15). We have also shown that the effects of excess NE on sympathetic nerve terminal function could be prevented by antioxidant vitamins (23a), suggesting that this action of NE probably is mediated via the formation of NE-derived metabolites of oxygen free radicals.

We found a decrease of cardiac adenyl cyclase response to isoproterenol stimulation in rabbits 2 wk after rapid cardiac pacing, followed by a reduction of myocardial β-adrenoceptors at week 4 of pacing. Because the increase in plasma NE precedes the onset of changes in regulatory G proteins and β-adrenoceptor density in the development of heart failure, it has been postulated that the changes in the β-adrenoceptor-coupled G protein adenyl cyclase system are a result of generalized adrenergic stimulation. However, our prior studies in an animal model with right-sided heart failure have shown that myocardial β-adrenoceptor density is reduced only in the failing right ventricle (23). The left ventricle shows no changes in myocardial β-adrenoceptor density despite exposure to the same levels of elevated circulating NE as the right ventricle. Thus the β-adrenoceptor changes would have to be explained by increased local cardiac-derived NE (4), produced by either the preferential sympathetic stimulation to the failing heart, or a defect in the neuronal NE uptake mechanism, or both. A recent study (38) indicates that intact ventricular innervation is essential for the expression of myocardial β-adrenergic subsensitivity during the development of pacing-induced heart failure. We have shown previously that myocardial β-adrenoceptor density correlated inversely with cardiac interstitial NE (7), suggesting localized chamber-specific agonist-induced homologous desensitization in heart failure (14).

Unlike β-adrenoceptor density, the decreases of adenyl cyclase activity in response to isoproterenol, Gpp(NH)p, and forskolin were diminished in early heart failure, occurring within 2 wk of rapid cardiac pacing. The decrease in the response to isoproterenol may be related, at least in part, to the uncoupling of the β-receptors to G proteins, which is known to occur after NE infusion (43). The coupling of β-receptors to G proteins probably is mediated by G protein receptor kinase (33). In addition, rapid cardiac pacing has been shown to reduce Gs protein expression in the left ventricle (33, 34). Changes in Gs proteins, however, have been conflicting. Kiuchi et al. (18) showed an increase in Gα2 protein in heart failure, but other investigators (34, 36) have demonstrated a decrease in Gα2. Roth et al. (36) found that cardiac Gs content did not correlate with adrenergic responsiveness and that the decreased Gs was caused by increased degradation rather than decreased synthesis.

Rabbits are fragile and do not tolerate acute manipulations (such as catheterization) well unless they are adequately anesthetized. Ketamine, midazolam, and xylazine are the preferred parenteral anesthetics compared with pentobarbital (3). We employed ketamine and midazolam to facilitate insertion of a Millar catheter into the left ventricle. Although this anesthetic combination produces minimal cardiorespiratory effects, it increases heart rate in normal animals and humans (17, 24). The increase in heart rate produced by anesthetics might have minimized the difference in heart rate between control and heart failure rabbits and thus obscured the tachycardia known to occur in heart failure.

The majority of NE in the tissue is stored in the adrenergic neuronal terminal vesicles. The cardiac NE content is determined by the rates of NE release, turnover, and synthesis. NE release and turnover are influenced by cardiac sympathetic tone and NE uptake capacity, whereas the rate of NE synthesis is affected largely by tyrosine hydroxylation, which is the rate-limiting step involved in the catecholamine biosynthesis (30). Results of our present study indicate that despite apparent increased sympathetic discharges, cardiac NE content was preserved in an early phase of heart failure, probably because the tyrosine hydroxylation and NE reuptake were relatively intact to compensate for the increased NE discharge. However, as heart failure advanced, both tyrosine hydroxylase and NE uptake-1 carrier site density decreased. As a result, cardiac NE content, as judged by the NE histofluorescence, decreased at 8 wk of rapid cardiac pacing. The relative contributions of the various factors to cardiac NE depletion in heart failure, however, may vary depending on the experimental conditions. An early study in animals showed reduced tyrosine hydroxylase activity played a role in the depletion of

![Fig. 9. Hypothetical cause-and-effect relationship regarding temporal changes in LV function, sympathetic stimulation, NE uptake activity, tyrosine hydroxylase, β-adrenergic sensitivity, β-adrenoceptor density, and NE content in the heart after rapid ventricular pacing.](http://ajpheart.physiology.org/Downloadedfrom)
cardiac NE stores in heart failure (35). On the other hand, Eisenhofer et al. (9) showed that the decreased cardiac NE stores in patients with heart failure was caused by chronically increased NE turnover and reduced efficiency of NE reuptake and storage rather than by insufficient tyrosine hydroxylation.

In conclusion, our present study is the first systematic longitudinal investigation of the temporal alterations in cardiac sympathetic nerve terminal function and its associations with myocardial β-adrenoceptor density in pacing-induced cardiomyopathy. Such a study is important not only because it illustrates that both qualitative and quantitative changes may occur at different stages of heart failure but also because it provides a temporal sequence of events that may allow us to speculate on the mechanisms of changes in early heart failure. Discrepancies in results in the heart failure literature may be related in part by the differences in duration of heart failure studied by the various investigators. Figure 9 summarizes the changes observed in this study, and a hypothesis that we have developed regarding the initial changes in heart failure. It is believed that rapid ventricular pacing causes immediate left ventricular dysfunction and sympathetic nervous system stimulation. There is an early and preferential activation of the cardiac sympathetic nerves. This results in an increase in cardiac interstitial NE concentration. Early in the course of development of heart failure (within the first 2 wk), adenyl cyclase responses to isoproterenol, Gpp(NH)p, and forskolin are reduced, suggesting a defect in the β-receptor coupling mechanism and its associated regulatory G proteins. Myocardial NE uptake activity is also reduced. This impairs the ability of the heart to remove NE in the synaptic cleft, leading to a higher interstitial NE concentration and greater spillover of NE from the heart. By 4 wk of pacing, we found reductions of NE concentration and greater spillover of NE in the synaptic cleft, leading to a higher interstitial NE content. This impairs the ability of the heart to remove NE in the synaptic cleft, leading to a higher interstitial NE concentration and greater spillover of NE from the heart. By 4 wk of pacing, we found reductions of NE concentration and greater spillover of NE from the heart.

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