Myocardial β-Adrenergic Receptor Expression and Signal Transduction After Chronic Volume-Overload Hypertrophy and Circulatory Congestion

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Background. The volume-overload, high-output state induced by aortocaval fistula is unique because it is not generally associated with marked abnormalities of contractile function. Thus, changes in β-adrenergic receptor (βAR) expression should reflect more directly the influence of neurohumoral adrenergic tone, clarifying the manner in which peripheral (neurohumoral) versus primary myocardial factors are operative in decreased βAR-dependent signal transduction.

Methods and Results. We examined the β-adrenergic receptor–responsive adenyl cyclase pathway in hearts from pigs subjected to volume-overload hypertrophy with circulatory congestion. Nine pigs underwent initial pharmacological and hemodynamic studies, and, 5 weeks after aortocaval fistula placement, when signs of circulatory congestion were evident, these measurements were repeated. Biochemical analyses of plasma and myocardium from these animals and seven normal animals were compared. Experimental animals showed signs of circulatory congestion (tachypnea, weight gain, pulmonary rales) within 3–4 weeks of fistula placement. Necropsy showed ascites and biventricular cardiac hypertrophy, but no fibrosis or inflammation was present on histological inspection. Heart rate responsiveness to βAR stimulation was blunted, with $E_{D_{50}}$ for isoproterenol increased 133% ($p<0.001$) after development of circulatory congestion. Biochemical analyses of the βAR-responsive adenyl cyclase pathway showed uniform decreases in βAR number in right atrium, right ventricle, and left ventricle (36–41% decreases, $p<0.005$). Downregulation was selective for β-receptors, and remaining receptors in the right and left ventricles showed low-affinity agonist binding, suggesting an uncoupling from $G_{\beta\gamma}$. All measures of adenyl cyclase activity were diminished significantly in membrane homogenates from the right atrium (mean reduction, 50±10%) and left ventricle (mean reduction, 44±8%) after volume overload. Finally, we found that amounts of cardiac $G_{\beta\gamma}$, as measured in reconstitution assays, were decreased in both the right atrium ($p<0.02$) and the left ventricle ($p<0.01$) of volume-overloaded animals but that levels of pertussis toxin substrate were unchanged.

Conclusions. Biochemical findings occurred in the absence of myocardial inflammation or fibrosis and without pharmacological interventions, suggesting that circulatory congestion, with attendant elevation in plasma norepinephrine, may be a sufficient stimulus to induce such changes. The data are compatible with a catecholamine-driven βAR pathway desensitization. Thus, a primary defect in intrinsic contractile function is not a necessary component for abnormalities of the myocardial βAR-responsive adenyl cyclase pathway. (Circulation 1992;85:269–280)
Over two decades have passed since abnormalities in plasma and myocardial catecholamines in patients with congestive heart failure were described. These observations now have been confirmed in a variety of models of myocardial hypertrophy and heart failure. However, the relations between myocardial β-adrenergic receptor (BAR) number and physiological responsiveness in these models are variable, depending on the species studied, the stress used to produce myocardial hypertrophy and/or heart failure, and the time course of the perturbation. Thus, exact molecular mechanisms linking myocardial hypertrophy and heart failure with altered BAR expression and signal transduction remain elusive.

Another topic in which data are conflicting is how stimulatory (G
S) and inhibitory (G
i) guanosine triphosphate (GTP) binding proteins are altered in myocardial hypertrophy and circulatory congestion. Studies have demonstrated increased levels of per-tussis toxin substrate, suggesting increased G
i in myocardial membranes from hearts explanted from patients with end-stage idiopathic dilated cardiomyopathy; no changes in cardiac G
S were found. In contrast, decreased cardiac G
S was found in association with pressure overload–induced circulatory congestion in dogs.

Although myocardial BAR expression has been examined in humans with mitral valve disease, no previous studies have examined the BAR-responsive adenyl cyclase pathway (BAR, G-proteins, and adenyl cyclase) from hearts subjected to volume-overload hypertrophy with circulatory congestion. The volume-overload, high-output state induced by aortic fistula is unique because it has not generally been associated with abnormalities of contractile function. Thus, changes in BAR expression should reflect more directly the influence of neurohumoral adrenergic activation, clarifying the manner in which peripheral (neurohumoral) versus primary myocardial factors are operative in decreased BAR-dependent signal transduction. In the present study, we determined the effects of chronic volume-overload hypertrophy with circulatory congestion on myocardial BAR number and BAR-stimulated adenyl cyclase activity. Our hypotheses were 1) increased adrenergic activation would promote BAR downregulation and desensitization of BAR-stimulated adenyl cyclase activity, and 2) the amounts of G
S would be decreased in myocardial membranes from animals with circulatory congestion.

Methods

Animals and Surgical Procedures

Experimental animals included nine female pigs (Sus scrofa) weighing 45 ± 23 kg (mean ± 1 SD; range, 15–88 kg). After acclimatization to human handling, animals received ketamine (50 mg/kg i.m.) and atropine sulfate (0.1 mg/kg i.m.) followed by sodium amytal (100 mg/kg i.v.). Animals underwent endotra-
sion equation. Two animals were studied after intravenous administration of hexamethonium bromide (100 mg/kg), atropine sulfate (0.075 mg/kg), and prazosin (0.1 mg/kg) to eliminate variations in reflex autonomic tone and peripheral vasoconstriction known to accompany the congested circulatory state.

Terminal Surgery
Forty-eight to 72 hours after completion of all pharmacological and physiological testing, animals were anesthetized and intubated with prazosin (0.1 mg/kg) and tested for autonomic tone. The heart was removed and placed in iced saline (4°C), and the RA, left atrium, and right ventricular free wall were removed. The right ventricle (RV), LV, and septum were weighed. RA samples were obtained from identical regions in all animals. Transmural samples of LV free wall were taken midway from base to apex near the midportion of the left anterior descending coronary artery. Myocardial samples were rinsed free of blood and frozen (−70°C). Time from heart removal to placing samples in liquid nitrogen was 10–15 minutes.

Membrane Preparation
Frozen (−70°C) transmural samples were powdered in a stainless steel mortar and pestle (also −70°C), placed in Tris buffer, glass-glass homogenized, and contractile proteins were extracted (0.5 mol/l KCl; 20 minutes, 4°C). The pellet of a 45,000g centrifugation was resuspended in buffer, and radioligand binding experiments, adenylyl cyclase studies, and reconstitution studies were performed.

β-Adrenergic Receptor Binding Studies
β-Adrenergic receptors were identified using the radioligand [125I]iodocyanopindolol (ICYP; 5–700 pmol/l) in saturation isotherm experiments conducted on crude membrane preparations as previously described. Determinations of the Kᵢ for iso-proterenol and the proportion of β-receptors in high- or low-affinity states were performed in competition binding experiments by incubating 100 pmol/l ICYP with 10⁻¹⁰–10⁻⁴ mol/l (−)-iso-proterenol with and without 5′-guanylylimidodiphosphate (Gpp[NH]p; 100 μmol/l), a nonhydrolyzable guanosine triphosphate analogue. The proportions of β₁ and β₂ subtype receptors were determined in competition experiments with the highly selective β₁-adrenergic receptor antagonist bisoprolol (10⁻¹⁰–10⁻⁴ mol/l). Protein concentrations were determined by the method of Bradford, and assessment of membrane protein yield per milligram of crude membrane homogenate was performed using the cardiac sarcolemmal membrane marker, K⁺-stimulated p-nitrophenylphosphatase (K⁺-pNP-Pase), after the method of Bers. We performed assays on membranes from experimental animals and control animals side by side to minimize variation in cyclase activity caused by minor changes in assay conditions.

Experiments were conducted to establish that β-receptors were not lost to the supernatant. When LV membranes were prepared (as above), K⁺-pNP-Pase activity, a marker for sarcolemmal membrane, was negligible in the supernatant. We therefore concluded that receptors, if present in the supernatant, must be unassociated with sarcolemmal membrane fragments. To detect solubilized receptors, we dialyzed 10 ml of supernatant from a 45,000g centrifugation with 1.0 l of potassium-deficient Tris buffer overnight at 4°C to reduce KCl concentration, to prevent interference with the receptor-radioligand interaction. Supernatants (0.3 mg/ml) then were used in saturation isotherm experiments (n=2; LV from control and volume-overloaded animals). GF/C filter paper was pretreated with 2% polyethyleneimine to trap solubilized receptors. There was no specifically bound ICYP in the supernatant fraction of control or volume-overloaded animals. Thus, we believe that data obtained from saturation isotherms conducted on the membrane preparation used accounts for all of the β-receptors.

Adenylyl Cyclase Assays
Methods were modified from Salomon. Adenylate cyclase activity was determined in a final volume of 100 μl and the assay mixture included final concentrations of 0.5 mmol/l ATP, 5 mmol/l creatine phosphate, 50 U/ml creatine phosphokinase, 0.1 mmol/l cAMP, 10 mmol/l HEPES buffer (pH 7.4), 2.5 mmol/l MgCl₂, 0.25 mmol/l EDTA, and 0.74 μmol/l [α-³²P]-ATP (800,000 cpm). The following agents were used to stimulate cAMP (cyclic adenosine monophosphate) production (final concentrations): manganese, 10 mmol/l; Gpp[NH]p, 100 μmol/l; isoproterenol, 10 μmol/l; and NaF, 10 mmol/l. Reactions were initiated by adding 120–140 μg of myocardial membrane homogenate to ice-cold reactants, then incubating at 37°C for 15 minutes. We found that cAMP production under these conditions was linear with respect to time and protein concentration, and that 3-isobutyl, 2-methylxanthine (1.0 mmol/l), adenosine deaminase (5 U/ml), or both, had no effect on basal or maximally stimulated cAMP production. Reactions were terminated by adding 0.5 ml of a 0.1 N HCl solution containing 100 μmol/l ATP, 1.4 mmol/l cAMP, [³²P]-cAMP (7,000 cpm), 50 mmol/l Tris-HCl (pH 7.5), and sodium dodecyl sulfate (2%) to each tube, and heated to 100°C for 3 minutes. Volumes then were brought to 1 ml by adding 800 μl of water, and cAMP was fractionated using Dowex-alumina sequential column chromatography. Recovery was 75–92% using these methods with replicate variation less than 10%.

Experiments were conducted to establish that adenylyl cyclase activity is not lost in the supernatant of a 45,000g centrifugation. We found that forskolin-stimulated cAMP production was very low in the supernatant of a 45,000g centrifugation prepared from LV membranes (supernatant, 18 pmol/mg/min; pellet, 541 pmol/mg/min; n=2). Thus, a negligible amount of
adenyl cyclase activity is found in the supernatant fraction of routine membrane preparations.

Assessment of Gs

We modified a reconstitution assay for use with porcine myocardial membranes.18 The capacity of a cholate extract of myocardial membrane homogenate to reconstitute Gs-mediated production of cAMP in membranes of Gs-deficient (cyc-) murine S49 lymphoma cells served as a functional assay for Gs. cyc- cells (strain 94.15.1) were grown at 37°C in Dulbecco's modified Eagle's medium containing 25 mmol/l Na HEPES (pH 7.4) and 10% heat-inactivated horse serum. Plasma membranes were prepared after the methods of Ross,19 using a nitrogen cavitation apparatus to lyse cells by rapid decompression after equilibration for 10 minutes with N2 at 450 psi at 4°C. cyc- membranes were suspended (3 mg/ml) in buffer (20 mmol/l Na HEPES (pH 8.0), 2 mmol/l MgCl2, and 1 mmol/l EDTA). The preparation then was stored at −70°C.

Cardiac membrane homogenates (RA and LV) were suspended (3 mg/ml) in Tris buffer and solubilized in 1% Na cholate for 1 hour at 4°C. The supernatant from a 20,000 g spin (4°C, 30 minutes) was heated (30°C, 10 minutes) to inactivate solubilized catalytic subunit of adenyl cyclase. This extract then was diluted in 0.1% Lubrol PX (in the same buffer) to stabilize Gs. 15 μl of extract or serially diluted extract was then added to 25 μl of lysed cyc- membranes (75 μg of cyc- membrane protein) and agitated (30 minutes, 4°C). To maintain comparable concentrations of protein and detergent, diluted extract mixtures were supplemented with undiluted extract (heated at 100°C for 10 minutes to inactivate Gs). Preactivation was initiated by adding 10 μl of 1.0 mmol/l GTP, 10 μl of 100 mmol/l NaF, and 16.5 μl of reaction buffer, and incubating for 20 minutes at 30°C. Adenylate cyclase activity was assayed by adding [α-32P]-ATP (800,000 cpm) in 23.5 μl of water to each sample tube and incubating for 10 minutes at 30°C. The reaction then was terminated and cAMP production was measured as described above. Reconstitution assays were performed on undiluted extract and several serial dilutions, giving a wide range of protein content. We have found that intrinsic adenyl cyclase activity in extract and cyc-membranes is negligible, and that NaF stimulation of cyc- membranes yields no cAMP production. In preliminary studies, we found cAMP production to be proportional to the amount of extract added (from 1 to 120 μg) and that the rate of cAMP synthesis remains linear with time for 40 minutes. We performed assays on extracts from experimental animals side by side with appropriate control extracts, using the same batch of cyc- membranes to minimize the potential confounding influence of variation in catalytic subunit concentration in cyc- membranes. Data are expressed as picomole of cAMP produced per 10 minutes as a function of membrane protein used for the detergent extraction.

Assessment of Gi

We used pertussis toxin–dependent ADP ribosylation to assess Gi in membrane homogenates from RA and LV. [32P]ADP ribose incorporation into Gi in the presence of pertussis toxin using [32P]NAD substrate (specific activity, 40 Ci/mmole) was modified from the method of Bokoch et al.20 Ten microliters of sarcolemmal membrane protein (1 mg/ml) was incubated for 60 minutes at 30°C with 28 μl of a solution containing 10 mmol/l DTT, 100 mmol/l Tris, 10 mmol/l thymidine, 100 μmol/l GTP, 1.0 mmol/l ATP, 2.5 mmol/l MgCl2, 1.0 mmol/l EDTA, 0.5 mmol/l B-NADP, 1.0 μmol/l [32P]NAD, and 0.2 μg pertussis toxin (pH 8.0). Preactivation of pertussis toxin was not necessary to achieve maximal ribosylation if toxin was added simultaneously with 10 mmol/l DTT. Optimal incubation time and protein and NAD concentrations were determined in preliminary experiments.

Plasma and Tissue Catecholamine Measurements

Blood samples were obtained from animals in the basal state 2–3 weeks after thoracotomy (before aortocaval fistula) and again just before they were killed. Levels of epinephrine and norepinephrine in plasma and myocardium were determined using a previously described sensitive radioenzymatic assay21 and were expressed as catecholamine per milligram of wet tissue.

Histological Examination

LV samples were prepared for light microscopy and stained with hematoxylin and eosin for general histological assessment or with Masson’s trichrome stain to assess degree of fibrosis.

Data Analysis

Data are expressed as mean±1 SD. Specific measurements are compared before and after the volume-overloaded state in each animal using Student’s t test for paired data. The null hypothesis was rejected when probability was less than 0.05 (two-tailed). The Pearson product-moment correlation coefficient (r) is reported as a measure of the strength of association between change in heart rate and logarithm of dose of isoproterenol determined by linear regression analysis. Saturation isotherm experiments underwent Scatchard analysis, and competition binding experiments were analyzed with the nonlinear regression program found in Graphpad (Harvey Motulsky, University of California San Diego). F ratios were used to test whether one- or two-component curves better fit the data, and when equivalent, the simpler model was selected.

Results

Characterization of the Model

Table 1 shows that 4–5 weeks after creation of aortocaval fistula, animals had increased basal heart rate: Control (CON) was 93±10 beats per minute, volume overload (VOL) was 130±20 beats per
minute ($p=0.005$), and LV end-diastolic pressure for CON was 10±2 mm Hg and for VOL was 29±9 mm Hg ($p=0.005$). Signs of circulatory congestion (tachypnea, abnormal weight gain, ascites) were evident in most experimental animals by this time. Pulse pressure was increased after creation of the fistula (CON, 47±10 mm Hg; VOL, 70±21 mm Hg; $p<0.05$). The arterial–mixed venous oxygen content difference was decreased, as expected with high cardiac output in the presence of a left-to-right shunt. Indeed, this value decreased progressively during the study, indicating a sequential increase in cardiac output with CON, 4.1±1.1 vol/100 ml; VOL (1 day after shunt placement), 2.6±0.9 vol/100 ml ($p<0.05$); and VOL (32±6 days after shunt placement), 1.3±0.5 vol/100 ml ($p<0.002$). These data strongly suggest that cardiac function, as reflected by cardiac output, remained supranormal throughout the study despite the presence of circulatory congestion.

At necropsy (37±18 days after fistula), these animals had ascites (mean amount, 552 ml; range, 85–2,000 ml) and large hearts, with all four chambers appearing grossly enlarged. Both right and left ventricular/body weight ratios were increased, confirming biventricular hypertrophy (Table 1). Fistula size (maximal diameter) was 1.2±0.4 cm.

**TABLE 1. Left Ventricular End-Diastolic Pressure, Basal Heart Rate, and Myocardial Hypertrophy**

<table>
<thead>
<tr>
<th></th>
<th>LVEDP (mm Hg)</th>
<th>Basal HR (bpm)</th>
<th>LV/BW (g/kg)</th>
<th>RV/BW (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10±2</td>
<td>93±10</td>
<td>2.8±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>VOL</td>
<td>29±9†</td>
<td>130±20†</td>
<td>3.5±0.5§</td>
<td>1.6±0.8*</td>
</tr>
</tbody>
</table>

*p<0.02, †p<0.005, §p<0.001.

Values represent mean±1 SD (n=6–9). LVEDP, left ventricular end-diastolic pressure; HR, heart rate; bpm, beats per minute; LV/BW, (left ventricle+septum)/body weight; RV/BW, right ventricle/body weight; VOL, volume-overload hypertrophy.

Five to 6 weeks after aortocaval fistulas were created, striking increases in LVEDP and basal HR were observed in association with biventricular enlargement.

**Plasma and Myocardial Catecholamine Content**

Figure 1 shows that 4–5 weeks after creation of aortocaval fistula, animals had increased levels of both plasma epinephrine (CON, 36±30 pg/ml; VOL, 384±255 pg/ml; $p<0.05$) and norepinephrine (CON, 191±133 pg/ml; VOL, 1,054±443 pg/ml; $p<0.01$). In contrast, compared with the control state, myocardial levels of norepinephrine were reduced both in RA (CON, 5,165±1,834 pg/mg; VOL, 1,292±1,034 pg/mg; $p<0.01$) and LV (CON, 2,549±847 pg/mg; VOL, 514±351 pg/mg; $p<0.001$). Levels of myocardial epi-

**Isoproterenol-Stimulated Chronotropic Response**

Figure 2 shows results of dose–response studies examining the ability of graded bolus doses of (−)isoproterenol to increase heart rate. These studies were performed before and after development of circula-

ty congestion in nine animals. Basal heart rate increased (Table 1), but maximal isoproterenol-stim-

**Figure 1. Graph shows radioenzymatic measurement of plasma catecholamine levels. After signs of circulatory conges-

**β-Adrenergic Receptor Binding Studies**

Figure 3 shows the results of saturation isotherm experiments performed in seven animals; serial biopsies of RA and LV were available in five of these animals. The changes in βAR number were consistent among animals so that paired analyses and unpaired analyses were similar; we therefore report unpaired data. βAR number was decreased after aortocaval fistula. The extent of βAR downregulation was 36% in RA (CON, 55±11 fmol/mg; VOL,
TABLE 2. Results From Radioligand Binding Experiments Using Bisoprolol to Compete for ^125^I-Iodocyanopindolol Cyanopindolol Binding Sites in Right Atrial, Left Ventricular, and Right Ventricular Membranes

<table>
<thead>
<tr>
<th></th>
<th>Control (n=7)</th>
<th>VOL (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA (fmol/mg)</td>
<td>29±7</td>
<td>14±5f</td>
</tr>
<tr>
<td>LV (fmol/mg)</td>
<td>40±7</td>
<td>17±7f</td>
</tr>
<tr>
<td>RV (fmol/mg)</td>
<td>44±19</td>
<td>23±6*</td>
</tr>
<tr>
<td>(K_i (nmol/l))</td>
<td>63±53</td>
<td>90±70</td>
</tr>
<tr>
<td>(K_i (pmol/mg/hr))</td>
<td>15±13</td>
<td>47±80</td>
</tr>
<tr>
<td>(K_i (mmol/l/hr))</td>
<td>90±91</td>
<td>103±63</td>
</tr>
<tr>
<td>(K_i (fmol/mg))</td>
<td>14±5f</td>
<td>47±80</td>
</tr>
<tr>
<td>(K_i (pmol/mg/hr))</td>
<td>17±7f</td>
<td>46±33</td>
</tr>
<tr>
<td>(K_i (fmol/mg/hr))</td>
<td>23±6*</td>
<td>220±90</td>
</tr>
</tbody>
</table>

*p<0.02, f<p<0.005 (vs. control).

VOL, Volume-overloaded animals; \(K_i\), dissociation constant for high-affinity competition (\(\beta_1\)-receptors); \(K_i\), dissociation constant for low-affinity competition (\(\beta_2\)-receptors); RA, right atrium; LV, left ventricle; RV, right ventricle.

35±12 fmol/mg; \(p<0.005\), 38% in RV (CON, 56±14 fmol/mg; VOL, 35±5 fmol/mg; \(p<0.005\)), and 41% in LV (CON, 51±12 fmol/mg; VOL, 30±9 fmol/mg; \(p<0.001\)). Data shown were obtained from a mean of two saturation isotherms per tissue per animal, performed with triplicate points for each of eight concentrations of ICYP. \(K_d\) for ICYP was invariant with volume-overload hypertrophy in membranes from RA (CON, 118±56 pmol/l; VOL, 76±16 pmol/l), RV (CON, 45±41 pmol/l; VOL, 62±23 pmol/l), and LV (CON, 62±22 pmol/l; VOL, 51±21 pmol/l). Mean \(r^2\) values for the Scatchard analyses were 0.97±0.03.

Because heart weight/body weight ratios were increased in volume-overloaded animals (Table 1), we used the specific activity of \(K^+\)-pNPPase as a marker for sarcolemmal membrane\(^{15}\) to confirm that expression of receptor binding per milligram of protein was an appropriate means to measure loss of receptors. There was no significant difference in specific activities of this marker between crude membrane homogenates from control and volume-overloaded animals either in RA (CON, 513±28 nmol/mg/hr; VOL, 497±24 nmol/mg/hr) or LV membranes (CON, 461±37 nmol/mg/hr; VOL, 453±33 nmol/mg/hr).

Competitive binding studies with bisoprolol, a selective \(\beta_1\)-antagonist, demonstrated that \(\beta AR\) subtype proportions were different after development of myocardial hypertrophy (Table 2). After volume overload, animals showed a lower proportion of \(\beta_1\) subtype receptors in myocardial membranes from RA, RV, and LV. The number of \(\beta_1\) and \(\beta_2\) receptors was calculated using the proportion of \(\beta AR\) subtypes obtained from bisoprolol competition experiments. In RA, RV, and LV, downregulation

FIGURE 2. Graph shows isoproterenol-stimulated heart rate (HR) change as a function of dose. Data were obtained before and after aortocaval fistula placement in each of nine animals. The dose of isoproterenol required for a 50% maximal heart rate response (\(ED_{50}\); left side of figure) was increased 133%, and the slope of the line relating isoproterenol dose with heart rate change was reduced by 49% (right side of figure) after signs of circulatory congestion had developed. Open bars represent mean values before fistula placement (control); crosshatched bars represent mean values after fistula placement (volume overloaded); error bars denote 1 SD (n=9). Inset: Data from a representative animal, obtained before and after placement of aortocaval fistula. The entire curve was right-shifted after circulatory congestion, documenting decreased heart rate responsiveness to isoproterenol stimulation. Closed circles represent control values, open circles represent values after fistula placement. ISO, isoproterenol; LOG, logarithm (base 10); MCG, microgram; BPM, beats per minute.

FIGURE 3. Graph shows results of saturation isotherms performed on myocardial tissue. After volume overload and circulatory congestion, \(\beta\)-adrenergic receptor number was reduced in right atrium (RA), right ventricle (RV), and left ventricle (LV). Data are expressed in femtomole per milligram of protein. Open bars represent mean values from control animals, crosshatched bars represent mean values from volume-overloaded animals; error bars denote 1 SD (n=7, both groups). \(K^+\)-stimulated \(\beta\)-nitrophenolphosphatase, a sarcolemmal membrane marker, was used to establish that sarcolemmal yield was similar between groups; histological analyses showed no fibrosis or inflammation in myocardial samples from volume-overloaded animals.
occurred among β1-receptors only; β2-receptors were unchanged (Table 2).

Table 3 and Figure 4 summarize data from competitive radioligand binding experiments using (-)-isoproterenol. The affinity of β-receptors for (-)-isoproterenol (with Gpp[NH]p) was similar in control and volume-overloaded RA (CON, Kᵣ=0.6±0.4 μmol/l; VOL, Kᵣ=0.6±0.3 μmol/l; p=NS); RV (CON, 0.3±0.2 μmol/l; VOL, 0.6±0.2 μmol/l; p<0.05), and LV membranes (CON, Kᵣ=0.5±0.4 μmol/l; VOL, Kᵣ=0.5±0.2 μmol/l; p=NS). However, the proportion of high-affinity binding sites in RV and LV membranes, determined by competitive binding with isoproterenol in the absence of Gpp(NH)p, was decreased by volume overload. After circulatory congestion, there were 64% and 73% decreases in the proportion of β-receptors in the high-affinity state in LV and RV, respectively, demonstrating an uncoupling of βAR and Gₛ.

**Adenylyl Cyclase Assays**

βAR-dependent and Gₛ-dependent stimulation of adenylyl cyclase were diminished markedly in RA and LV membranes from pigs after volume-overload hypertrophy and circulatory congestion (Table 4). Mean reduction in cAMP production in RA membranes was 59±10% (range, 43–69%), and mean reduction in LV membranes was 44±8% (range, 34–57%). Stimulation by Mn²⁺, which is thought to reflect catalytic subunit activity, was reduced in volume-overloaded animals, suggesting that the decrement in cAMP production was due, in part, to decreased activity of the catalytic subunit.

**Assessment of Gₛ**

To determine if reduced function of Gₛ contributed to the diminution in adenylyl cyclase activity, we performed reconstitution assays using cholate extracts (Gₛ rich) from RA and LV membranes (Figures 5 and 6). Sodium fluoride stimulation (Gₛ-dependent) of sarcolemmal membrane extracts from volume-overloaded animals was decreased both in RA and in LV membranes, suggesting that Gₛ activity was decreased after the development of myocardial hypertrophy and circulatory congestion. For example, the amount of RA sarcolemmal protein required for 20 pmol/10 minutes of cAMP production (Figure 5) was quite different after the development of circulatory congestion: (CON, 30 μg; VOL, 50 μg). Similarly, the amount of LV sarcolemmal protein required for 60 pmol/10 minutes of cAMP production (Figure 6) was quite different after the development of circulatory congestion (CON, 90 μg; VOL, 120 μg).

**Assessment of Gᵢ**

To determine whether diminution in adenylyl cyclase activity in myocardial membranes from volume-overloaded animals was the result of increased Gᵢ, we performed pertussis toxin–mediated ADP-ribosylation studies to assess Gᵢ in sarcolemmal mem-

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**Table 3. Results From Radioligand Binding Experiments Using (-)-Isoproterenol in the Absence of Guanine Nucleotides to Compete for ¹²⁵I-Iodocyanopindolol Binding Sites**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=7)</th>
<th></th>
<th>VOL (n=7)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>% High</td>
<td>Kᵣ (nmol/l)</td>
<td>Kₐ (μmol/l)</td>
<td>% High</td>
</tr>
<tr>
<td>RA</td>
<td>14±10</td>
<td>5±5</td>
<td>0.8±0.8</td>
<td>7±12</td>
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<tr>
<td>LV</td>
<td>22±5</td>
<td>2±1</td>
<td>0.4±0.4</td>
<td>8±10†</td>
</tr>
<tr>
<td>RV</td>
<td>45±30</td>
<td>31±19</td>
<td>2.6±2.3</td>
<td>12±14†</td>
</tr>
</tbody>
</table>

*tp<0.01, †tp<0.02 (vs. control).

VOL, volume-overloaded animals; Kᵣ, dissociation constant for high-affinity competition (coupled receptors); Kₐ, dissociation constant for low-affinity competition (uncoupled receptors); RA, right atrium; LV, left ventricle; RV, right ventricle.

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**Table 4. Stimulation of Adenylyl Cyclase in Right Atrial and Left Ventricular Membranes**

<table>
<thead>
<tr>
<th></th>
<th>Right atrium</th>
<th>Left ventricle</th>
<th></th>
<th>Right atrium</th>
<th>Left ventricle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>VOL</td>
<td>p</td>
<td>CON</td>
<td>VOL</td>
</tr>
<tr>
<td>Basal</td>
<td>36±4</td>
<td>24±11</td>
<td>&lt;0.10</td>
<td>116±22</td>
<td>68±18</td>
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<td>ISO+Gpp</td>
<td>74±16</td>
<td>23±9</td>
<td>&lt;0.001</td>
<td>663±53</td>
<td>394±139</td>
</tr>
<tr>
<td>Gpp</td>
<td>64±7</td>
<td>25±7</td>
<td>&lt;0.0001</td>
<td>177±49</td>
<td>93±16</td>
</tr>
<tr>
<td>NaF</td>
<td>100±9</td>
<td>57±15</td>
<td>&lt;0.01</td>
<td>201±23</td>
<td>132±38</td>
</tr>
<tr>
<td>FORSK</td>
<td>219±22</td>
<td>88±18</td>
<td>&lt;0.0001</td>
<td>716±58</td>
<td>408±53</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>64±28</td>
<td>20±10</td>
<td>&lt;0.05</td>
<td>69±32</td>
<td>41±5</td>
</tr>
</tbody>
</table>

Membranes from control (n=7) and volume-overloaded animals (n=7) were studied simultaneously, stimulated with β-receptor–dependent and Gₛ-dependent agents, and cyclic adenosine monophosphate (cAMP) production was measured. In all cases, myocardium from volume-overloaded animals showed marked diminution in cAMP production.

Data represent cAMP produced in pmol/min/mg protein±1 SD, and are net values (basal subtracted). p, control vs. volume overload, two-tailed t test; CON, control; VOL, volume overload; ISO, 10 μmol/l isoproterenol; Gpp, 100 μmol/l 5'-guanylylimidodiphosphate; NaF, 10 mmol/l sodium fluoride; FORSK, 100 μmol/l forskolin; Mn²⁺, 10 mmol/l managanese ion.
branes from RA and LV (Figure 7). We found that pertussis toxin–catalyzed incorporation of $[^{32}]$P into sarcolemmal membranes was similar in both RA (CON, 2,414±526 fmol/mg; VOL, 2,039±526 fmol/mg; $p=NS$) and LV (CON, 1,570±390 fmol/mg; VOL, 1,470±369 fmol/mg; $p=NS$). These data suggest that depressed cAMP production was not due to an enhanced inhibition of adenylate cyclase activity by $G_s$.

**Specific Effects of Thoracotomy**

We have shown previously that myocardial $\beta$AR number and heart rate responsiveness to isoproterenol stimulation are not affected by thoracotomy. In the present study, seven animals were used to determine whether adenyl cyclase activity was altered by thoracotomy and instrumentation independently from effects of aortocaval fistula. Three pigs underwent thoracotomy and instrumentation and were killed 50±10 days later (THOR). These animals were compared in side-by-side assays with four additional control animals that were killed without prior thoracotomy or instrumentation (CON-THOR). Adenylyl cyclase activity in LV membrane homogenates from pigs that had undergone thoracotomy alone was not decreased; measurements were as follows: basal (CON-THOR, 87±8 pmol/min/mg; THOR, 87±12 pmol/min/mg); isoproterenol and Gpp[NH]p-stimulated (CON-THOR, 407±49 pmol/min/mg; THOR, 373±208 pmol/min/mg); NaF-stimulated (CON-THOR, 344±18 pmol/min/mg; THOR, 315±141 pmol/min/mg); forskolin-stimulated (CON-THOR, 853±167 pmol/min/mg; THOR, 718±113 pmol/min/mg); Mn$^{2+}$-stimulated (CON-THOR, 79±6 pmol/min/mg; THOR, 73±8 pmol/min/mg). Therefore, differences in $\beta$AR expression and adenyl cyclase activity in this study were not due to the effects of thoracotomy alone.

**Histological Analyses**

Histological analyses of myocardium from volume-overloaded animals did not show inflammatory infiltrates or fibrosis.

**Discussion**

There are four principal findings of this work. First, chronic volume-overload–induced cardiac hypertrophy with circulatory congestion is associated with multichamber myocardial desensitization affecting many elements of the $\beta$AR-dependent adenylate cyclase pathway from initial interaction of agonist with receptor to final physiological response in vivo. These data suggest a catecholamine-driven desensitization of cardiac $\beta$-adrenergic receptors, adenyl cyclase, and cardiac $G_s$. Second, our data imply that abnormalities in myocardial $\beta$AR-dependent signal transduction can occur despite normal contractile function, in the absence of myocardial fibrosis, and without associated pharmacological therapy. These findings indicate that activation of the sympathetic nervous system with enhanced release of endogenous catecholamines is associated with prominent changes in myocardial signal transduction and that these changes do not require precedent abnormalities in myocyte function. Third, downregulation of myocardial $\beta$AR number by volume-overload hypertrophy is selective for the $\beta_1$-receptor subtype and is similar in magnitude in membranes from RA, RV, and LV. These data imply that norepinephrine, released from myocardial adrenergic nerves and abundant in plasma from volume-overloaded animals, may be of greater importance mechanistically in downregula-
tion than epinephrine because norepinephrine would be expected to preferentially downregulate β_{1}-receptors.23 Alternatively, the β_{1}-receptor may be inherently more susceptible to downregulation from either norepinephrine or epinephrine, both of which were significantly elevated. Finally, levels of cardiac G_{s} in RA and LV membranes are decreased after volume-overload–induced hypertrophy with circulatory congestion. In contrast, no change was observed in pertussis toxin–mediated labeling, suggesting that levels of cardiac G_{i} may not be a key component of decreased signal transduction in this model of circulatory congestion.

It is well known that congestive heart failure is associated with significant alterations in sympathetic nervous system function. βAR number and measures of βAR responsiveness are reduced in hearts of patients with severe congestive heart failure.2 βAR downregulation is associated with high levels of serum norepinephrine but with lower myocardial catecholamine levels. Norepinephrine levels are correlated with prognosis and severity of heart failure. Progressive heart failure is associated with increasing levels of plasma catecholamines and decrease in myocardial βAR number, primarily due to selective β_{1}-receptor downregulation.24 More recent studies have demonstrated increased levels of G_{i} in myocardial membranes from hearts explanted from patients with end-stage idiopathic dilated cardiomyopathy,3 suggesting that decremental contractile function may be due, in part, to inhibition of adenylyl cyclase through G_{i}.

Although carefully performed, these clinical studies have certain inherent limitations. For example, they were conducted on patients with heart failure caused by cardiomyopathy or coronary heart disease, entities associated with myocardial fibrosis. Reduced βAR number may have been due in part to fibrosis rather than to an actual decrease in βAR number on cardiac myocytes. In addition, such patients take medications that interact with β-receptors, so that changes in βAR expression may have been due in part to medical therapy. Animal models of myocardial hypertrophy and heart failure can complement clinical studies because they provide a means of circumventing some of these problems.

Several studies in animals have used aortic banding to study the effects of pressure-overload hypertrophy on βAR expression; these studies have yielded quite variable results. Karlner et al25 found increased myocardial βAR number in pressure-overload hypertrophy with associated heart failure in guinea pigs. Vatner et al26 also found upregulation of myocardial βAR number in dogs with pressure-overload hypertrophy from aortic banding. Later studies from their laboratory27 confirmed the finding of βAR upregulation even when animals showed signs of circulatory congestion. Recently, this same group reported that diminution of adenylyl cyclase activity in myocardial membranes from dogs with pressure-overload hypertrophy may be due to decreased levels of G_{s} as assessed by cholera toxin labeling and by reconstitution studies using cells deficient in G_{s}.4 In contrast, other models of pressure-overload hypertrophy, including rats with genetic hypertension28 or renal artery clipping,29 are associated with myocardial βAR downregulation.

Thus, a cohesive picture linking myocardial hypertrophy and heart failure with βAR expression, G-proteins, and adenylyl cyclase activity has not emerged. Pressure-overload hypertrophy can be associated with either βAR upregulation or downregulation, depending on the species used. Heart failure...
in humans appears to be associated with increased $G_s$, whereas the congested circulatory state associated with long-term pressure overload in dogs is associated with decreased $G_s$. Reports of myocardial $\beta$AR upregulation in the presence of increased neurohumoral adrenergic activation\(^{25,26}\) contradicts the widely accepted dogma that receptor number varies inversely with agonist tone. Two conclusions derive from these observations: 1) There may not be a simple relation between myocardial cell surface receptor number and resultant signal transduction, and 2) the mechanisms by which myocardial hypertrophy and heart failure result in altered receptor expression have not yet precisely been determined.

We chose to study pigs because their hearts are sufficiently large to permit biochemical characterization of multiple cardiac chambers and because of the relative ease of obtaining physiological data from conscious animals. The current study shows that in this model, the magnitude of decreased myocardial $\beta$AR number (in multiple chambers), adenyl cyclase activity, and cardiac $G_s$ are remarkably similar.

Although circulatory congestion resulting from volume-overload hypertrophy secondary to aortocaval fistula is fundamentally different from circulatory congestion seen in clinical dilated heart failure, there are striking similarities. For example, plasma catecholamines are elevated, and myocardial norepinephrine levels are decreased in both entities. In both circumstances, the symptoms and signs of circulatory congestion are dominant (tachypnea, ascites, tachycardia), and in both cases, cardiac dilation without striking concentric hypertrophy characterize the anatomic changes of the heart. There are important differences between the two models. First, plasma epinephrine levels are higher in the current model than in clinical dilated heart failure, a feature that may have contributed to changes in myocardial $\beta$AR expression. Second, dilated heart failure is usually irreversible; in contrast, in the aortocaval fistula model, after the fistula is closed, all signs of circulatory congestion disappear.\(^6\) Furthermore, despite circulatory congestion, papillary muscle taken from animals with circulatory congestion caused by aortocaval fistula show entirely normal or increased contraction characteristics in vitro.\(^6-11\) Thus, intrinsic cardiac function seems well preserved despite marked circulatory congestion. It is not until much longer after shunt placement, when cardiac output begins to fall, that small decreases in contractile function have been seen with this model.\(^30,31\) Although we did not measure contractile function per se, cardiac output, as reflected by the basal arterial-mixed venous oxygen content difference, remained high for the duration of the study. Furthermore, it was demonstrated recently that long-term substantial left-to-right shunt in pigs is not associated with abnormalities in left ventricular contractile function.\(^11\)

We found coordinate changes in biochemical and physiological measures of $\beta$AR function. We have used heart rate response to isoproterenol stimula-
tion, a reproducible and relatively easy measurement to obtain, as a means to correlate biochemical measures (particularly studies of RA membranes) with physiological responsiveness in vivo. In previous studies, we determined that directional changes of RA $\beta$AR number (and, by inference, sinoatrial node $\beta$AR number) and isoproterenol-stimulated heart rate responsiveness are not always similar. For example, in exercise-induced RA $\beta$AR downregulation,\(^12\) isoproterenol $ED_{50}$ is, paradoxically, decreased rather than increased.\(^32\) We found that this phenomenon was associated with a significant increase in cardiac $G_s$, suggesting that, under some circumstances, $G_s$ may predict physiological responsiveness better than $\beta$AR number does. The diminution in heart rate responsiveness to isoproterenol stimulation in the current study is striking, affecting maximal response, $ED_{50}$, and slope of the dose-response relation. Biochemical correlates of blunted heart rate responsiveness include decreased $\beta$AR number, decreased cAMP production, and decreased cardiac $G_s$ in RA membranes after volume-overload hypertrophy and circulatory congestion. Thus, in contrast to the exercise model, the current study shows coordinate decreases in both receptor number and cardiac $G_s$, and the physiological impact is a striking diminution in heart rate responsiveness.

Recent work in heart failure has focused on the stimulatory guanine nucleotide regulatory protein $G_s$. This transducing protein, which links $\beta$AR activation with cAMP production, may be a pivotal element in altered signal transduction in heart failure. A major area in which there is conflicting data is how stimulatory ($G_s$) and inhibitory ($G_i$) guanosine triphosphate (GTP) binding proteins are altered in heart failure. Studies have demonstrated increased levels of pertussis toxin substrate, suggesting increased $G_i$ in myocardial membranes from hearts explanted from patients with end-stage idiopathic dilated cardiomyopathy;\(^3\) levels of $G_s$, as assessed by cholera toxin–mediated ADP ribosylation, were unchanged. These data suggest that decrements in contractile function may have been due in part to inhibition of adenylate cyclase through $G_i$. Horn et al\(^33\) described decreased cholera toxin substrate ($G_s$) in lymphocyte membranes prepared from patients with heart failure but found no change in pertussis toxin substrate ($G_i$). It is possible, because cholera toxin–mediated ADP ribosylation depends on many cofactors and is very sensitive to changes in assay conditions, that such methods may be less sensitive than other techniques in detecting altered levels of $G_s$, thus accounting for conflicting data regarding amounts of cardiac $G_s$ in heart failure. In this regard, when $G_s$ is assessed functionally by reconstituting cholate-extracted ($G_s$-rich) cardiac membranes into cells deficient in $G_s$ (the $cyc^+$ mutant S49 lymphoma cell), decreased $G_s$ is found in cardiac tissue from an animal model of circulatory congestion.\(^4,27\)

In the current study, we have used reconstitution assays to assess cardiac $G_s$. Our studies examined
cholate-extracted membranes from both RA and LV through a wide range of sarcolemmal protein amounts (Figures 5 and 6). Preliminary studies using an antibody technique to quantify cardiac G_{i}^{5} indicate that levels of G_{i} were decreased in ventricular membranes from volume-overloaded animals (18.2±1.4 versus 13.5±4.7 pmol/mg, p<0.05). Thus, both immunological and functional measurements of cardiac G_{i} agree: There appears to be a 25–30% decrease in cardiac G_{i} in myocardial membranes from volume-overloaded animals with circulatory congestion. In contrast, we found no change in pertussis toxin–mediated ADP ribosylation in RA or LV membranes from volume-overloaded animals, suggesting that cardiac G_{i} was not altered in this model of circulatory congestion. However, pertussis toxin labeling was performed on tissue without cholate extraction. Recently it was shown that cholate unmasks pertussis toxin substrate, thereby resulting in increased labeling. We had insufficient tissue to repeat these studies using cholate. Nevertheless, we doubt that volume-overload hypertrophy per se would alter extractability of pertussis toxin substrate, so our conclusion regarding cardiac G_{i} would not change. We had insufficient tissue to perform immunoblotting, perhaps a more precise means of quantifying cardiac G_{i}.

In summary, despite normal cardiac muscle function in vitro, volume-overload–induced myocardial hypertrophy is associated with physiological and biochemical abnormalities that mimic clinical dilated heart failure. These features include circulatory congestion, blunted heart rate responsiveness to adrenergic stimulation, elevated plasma norepinephrine, decreased myocardial norepinephrine, and uniform decreases in βAR number, cAMP production, and cardiac G_{i}. The biochemical findings occurred in the setting of normal cardiac histology and in the absence of pharmacological interventions, suggesting that circulatory congestion, with attendant elevation in plasma norepinephrine, may be a sufficient stimulus to induce such changes. The data are compatible with a catecholamine-driven βAR pathway desensitization. Thus, a primary defect in intrinsic contractile function is not a necessary component for abnormalities of the myocardial βAR-responsive adenyl cyclase pathway.

References


KEY WORDS • G-proteins • heart failure • aortocaval fistula • β-adrenergic receptor • adenylate cyclase • desensitization
Myocardial beta-adrenergic receptor expression and signal transduction after chronic volume-overload hypertrophy and circulatory congestion.

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Circulation. 1992;85:269-280
doi: 10.1161/01.CIR.85.1.269

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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