

Myocardial β -Adrenergic Receptor Function during the Development of Pacing-induced Heart Failure

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Abstract

The development of pacing-induced heart failure was studied in chronically instrumented, conscious dogs paced at a rate of 240 beats/min for 1 d ($n = 6$), 1 wk ($n = 6$), and 3–4 wk ($n = 7$). Left ventricular (LV) dP/dt was decreased ($P < 0.0125$) at 1 d, LV end-diastolic pressure and heart rate were increased ($P < 0.0125$) at 1 wk, but clinical signs of heart failure were only observed after 3–4 wk of pacing. Plasma norepinephrine rose ($P < 0.0125$) after 1 d of pacing, whereas LV norepinephrine was reduced ($P < 0.0125$) only after 3–4 wk of pacing. Both the fraction of β -adrenergic receptors binding agonist with high affinity and adenylyl cyclase activity decreased ($P < 0.0125$) after 1 d of pacing. Total β -adrenergic receptor density was not changed at any time point, but β_1 -adrenergic receptor density was decreased ($P < 0.0125$) after 1 wk. The functional activity of the guanine nucleotide binding protein, G_s , was not reduced, but the $G_{i\alpha 2}$ isoform of the α subunit of the GTP-inhibitory protein rose after 3–4 wk of pacing. Thus, myocardial β -adrenergic signal transduction undergoes change shortly (1 d) after the initiation of pacing, before heart failure develops. The mechanism of β -adrenergic receptor dysfunction in pacing-induced heart failure is characterized initially by elevated plasma levels of catecholamines, uncoupling of β -adrenergic receptors, and a defect in the adenylyl cyclase catalytic unit. Selective down-regulation of β_1 -adrenergic receptors, increases in $G_{i\alpha 2}$, and decreases in myocardial catecholamine levels occur as later events. (*J. Clin. Invest.* 1993. 91:907–914.) Key words: adenylyl cyclase • β_1 -adrenergic receptors • β_2 -adrenergic receptors • congestive heart failure • norepinephrine

Introduction

It is generally acknowledged that heart failure is characterized by several disorders in cardiac autonomic properties (1), including sympathetic (2), parasympathetic (3), and barorecep-

tor responses (4). It is conceivable that part of the mechanism of altered autonomic cardiovascular control and reduced catecholamine responsiveness in heart failure involves changes in end organ responsiveness mediated via β -adrenergic receptors. To understand the mechanism of reduced catecholamine responsiveness in heart failure, several experimental animal models and in vitro studies of failing human ventricular muscle have addressed the question of altered β -adrenergic receptor function. Myocardial β -adrenergic receptor density has been demonstrated to be increased (5, 6), unchanged (7), or decreased (8–11) in experimental animals with heart failure. Bristow and colleagues (12–14) and others (15–17) have consistently shown a reduction in the number of β -adrenergic receptors, particularly of the β_1 subtype (11, 12, 17), in the left ventricle of failing human hearts. Most prior studies have examined the advanced or terminal stages of heart failure, or examined a single component of the β receptor–GTP-stimulatory protein (G_s)¹–adenylyl cyclase pathway, leaving an incomplete picture as to the development of the abnormalities in β -adrenergic signal transduction. It can be argued that understanding the inciting events may even be more important than the terminal events, in that novel therapeutic strategies designed at preventing these derangements may be more effective than strategies designed at reversing the derangements at end stages where other complications have also developed.

To examine potential differences in altered myocardial β -adrenergic receptor mechanisms during the development of heart failure, rapid ventricular pacing was initiated for 1 d, 1 wk, and 3–4 wk during which time progressive severe congestive cardiomyopathy evolved (18, 19). Then cardiac sarcolemma were prepared and used to determine β -adrenergic receptor density, including β_1 and β_2 subpopulations and adenylyl cyclase activity. Furthermore, agonist competition curves were used to quantitate the percentage of G protein-coupled receptors, i.e., those binding agonist with high affinity. Finally, the various guanine nucleotide-binding regulatory proteins that stimulate (G_s) or inhibit (G_i) the catalytic subunit were also measured.

Methods

Surgical instrumentation. 28 adult mongrel dogs of either sex were anesthetized with halothane (0.5–1.5 vol/100 ml in oxygen) and ventilated with a respirator (Harvard Apparatus, S. Natick, MA) after induction with thiethylal sodium (10–15 mg/kg i.v.). A left thoracotomy

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1. **Abbreviations used in this paper:** cyp, cyanopindolol; G_i , GTP-inhibitory protein; $G_{i\alpha 1}$, $G_{i\alpha 2}$, $G_{i\alpha 3}$, isoforms of the α subunit of the GTP-inhibitory protein; Gpp(NH)p, 5'-guanylylimidodiphosphate; G_s , GTP-stimulatory protein; K_H , high-affinity site, K_L , low-affinity site, K_i , dissociation constants; LV, left ventricular.

was performed through the fifth intercostal space using sterile technique. Tygon catheters were placed in the descending thoracic aorta and left atrium. A solid-state miniature pressure transducer was implanted in the apex of the left ventricle. At the time of instrumentation, stainless steel pacing wires were placed on the right ventricle. Experiments were initiated 2–3 wk after recovery from surgical instrumentation. Hemodynamic measurements were made with the dogs fully awake, lying quietly on their right side before pacing was initiated, and at intervals during the pacing protocol, but after a 30-min stabilization period subsequent to deactivation of pacing. Rapid ventricular pacing was induced at a rate of 240 beats/min and controlled using a programmable pacemaker (model EV4543; Pace Medical, Inc., Waltham, MA), which was worn externally in a vest. Animals used in this study were maintained in accordance with guidelines of the Committee on Animals of the Harvard Medical School and the Guide for Care and Use of Laboratory Animals (Department of Health and Human Services Publication No. [National Institutes of Health] 83-23, revised 1985).

Membrane preparation. Six dogs were paced for 1 d, six dogs were paced for 1 wk, and seven dogs were paced for 3–4 wk. The data from dogs after 1 d, 1 wk, and 3–4 wk of pacing were compared with nine sham-operated dogs. The sham-operated control animals all underwent thoracotomy. After the dogs were anesthetized with sodium pentobarbital (20–30 mg/kg), their hearts were immediately excised and placed in iced saline. The left ventricle and septum were weighed, trimmed of fat and connective tissue, minced, and homogenized in 4 ml/gm tissue of buffer I (0.75 M NaCl, 10 mM histidine, pH 7.5) with the Polytron S-20 (Brinkmann Instruments, Inc., Westbury, NY) for 5 s at a setting of 6. The suspension was centrifuged at 14,000 *g* for 20 min. The supernatant was discarded, and the pellet was resuspended in buffer I. The tissue was homogenized and centrifuged two more times as described above. Then the pellet was resuspended in buffer II (10 mM NaHCO₃, 5 mM histidine, pH 8.0), homogenized three times for 30 s with the Polytron at a setting of 6, and centrifuged at 14,000 *g* for 20 min. The pellet was resuspended in Tris buffer (100 mM Tris, 1 mM EGTA, 5 mM MgCl₂, pH 7.2). The suspension was filtered through one layer of Japanese silk screen (size 12). Then the pellet was centrifuged at 1,000 *g* for 15 min and saved at –70°C as the crude membrane fraction. The supernatant from the 14,000-*g* centrifugation was centrifuged at 44,000 *g* for 30 min. The pellet was resuspended in 10 ml of cold deionized water. An equal volume of 2.0 M sucrose was then added. A discontinuous sucrose density gradient was prepared with 0.25, 0.6, and 1.0 M sucrose layers. The tissue homogenate was suspended in 1.0 M sucrose (final concentration) and layered at the bottom of the gradient, and then centrifuged at 170,000 *g* for 85 min. The layer that floated up between the 0.6 and 0.25 M sucrose interface was removed and diluted in deionized water. This suspension was centrifuged at 170,000 *g* for 30 min. The resulting pellet was resuspended in 0.25 M sucrose and 10 mM histidine and stored at –70°C as the “partially purified sarcolemma” fraction (20). All biochemical studies were performed using a crude cardiac sarcolemmal preparation, except G_i immunoblotting, which required both crude and partially purified preparations.

β-Adrenergic receptor antagonist binding studies. β-Adrenergic receptor antagonist binding studies were performed using 25 μl of [¹²⁵I]-cyanopindolol (¹²⁵I-cyp; 0.010–1.0 nM), 25 μl of isoproterenol (100 μM) or Tris buffer, and 100 μl of membrane protein (10 μg per assay). Assays were performed in triplicate, incubated at 37°C for 40 min, terminated by rapid filtration on GF/C filters (Whatman Inc., Clifton, NJ), and counted in a gamma counter (Tracor, Inc., Austin, TX) for 1 min. The binding data were analyzed by the interactive LIGAND program of Munson and Rodbard (21). A linear regression was performed on the amount bound vs. bound/free ligand. An *r*² value of 0.85 or greater was the criterion used for acceptability of the data.

Agonist binding studies. Competitive inhibition agonist binding curves were performed using 85 μl of the crude (10 μg per assay) sarcolemma, 25 μl of [¹²⁵I]-cyp (0.07 nM), 25 μl of isoproterenol (10^{–9} to 5 × 10^{–4} M) with 22 concentrations of isoproterenol, and 15 μl of Tris

buffer. Assays were performed in duplicate, incubated at 37°C for 40 min, terminated by rapid filtration on GF/C filters, and counted in a gamma counter for 1 min. The binding data were analyzed by the LIGAND program (21). In the computer analysis the *F* test was used to compare the best fit for the ligand binding competition data. The best fit, two-site vs. one-site, was determined by the *P* value for the *F* test. When the data were best fit to a single low-affinity site, the number of receptors in the high-affinity state was set to zero.

Adenylyl cyclase activity. Adenylyl cyclase activity was assayed according to the method of Salomon et al. (22) as previously described (23). Cardiac membranes (25–50 μg of protein) were added to a solution containing 1 mM ATP (2–3 × 10⁶ counts/min of [α-³²P]ATP), 20 mM creatine phosphate, creatine phosphokinase (1 U), 1 mM cAMP (2,000–3,000 counts/min of [³H]cAMP as an internal standard), 25 mM Tris, 5 mM MgCl₂, 1 mM EDTA, and the test substance to measure adenylyl cyclase activity [GTP (0.1 mM), isoproterenol (0.1 mM), Gpp(NH)p (0.1 mM), NaF (10 mM), and forskolin (0.1 mM)]. 10 μl of stopping solution (20 mM ATP, 10 mM cAMP, 2% sodium dodecyl sulfate [SDS]) were added to each tube to terminate the reaction, and the tubes were heated on a dry bath at 100°C for 3 min. Cyclic AMP was separated, as previously described (23). Recovery of added cAMP was 40–80%.

G_s reconstitution. G_s functional activity in the solubilized crude myocardial membranes was assayed by a reconstitution protocol using mouse lymphoma S49 *cyc*[–] membranes which are devoid of G_s activity (24), a technique previously employed in our laboratory (25).

Immunoblotting (G_i). G-proteins were quantitated by immunoblotting using rabbit antisera against synthetic peptides that correspond to defined regions of G-proteins as previously described (26, 27). Briefly, the P₄ antisera recognizes the common carboxy-terminal sequence KNNLKDCGLF (345–354 and 346–355) of G_{ia1} and G_{ia2}, isoforms of the α subunit of the GTP-inhibitory protein. The EC2 antisera (DuPont Pharmaceuticals, Wilmington, DE) recognizes the carboxy-terminal sequence KENLKECGLY (346–355) of G_{ia3} but it also cross-reacts with G_o (Amersham Corp., Arlington Heights, IL). The P₆ antisera recognizes the internal amino acid sequence LDRIAQPNYI (160–169) of G_{ib1}. (P₄ and P₆ antisera were provided by Dr. Homcy.) Proteins from crude sarcolemma, partially-purified sarcolemma, and known amounts of recombinant G_{ia2} and G_{ia3} (kindly provided by Drs. L. Birnbaumer and J. Codina, Baylor University, Houston, TX) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide running gel, 4% acrylamide stacking gel) (28). The proteins were electrophoretically transferred onto Westran-polyvinylidene difluoride protein blotting membrane (1 h at 1.5 mA/cm²; Sema-phor Semi-dry Electrophoretic Transfer Apparatus, Integrated Separation Systems, Hyde Park, MA) and incubated in a blocking solution (150 mM NaCl, 8.4 mM Na₂HPO₄, 1.6 mM NaH₂PO₄, 0.1% Tween-20, 5% nonfat dry milk, 0.1% Thimerosal) at 4°C overnight. The membranes were then incubated with specific antisera (1:1,000–1:2,000 dilution) for 2 h, washed four times with blocking solution, and incubated with goat anti-rabbit horseradish peroxidase antibody (1:1,000 dilution) for 45 min, washed four times with the same solutions as above without milk. Autoradiography was performed using the Enhanced Chemiluminescence System (Amersham Corp.) at 23°C for 90 seconds. Autoradiographic densities were determined by two-dimensional laser densitometry (Ultrosan XL Laser Densitometer, LKB Produkter AB, Bromma, Sweden). In order to adjust for variation in autoradiographic density owing to differences in film exposure and transfer efficiency, two identical reference samples were included on each gel to serve as an internal reference. The relative quantities of immunodetectable G_{ia} were calculated as the percentage of the mean of the reference samples which were included on each gel to serve as an internal reference. This method of comparing G-protein levels from different individuals has been used extensively by others (29–31). Values reported are the mean of three or more separate experiments for each membrane sample.

Proportion of β-adrenergic receptor subtypes. Analysis of the percent of β₁- and β₂-adrenergic receptors in a membrane preparation has

been described by Molinoff et al. (32). The percentage of these subtypes was determined by competitive inhibition studies with the nonselective antagonist radioligand ^{125}I -cyp (0.07 nM), with the selective β_1 -antagonist betaxolol, and with the selective β_2 -antagonist ICI-118,551. The concentrations of antagonists tested ranged from 10^{-9} to 5×10^{-4} M. The reaction tubes contained 100 μl of 10 μg membrane homogenate, 25 μl of ^{125}I -cyp (0.07 nM), and 25 μl of the specific β_1 - or β_2 -adrenergic antagonist. The incubation was the same as for antagonist-binding experiments. The high- and low-affinity binding constants (K_H and K_L) and the ratio of high- and low-affinity binding sites were determined by computer modeling as described by Munson and Rodbard (21). In the computer analyses, the variables for the β_1 - and β_2 -specific drugs were allowed to float to a value for the best fit, while the K_D for ^{125}I -cyp was constrained at 0.07 nM. By the F -test comparison, this approach showed the data fit better to a two-site model than to a one-site model.

Tissue and plasma catecholamine levels. Plasma catecholamine samples were taken before pacing and at the time of sacrifice. At the time of sacrifice, left ventricular (LV) tissue was removed. Tissue and plasma catecholamine levels were measured by the radioenzymatic assay of Peuler and Johnson (33).

Sodium, potassium-ATPase activity. Sodium, potassium-ATPase activity was determined according to the method of Jones and Besch (34). Protein concentration for each sample was determined by the method of Lowry et al. (35).

Analysis. Data were analyzed using the SAS program on an IBM-PC 386. Data were expressed as mean \pm SEM. For multiple comparisons, Student's t test with Bonferroni (Dunn) correction were used to determine statistical significance of the differences among the four groups (36). Thus, when four comparisons were made, a P value of < 0.0125 was used to determine significance.

Results

Hemodynamics. As shown in Table I, there were no significant differences in baseline hemodynamics before pacing among the four groups studied. As shown in Table II, heart rate and LV end-diastolic pressure began to increase at 1 d, but were not increased significantly until 1 wk and then increased further after 3–4 wk of pacing. Similarly, mean arterial pressure and LV systolic pressure began to decrease at 1 d of pacing, but were not decreased significantly until 1 wk of pacing. In contrast, LV dP/dt was significantly decreased ($P < 0.0125$) at 1 d and was depressed further at 1 and 3–4 wk of pacing. Notably, exertional dyspnea, ascites, and pulmonary and peripheral edema were evident only in dogs studied after 3–4 wk of pacing.

Catecholamine levels. Plasma norepinephrine levels rose ($P < 0.0125$) after 1 d to 552 ± 63 pg/ml from a control level of 197 ± 14 pg/ml and remained elevated after 3–4 wk of pacing

Table I. Baseline Hemodynamics before Pacing in the Four Groups Studied

	Sham operated	1-d pacing	1-wk pacing	3–4-wk pacing
LV systolic pressure (mmHg)	120 \pm 3	123 \pm 7	117 \pm 5	118 \pm 3
LV end-diastolic pressure (mmHg)	6.9 \pm 0.4	6.7 \pm 0.5	7.3 \pm 0.9	7.2 \pm 1.0
LV dP/dt (mmHg/s)	2941 \pm 92	3082 \pm 107	2856 \pm 231	2946 \pm 87
Mean arterial pressure (mmHg)	95 \pm 2	102 \pm 5	92 \pm 5	94 \pm 3
Heart rate (beats/min)	94 \pm 3	88 \pm 3	96 \pm 6	90 \pm 4

Table II. Effects of Pacing on Hemodynamics

	Before pacing	1-d pacing	1-wk pacing	3–4-wk pacing
LV systolic pressure (mmHg)	119 \pm 3	102 \pm 5	94 \pm 6*	99 \pm 4*
LV end-diastolic pressure (mmHg)	7.1 \pm 0.5	9.6 \pm 0.8	15.1 \pm 2.8*	27.7 \pm 2.4*
LV dP/dt (mmHg/s)	2960 \pm 84	1840 \pm 102*	1592 \pm 137*	1422 \pm 123*
Mean arterial pressure (mmHg)	96 \pm 2	84 \pm 4	76 \pm 5*	81 \pm 4*
Heart rate (beats/min)	91 \pm 3	104 \pm 5	119 \pm 3*	123 \pm 6*

* $P < 0.0125$ difference from before pacing.

(611 ± 95 pg/ml). After 1 wk of pacing, plasma norepinephrine was elevated at an intermediate level (321 ± 34 pg/ml) (Fig. 1). Plasma epinephrine levels rose ($P < 0.0125$) after 1 d to 344 ± 23 pg/ml from a control level of 127 ± 13 pg/ml and remained elevated at 338 ± 60 pg/ml at 3–4 wk after pacing. LV tissue norepinephrine levels in dogs after 3–4 wk of pacing were decreased significantly as compared with sham-operated dogs (146 ± 28 vs. 591 ± 34 pg/mg, $P < 0.0125$). However, LV tissue norepinephrine levels in dogs after 1 d (542 ± 80 pg/mg) and 1 wk (624 ± 122 pg/mg) of pacing were similar to those values observed in the sham-operated group (591 ± 34 pg/mg) (Fig. 1). LV tissue epinephrine levels in dogs after 3–4 wk of pacing were also decreased as compared with sham-operated dogs (7 ± 0 vs. 25 ± 3 pg/mg, $P < 0.0125$), but not after 1 d (25 ± 3 pg/mg) and 1 wk (25 ± 5 pg/mg) of pacing.

β -Adrenergic receptors. There were no significant differences in total β -adrenergic receptor density among the four groups (sham-operated, 71 ± 1 ; 1 d, 70 ± 3 ; 1 wk, 73 ± 3 ; 3–4 wk, 71 ± 5 fmol/mg). The K_D for ^{125}I -cyp was also not significantly different among the four groups (sham-operated, 0.07 ± 0.01 ; 1 d, 0.07 ± 0.01 ; 1 wk, 0.07 ± 0.02 ; 3–4 wk, 0.07 ± 0.01 nM).

β_1 - and β_2 -adrenergic receptor subpopulations. To determine the proportion of β_1 - to β_2 -adrenergic receptors, competitive inhibition binding with ^{125}I -cyp and subtype selective antagonists were performed. Computer modeling of the data indicated that a two-site model was preferred for these competition curves (Table III). There were no significant differences in the proportion of β_1 - and β_2 -adrenergic receptors in dogs after 1 d of pacing (β_1 , $65 \pm 4\%$; β_2 , $35 \pm 4\%$) as compared with sham-operated dogs (β_1 , $64 \pm 1\%$; β_2 , $36 \pm 1\%$) despite the fact that plasma catecholamine levels had already increased by 1 d of pacing. However, β_1 -adrenergic receptors were depressed significantly ($P < 0.0125$) in dogs after 1 wk ($42 \pm 3\%$) and 3–4 wk ($44 \pm 2\%$) of pacing compared with sham-operated dogs. Conversely, β_2 -adrenergic receptor density actually increased in dogs after 1 wk ($58 \pm 3\%$) and 3–4 wk ($56 \pm 2\%$) of pacing compared with sham-operated dogs ($36 \pm 1\%$). An example of the shift in betaxolol binding is shown in Fig. 2.

β -Adrenergic receptor agonist binding. Isoproterenol competition curves for the all sham-operated dogs were fitted to a two-site model, i.e., a high-affinity site (K_H), and a low-affinity site (K_L). The K_i for the high-affinity site was 32 ± 11 nM and the K_i for the low-affinity site was 346 ± 56 nM (Table IV). In one of six dogs after 1 d of pacing the agonist binding data was best fit to a single low-affinity site model with a K_i of 236 nM. In one of six dogs after 1 wk of pacing the agonist binding data was best fit to a single low-affinity site model with a K_i of 231

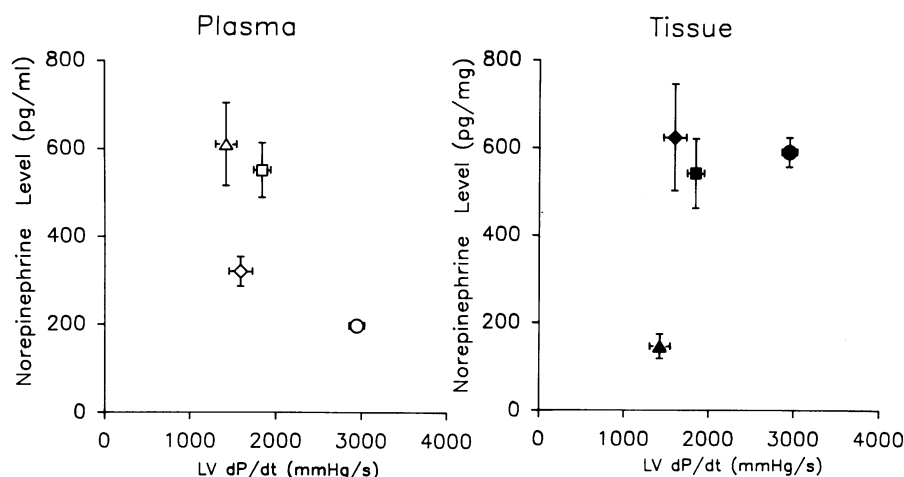


Figure 1. Relationship between LV function and plasma or myocardial norepinephrine content is shown. There was no change in tissue norepinephrine until 3–4 wk after pacing (triangles), even though marked changes in myocardial contractility were observed after 1 d of pacing (squares). Although plasma norepinephrine was elevated at 1 d after pacing, there was not a progressive rise: in fact, plasma catecholamines dipped at 1 wk (diamonds) and then rose further, despite the progressive decline in LV function.

nM. In three of the seven animals with 3–4 wk pacing the agonist binding data was best fit to a single low-affinity site model with a K_i of 236 ± 54 nM. The average percent of β -adrenergic receptors binding agonist with high affinity and low affinity are shown in Table IV. After 1 d of pacing there was a significant loss ($P < 0.0125$) of high-affinity receptor sites (sham-operated, $64 \pm 7\%$; 1 d, $37 \pm 8\%$). These changes were further depressed at 1 and 3–4 wk of pacing ($16 \pm 4\%$ vs. $8 \pm 5\%$). An example of the shift in agonist binding is shown in Fig. 3.

Adenylyl cyclase activity. Adenylyl cyclase activity was significantly depressed ($P < 0.0125$) even after 1 d of pacing and further depressed at 1 and 3–4 wk after pacing. This included progressive decreases in basal and all stimulated activities except for sodium fluoride as shown in Table V. Sodium fluoride-stimulated adenylyl cyclase activity was depressed only at 1 and at 3–4 wk after pacing.

When adenylyl cyclase activity was recalculated with basal activity subtracted, significant decreases in adenylyl cyclase were still observed. However, under these conditions, it was evident that the fall in sodium fluoride-stimulated cyclase activity was less marked and occurred later.

G_s functional levels. The functional activity of G_s was assessed using membranes prepared from *cyc⁻* S49 lymphoma cells that are genetically deficient in G_{sa} . There were no significant differences in the functional activity of G_{sa} after pacing (sham-operated, 5.8 ± 0.4 ; 1 d, 5.5 ± 0.7 ; 1 wk, 6.8 ± 0.4 ; 3–4 wk, 5.1 ± 0.7 pmol cyclic AMP/ μ g per 10 min).

G_i levels. Immunoblotting of crude cardiac membranes and rat brain with the P_4 antisera demonstrated a single 40-kD band in the cardiac preparation and both a 40- and a 41-kD

band in rat brain. With the P_6 (G_{ia1} specific) antisera, there was no band detected in the cardiac preparation and only a single 41-kD band in rat brain. Pretreatment with an excess of the specific internal amino acid sequence of G_{ia1} resulted in loss of the 41-kD band recognized by P_6 . Pretreatment with an excess of the common carboxy-terminal amino acid sequence of G_{ia1} and G_{ia2} resulted in loss of both the 40- and 41-kD bands recognized by P_4 . Together these findings indicate that P_4 is specific for both G_{ia1} and G_{ia2} while P_6 is specific for only G_{ia1} . Therefore, the single 40-kD band recognized by P_4 (and not P_6) in canine myocardium represents only G_{ia2} . The EC/2 antisera recognized a single 40-kD band in crude cardiac membrane and both a 40- and a 39-kD band in rat brain. These data, therefore, suggest that crude canine cardiac tissue contains G_{ia2} and G_{ia3} but minimal, if any, G_{ia1} or G_o . To determine the relative amounts of G_{ia2} and G_{ia3} present in our samples, standard curves were generated by loading increasing known amounts of recombinant G_{ia2} and G_{ia3} onto the gel along with samples of partially purified sarcolemma from failing and control dogs. The autoradiographic intensity of specific antibody binding to the samples was then compared to the linear portion of the standard curve. By this method, the amounts of G_{ia2} and G_{ia3} (in partially purified sarcolemma) were 1.62 ± 0.03 and 0.22 ± 0.02 μ g/mg of partially purified sarcolemma. The autoradiographic intensity of antibody binding to the 40-kD protein band identified as G_{ia2} was linearly related to the amount of membrane protein applied to the gel over a range of 5–40 μ g. We, therefore, loaded 20–25 μ g of crude membrane protein per lane in order to determine the relative quantities of G_{ia2} per microgram of crude sarcolemma in the sham-operated dogs, 1-d pacing dogs, and 3–4-wk pacing dogs. Relative to the con-

Table III. Analysis of Subtype-selective Competition Binding Curves

		Sham operated		1-d pacing		1-wk pacing		3–4 wk pacing	
		Fraction	K_i	Fraction	K_i	Fraction	K_i	Fraction	K_i
		%	μ M	%	μ M	%	μ M	%	μ M
Betaxolol	β_1	64 ± 1	0.06 ± 0.01	65 ± 4	0.04 ± 0.01	$42 \pm 3^*$	0.04 ± 0.01	$44 \pm 2^*$	0.08 ± 0.02
	β_2	36 ± 1	67 ± 8	35 ± 4	55 ± 7	$58 \pm 3^*$	61 ± 9	$56 \pm 2^*$	55 ± 16
ICI 118,551	β_1	60 ± 2	11 ± 2	61 ± 4	11 ± 2	$36 \pm 3^*$	14 ± 3	$40 \pm 3^*$	13 ± 1
	β_2	40 ± 2	0.22 ± 0.06	39 ± 4	0.22 ± 0.06	$64 \pm 3^*$	0.26 ± 0.02	$60 \pm 3^*$	0.28 ± 0.04

* $P < 0.0125$ difference from sham-operated group.

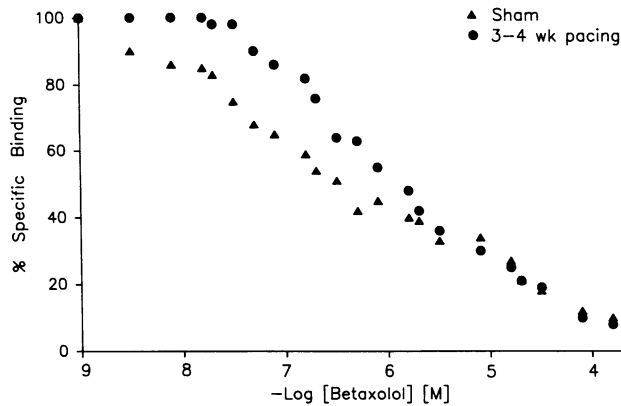


Figure 2. ^{125}I -cyp (0.07 nM) with increasing concentrations of betaxolol is shown for the left ventricle from a sham-operated (\blacktriangle) and an animal after 3–4 wk of pacing (\bullet). The curve from the 3–4-wk pacing animal is shifted to the right, indicating a reduction in β_1 -adrenergic receptors.

rol hearts, the level of $G_{\text{ia}2}$ increased by $44 \pm 6\%$ ($P < 0.017$) in pacing-induced heart failure (Fig. 4). Owing to the low levels of $G_{\text{ia}3}$ present in crude cardiac sarcolemmal preparation, a similar experiment to determine the effect of pacing-induced heart failure on $G_{\text{ia}3}$ could not be performed.

Sodium, potassium-ATPase. As a measure of the consistency of membrane preparations, the sarcolemmal marker, sodium, potassium-ATPase, was measured in the crude preparation and was not found to be significantly different among the four groups, i.e., 2.83 ± 0.15 in sham-operated dogs, 2.75 ± 0.24 in dogs after 1 d of pacing, 2.85 ± 0.34 in dogs after 1 wk of pacing, and 2.97 ± 0.23 $\mu\text{mol P}_i/\text{h}$ per mg in dogs after 3–4 wk of pacing.

Discussion

Many investigators have shown that heart failure is characterized by elevated plasma catecholamine levels (37–40) and a reduction in myocardial catecholamine stores (41, 42) and that these measures correlate closely with the degree of LV dysfunction (37–39). These concepts have been derived primarily from clinical studies, in which the patients already had well-developed heart failure. The data from the current investigation are in agreement with these concepts only in general terms. Interestingly, we observed no change in tissue catechol-

Table IV. Agonist Binding Studies

	Sham operated	1-d pacing	1-wk pacing	3–4-wk pacing
Isoproterenol binding affinity (nM)				
K_H	32 ± 11	23 ± 17	20 ± 13	27 ± 20
K_L	346 ± 56	284 ± 38	306 ± 64	339 ± 57
High-affinity receptor sites (%)				
	64 ± 7	$37 \pm 8^*$	$16 \pm 4^*$	$8 \pm 5^*$
Low-affinity receptor sites (%)				
	36 ± 7	$63 \pm 8^*$	$84 \pm 4^*$	$92 \pm 5^*$

* $P < 0.0125$ difference from sham-operated group.

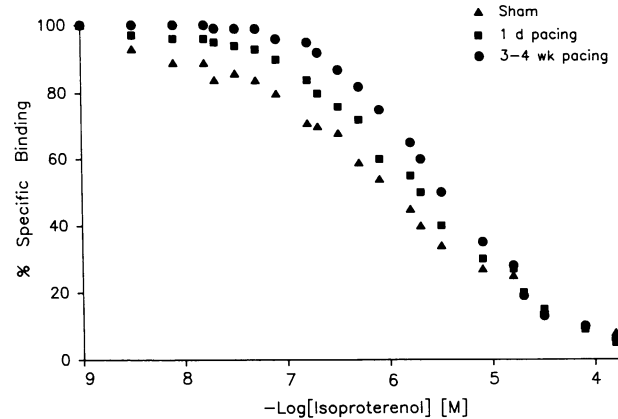


Figure 3. Agonist binding with 0.07 nM ^{125}I -cyp and with increasing concentrations of isoproterenol is shown for the left ventricle from a sham-operated (\blacktriangle), an animal with 1 d of pacing (\blacksquare), and an animal with 3–4 wk of pacing (\bullet). The 1-d and 3–4-wk pacing curves are shifted to the right, indicating a shift toward low-affinity sites.

amines until 3–4 wk after pacing, even though marked changes in myocardial contractility were observed after 1 d of pacing. While plasma catecholamines were elevated at 1 d after pacing, there was not a progressive rise; in fact, plasma catecholamines dipped at 1 wk and then rose further, despite the progressive decline in LV function (Fig. 1). Clearly, increases in circulating catecholamines and decreases in myocardial catecholamines are important markers of heart failure, but because their regulation is linked to a variety of complex uptake and metabolic mechanisms, their levels cannot predict precise levels of cardiac dysfunction. However, it is possible to conclude from these data that the decline in tissue catecholamines is a result rather than a cause of the heart failure process, whereas the elevated plasma catecholamine levels may actually be important in the pathogenesis of heart failure by inducing desensitization (12–17).

In the current investigation, the rise in plasma catecholamines clearly preceded the down-regulation of β_1 -adrenergic receptors by at least 1 wk, suggesting that either classical desensitization mechanisms, which occur almost immediately in vitro (43), are not responsible for the down-regulation of β -adrenergic receptors in heart failure, or that desensitization in the intact organism is more complex (44–46). The complexity may be related to the presence of intact cardiac nerves in vivo. Because of this, the circulating levels of plasma catecholamines

Table V. Adenylyl Cyclase Activity

	Sham operated	1-d pacing	1-wk pacing	3–4 wk pacing
pmol cAMP/mg per min				
Basal	104 ± 4	$74 \pm 10^*$	$69 \pm 5^*$	$49 \pm 2^*$
GTP	147 ± 5	$110 \pm 15^*$	$99 \pm 7^*$	$77 \pm 4^*$
GTP + isoproterenol	257 ± 10	$191 \pm 16^*$	$172 \pm 16^*$	$136 \pm 9^*$
Gpp(NH)p	441 ± 15	$313 \pm 25^*$	$320 \pm 26^*$	$280 \pm 17^*$
NaF	374 ± 20	321 ± 28	$283 \pm 19^*$	$263 \pm 9^*$
Forskolin	795 ± 43	$578 \pm 75^*$	$610 \pm 38^*$	$484 \pm 14^*$

* $P < 0.0125$ difference from sham-operated group.

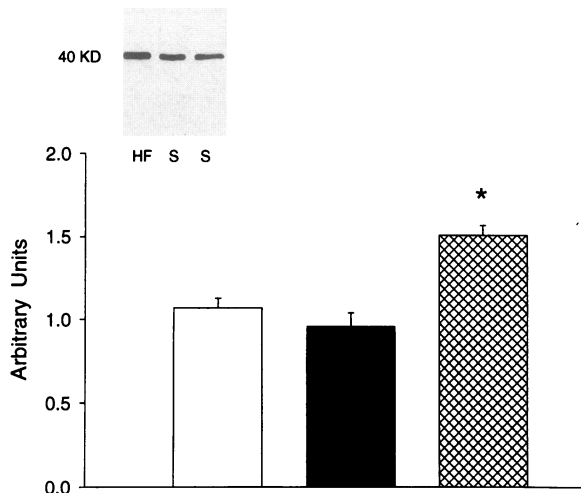


Figure 4. Relative levels of immunodetectable G_{ia2} are compared in sham-operated animals (open bar), 1-d pacing animals (solid bar), and 3-4-wk pacing animals (cross-hatched bar). G_{ia2} levels are expressed in arbitrary units. As denoted by the asterisk, there was a significant increase ($P < 0.017$) in G_{ia2} levels in 3-4-wk pacing animals compared with either sham-operated animals or animals after 1 d of pacing. A representative Western blot is shown in the upper left corner, depicting enhanced expression of the 40-kD band, representing G_{ia2} in membranes from the dogs with 3-4 wk of pacing (HF) compared to two of the sham-operated controls (S).

may provide an insensitive index of cardiac sympathetic activity at the sarcolemmal receptor, in that neural mechanisms may be more important in the regulation of catecholamine concentration at the receptor site. It is well documented that there are several steps in the process of catecholamine-induced desensitization (44-46). The first phase appears to be a rapid uncoupling of the receptor from activation of adenylyl cyclase, which is in keeping with our observations (44-46). Our observation is that the earliest events in the alteration of myocardial β -adrenergic receptor function during the development of pacing-induced heart failure are characterized by uncoupling of the β -adrenergic receptor and reduced adenylyl cyclase activation, rather than β_1 -adrenergic receptor down-regulation.

There are also specific alterations in the responsiveness to both endogenous and exogenous catecholamines, i.e., the sensitivity of the myocardium to β -adrenergic stimulation is reduced (14, 38, 47). To understand the pathogenesis and mechanism of reduced catecholamine responsiveness in heart failure, several experimental animal models and studies of failing human ventricle have addressed the question of altered β -adrenergic receptor function. Studies in human hearts have shown a reduction in the number of β -adrenergic receptors (12-17), particularly of the β_1 subtype (11, 12, 17), from the left ventricle of failing hearts. Recently, considerable attention has been directed to the pacing-induced heart failure model (8, 9), in that it exhibits many features common to human heart failure, e.g., the changes in β -adrenergic receptor signaling in this model approximate those in human studies (12-17). The majority of these studies as well as all of the studies using human myocardium have focused on later stages in the disease process, after congestive heart failure has been established for some time. The primary goal of the present investigation was to examine the initial inciting events in altered β -adrenergic re-

ceptor signaling during the development of heart failure. The pacing-induced heart failure model lends itself to this experimental design since the animals can be studied sequentially as the disease state develops.

The major finding of the present investigation was that alterations in two components of the β -adrenergic receptor-signaling pathway occur as early as 1 d after pacing, as well as elevation of plasma catecholamines. At that time, there were already significant alterations in adenylyl cyclase activity and β -adrenergic receptors binding agonist with high affinity. These changes occurred prior to development of heart failure, i.e., LV end-diastolic pressure had not increased significantly, and there were no clinical signs of congestive heart failure. At this time, LV dP/dt was significantly depressed, and responsiveness to sympathomimetic amines was also significantly attenuated (48). Because all stimulators of adenylyl cyclase were reduced in their ability to activate the enzyme, this suggests that a defect in the catalytic unit of adenylyl cyclase, however, had occurred at this time. In contrast, there was no change in total β -adrenergic receptor density, no change in β_1/β_2 receptor ratio, no change in G_s or G_{ia2} . These data suggest that the initial alterations in β -adrenergic signalling involve a loss of high-affinity β -adrenergic receptors and a defect in the adenylyl cyclase catalytic unit. In that these effects occurred early and intensified with further development of the disease, it is interesting to speculate that these mechanisms may be important in the pathogenesis of autonomic dysfunction in heart failure and possibly even in the pathogenesis of heart failure.

Down-regulation of β -adrenergic receptors has been implicated as the dominant mechanism to account for reduced catecholamine responsiveness in heart failure (12-17). Because inotropic and chronotropic responses to catecholamines are thought to be mediated through β_1 -adrenergic receptor subtype activation, selective down-regulation of β_1 -adrenergic receptors would be required for this mechanism to be operative. This has been observed by Bristow et al. (12). In the current study, no significant change in total β -adrenergic receptor density was observed. However, β_1 -adrenergic receptor down-regulation was observed at 1 and 3-4 wk after pacing. At the time that down-regulation of β_1 -adrenergic receptors was apparent in dogs, after 1 and 3-4 wk of pacing, the β_2 -adrenergic receptor subpopulation density was actually increased in this model. The mechanism of the increase in β_2 -adrenergic receptors is not known. It remains to be determined if these additional β_2 -adrenergic receptors are located on myocytes and are functionally coupled and subtend a physiologic action in heart failure. In this regard, Bristow et al. (12) have shown in isolated right ventricular strips from patients with heart failure, that the β_1 selective agonist denopamine was ineffective in inducing positive inotropic effects, whereas a selective β_2 -adrenergic receptor agonist retained substantial inotropic activity. Murphree and Saffitz (49) found that down-regulation of β -adrenergic receptors was due primarily to a selective reduction of β receptors of subendocardial myocytes. Perhaps if the transmural distribution had been assessed in the current study, greater differences would have been observed subendocardially.

Another mechanism by which catecholamine responsiveness can be reduced in heart failure may involve the coupling between β -adrenergic receptors and G proteins. It is the high affinity form of the receptor that is functionally coupled to the GTP-stimulatory protein, G_s , and therefore, represents the

most physiological relevant form of the receptor (50, 51). Coupling of the β -adrenergic receptor can be evaluated utilizing agonist binding techniques (6). A prior study from our laboratory (6) and others (11) demonstrated uncoupling of the β -adrenergic receptor in heart failure with chronic pressure or volume-overload hypertrophy. However, β -adrenergic receptor coupling has not been assessed in other models of heart failure. A major finding of the present investigation was that the number of β -adrenergic receptors in the high-affinity state decreased progressively in dogs following 1 d, 1 wk, and 3–4 wk of pacing. This appears to be one of the earliest abnormalities in the β -adrenergic receptor signaling pathway that occurs during the development of heart failure. The changes in the fraction of β -adrenergic receptors binding agonist with high affinity may be related to the degree of blunted responsiveness to catecholamines as well as to the eventual reduction in myocardial β -adrenergic receptor density. Although prior studies did not assess the relationship between the degree of heart failure and agonist binding, Fowler et al. (52) noted that human myocardial β -adrenergic receptor down-regulation is related to the degree of heart failure, and is associated with specific impairment of the β -agonist-mediated contractile response.

Furthermore, our data indicate that adenylyl cyclase activity whether stimulated via the β -receptor (isoproterenol), G protein (Gpp[NH]p), or more directly at the catalytic unit itself (forskolin), began to decrease in dogs after 1 d of pacing, and decreased progressively at 1 wk, and 3–4 wk of pacing. This suggests a defect in the catalytic unit of adenylyl cyclase, which was also observed by Calderone et al. (8) and Marzo et al. (9). Bristow et al. (53), and others (10, 11, 15) have also documented that adenylyl cyclase activity is reduced in response to activation by various pharmacological agents.

The current study did not demonstrate reduced G_s levels in the sarcolemma of animals with pacing-induced heart failure. Feldman et al. (31) also found no reduction in G_s levels in failing human myocardium either by the ADP-ribosylation or by reconstitution assays. Horn et al. (54) have reported that G_s was reduced in the lymphocytes of patients with heart failure using cholera toxin-stimulated labeling, and documented that patients who have a low ratio of G_s to G_i in peripheral lymphocytes have more severe hemodynamic abnormality and a worse one year prognosis than patients with a higher ratio of G_s to G_i (55).

Whereas the functional activity of G_s was unchanged after pacing, an increase in $G_{i\alpha 2}$ as measured by immunoblotting after 3–4 wk of pacing was observed in the present study. Calderone et al. (8) demonstrated that G_i as measured by ADP-ribosylation with pertussis toxin was decreased in pacing-induced heart failure. In contrast, Marzo et al. (9) showed an increase in G_i level by both ADP-ribosylation with pertussis toxin and immunoblotting in the same model. Most studies in human heart failure, e.g., those of Feldman et al. (31) and Böhm et al. (56), Eschenhagen et al. (57) have also demonstrated an increase of G_i . Thus, our data, as well as those of Marzo et al. (9) are consistent with the human data (31, 56, 57). We cannot explain why Calderone et al. (8) found a decrease in G_i . However, it is worth noting that except for α_1 -adrenergic receptors, all membrane markers including sodium, potassium-ATPase are reduced in that study (8), which differs from the results obtained in all other studies.

Prior studies (31, 56) have suggested that in the late stages of heart failure an increase in the level of G_i may be responsible

for depressed basal and Gpp(NH)p-stimulated adenylyl cyclase activity. Although we found that isoproterenol-stimulated adenylyl cyclase was depressed at 1 d, $G_{i\alpha 2}$ was not elevated at this point. Therefore, it is unlikely that the increase in G_i is solely responsible for the depressed adenylyl cyclase, and alterations in the catalytic unit of adenylyl cyclase still appear to play an important role. Interestingly, recent studies by Ishikawa et al. (58) indicate that the content of adenylyl cyclase isoforms are altered during the development of heart failure.

In summary, the process of β -adrenergic receptor dysfunction in pacing-induced heart failure involves a complex mechanism associated with a reduction in adenylyl cyclase activity, loss of high affinity cardiac β -adrenergic receptors, and a selective down-regulation of β_1 -adrenergic receptors. These changes are not static but are dynamic and progressive. Increases in plasma catecholamines, uncoupling of the β -adrenergic receptor and a defect in adenylyl cyclase occurs initially, i.e., even after 1 d of pacing, while selective down-regulation of β_1 -adrenergic receptors, and decreases in myocardial catecholamines and increases in $G_{i\alpha 2}$ are later events. Thus, the loss of high-affinity cardiac β -adrenergic receptors appears to be more important than simple down-regulation of receptor number in mediating physiological changes in β -adrenergic receptor function during the initial development of heart failure.

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