Myocardial β-Adrenergic Adenylate Cyclase Complex in a Canine Model of Chagasic Cardiomyopathy

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Infection of beagles with an opossum-derived strain of Trypanosoma cruzi (Tc-O) results in features of early and chronic chagasic cardiomyopathy, that is, increases in PR interval, atrioventricular block, and frequent ventricular premature contractions, ventricular tachycardia, and decreased left ventricular ejection fraction. These signs are not observed in animals infected with a canine strain of T. cruzi (Tc-D). To understand the biochemical basis for these early cardiac effects, we examined the β-adrenergic adenylate cyclase complex in myocardial membranes prepared from animals infected with either of the two strains. In animals infected with Tc-O (symptomatic), the maximum velocity (Vmax) decreased and concentration of agonist resulting in 50% of Vmax (Kact) increased for isoproterenol-dependent adenylate cyclase activity; in animals infected with Tc-D (asymptomatic), Vmax and Kact for isoproterenol were unchanged from control, uninfected animals. β-Receptor density decreased by 20% in symptomatic animals with no change in affinity, whereas no differences were observed between uninfected and infected asymptomatic animals. A complex pattern of changes was apparent in the guanine nucleotide binding protein, Gα, in the setting of infection. Alterations in cholera toxin-dependent ADP-ribosylation patterns as well as immunochemical detection with anti-Gαi antisera suggested a change in the biochemical nature of the Gα species and not necessarily a physical loss of this protein. Reconstitution of adenylate cyclase activity in cyc-M membranes demonstrated a decrease in hormone-sensitive Gα activity in membranes prepared from symptomatic animals without a change in activity demonstrable in the presence of Gpp(NH)p. Collectively, the results suggest that the depression in β-adrenergic adenylate cyclase activity associated with symptomatic infection of beagles with T. cruzi occurs primarily as a result of changes in the Gα protein complex, most likely resulting in an uncoupling of the β-adrenergic receptor from the Gα protein. (Circulation Research 1991;69:185-195)

An outstanding characteristic of myocardial dysfunction in congestive heart failure is a decline in responsiveness to the inotropic and chronotropic actions of β-adrenergic catecholamines. At a time when the failing heart has become increasingly dependent on adrenergic support to maintain its contractile state, responsiveness to the actions of endogenous and exogenous catecholamines is reduced. The mechanistic basis for the decline in β-adrenergic responsiveness has yet to be established. Experimental models used in this regard cannot clearly distinguish a cause-and-effect relation between the alterations in the β-adrenergic receptor complex and the compromise in myocardial function. This is so because in most cases, analysis of myocardial dysfunction occurs when animals are in frank congestive heart failure. One hypothesis proposed has been that elevations in plasma catecholamines, a consistent feature of human congestive heart failure, may perpetuate a state of adrenergic desensitization, thereby uncoupling β-adrenergic receptors and attenuating their capacity to activate adenylate cyclase. However, levels of plasma and tissue catecholamines are also usually measured late in the expression of congestive heart failure, again...
obscur[ing] their relative contribution to the expression of disease.

We recently have studied myocardial dysfunction in the setting of Chagas’ disease in mice after infection of the host animal with the parasite Trypanosoma cruzi.6 The results indicate that many features of this experimental model are shared in common with other cardiac diseases of microvascular etiology. For example, myocardial pathology in chagasic mice was remarkable for inflammation, fibrosis, and microvascular aneurysmal formation and dilation,7 features commonly observed in the congenital Syrian hamster model of congestive heart disease. Indeed, in both the Syrian hamster and mice infected with T. cruzi, concomitant verapamil administration prevented the expression of disease.8,9 More important, throughout the course of infection and associated cardiac manifestations of Chagas’ disease in mice, we observed profound alterations in the β-adrenergic adenylate cyclase complex, including a decrease in β-adrenergic receptor density and affinity, a decrease in agonist-dependent activation of adenylate cyclase activity, and a decrease in the magnitude of choleragenid pertussis toxin-dependent ADP-ribosylation, a marker for the guanine nucleotide binding proteins that couple the receptor to the effector cyclase unit.6 Similar changes have been observed in other models of cardiac dysfunction and congestive heart failure.4,5 Many of the changes in the β-adrenergic receptor complex predated the appearance of significant cardiac dysfunction, suggesting that such changes may have been important early participants in the development of congestive heart failure. However, the murine model of Chagas’ disease does not permit accurate and subtle determinations of cardiac dysfunction during the development of Chagas’ disease. To circumvent these limitations, in this report, we have used a canine model of Chagas’ disease that includes an additional feature, namely, the use of a strain of T. cruzi that produces parasitemia but no apparent myocardial involvement. Thus, in addition to uninfected controls, we can contrast changes observed in asymptomatic and symptomatic infected animals and identify infection- versus disease-associated alterations in myocardial β-adrenergic adenylate cyclase activity. Furthermore, the model permitted us to evaluate a time point in the evolution of cardiac dysfunction in the absence of congestive heart failure, thereby permitting us to test the hypothesis that changes in the β-adrenergic receptor complex are important participants in the development of congestive heart failure per se.

Materials and Methods

Materials

[125I]iodocyanopindolol (ICYP), [α-32P]ATP, and [3H]cyclic AMP (cAMP) were obtained from New England Nuclear–DuPont, Boston. Gpp(NH)p was obtained from Sigma Chemical Co., St. Louis. Cholera toxin was obtained from Schwartz Mann Laborat-ories, Cambridge, Mass. All other reagents were of the highest purity commercially available.

Dogs

Pure-bred beagles were purchased from Hazleton Research Products, Inc., Denver, Pa. A total of 12 bred beagles was used in the study (age, 15–40 weeks). In each group to be studied, four animals served as controls, four animals were infected with the Tc-O strain of T. cruzi, and four animals were infected with the Tc-D strain of T. cruzi. Dogs were vaccinated as puppies against Bordetella, parainfluenza, parvovirus, distemper virus, hepatitis virus, rabies, and leptospirosis. They were dewormed regularly and had three consecutively negative negative fecal examinations before entering the study. Animals were housed singly in cages in the School of Veterinary Medicine Laboratory Animal Resource Facility for a 4-week acclimatization period before and during the study except for a period each day when they exercised in individual runs. They were fed a commercial dry dog chow.

Parasites and Infection

The T. cruzi isolates were recovered from a dog (Tc-D) and an opossum (Tc-O) and were maintained in our laboratory as previously described.10 Trypomastigotes were maintained in and harvested from African green monkey kidney cells (Vero cells), washed three times in Eagle’s minimum essential media (MEM; GIBCO, Grand Island, N.Y.), counted in a modified Neubraum hemocytometer, and resuspended to a concentration of 10⁷ per milliliter in MEM. Dogs from each group were inoculated subcutaneously between the shoulder blades with 5×10⁴ trypomastigotes per gram body weight up to a maximum dose of 2×10⁷ organisms from either Tc-O or Tc-D, or with MEM (uninfected controls). The quantity of parasites inoculated and the age of the animal at which inoculation was performed were chosen after preliminary studies that indicated these conditions were associated with little or no mortality during the acute phase of the disease, that is, acute Chagas’ disease, which occurs within 30 days of infection.11 Infected animals displayed similar humoral (antibody titers) and cellular (peripheral blood mononuclear cell blastogenesis responses) immune responses to infection.10

Parasitemia

Parasitemia was determined every third day for 30 days (acute period of infection), then every 2 weeks using a quantitative thick smear procedure as described previously.12 When smears became negative, parasitemia was confirmed by finding parasites in dog blood cultured in liver infusion tryptose medium supplemented with 10% fetal calf serum.13

Electrocardiography

The standard 10-lead system for canine electrocardiographic (ECG) analysis using a polygraph record-
ing system (model 79D, Grass Instruments Inc., Quincy, Mass.) was recorded before and approximately every 10 days after infection.\textsuperscript{14} Briefly, three bipolar standard leads (I, II, and III), three augmented unipolar limb leads (aVR, aVL, and aVF) and four unipolar precordial chest leads (CV\textsubscript{3}RL, CV\textsubscript{4}LL, CV\textsubscript{4}LU, and V\textsubscript{6}) were recorded. Nonseparated dogs were placed in right lateral recumbency, and needle electrodes were placed subcutaneously over the olecranon process of the elbow and the patella of the stifle of both legs. For each tracing, the voltage was standardized at 1 mV/cm and a paper speed of 50 mm/sec was used except for an extended lead II strip that was recorded at 25 mm/sec for the purpose of detecting intermittent arrhythmias. ECG measurements were made using standard methods as described previously.\textsuperscript{15}

**Echocardiography**

Echocardiography was performed on a regular basis using a Model 300C two-dimensional and M-mode echocardiogram (Advanced Technology Laboratories, Bellevue, Wash.) and was recorded on an ATL multi-image camera. Observers unaware of the identity or state of the experimental dogs interpreted the measurements of the echocardiographic tracings. Nonseparated dogs were placed in left lateral recumbency, and a transducer (S or 7.5 MHz) was applied directly to the animals’ shaved chests. Cardiac structures were recognized with two-dimensional echocardiograms using the parasternal short-axis, long-axis, and apical four-chamber views before M-mode still-frame radiographs were taken, from which measurements were determined in accordance with the guidelines provided by the Committee on M-mode Standardization, American Society of Echocardiography.\textsuperscript{16}

**Preparation of Myocardial Membranes and Tissue**

Animals were anesthetized with pentobarbital, and their hearts were excised rapidly and immediately placed in ice-cold 0.15 M saline. Within 10 minutes, the hearts were freed of large vessels, atria, fat, and blood and were minced in buffer (0.25 M sucrose, 0.03 M histidine, pH 7.4, with 5 mM EDTA). The ventricular tissue was homogenized with a polytron (Brinkmann Instruments, Inc., Westbury, N.Y.) at 4°C. The resulting crude homogenate was centrifuged at 11,000g for 20 minutes at 4°C. Supernatant was immediately centrifuged at 43,000g for 30 minutes at 4°C. This final pellet was resuspended in the homogenizing buffer without EDTA and stored at −60°C. This membrane preparation was used for all assays performed in this report. Membranes retained adenylate cyclase activity without change for up to 3 months.

**Determination of β-Receptor Sites**

The binding assay used $[^{125}\text{I}]$ICYP. Cyanopindolol was iodinated and purified to a specific activity of 2,200 Ci/mmol according to methods previously published.\textsuperscript{6} Aliquots of myocardial membranes (100–300 μg) were distributed to tubes containing $[^{125}\text{I}]$ICYP in a final volume of 1 ml whole-cell buffer (0.15 M NaCl, 0.01 M Tris, pH 7.5, 0.01 M KCl, and 1 mg/ml bovine serum albumin with 2 mg/ml dextrose). The binding assay was carried out in a shaking water bath for 20 minutes at 37°C. By 20 minutes of incubation under these conditions, we determined that binding by $[^{125}\text{I}]$ICYP had reached saturation (results not shown); values obtained from longer incubation periods (30–60 minutes) were identical to those reported here. The reaction was terminated by filtering the suspension over A/E glass fiber filters (Gelman Sciences Inc., Ann Arbor, Mich.). The filters were washed with 10 ml 0.01 M Tris at room temperature, and radioactivity was determined in an Auto-Gamma scintillation counter (Packard Instrument Co., Meriden, Conn.). Specific binding was defined as the difference between total binding and binding inhibited by (−)-propranolol (0.1 μM). Maximal binding capacity was determined by Scatchard analysis\textsuperscript{6} using the LIGAND binding analysis program.\textsuperscript{17} For determination of isoproterenol displacement curves, binding assays were carried out for 60 minutes at 37°C under otherwise identical reaction conditions.

**Preparation of Cyc− Cells and Reconstitution Assays**

The Cyc− mutant of S49 lymphoma cells were grown as described previously.\textsuperscript{18} On the day of harvesting, cells were homogenized in a 50 mM Tris/0.25 M sucrose buffer containing 5 mM EDTA. Homogenates were spun at 1,000g for 10 minutes at 4°C, the pellet discarded, and the supernatant spun at 30,000g for 30 minutes at 4°C. This pellet was resuspended in 50 mM Tris/0.25 M sucrose and stored at −60°C until further use. For reconstitution assays, 200 μl of crude membrane protein (2 mg/ml) was mixed with 100 μl of a 50 mM Tris/200 mM NaCl buffer containing 0.6% cholate and was incubated over ice, with frequent vortexing. After a 30-minute incubation period, the suspension was centrifuged at 100,000g for 45 minutes; 100 μl of the supernatant was added to 200 μl of cyc− membrane suspension and was incubated over ice with frequent vortexing for 20 minutes, after which aliquots of this material were added directly to an adenylate cyclase assay as described above. Addition of isolated cholate extracts or cyc− membranes to the adenylate cyclase reaction mixture resulted in no isoproterenol- or Gpp(NH)p-dependent adenylate cyclase activity.

**Adenylate Cyclase Assay**

The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 2.5 mM magnesium unless otherwise noted, 0.143 mM ATP, an ATP regenerating system (creatinine phosphate/creatine phosphokinase), ATP ($[^{32}\text{P}]$ATP, 1–2×10$^6$ cpm/assay tube), and 6 mM theophylline in a final volume of 75 μl.\textsuperscript{19} Agents dissolved in 50 mM Tris HCl (pH 7.5) were added in concentrations noted in individual experiments. The adenylate cyclase reaction was terminated by the
addition of an ATP-cAMP “stopping solution.” Isolation of [32P]cAMP was accomplished by sequential Dowex and alumina chromatography using [3H]cAMP as a recovery marker.20

**ADP-Ribosylation Studies**

ADP-ribosylation of G proteins was performed according to the method of Kaslow et al21 with modifications.22 The reaction was initiated with 25 µl of the membrane suspension added to 65 µl of buffer consisting of trisglycin (10 mM), thymidine (20 mM), K2PO4 (50 mM, pH 7.5), ADP-ribose (5 mM), arginine (20 mM), [32P]NAD (23–60 Ci/mmol), and other agents as noted. Before addition to the reaction mixture, cholera toxin (5 µg) was activated by incubation with dithiothreitol (20 mM) for 10 minutes at 30°C. For incubations with cholera toxin, Gpp(NH)p was added (100 µM). The complete reaction mixture with or without cholera toxin then was incubated for 20 minutes at 30°C and was terminated by the addition of 1 ml ice-cold 7% trichloroacetic acid and centrifuged at 12,000g. The pellet was resuspended in 1% trichloroacetic acid, centrifuged at the same speed, and solubilized in “sample buffer” (10 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, pH 6.8). Samples were boiled for 2 minutes. Electrophoresis was performed on vertical slab gels (main gel, 10%; stacking gel, 1.25% acrylamide) at 150 V for 2 hours. Gels were stained subsequently with Coomassie blue and analyzed by autoradiography. Intensity of bands corresponding to molecular weights of the G proteins of interest were determined spectrophotometrically as the optical density (OD/pixel) using Quantimat 970 (Cambridge Instruments, Inc., Cambridge, Mass.).5

**Preparation of Gαs, Antisera and Performance of Western Blots**

The antigen used to raise antisera contained a 16–amino acid sequence (Cys-Lys-Glu-Leu-Oln-Lys-Asp-Lys-Oln-Val-Tyr-Arg-Ala-Thr-His-Arg) unique to the Gαs subunit of Gα (Peninsula Laboratories, Inc., San Carlos, Calif). The polypeptide was conjugated to keyhole limpet hemocyanin (KLH) via the amino terminal cysteine residue of the polypeptides using m-maleimido-benzoyl-N-hydroxysuccinimide ester as described.23 The peptide-KLH conjugate was used to raise polyclonal, monospecific antisera in rabbits. A total of 100 µg peptide-KLH emulsified in complete Freund’s adjuvant was injected intradermally at 15–20 sites on the rabbit and was followed by a booster injection of 50 µg peptide-KLH in incomplete Freund’s adjuvant after 4 weeks. Sera from various rabbits were tested for binding to the antigen using an immunoblot technique, and those samples giving positive reaction at 1:500 dilution were used. Western blot analysis was performed after overnight transfer of proteins, run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and prepared as described above, to nitrocellulose in a buffer containing 20% methanol, glycine (150 mM), and Tris buffer (20 mM) at 75 V and 250 mA. Nitrocellulose blots then were incubated with rabbit anti-Gαs antisera as previously described, and [32P]Istaph A protein was used to detect bound antibody. Specificity of the antisera to Gαs was evaluated by several criteria. First, no antibody binding in the 40–65-kDa region was detectable in membranes prepared from cyc– cells, mutants of S49 lymphoma cells lacking Gα.24 Second, in simultaneous assays performed under identical reaction conditions, cholera toxin–dependent ADP-ribosylation of dog heart membranes occurred in a molecular weight region (42 kDa) completely superimposable with antibody binding. Third, our antisera recognized purified Gα obtained from rabbit liver as a single 42-kDa band. Finally, using Gα antisera 234725 (obtained from Dr. D. Manning, Department of Pharmacology, University of Pennsylvania Medical School, Philadelphia), we observed qualitatively identical binding patterns to dog heart membranes as observed using our own antisera.

**Statistical Analysis and Significance**

Biochemical and physiological assays were performed from one to three times on each individual animal or associated membrane preparation, and the data were pooled for each of the three groups (uninfected; infected asymptomatic, Tc-D; infected symptomatic, Tc-O). Within each group, the variation from one individual animal to the other with regard to all biochemical and physiological parameters under consideration was always less than 15% of the mean value obtained for the group as a whole. Intergroup variations were determined from the means and standard deviations of all relevant assays performed, and significance was determined using Student’s t test as previously described.5

**Determination of Na⁺,K⁺-ATPase Activity**

Determination of total, Na⁺,K⁺-ATPase, and Mg⁺⁺-ATPase activity in membranes alone or in the presence of cholate was performed on membranes prepared as above and as previously reported.20

**Results**

Our laboratory previously has described two isolates of T. cruzi, Tc-O and Tc-D, that produce different clinical responses when inoculated in the mouse.27 The availability of these two strains provided us with the opportunity to distinguish infection with myocardial features (referred to as asymptomatic) from infection in the absence of any overt cardiac signs (referred to as asymptomatic). Beagles inoculated with either Tc-O or Tc-D strains of T. cruzi displayed similar humoral (antibody titers) and cellular (peripheral blood mononuclear cell blastogenesis responses) immune responses to infection.10 Dogs infected with Tc-O developed acute myocarditis associated with increases in PR interval (Table 1), atrioventricular block, depression of R wave amplitude, and shifts in the mean electrical axis by day 20 after infection. Echocardiograms were normal during this stage. After the acute stage, Tc-O–
infected dogs entered a stage at which ECGs were normal. Progression to the chronic stage was indicated by the development of ventricular-based arrhythmias, including ventricular premature contractions between 60 and 70 days after infection. Ventricular premature contractions often became multifocal and eventually progressed to various forms of ventricular tachycardia.

Evidence of chronic myocardial involvement was demonstrable in animals infected with the Tc-O strain of *T. cruzi* using echocardiographic measurements and included a progressive decline in the ejection fraction 60 days after and for the duration of infection (Table 2). In contrast, despite parasitemia, dogs infected with the Tc-D strain showed no evidence of myocardial dysfunction. Accordingly, animals infected with the Tc-O strain are termed symptomatic and dogs infected with the Tc-D strain are termed asymptomatic, despite the fact that at the time of necropsy (250 days after infection), infected and uninfected animals were clinically indistinguishable on the basis of weight, eating habits, or spontaneous activity. In light of the established relation between the β-adrenergic receptor adenylate cyclase complex and the maintenance of normal myocardial function, we next determined to what extent these physiological manifestations of myocardial disease were reflected in the activity and interrelation of the β-adrenergic receptor complex. We first examined adenylate cyclase activity in response to isoproterenol.

The maximum velocity (Vmax) for isoproterenol in the presence or absence of Gpp(NH)p was virtually indistinguishable between uninfected and asymptomatic infected animals (38 and 87 pmol/mg/min in uninfected controls for isoproterenol alone or in the presence of Gpp[NH]p, respectively. In myocardial membranes prepared from infected asymptomatic animals, despite the absence of a change in basal adenylate cyclase activity or activity in response to Gpp(NH)p (results not shown), the Vmax for isoproterenol alone or in the presence of Gpp(NH)p decreased to 12 and 36 pmol/mg/min, respectively (Figure 1). In membranes prepared from symptomatic infected animals, there was a marked increase in the apparent concentration of agonist resulting in 50% of the Vmax (Kcat) for isoproterenol in the absence or presence of Gpp(NH)p, to 1.4 and 0.62 μM, when contrasted with values obtained in uninfected or infected asymptomatic animals (0.46 and 0.22 μM, respectively). We next examined the com-

### Table 1. Infection-Associated Changes in Electrocardiographic Parameters of Dogs

<table>
<thead>
<tr>
<th>Electrocardiographic parameter</th>
<th>Infected symptomatic</th>
<th>Infected asymptomatic</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR interval (sec)</td>
<td>0.123±0.021*</td>
<td>0.082±0.013</td>
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<td>R wave amplitude (mV)</td>
<td>0.920±0.550*</td>
<td>1.630±0.470</td>
<td>1.900±0.350</td>
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</table>

Values are mean±SD. Data were obtained from electrocardiographic lead II in dogs 20 days after infection with a pathogenic *T. cruzi* isolate (Tc-O, infected symptomatic; four animals) or a nonpathogenic *T. cruzi* strain (Tc-D, infected asymptomatic; four animals) and in uninfected dogs (four animals).

*Significantly different from values from control dogs (p<0.05).

### Table 2. Canine Cardiac Ejection Fraction as Function (Percent) of Duration of *T. cruzi* Infection

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Condition</th>
<th>0</th>
<th>110</th>
<th>185</th>
<th>245</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td></td>
<td>77±2</td>
<td>78±3</td>
<td>76±5</td>
<td>79±3</td>
</tr>
<tr>
<td>Asymptomatic infected</td>
<td></td>
<td>75±1</td>
<td>73±6</td>
<td>76±8</td>
<td>75±2</td>
</tr>
<tr>
<td>Symptomatic infected</td>
<td></td>
<td>76±3</td>
<td>66±5</td>
<td>55±10*</td>
<td>50±4*</td>
</tr>
</tbody>
</table>

*Significantly different from value obtained at day 0 (p<0.01).

FIGURE 1. Isoproterenol-dependent adenylate cyclase activity in myocardial membranes prepared from dogs: Influence of infection. Maximum velocity (Vmax) and Kact for isoproterenol in the presence (shaded bars) and absence (open bars) of 1 μM Gpp(NH)p were determined as described in “Materials and Methods.” Results shown are means of three separate determinations made at various times from myocardial membranes prepared from each of three groups of dogs (controls, asymptomatic infected, symptomatic infected). Values for Kact and Vmax were determined from 17 individual concentrations of isoproterenol between 0.01 and 10 μM, each done in triplicate, and analyzed using a linear regression analysis of 1/V−Vo vs. 1/[S] as described previously. Standard deviations are less than 15% of the means, and are not shown here. Vmax is picomoles cyclic AMP generated per milligram protein per minute. *Significantly different from identical measurement made in uninfected or infected asymptomatic, at p<0.01; †significantly different from identical measurement made in infected asymptomatic or uninfected, at p<0.005.
components of the adenylate cyclase complex to determine a site that may account for these changes.

β-Adrenergic receptor density and affinity in myocardial membranes prepared from asymptomatic infected or uninfected animals showed little difference (165 fmoI/mg protein and 230 pM, respectively; Figure 2). In contrast, β-adrenergic receptor density decreased in symptomatic infected animals to 130 fmoI/mg protein, without a significant change in receptor affinity from that determined in uninfected animals. To analyze the coupling of the β-adrenergic receptors to the guanine nucleotide binding units, we determined the ability of isoproterenol to displace bound [125I]ICYP from myocardial membranes in the presence and absence of Gpp(NH)p (Figure 3). By itself, the concentration of isoproterenol required to displace 50% of bound [125I]ICYP (IC50) in myocardial membranes prepared from uninfected animals was 0.05 μM (Table 3); in the presence of Gpp(NH)p, IC50 for isoproterenol increased approximately fourfold to 0.22 μM (p<0.01). Virtually identical results were obtained in membranes prepared from asymptomatic infected animals (Figure 3). However, the results obtained in symptomatic infected animals differed in several ways. First, IC50 for isoproterenol increased to 0.25 μM (p<0.05 compared with similar values obtained for control or infected asymptomatic animals). Second, although Gpp(NH)p still resulted in a decrease in sensitivity to isoproterenol (IC50, 0.33 μM; p<0.05), the relative fold increase in the concentration of isoproterenol to achieve the same degree of inhibition in the presence of Gpp(NH)p was markedly decreased (from fourfold in uninfected and asymptomatic infected to less than twofold in symptomatic infected). When the isoproterenol displace-

**Figure 2.** β-Adrenergic receptor density in myocardial membranes of dogs: Influence of infection. β-Adrenergic receptor density was determined using [125I]iodocyanopindolol in a binding assay as described, with membranes from uninfected (open triangle), infected asymptomatic (closed circle), and infected symptomatic (open circle) animals. Data points are means determined from three separate determinations as described in Figure 1 legend; lines drawn are from linear regression analysis. Correlation coefficients were determined to be 0.95. Standard deviations were less than 12% of the means and are not represented here for clarity.

**Figure 3.** Isoproterenol affinity in the absence and presence of Gpp(NH)p in dog heart membranes: Influence of infection. Dog heart membranes were incubated with [125I]iodocyanopindolol (ICYP) and increasing concentrations of isoproterenol in the absence (open circles) or presence (closed circles) of 1 μM Gpp(NH)p Data for uninfected (panel A), infected asymptomatic (panel B), and infected symptomatic (panel C) represent means of two separate determinations on each animal. Arrows represent concentration of isoproterenol resulting in 50% maximal binding of [125I]ICYP in the presence and absence of Gpp(NH)p. Pooled values for the determinations of IC50 in the presence and absence of Gpp(NH)p for isoproterenol and slope factors17 are shown in Table 3.

**Table 3.** LIGAND17 Analysis of Figure 3

<table>
<thead>
<tr>
<th>Group</th>
<th>IC50 (μM)</th>
<th>Slope factor</th>
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<tbody>
<tr>
<td>Uninfected</td>
<td>0.05±0.01</td>
<td>0.22±0.05</td>
</tr>
<tr>
<td>Infected asymptomatic</td>
<td>0.05±0.03</td>
<td>0.29±0.04</td>
</tr>
<tr>
<td>Infected symptomatic</td>
<td>0.25±0.07</td>
<td>0.33±0.05</td>
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</table>

IC50 Isoproterenol concentration required to displace 50% of bound [125I]iodocyanopindolol.
suggested that a prominent lesion in the adenylate cyclase complex, accounting for the decline in β-adrenergic adenylate cyclase activity observed in symptomatic infected animals, may be the stimulatory guanine nucleotide binding protein Gs, which couples the receptor to the catalytic unit. Accordingly, we next analyzed the Gs subunit more directly.

ADP-ribosylation by cholera toxin is a useful marker for the stimulatory guanine nucleotide binding protein Gs. As shown in Figure 4, the magnitude of cholera toxin-catalyzed ADP-ribosylation in a 42-kDa band in uninfected and asymptomatic infected animals is essentially equal to, and slightly, but not significantly, increased in the presence of NADP. In contrast, cholera toxin-dependent ADP-ribosylation in membranes prepared from symptomatic infected animals was less than 50% of that observed in uninfected animals. Furthermore, unlike the modest increase in cholera toxin-dependent ADP-ribosylation observed in the presence of NADP in both control and infected asymptomatic animals (<20%), the presence of NADP permitted twice as much cholera toxin-dependent ADP ribosylation to occur in infected symptomatic animals.

To complement the cholera toxin–dependent ADP-ribosylation studies, we examined the putative Gs coupling units more directly using rabbit antisera directed against specific amino acid sequences of Gαs developed as described.23 In a Western blot analysis of SDS-PAGE–treated myocardial membranes (Figure 5), the Gαs antisera bound to a number of bands of molecular weight greater than 36 kDa in a manner both common to all membrane preparations and specifically related to the state of the animal from which the membranes were prepared. We also examined the extent to which incubation of the dog heart membranes with cholera toxin or GTP-γ-S and Mg2+, conditions associated with the activation and disso-

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Cholera toxin–dependent ADP-ribosylation of 42-kDa proteins from dog heart membranes: Influence of infection. Cholera toxin–dependent ADP-ribosylation in the presence (hatched bars) or absence (shaded bars) of 10 μM NADP in the reaction mixture was carried out as described. Quantimet analysis of autoradiograms was performed as described in “Materials and Methods”; all values are relative to cholera toxin–dependent ADP-ribosylation in uninfected membranes in the absence of NADP, which is assigned a value of 100%. Results are means and standard deviations of four separate determinations. O.D., optical density. *Significantly different from identically treated value obtained in uninfected or infected asymptomatic, at p<0.01; †significantly different from value obtained in the same preparation but in the absence of NADP, at p<0.05.

![Figure 5](http://circres.ahajournals.org/)

**Figure 5.** Analysis of dog heart membrane proteins using anti-Gαs antisera: Influence of infection. Dog heart membranes from infected symptomatic (lanes 1–3), infected asymptomatic (lanes 4–6), and uninfected animals (lanes 7–9) were incubated in ADP-ribosylation assay mixture without [32P]NAD as described in “Materials and Methods,” with no toxin or activators (lanes 1, 4, and 7), cholera toxin and Gpp(NH)p (1 μM) (lanes 2, 5, and 8), GTP-γ-S (1 μM) and Mg2+ (5 mM) (lanes 3, 6, and 9) and were separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a manner identical to that used for results of Figure 4. Western blot analysis of nitrocellulose blot transfers was performed as described, and anti-Gαs antisera binding was detected using [32P]Staph A protein, as described. Results shown here are from one of five separate identical determinations.
Detergent-extracted G, obtained from dog heart membranes was used to reconstitute cyc– mutant lymphoma cells, and adenylate cyclase activity in response to isoproterenol or Gpp(NH)p was determined. In experiments not shown, we determined that under the conditions of detergent extraction used here, little or no significant cholera toxin–dependent ADP-ribosylation was demonstrable in the pellet retrieved after high-speed centrifugation of the detergent-treated membranes, suggesting an equivalent degree of G, extraction. Moreover, using a wide range of cholate concentration (from 0.1 to 2%), we observed the identical relations with membrane source and activity of G, in cyc– reconstitution assays. As shown in Figure 6, isoproterenol-dependent adenylate cyclase activity in cyc– membranes reconstituted with cholate extracts obtained from infected symptomatic animals was approximately 30% less than that observed in cyc– membranes reconstituted with cholate extracts obtained from infected asymptomatic or control animals. In contrast, there appears to be no substantial difference in the adenylate cyclase activity in response to Gpp(NH)p in cyc– cells with donor G, from infected or uninfected animals when determined under otherwise identical reaction conditions (results not shown).

An important consideration in the analysis of plasma membrane β-adrenergic receptor complex in the setting of asymptomatic and symptomatic infection with T. cruzi is the contribution of noncardiac cells to the total plasma membrane population. This is particularly important in Chagas’ disease, in which myocardial involvement includes varying degrees of inflammation and fibrosis. Histological analysis (data not shown) of myocardial tissue obtained from asymptomatic and symptomatic infected animals failed to reveal substantial inflammatory responses, which are characteristic of the later stages of Chagas’ disease. Nonetheless, we determined levels of Na+,K+-ATPase activity as a marker for the purity of our membrane preparation, in both the presence and absence of cholate. As shown in Table 4, Na+,K+-ATPase activity in myocardial membranes prepared from symptomatic infected animals was significantly higher than the activity determined in membranes prepared from both symptomatic and control animals.

Discussion

Infection of beagle dogs with an opossum-derived strain of T. cruzi results in physiological, pathological, and biochemical consequences consistent with the expression of acute and chronic cardiac dysfunction in the setting of human Chagas’ disease. As we have documented here, conduction abnormalities occur within 20 days after infection, including lengthening of the PR interval. After a brief asymptomatic period, myocardial irritability manifests itself by the presence of frequent ventricular premature contractions and runs of ventricular tachycardia. Complementary ECG data documented myocardial dysfunction early in the chronic stage of disease, again characteristic of the expression of cardiac dysfunction in human Chagas’ disease. Similar to observations made in other experimental studies of cardiomyopathy, we also report a depression of myocardial β-adrenergic adenylate cyclase activity in symptomatic infected animals. However, in our model, symptomatic animals had no clinically overt evidence of congestive heart failure. Our data are consistent with the hypothesis that β-adrenergic adenylate cyclase activity is affected early in the progression of cardiac disease before clinically overt symptoms of congestive heart failure develop, and hence suggest a mechanistic relation between early changes in the β-adrenergic receptor complex and subsequent cardiac dysfunction. In this regard, our results are striking in the degree to which they demarcate the progression of cardiac disease.

In uninfected animals, our results documenting canine myocardial β-adrenergic receptor density af-
finity and adenylate cyclase activity are in general agreement with other reports.\textsuperscript{32,33} Infection of dogs with a symptomatic strain of *T. cruzi* produces a number of changes in the β-adrenergic receptor complex, most notably a decline in isoproterenol-dependent adenylate cyclase activity. In the analysis of the role of the β-adrenergic receptor per se, the finding of relatively insignificant changes in β-adrenergic receptor density and affinity observed in symptomatic animals is to be contrasted with the observations made in the murine model of Chagas' disease,\textsuperscript{6} in which 6 days after infection, β-receptor density increases and affinity decreases. Species specificity may account for these differences. However, in a canine model of left ventricular failure (1 year of left ventricular outflow obstruction in dogs, resulting in frank congestive heart failure), β-adrenergic receptor density increased and affinity decreased. Hence, the physiological basis for the cardiomyopathy may dictate what changes in β-receptors are to be seen. It is important to note, however, that β-adrenergic receptor density need not follow alterations in β-adrenergic adenylate cyclase activity. For example, in Yucatan miniswine, long-term treadmill running resulted in a markedly increased chronotropic response to isoproterenol.\textsuperscript{34} Paradoxically, β-adrenergic receptor number decreased, whereas the density of the guanine nucleotide binding protein G<sub>α</sub> in exercised animals increased. Thus, measurement of β-receptor density alone may fail to provide an adequate biochemical explanation for altered adenylate cyclase activity.

In symptomatic infected animals, the affinity of the β-receptor for its agonist is reduced, and there is a marked decrease in the ability of Gpp(NH)p to alter agonist binding. Caution must be exercised in the analysis of this data, particularly because the K<sub>d</sub> value for the β-adrenergic receptor in control animals reported here is twofold higher than that reported elsewhere.\textsuperscript{33} This renders it difficult to assign with certainty the implication of the isoproterenol displacement studies vis-à-vis coupling to the G protein when contrasted with other reports. Nonetheless, the studies reveal important differences when viewed within the comparative context as presented here. Thus, the shallow nature of the isoproterenol displacement curves (slope factor close to unity) generated in the presence of Gpp(NH)p suggests that at this sensitive but subsaturating concentration of the guanine nucleotide (1 μM), not all available high-affinity sites are converted to low affinity.\textsuperscript{35} This appears to be the case for all groups of animals studied. The reduction in both the affinity for and the influence of Gpp(NH)p on the binding of isoproterenol to the β-adrenergic receptor that occurs only in symptomatic infected animals, evidence consistent with a compromise in the coupling relation between the receptor and G<sub>α</sub>, is therefore unique to myocardial involvement and not infection per se. These results are consistent with a reduction in high-affinity β-adrenergic receptors, as reported in the canine outflow obstruction model,\textsuperscript{4} although our data do not permit us to accurately quantify the proportion of high- and low-affinity receptors, hence limiting the extent to which the degree of uncoupling can be quantified.

Additional evidence implicates a primary abnormality in G<sub>α</sub> in the symptomatic infected animals. In the current study, as well as in other studies of cardiomyopathy such as the murine model of chagasic cardiomyopathy,\textsuperscript{6} cholera toxin–dependent ADP-ribosylation was decreased in the symptomatic animal. However, cholera toxin–dependent ADP-ribosylation can be influenced, in part, by NAD glycohydrolase activity. Thus, only in membranes prepared from infected symptomatic animals, cholera toxin–dependent ADP-ribosylation in response to the presence of NADP, an inhibitor of NAD glycohydrolase activity, was substantially (more than twofold) greater. The influence of NADP was considerably less in both infected asymptomatic and uninfected animals, results similar to the murine model of Chagas' disease.\textsuperscript{6} This observation suggests that symptomatic infection is associated with a change in the activity of myocardial NAD glycohydrolase. It would be of great interest to determine if altered myocardial NAD glycohydrolase activity occurs in other states of cardiac dysfunction, suggesting an important but as yet to be identified role for the enzyme in myocardial function.

Some limitations in the application of cholera toxin to quantitate G<sub>α</sub> have been overcome using antisera directed against the guanine nucleotide binding protein.\textsuperscript{23} As applied to the developing heart, specific anti-G antisera recognized temporal changes in the expression of the various G proteins.\textsuperscript{9} In our model, there appeared to be a decrease in binding of anti-G<sub>α</sub> antisera in the 42-kDa region as well as a decrease in cholera toxin–dependent ADP-ribosylation in symptomatic animals. However, in the presence of NADP, cholera toxin–dependent ADP-ribosylation in symptomatic animals is not significantly different from levels observed in asymptomatic or control animals. Moreover, the loss in the 42-kDa region of G<sub>α</sub> antibody binding observed in symptomatic animals may be compensated by an increase in binding to the 45-kDa species. Collectively, the data suggest that the symptomatic infection–associated change in G<sub>α</sub> may not be quantitative, but rather qualitative in aspects of its structure and function, particularly with regard to its coupling to the β-adrenergic receptor per se. The possibility that qualitative changes in G<sub>α</sub> may extend to the asymptomatic infected animals as well comes from the observation that both cholera toxin–dependent ADP-ribosylation and particulate adenylate cyclase activity appear to be identical with that observed in the uninfected controls, whereas the levels of G<sub>α</sub> antisera binding are markedly increased over uninfected controls. Hence, G<sub>α</sub> immunobots alone do not necessarily correlate with adenylate cyclase activity. However, it remains to be determined whether compensatory changes in G<sub>α</sub> or other influences affect adenylate cyclase activity in states of cardiac dysfunction, as has been report-
Our results here are to be contrasted with a recent report indicating no change in anti-Gαs antisera binding in a 45-kDa region in myocardial membranes prepared from cardiomyopathic Syrian hamsters, despite a decrease in isoproterenol-dependent adenylyl cyclase activity. In that study, however, the authors were unable to determine chola toxin-dependent ADP-ribosylation, although a functional decrease in G3 activity was demonstrated.

Binding of antisera to G proteins also may be influenced by the state of activation of the G protein, as has been reported. Incubation of membrane preparations with cholera toxin or GTP-γ-S and Mg2+ results in activation and hence dissociation of Gαs. In our hands, pretreatment of myocardial membranes with these Gα activating agents consistently increased antisera binding in all three different myocardial membrane preparations, suggesting that our antisera may bind more avidly to the isolated αs subunit, as reported. This possibility remains to be confirmed. In such an event, our results would be consistent with the notion that the infection-associated state of cardiac dysfunction does not alter the actual activation or dissociation of Gαs, but rather its interaction with the β-receptor per se.

It also is of interest to note that our antisera bind to a number of higher molecular weight proteins not thought to be associated with Gαs, whereas in other reports, anti-Gαs antisera bind predominantly to the 42–52-kDa region. In this regard, polyclonal rabbit antisera raised against various antigens often contain antibodies against keratin, which appears as spurious bands in the molecular weight region greater than 55 kDa. The use of affinity-purified Gαs antisera (results not shown) did not significantly affect the binding patterns obtained with these membranes. Although preliminary experiments (results not shown) suggest that purification of membranes favors, but does not result in, exclusive antisera binding to the bands of interest, that is, the 42-kDa region, for this study, in the interests of consistency, a uniform membrane preparation was used for all analyses. Central to the concerns of this communication is the striking observation that the infection of the binding of Gαs antisera is restricted exclusively to relevant bands of molecular weights less than 45 kDa.

As in all studies of the behavior of the β-adrenergic receptor complex in states of myocardial dysfunction, it must be recognized that myocardial membrane preparations contain substantial quantities of membranes from noncardiocyte cell types. Indeed, myocardial dysfunction frequently is associated with varying degrees of inflammation and fibrosis, further contributing to the heterogeneous makeup of the membrane preparation. Our analysis of Na+,K+-ATPase activity indicates that myocardial membranes prepared from symptomatic infected animals actually demonstrate an increase in this activity. This increase in activity occurs only in the setting of infection associated with cardiac dysfunction, implying that it may be specific to myocardial dysfunction and not infection per se. The biochemical basis for this observation has yet to be determined. Nonetheless, by virtue of the extent to which Na+,K+-ATPase activity reflects membrane purity, our data suggest that at this stage of chronic Chagas’ disease, the low levels of inflammation observed histologically do not diminish in a demonstrable manner the concentration of myocardial membranes in our preparations. Hence, it would appear unlikely that the decrease in β-adrenergic-mediated adenylyl cyclase activity can be accounted for simply by the decrease in the concentration of myocardial membranes in symptomatic infected animals. Moreover, only isoproterenol-dependent adenylyl cyclase activity appears to be compromised in cyclo- assays with donor Gαs from symptomatic infected animals. In contrast, adenylyl cyclase activity in response to Gpp(NH) p was un influenced by the source of donor Gαs. Indeed, the relatively modest decrease in isoproterenol-associated Gαs activity in cyclo-assays reported here underscores the notion that the symptomatic infected animals demonstrate scant evidence of cardiac dysfunction, consistent with the minimal pathological changes observed. Similarly, other qualitative changes that are independent of protein concentration attend infection with or without symptomatic myocardial involvement and include patterns of Gαs antisera binding, alterations in isoproterenol receptor affinity and influence of Gpp(NH)p, as well as the unique influence of NADP on cholera toxin-dependent ADP-ribosylation. Consistent with our immunochemical and cholera toxin-dependent ADP-ribosylation studies, the adenylyl cyclase data may be interpreted as reflecting the possibility that uncoupling of the β-adrenergic receptor from the Gαs protein may be the predominant feature of early changes that antedate cardiac dysfunction in Chagas’ disease.

In summary, the infection of dogs with a symptomatic strain of T. cruzi offers the important opportunity to study the development of a cardiomyopathic state wherein subtle indexes of cardiac dysfunction can be accurately assessed and convenient biochemical measurements of β-adrenergic receptor activity determined. Our studies suggest that early during the course of cardiac involvement in Chagas’ disease, β-adrenergic adenylyl cyclase activity declines specifically in response to isoproterenol. This probably reflects a functional uncoupling of the β-adrenergic receptor to the Gαs protein, probably as a consequence of an as yet unidentified alteration in Gαs. We propose that these early changes in the activity of the β-adrenergic receptor complex relate to infection-specific changes in the microvasculature of the heart, a direct consequence of infection with T. cruzi. The biochemical alterations detailed here may be observed in any pathological state associated with a compromise in microvascular perfusion.

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References


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