Mechanisms of Supersensitivity to Sympathomimetic Amines in the Chronically Denervated Heart of the Conscious Dog

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SUMMARY. Mechanisms of denervation supersensitivity to sympathomimetic amines were studied in conscious animals. Norepinephrine, 0.1 μg/kg, increased left ventricular dP/dt significantly more (4208 ± 828 mm Hg/sec) in dogs with cardiac denervation than in intact dogs (1029 ± 280 mm Hg/sec), P < 0.01, whereas responses to isoproterenol were similar in both groups. Denervation supersensitivity to isoproterenol could be demonstrated only after opposing reflex effects were blocked. After ganglionic blockade, norepinephrine still induced 2- to 3-fold greater increases in left ventricular dP/dt and 3- to 7-fold greater increases in heart rate in cardiac-denervated dogs, whereas isoproterenol and prenalterol, not taken up by presynaptic nerve endings, elicited only 40%-50% greater increases in left ventricular dP/dt and heart rate in dogs with cardiac denervation. The density of β-adrenergic receptors ([3H]dihydroalprenolol) was elevated (P < 0.01) in denervated left ventricles (105 ± 6.9 fmol/mg protein, n = 8) compared to normal left ventricles (70 ± 6.3 fmol/mg protein, n = 12). This was accompanied by enhanced isoproterenol-mediated adenylate cyclase activity. However, muscarinic cholinergic receptor density, ([3H]quinuclidinyl benzilate), decreased from control levels of 251 ± 11 fmol/mg protein (n = 7) to 193 ± 14 fmol/mg protein (n = 6). Thus, chronic cardiac denervation results in up-regulation of the β-adrenergic receptor and down-regulation of the muscarinic receptor. The increased β-adrenergic receptor density and adenylate cyclase stimulation correlated well with the amount of denervation supersensitivity to isoproterenol and prenalterol, but accounted for only a minor fraction of denervation supersensitivity to norepinephrine. The major mechanism of denervation supersensitivity to norepinephrine appears to involve lack of the norepinephrine reuptake. (Circ Res 57: 55-64, 1985)

THE mechanism of denervation supersensitivity has intrigued physiologists for a considerable time (Jackson, 1884; Anderson, 1904; Meltzer and Auer, 1904; Hampel, 1935; Cannon, 1939), and has been ascribed to the absence of norepinephrine uptake (Trendelenburg, 1966; Dempsey and Cooper, 1968; Nadeau et al., 1971), and to a post-synaptic mechanism, such as increased density of β-adrenergic receptors (Glaubiger et al., 1978; Nomura et al., 1980). An understanding of the mechanisms controlling the function of the denervated heart has taken on new significance because of the greater clinical utilization of cardiac transplantation with the advent of improved immunosuppressive therapy. In addition, it has been shown by Barber et al. (1982) that transmural myocardial infarction interrupts the sympathetic nerves, resulting in local cardiac denervation.

The present investigation characterizes β-adrenergic receptor regulation of the heart with chronic, surgical denervation, without interrupting the innervation to the remainder of the circulation. First, inotropic (changes in LV dP/dt) and chronotropic (changes in heart rate) responses to the neurotransmitter, norepinephrine, and to isoproterenol and prenalterol, which are not taken up by the presynaptic nerve terminals, were compared in two groups of chronically instrumented conscious dogs, one intact and the other with total surgical cardiac denervation (Randall et al., 1980). A second goal was to determine whether the absence of reflex effects on myocardial function contributes to the mechanism of denervation supersensitivity. To accomplish this, we examined the responses to sympathomimetic amines of conscious animals with reflexes intact and also after ganglionic blockade. These experiments were conducted in conscious animals to avoid the complicating effects of anesthesia and recent surgery, particularly on autonomic control (Vatner, 1978), and also to allow the full effects of denervation supersensitivity to become manifest (Hampel, 1935; Trendelenburg, 1966). The next goal was to determine if the physiological responses could be explained by biochemical changes, specifically, whether there was a commensurate increase in β-adrenergic receptor density, and whether these changes were coupled to alterations in adenylate cyclase activity. A final goal was to determine
whether similar changes occurred in muscarinic receptors in the denervated heart, since effects of chronic parasympathetic denervation have not been examined previously in detail.

Methods

Preparation of the Model

Mongrel dogs of either sex were anesthetized with sodium pentobarbital, 30 mg/kg, and ventilated with a respirator. The pericardium, exposed via a left thoracotomy, was opened, and surgical cardiac denervation was performed according to the method of Randall et al. (1980). The intrapericardial denervation technique consists of (1) section of the ventrolateral cardiac nerve, (2) adventitial stripping of the left superior pulmonary vein and of the right and common pulmonary artery, and (3) section of pericardial reflections in the transverse sinus and around the superior vena cava, as well as ligation and division of theazygos vein. In addition, the ansae subclaviae were divided bilaterally.

Intravascular solid state pressure gauge (P22, Konigsberg Instruments, Inc.) was implanted in the left ventricle via an apical stab wound. Tygon catheters were implanted in the descending thoracic aorta and left atrium. The incision was closed in layers, the pneumothorax was reduced, and the animals were allowed to recover for 2-4 weeks before experimentation. Another group of dogs underwent similar operations but without the surgical cardiac denervation procedure.

Arterial and left atrial pressures were measured by means of the implanted catheters and Statham P23ID strain gauge manometers (Statham Instruments). Left ventricular (LV) pressure was measured with the solid state miniature pressure gauge, which was calibrated in vitro against a mercury manometer and in vivo against the arterial and left atrial pressure measurement devices.

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council [DHAW Publication (NIH) 78-23, revised 1978].

Protocols

At first, the effects of graded bolus injections of norepinephrine (0.01, 0.05, 0.10, 0.20, and 1.00 μg/kg) and isoproterenol (0.01, 0.05, 0.10, and 0.30 μg/kg) on measurements of arterial and LV pressures, LV dP/dt, and heart rate in two groups of conscious dogs (seven intact and five denervated) were examined. It is important to recognize that all reflex mechanisms were operating in intact dogs, but that the efferent reflex control of the heart from arterial baroreceptor afferents was absent in the dogs with cardiac denervation. Accordingly, two other groups of conscious dogs (eight normal and nine denervated) were studied with autonomic reflex effects blocked with hexamethonium bromide 30 mg/kg (referred to as hexamethonium), and atropine, 0.1 mg/kg. Absence of reflex heart rate change in response to changes induced in arterial pressure by phenylephrine and nitroglycerin confirmed the adequacy of the blockade. In these experiments the effects of a selective β1-adrenergic agonist, prenalterol, 4.0 μg/kg, were examined, as well as infusions of isoproterenol and norepinephrine administered on separate days. LV function was allowed to return completely to baseline values before the next dose was administered. Reproducibility was assured by administering the same doses of isoproterenol, prenalterol, and norepinephrine on different days to the same animals at least 3-7 days later. Prenalterol was selected because it is not taken up by presynaptic nerve terminals, and because it is primarily specific to β1-adrenergic receptors, and therefore, does not alter arterial pressure (Manders et al., 1980). To confirm that prenalterol does not have a tyramine-like effect and is not taken up by the nerve terminals, we administered the agonist to intact dogs before and after norepinephrine uptake blockade with desmethylimipramine (DMI), 1.0 mg/kg, which abolished the effects of tyramine, 50 μg/kg. In these experiments, prenalterol increased heart rate (48 beats/min) and LV dP/dt (1757 mm Hg/sec) before DMI, as compared to an increase in heart rate of 39 beats/min and in LV dP/dt of 1309 mm Hg/sec after administration of DMI. These experiments indicate that prenalterol does not act through release of neuronal catecholamines.

On a separate day, 5-minute infusions of isoproterenol 0.1 and 0.2 μg/kg per min, were also studied. However, since isoproterenol (via its β1 vasodilator properties) reduces arterial pressure and could affect the utility of LV dP/dt as an index of the inotropic state, phenylephrine, an α1-adrenergic agonist, was infused simultaneously to maintain arterial pressure at baseline values. Finally, on another day, 5-minute infusions of the neurotransmitter, norepinephrine, 0.02, 0.10, 0.20, 0.40, and 0.60 μg/kg per min, were examined in the presence of hexamethonium and atropine. Prazosin, 1.0 mg/kg, was also administered as a pretreatment in these experiments to prevent norepinephrine-induced α-adrenergic vasoconstriction. This protocol was utilized to allow full expression of the β1 effects of norepinephrine, which was the main focus of the present investigation. In six intact dogs and six with cardiac denervation, blood samples were collected before and during steady state responses to the infusions of norepinephrine, 0.02, 0.10, 0.20, 0.40, and 0.60 μg/kg per min, for later assay of plasma norepinephrine concentration, by the radioenzymatic method of DaPrada and Zurcher (1976). Using the measured levels of plasma norepinephrine, we correlated the responses of LV dP/dt and heart rate with plasma concentrations achieved, as well as with the dose of norepinephrine administered. In addition, norepinephrine (0.20 μg/kg per min) was infused before and after DMI in the presence of hexamethonium, atropine, and prazosin in three intact dogs and three dogs with cardiac denervation.

The data were recorded on a multichannel tape recorder (Honeywell) and played back on a direct-writing oscillograph (Gould-Brush). A cardiograph triggered by the pressure pulse provided instantaneous and continuous recordings of heart rate. Continuous recordings of LV dP/dt were derived from LV pressure signals using an operational amplifier, connected as a differentiator, with a frequency response of 700 Hz. A triangular wave signal was substituted for the pressure signal to calibrate the differentiator directly.

Biochemical Studies

These studies were conducted at least 3 days after the last infusion of isoproterenol, prenalterol, and norepinephrine. After the dogs were anesthetized with sodium pentobarbital, 30 mg/kg, the hearts were excised immediately and placed into iced normal saline. All subsequent...
procedures were carried out at 4°C. Approximately 1 mm of epicardium and endocardium was trimmed with a scissors and discarded. LV myocardium was minced coarsely in buffer A (0.25 M sucrose, 1 mM MgCl₂, and 1 mM K₂HCO₃), and homogenized with a PT-10 ST Polytron (Brinkmann Instruments, Inc.) tissue disruptor. The homogenate was filtered through one layer of Japanese silk screen, size 12, and centrifuged (Sorvall RC-2, DuPont Instruments, DuPont Co.), at 1,000 g for 15 minutes. The supernatant was resuspended at 45,000 g for 15 minutes, and the pellet was resuspended in buffer B (100 mM Tris, 5 mM MgCl₂, 1 mM EDTA, pH 7.2, 4°C), using a glass homogenizer. The homogenization and 45,000 g spin were repeated twice. The pellet was resuspended in buffer B to a protein concentration of 3 mg/ml and stored in liquid nitrogen until assayed. Assays were performed within 8 weeks of storage, at which time, the membranes were again washed in buffer B and centrifuged.

All studies were performed in triplicate. For the determination of β-adrenergic receptor-binding saturation, 100 μl of the cardiac membrane preparation (2–3 mg protein/ml) were incubated at 37°C for 30 minutes with increasing concentrations (1.0–30 nM) of [3H]dihydroalprenolol ([3H]DHA, [3H]NBP) (New England Nuclear), with or without unlabeled d,l-propranolol (10 μM), in a final reaction volume of 150 μl. Isoproterenol (10 μM) was also used instead of propranolol in one series of experiments. Isoproterenol and propranolol inhibited specific [3H]DHA binding identically. A 10-μM concentration of propranolol was required to inhibit specific binding of ligand which competed at the higher concentration of [3H]DHA. After incubation, 150 μl of reaction mixture were filtered rapidly under vacuum onto Whatman GF/C glass fiber filters (Whatman, Inc.). The filters were washed quickly (<10 seconds) three times with 4 ml of buffer B at 4°C. The filters were counted for 5 minutes in 10 ml of Hydrofluor (New England Nuclear) in a Packard Tricarb 300 scintillation counter with a counting efficiency of 45%. Analyses of saturation binding assays were performed according to the method of Scatchard (1949). The data were also analyzed with the iterative curve-fitting program "Ligand" of Munson and Rodbard (1980).

The muscarinic, cholinergic receptor was analyzed as an independent marker, using binding studies with increasing concentrations (0.05–6.0 nM) of [3H]quinuclidinyl benzilate ([3H]QNB) (New England Nuclear), with or without atropine, 1 μM (Yamada et al., 1980). Methacholine, 100 μM, provided results similar to those with atropine. Assay conditions were the same as for the [3H]DHA-binding studies.

For the adenylate cyclase assay, cardiac membranes containing 200–300 μg of protein were incubated for 10 minutes in a shaking water bath at 37°C. The adenylate cyclase assay contained 50 μl of a solution with 1 mM adenosine triphosphate (ATP) (2–3 × 10⁶ counts/min of α-[32P]ATP), 20 mM creatine phosphate, creatine phosphokinase (1 unit), 1 mM cyclic adenosine 3′,5′-monophosphate (cAMP) (2000–3000 counts/min of [3H]cAMP as an internal standard), 25 mM Tris, 5 mM MgCl₂, 1 mM EDTA, the test substance, i.e., 0.1 mM isoproterenol, and 1.0 mM GTP. Ten microliters 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 in a dry bath at 100°C for 3 minutes. [3H]cAMP was separated according to the method of Symon et al. (1974). Recovery of added CAMP was 40–80%. Zero time controls approached background. Maximal adenylyl cyclase activity was assessed by measuring cAMP production in the presence of either 10 mM sodium fluoride or 1 μM Gpp(NH)p plus 0.1 mM isoproterenol.

5′-Nucleotidase (5′-mononucleotide phosphohydrolase) was assayed by the enzyme kinetic method of Arkesteijn (1976). The rate of NAD⁺ formation from coupled reactions involving AMP and 2-oxoglutarate was measured as a decrease in absorbance at 340 μM, which is directly proportional to 5′-nucleotidase activity.

We measured plasma epinephrine and norepinephrine levels in the conscious dogs, and tissue norepinephrine levels attained from samples before the animals were killed, by the method of DaPrada and Zurcher (1976). The myocardial samples were taken from the mid-anterior LV free wall. In some of the denervated hearts, multiple samples were taken from anterior, posterior, and septal areas.

The protein concentrations for each membrane assay were determined by the method of Lowry et al. (1951).

Statistical Analysis

Data were expressed as mean ± SEM. Data were stored in a digital computer (PDP-11/34), and statistical evaluation was performed by Student's t-test for group and paired comparisons (Armitage, 1975).

Results

Confirmation of Cardiac Denervation

The completeness of cardiac denervation was confirmed by three separate procedures, first at operation, then 2–4 weeks later, in the conscious dogs, and finally, after sacrifice. At operation, the cardiac denervation procedure, stimulation of the ansae subclaviae and the vagi failed to alter heart rate or LV dp/dt. In intact conscious dogs, phenylephrine (10 μg/kg) increased mean arterial pressure by 27 ± 2 mm Hg and reduced heart rate by 33 ± 6 beats/min, whereas nitroglycerin (15 μg/kg) reduced mean arterial pressure by 25 ± 2 mm Hg and increased heart rate by 71 ± 7 beats/min. In dogs with cardiac denervation, phenylephrine increased mean arterial pressure by 56 ± 6 mm Hg and did not decrease heart rate, whereas nitroglycerin reduced mean arterial pressure by 34 ± 2 mm Hg and did not change heart rate. After the animals were killed, LV norepinephrine levels were 2.2 ± 1.2 pg/mg wet weight (ww), in the cardiac-denervated group, whereas, in the normal group, LV norepinephrine levels were 575 ± 87 pg/mg ww. Before sacrifice, in dogs with cardiac denervation, plasma levels (pg/ml) of norepinephrine (228 ± 30) and epinephrine (125 ± 28) were similar to plasma levels of norepinephrine (214 ± 36) and epinephrine (69 ± 13) in intact dogs.

Physiological Studies

Experiments in Conscious Dogs with All Reflexes Intact and Denervated Conscious Dogs with Arterial Baroreceptor Afferent Reflexes Intact

Baseline values before administration of sympathomimetic amines were similar in the two groups for
mean arterial, LV systolic and diastolic pressures, and heart rate, whereas LV dP/dt was slightly less, \( P < 0.05 \), in dogs with cardiac denervation. Norepinephrine increased LV dP/dt 3- to 7-fold more, \( P < 0.01 \), in dogs with cardiac denervation (Fig. 1). For example, norepinephrine (0.1 \( \mu \)g/kg) increased LV dP/dt by 3220 ± 557 from 3629 ± 222 mm Hg/sec in normal dogs and by 3118 ± 439 from 3078 ± 170 mm Hg/sec in dogs with cardiac denervation.

Experiments in Conscious Dogs (Normal and Denervated) after Ganglionic Blockade

Baseline values after hexamethonium (30 mg/kg) and atropine (0.1 mg/kg) and before administration of sympathomimetic amines were similar in the two groups, except for heart rate, which was higher in the normal dogs (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Norepinephrine* (0.2 ( \mu )g/kg per min)</th>
<th>Isoproterenol† (0.1 ( \mu )g/kg per min)</th>
<th>Prenalterol‡ (4 ( \mu )g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control ( \Delta )</td>
<td>Control ( \Delta )</td>
<td>Control ( \Delta )</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>121 ± 8.2</td>
<td>122 ± 4.4</td>
<td>138 ± 4.7</td>
</tr>
<tr>
<td>Denervated</td>
<td>103 ± 3.2</td>
<td>98 ± 4.1†</td>
<td>116 ± 3.4†</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Normal</td>
<td>87 ± 3.6</td>
<td>91 ± 4.3</td>
<td>94 ± 5.5</td>
</tr>
<tr>
<td>Denervated</td>
<td>83 ± 4.2</td>
<td>93 ± 4.5</td>
<td>92 ± 6.4</td>
</tr>
<tr>
<td>LV systolic pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>99 ± 3.6</td>
<td>107 ± 4.6</td>
<td>105 ± 4.0</td>
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<tr>
<td>Denervated</td>
<td>100 ± 4.9</td>
<td>112 ± 5.1†</td>
<td>109 ± 5.5</td>
</tr>
<tr>
<td>LV end-diastolic pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>6.8 ± 0.7</td>
<td>7.0 ± 0.4</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>Denervated</td>
<td>7.7 ± 0.6</td>
<td>7.2 ± 0.3</td>
<td>7.4 ± 0.6</td>
</tr>
<tr>
<td>LV dP/dt (mm Hg/sec)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>2530 ± 176</td>
<td>2496 ± 101†</td>
<td>2605 ± 114( |)</td>
</tr>
<tr>
<td>Denervated</td>
<td>2692 ± 151( |)</td>
<td>2673 ± 256†</td>
<td>2899 ± 212( |)</td>
</tr>
</tbody>
</table>

* Normal, \( n = 8 \); denervated, \( n = 9 \).
† Normal, \( n = 6 \); denervated, \( n = 5 \).
‡ Normal, \( n = 8 \); denervated, \( n = 9 \).
Denervated different from normal, \( \| P < 0.01, \| P < 0.05 \).
FIGURE 2. The effects of norepinephrine infusion on increases in heart rate (top) and LV dP/dt (bottom) in the presence of ganglionic, $\alpha_1$-adrenergic, and cholinergic receptor blockades are compared for eight normal animals (circles) and nine animals with cardiac denervation (triangles). Heart rate rose by 3- to 7-fold more, while LV dP/dt rose by 2- to 3-fold more in the dogs with cardiac denervation.

When increases in heart rate and LV dP/dt (Fig. 3) were plotted against the measured plasma levels of norepinephrine, the differences between intact dogs and those with cardiac denervation were similar to those observed when the responses were plotted against the doses of norepinephrine administered (see Fig. 2).

Norepinephrine (0.20 $\mu$g/kg per min) was infused before and after DMI to three intact dogs and three dogs with cardiac denervation. In the three intact dogs, this dose of norepinephrine increased heart rate and LV dP/dt by an average of 12 beats/min and 1787 mm Hg/sec, respectively. After DMI had been administered to these three intact dogs, the same dose of norepinephrine increased heart rate and LV dP/dt by an average of 61 beats/min and 4825 mm Hg/sec, respectively. In the three dogs with cardiac denervation, the same dose of norepinephrine before DMI increased heart rate and LV dP/dt by an average of 67 beats/min and 4414 mm Hg/sec, respectively. After DMI in these three cardiac-denervated dogs, the same dose of norepinephrine increased heart rate and LV dP/dt by an average of 74 beats/min and 5290 mm Hg/sec, respectively.

Prenalterol (4 $\mu$g/kg) in the Presence of Hexamethonium (30 mg/kg) and Atropine (0.1 mg/kg): Prenalterol produced an increase in LV dP/dt in dogs with cardiac denervation that was 41% greater ($P < 0.05$) than that in normal dogs. The increases in heart rate were 24% greater in dogs with cardiac denervation (Fig. 4); however, the differences for heart rate were not significantly different (Table 1). Prenalterol did not alter mean arterial pressure in either group, and reduced LV end-diastolic pressure similarly in both groups.

Responses to Isoproterenol (0.1 and 0.2 $\mu$g/kg per min) in the Presence of Hexamethonium (30 mg/kg), Atropine (0.1 mg/kg), and Phenylephrine: Isoproterenol (0.1 and 0.2 $\mu$g/kg per min) also produced respective 40% and 32% greater increases in LV dP/dt ($P < 0.05$) in dogs with cardiac denervation compared to responses in normal dogs (Fig. 4). In parallel fashion, these doses of isoproterenol increased heart rate 51% and 36% more ($P < 0.05$) in dogs with cardiac denervation. For example, isoproterenol (0.1 $\mu$g/kg per min) increased heart rate by 45 ± 4.7 beats/min and LV dP/dt by 2495 ± 336 mm Hg/sec in normal dogs, and increased heart rate by 68 ± 7.6 beats/min and LV dP/dt by 3501 ± 89 mm Hg/sec in dogs with cardiac denervation. Mean arterial pressure was held constant with phenyleph-
FIGURE 5. β-Adrenergic receptor number (left), as determined by [3H]DHA binding (1.0–30 nM, and receptor affinity (Kd) (middle) and EC50 for isoproterenol-stimulated adenylate cyclase (right) are compared in membrane preparations from 12 intact (open bars) and eight denervated hearts (solid bars). β-Adrenergic receptor density in denervated hearts increased significantly, by 50%, whereas receptor affinity did not differ. The EC50 for isoproterenol-stimulated adenylate cyclase was also different in the membrane preparations from the denervated hearts.

Biochemical Studies

Myocardial β-Adrenergic Receptor-Binding Studies

Saturation plots of [3H]DHA binding demonstrated a significant increase in β-adrenergic receptor concentration in the membranes prepared from the denervated LV. Specific [3H]DHA binding to the myocardial membrane preparation was reversible, stereospecific, and demonstrated the potency order of isoproterenol>epinephrine>norepinephrine (Rockson et al., 1980). [3H]DHA binding to myocardial membranes was saturable, yielding a single component, according to the iterative nonlinear computer analysis of Munson and Rodbard (1980). Typical computer analyses of [3H]DHA binding to normal LV myocardial membranes and LV-denervated membrane preparations are shown in Figure 5. In these experiments, the affinity for [3H]DHA was similar in the LV of the denervated heart (Kd = 6.4 ± 1.1 nM, n = 8), compared to the normal LV preparation (Kd = 5.3 ± 0.8 nM, n = 12). The density of binding sites, as determined by the computer analysis, was significantly greater (P < 0.01) in the denervated LV preparations, compared to the normal membranes (105 ± 6.9 vs. 70 ± 6.3 fmol/mg protein) (Fig. 5). Specific binding was 60% of the total binding. Nonspecific binding was taken into account by Ligand, the Munson and Rodbard (1980) computer program.

To ensure that the increase in β-adrenergic receptor density in the denervated LV membranes did not reflect a difference in the content of plasma membrane, the membrane preparations were assayed to determine the content of another plasma membrane-associated protein uninvolved in receptor ligand binding. The membrane-associated activity of 5'-nucleotidase was similar in normal (116 ± 8.0 mU/mg protein) (n = 8) and in denervated LV preparations (112 ± 10.6 mU/mg protein) (n = 8).

Adenylate Cyclase Studies

β-Adrenergic receptor-mediated stimulation of adenylate cyclase was examined in the membrane preparations from both normal and denervated hearts. There were significant differences in the dose-response curves for isoproterenol-stimulated adenylate cyclase activity in LV-denervated hearts. The EC50 for isoproterenol-stimulated adenylate cyclase for normal hearts was 0.54 ± 0.15 μM, and for denervated hearts was 0.22 ± 0.03 μM (Fig. 5). In contrast, maximally stimulated adenylate cyclase activity was not different between normal and denervated left ventricles, whether stimulation was effected with Gpp(NH)p plus isoproterenol, or with sodium fluoride (Table 2). For example, sodium fluoride stimulation resulted in adenylate cyclase activity of 742 ± 85 pmol/min per mg protein in normal compared with 638 ± 72 pmol/min per mg protein in denervated hearts. With 1 mM Gpp(NH)p plus 0.1 mM isoproterenol, the maximum adenylate cyclase stimulation for the normal left ventricle was 537 ± 70 pmol/min per mg protein, compared with 438 ± 46 pmol/min per mg protein for denervated left ventricles. Control values with 1 mM Gpp(NH)p were 258 ± 32 pmol/min per mg protein for normal left ventricle and 206 ± 25 pmol/min per mg protein for denervated left ventricles.

Muscarinic Cholinergic Receptor-Binding Studies

Specific [3H]QNB binding to the myocardial membranes was reversible, stereospecific, saturable, and best characterized by a single binding site, according to the iterative nonlinear computer analysis of Munson and Rodbard (1980) (Fields et al., 1978). The

| TABLE 2 |
| Adenylate Cyclase Activity |
| cAMP (pmol/min per mg protein) |
| Normal (n = 8) | Cardiac denervation (n = 9) |
| Basal activity | | |
| Isoproterenol (0.1 mM) and GppNHp (1 mM) | | |
| Sodium fluoride (10 mM) | | |
stimulation of sympathetic or parasympathetic nerves, (2) in conscious dogs 2-4 weeks later, by the method of Randall et al. (1980). We confirmed the mammalian model, the dog, by the intrapericardial complete surgical denervation of the heart of a large dog, and observed further (Trendelenburg, Morison, 1934; Hampel, 1935). Trendelenburg and Weiner (1962) demonstrated that treatment with reserpine produced an effect similar to surgical denervation: hyperexcitability from the central nervous system (Jackson, 1884), accumulation of the neurotransmitter "sympathin" within the postsynaptic cell, and increased permeability of the postsynaptic cells (Rosenblueth and Bacq, 1931), or increased affinity of the postsynaptic α-adrenergic mechanisms were absent. Since the alterations induced by reflexes and denervation supersensitivity, which has not been addressed previously is that of altered reflex control of the denervated heart.

Our initial physiological experiments compared, in intact and denervated dogs, the effects of norepinephrine, the neurotransmitter which is taken up by presynaptic nerve terminals (Hertting, 1964a), and isoproterenol, which is not taken up by presynaptic nerve terminals (Hertting, 1964b). In these experiments, we observed marked supersensitivity to norepinephrine, but not to isoproterenol. Dempsey and Cooper (1968) showed increased sensitivity to norepinephrine, but not to isoproterenol, in surgically denervated cat hearts, and Nadeau et al. (1971), who studied the isolated rat heart after denervation with 6-hydroxydopamine, found supersensitivity to norepinephrine, but not to isoproterenol. Williams and Bradley (1983) also found no change in β-adrenergic agonist affinity after reserpine pretreatment. The results of these experiments, as well as ours in the intact, conscious animals, do not support a role for postsynaptic mechanisms, i.e., increased β-adrenergic receptors, in mediating denervation supersensitivity. However, in our investigation, we were able to demonstrate a role for postsynaptic mechanisms after complicating reflex actions were blocked. It must be kept in mind that chronotropic and inotropic responses to isoproterenol in intact, conscious dogs comprise the sum of the direct effects of the drug and the added action induced by stimulating autonomic reflexes. In dogs with cardiac denervation, the latter component was missing, because the efferent arc of the baroreflex is absent. Since the alterations induced by reflexes and in the postsynaptic β-adrenergic mechanisms were similar in magnitude but opposite in direction, it is probable that elimination of these baroreceptor reflex mechanisms offset the positive contribution of augmented β-adrenergic receptor numbers and adenylyl cyclase activity. Thus, the present investigation indicates that one mechanism of denervation supersensitivity which has not been addressed previously is that of altered reflex control of the denervated heart.

To verify this conclusion, experiments were conducted in conscious dogs with reflex effects blocked with hexamethonium. Under these conditions, isoproterenol (0.1 and 0.2 µg/kg) induced, respectively, 40% and 32% greater increases in LV dP/dt and 51% and 36% greater increases in heart rate in cardiac denervated dogs than were obtained in normal dogs, which were also treated with ganglionic blockade. In both groups of dogs, mean arterial pressure was held constant by simultaneous infusion of phenylephrine to counteract the vasodilator effects of β2-adrenergic receptor stimulation. Similar

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Cholinergic muscarinic receptor density (left), as determined by [3H]QNB binding (0.05-6.0 nM), and receptor affinity (Kd) (right) for normal (open bars) and six denervated hearts (solid bars). Cholinergic receptor density in denervated hearts decreased by 20% whereas receptor affinity was unchanged.

Discussion

Claude Bernard (1880) observed that "the excitability of all tissues seems to augment when they are separated from the nervous influence which dominates them." Exaggerated responses of denervated effector organs, particularly the muscles regulating contraction of the pupil, were demonstrated by several of the early investigators (Anderson, 1904; Meltzer and Auer, 1904; Hampel, 1935). Denervation supersensitivity was initially ascribed to many different mechanisms: hyperexcitability from the central nervous system (Jackson, 1884), accumulation of the neurotransmitter "sympathin" within the idle denervated cells (Cannon and Bacq, 1931), lowering of the threshold of the postsynaptic cell to the neurotransmitter (Bacq, 1933), or increased permeability of the postsynaptic cells (Rosenblueth and Bacq, 1931; Hampel, 1935). Trendelenburg and Weiner (1962) demonstrated that treatment with reserpine produced an effect similar to surgical denervation, and observed further (Trendelenburg, 1966) that there were two phases following denervation: (1) the cocaine-like presynaptic component which develops rapidly and completely during the second postoperative day, along with the depletion in norepinephrine tissue stores, and (2) subsequent further increase in sensitivity to norepinephrine secondary to the slow development of a postsynaptic component, which was later identified by Glaubiger et al. (1978).

In the present investigation, we accomplished complete surgical denervation of the heart of a large mammalian model, the dog, by the intrapericardial method of Randall et al. (1980). We confirmed the completeness of denervation (1) at surgery, by observing no heart rate change in response to electrical stimulation of sympathetic or parasympathetic nerves, (2) in conscious dogs 2-4 weeks later, by noting no reflex cardiac acceleration or slowing in response to acute hypotension or hypertension respectively, and (3) after sacrifice of the animals, by measuring a greater than 98% reduction of norepinephrine content in the heart. Thus, the interpretation of the data was not complicated by partial denervation or reinnervation during the course of these experiments.

The affinity levels of the denervated and normal hearts for [3H]QNB were similar (Kd, 0.36 ± 0.03, n = 6 vs. 0.31 ± 0.03 nM, n = 8), whereas cholinergic receptor numbers were decreased in the denervated group (193 ± 14 vs. 251 ± 11 fmol/mg protein) (Fig. 6).
results were observed with prenalterol, a selective β1-adrenergic agonist, which does not reduce arterial pressure by β2-adrenergic stimulation, and consequently does not require simultaneous infusion of phenylephrine. Prenalterol, like isoproterenol, does not undergo uptake by nerve terminals. This was confirmed in our experiments, as we noted similar responses to prenalterol before and after uptake blockade with DMI. Prenalterol, 4 μg/kg, induced a 41% greater increase in LV dP/dt and 24% greater increase in heart rate in denervated dogs, compared to intact dogs. However, the increased heart rate response was not statistically significant. Thus, even though prenalterol is a partial agonist (Hedberg et al., 1980), the augmented inotropic and chronotropic responses to prenalterol (ranging from 30%–50%) in cardiac-denervated dogs were almost identical to those obtained with isoproterenol.

To determine whether the physiological responses were secondary to changes in postsynaptic mechanisms, β-adrenergic receptor density and affinity were measured with [3H]DHA. In these experiments, a 50% increase in receptor density was observed in the cardiac-denervated hearts. An independent membrane marker 5'-nucleotidase demonstrated no differences in the intact and cardiac-denervated hearts, indicating that changes in β-adrenergic receptor density were not due to changes in the membrane preparation. The finding of β-adrenergic receptor up-regulation is consistent with most, but not all, of the prior work in this field, which was conducted in rodents. After treating rats with guanethidine, Glaubiger et al. (1978) found that β-adrenergic receptor number increased significantly, but affinity for the β-adrenergic receptor was unchanged. Similar results were obtained by Tenner et al. (1982) in rabbits after reserpine treatment, by Nomura et al. (1980) and by Lurie et al. (1983) in cats after guanethidine treatment, and by Bobik et al. (1980) and by Kajiyama et al. (1982) in rats after 6-hydroxydopamine treatment. Lurie et al. (1983) found increased β-adrenergic receptor density in rats and rabbits after cardiac transplantation. In contrast, a recent report by Torphy et al. (1982) indicated no change in β-adrenergic receptor density in guinea pigs after chronic reserpine treatment.

The changes in β-adrenergic receptor density found in the present investigation were also reflected in enhanced coupling to adenylate cyclase, since an approximate 2- to 3-fold reduction in the EC50 (50% maximal cyclase stimulation by isoproterenol) was measured (normals, 0.54 ± 0.15 μM vs. cardiac denervated, 0.22 ± 0.03 μM). Based on receptor occupancy theory, a decrease of this magnitude in the EC50 would have been predicted in the setting of a 50% increase in receptor concentration (Stiles et al., 1984). This assumes that the coupling stoichiometry between receptor and effector units is unaltered. Whereas adenylate cyclase kinetics were enhanced by cardiac denervation, maximal responses of adenylate cyclase to isoproterenol plus Gpp(NH)p and to sodium fluoride were not affected by cardiac denervation. The results of previous studies of the coupling of the β-adrenergic receptor to cyclase in the setting of denervation have been controversial. In part, this can be attributed to the fact that prior studies have examined only isolated points in the isoproterenol dose-response curve (Pik et al., 1977; Glaubiger et al., 1978; Chiu, 1979), or a single maximal concentration (Chiu, 1979; Bobik et al., 1980; Lurie et al., 1983). In the present investigation, we systematically evaluated both a complete dose-response curve for isoproterenol-stimulated adenylate cyclase and maximal response, utilizing two different methods of stimulation. Sodium fluoride, which induces maximal stimulation of the catalytic unit of adenylate cyclase, and isoproterenol plus Gpp(NH)p, which act through the β-adrenergic receptor to stimulate adenylate cyclase, induced similar increases in adenylate cyclase in membranes from the intact and denervated hearts.

We found that increases in receptor number and adenylate cyclase activity correlated well with the increases in inotropic and chronotropic responsiveness observed with isoproterenol and prenalterol only after autonomic reflex effects were blocked, but did not approach the magnitude of change observed with norepinephrine. This result is consistent with the concept that most norepinephrine supersensitivity was secondary to loss of the presynaptic reuptake mechanism. For example, norepinephrine (0.2 μg/kg per min) induced a 182% greater increase in LV dP/dt and a 711% greater increase in heart rate in dogs with cardiac denervation than was observed in intact dogs. Experiments in which norepinephrine levels were measured during infusion confirmed that these differences were not due to differences in blood levels of norepinephrine. Previous data which demonstrated similar supersensitivity to norepinephrine after uptake blockade with cocaine (Vatner et al., 1979) support this confirmation. Also, in the present investigation, three intact dogs and three dogs with cardiac denervation were infused with norepinephrine, before and after DMI. Before DMI, the increases in heart rate and LV dP/dt in response to norepinephrine were 5.8- and 2.5-fold greater, respectively, in the dogs with cardiac denervation. After DMI, the responses in three dogs with cardiac denervation to norepinephrine of heart rate and LV dP/dt were only 10% and 21% greater, respectively.

It was also considered that the greater increases in chronotropic response in the denervated hearts could have influenced the inotropic responses through the Bowditch mechanism. However, similar results were obtained whether heart rate was held constant or allowed to vary, as was observed during norepinephrine infusion in animals with cardiac denervation. This confirms earlier studies indicating...
that the Bowditch mechanism does not play an important role under physiological conditions in the conscious animal (Higgins et al., 1973).

The density of muscarinic receptors, measured in the present investigation with [3H]QNB binding, fell significantly in the denervated left ventricle, compared with the intact left ventricle. Thus, vagal denervation did not result in a corresponding increase in receptors, as was observed for β-adrenergic receptor density, but, rather, resulted in a decrease in muscarinic receptor density. However, it is important to note that the parasympathetic denervation is primarily preganglionic (Priola and Spurgeon, 1977), in contrast to the sympathetic denervation, which is primarily postganglionic. The reduction observed in muscarinic receptors could be attributed to a loss of muscarinic receptors located on presynaptic sympathetic nerve endings, as has been suggested by Sharma and Banerjee (1978). The reduction of these muscarinic receptors could be important in modulating responses to sympathomimetic amines at the cellular level (Watanabe et al., 1978; Vatner et al., 1979; Watanabe, 1984). Few data have been published previously on the effects of parasympathetic denervation on muscarinic receptors. There are prior data, however, on the effects on muscarinic receptors using chemical techniques for sympathetic denervation. The results of these studies have been controversial. Previous studies in rats, using chemical sympathectomy with 6-hydroxydopamine or reserpine, have shown increased (Yamada et al., 1980), decreased (Sharma and Banerjee, 1978; Nomura et al., 1979), or no change (Story et al., 1979; Waelbroeck et al.) in cholinergic receptor density with sympathetic denervation.

In summary, these experiments indicate that, in the conscious animal, there are several mechanisms responsible for the phenomenon of denervation supersensitivity. First, due to the absence of sympathetic and parasympathetic nerves, there is no reflex augmentation of the response to isoproterenol in the presence of cardiac denervation. This important mechanism affecting denervation supersensitivity previously has been largely ignored. With isoproterenol-induced hypotension, there are equally effective opposing activities of reflex effects and up-regulation of the β-adrenergic receptor/adenylate cyclase system. Furthermore, by conducting experiments in which reflex effects were blocked, we identified a significant component of the mechanism of cardiac denervation supersensitivity involving up-regulation of β-adrenergic receptors. Under these conditions, β-adrenergic receptor up-regulation results in an augmented response of adenylate cyclase to sympathomimetic amines, and can account entirely for the increased responsiveness to isoproterenol and prenalterol in the denervated model. However, the up-regulation of β-adrenergic receptors was responsible for only a minor fraction of the supersensitivity response to norepinephrine. The predominant mechanism appears to be presynaptic, and relates to the absence of norepinephrine uptake in the nerve terminal. Finally, the concomitant parasympathetic denervation, which is largely preganglionic, did not result in up-regulation of the muscarinic receptor, but the reverse, i.e., muscarinic receptor density was reduced in the left ventricle of the denervated hearts.

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