

Impairment of Cardiac β-Adrenoceptor Cellular Signaling by Decreased Expression of $G_{s\alpha}$ in Septic Rabbits

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Background: **Abnormalities in the β-adrenergic control of cardiac function play a role in the pathogenesis of several disease states. Because circulatory failure in patients with septic shock is known to be less responsive to catecholamines, we investigated whether the β-adrenoceptor–linked signal transduction mechanisms are altered in the heart of a septic animal model.**

Methods: **Rabbits were rendered endotoxemic by an intravenous injection of 100 μg/kg *Escherichia coli* lipopolysaccharide. Three and 6 h later, the myocardial tissues were used for the experiments.**

Results: **The positive inotropic response to isoproterenol was significantly impaired in papillary muscles isolated from septic rabbits compared with those from controls. The impaired inotropic responsiveness to isoproterenol was not prevented by the nitric oxide synthase inhibitor N^G-nitro-L-arginine, indicating no involvement of nitric oxide overproduction. Adenylate cyclase activity stimulated with isoproterenol and 5′-guanylyl imidodiphosphate was markedly reduced in septic myocardium. The contractile and adenylate cyclase responses to colforsin daropate, a direct adenylate cyclase activator, were unaffected by sepsis. Radioligand binding experiments with (−)[^{125}I]iodocyanopindolol revealed no significant alteration in myocardial β-adrenoceptor density or affinity in sepsis. Determination of cardiac $G_{s\alpha}$ level by Western blotting showed a reduction of approximately 50% in sepsis. The relative content of $G_{s\alpha}$ messenger RNA in septic myocardium also was reduced from the control level by about 50%, as determined by Northern blot analysis. Little change was found in protein and messenger RNA levels of $G_{i\alpha}$ in septic myocardium.**

Conclusions: **Impairment of myocardial functional responsiveness to β-adrenoceptor stimulation appears in the early stage of sepsis. The impaired response to β-adrenoceptor stimulation in the heart in this pathologic state may result in part from a decreased level of $G_{s\alpha}$ protein which occurs at the level of gene expression. (Key words: G proteins; heart; messenger RNA; septic shock.)**

ENDOTOXIN-INDUCED septic shock is associated with severe hypotension that leads to decreased organ perfusion, resulting in multiple organ failure and extremely high mortality.[1] Furthermore, cardiac contractility itself is depressed in patients with septic shock,[2,3] which can contribute to the hypotension observed. Decreased cardiac contractility has also been demonstrated in isolated hearts from animals following *in vivo* administration of endotoxin.[4–6] In addition, patients with septic shock suffer from hypotension and cardiac dysfunction refractory to high doses of intravenous catecholamines.[7] A decreased functional responsiveness to β-adrenoceptor stimulation has been shown in hearts from animal models of sepsis.[8,9]

The β-adrenoceptor signal transduction pathway consists of three distinct proteins: the β-adrenoceptor, which binds catecholamines; the catalytic moiety of adenylate cyclase, which synthesizes cyclic adenosine monophosphate (AMP) from adenosine triphosphate (ATP); and the stimulatory guanosine triphosphate (GTP)-binding regulatory protein (G_s), which couples the receptor to its effector.[10] Previous reports showing blunted catecholamine-stimulated adenylate cyclase activity in ventricular membranes[11] and attenuated isoproterenol-stimulated cyclic AMP accumulation in ventricular slices[12] and myocytes[13] in response to endotoxin administration *in vivo* suggest that endotoxin may induce impairment of the β-adrenoceptor signal transduction pathway in the heart. An increase in the level of the inhibitory GTP-binding regulatory protein (G_i), which antagonizes the action of G_s, is known to be involved in the diminished cardiac responses to β-adrenoceptor stimulation in disease states such as heart failure.[14] It is thus possible that endotoxin might increase myocardial expression of G_i and thereby diminish the β-adrenoceptor responsiveness in the heart.

The purpose of this study was to characterize the cardiac β-adrenoceptor signal transduction pathway in the rabbit which was rendered endotoxemic with *Escherichia coli* lipopolysaccharide. The inotropic responses to isoproterenol and cyclic AMP–related agents were examined in isolated right ventricular papillary muscles. Whether the density of β-adrenoceptors and adenylate cyclase activity are changed in sepsis was also investigated. Finally, alterations in α-subunits of G_s and G_i were assessed by simultaneously evaluating gene expression and protein amount.

Materials and Methods

Hemodynamic Measurements and Induction of Sepsis

All procedures were in accordance with the regulations laid down by the Hokkaido University School of

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Medicine Animal Care and Use Committee, Sapporo, Japan. Male New Zealand white rabbits (2–2.5 kg) were lightly anesthetized with diethyl ether. Polyethylene catheters (22 G; JELCO; Critikon, Tampa, FL) were inserted into the left-ear artery. Normal saline containing 2 U/ml heparin was continuously infused *via* the ear arterial catheter to maintain patency of the blood pressure cannula line. Following recovery of anesthesia, the arterial blood pressure (BP) and heart rate (HR) were continuously monitored using the Surgical Monitoring System (Nihon Kohden, Tokyo, Japan). The surface and core body temperatures of the rabbit were measured *via* the right ear and rectum by using Thermistor Monitor Model 4070 (Mon-a-therm; Mallinkrodt Medical, St. Louis, MO). Rabbits received a slow bolus injection of 100 μg/kg lipopolysaccharide (*E. coli* 055:B5; List Biological Laboratories, Campbell, CA) *via* the ear vein. The dose was contained in 3 ml of normal saline. In untreated (control) rabbits, the same amount of normal saline was given intravenously instead of lipopolysaccharide.

Organ Bath Experiments

Control and septic rabbits were anesthetized with diethyl ether and then killed by exsanguination. The hearts were rapidly excised and transferred to a dissection bath with oxygenated warm Krebs-Henseleit solution. The composition of the solution was: 119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 24.9 mM $NaHCO_3$, and 10.0 mM glucose. The right ventricular papillary muscles were carefully dissected from the heart. The muscle was mounted under 0.5 g of resting tension in a bath filled with 10 ml of Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2 at 32°C. The muscle was stimulated by rectangular pulses of 1 Hz in frequency, 5 ms in duration, and 1.5 times threshold voltage, delivered by a pair of spiral platinum electrodes connected to an electronic stimulator. Isometric tension was measured with a force transducer and recorded on a thermal-array recorder.

After an equilibration period of at least 60 min, concentration-response curves for the positive inotropic effects of isoproterenol, colforsin daropate, and dibutylic cyclic AMP (DBcAMP) were determined by the cumulative addition of increased agent concentrations. When N^G-nitro-L-arginine (L-NNA) was used, the incubation period was at least 30 min. The EC_{50} value (*i.e.*, the concentration required to produce 50% of the maximal response induced by the agent) was determined from log-probit plots of the individual response *versus* concentration.

Membrane Preparation

Rabbits were killed as stated previously. The hearts were removed and rinsed in ice-cold Tris-HCl buffer. Ventricles were dissected free of connective tissue, major vessels, and atria. The tissues were minced with scissors and homogenized in 5 volumes of ice-cold Tris-HCl buffer by the use of a polytron for 15 s. The buffer (pH 7.4, 4°C) contained 75 mM Tris-HCl, 25 mM $MgCl_2$, 5 mM EGTA, and 1 mM EDTA. The homogenates were centrifuged at $1{,}000g_{max}$ for 10 min at 4°C. The supernatant was filtered through a single layer of cheesecloth and retained. The pellet was suspended in 5 volumes of cold Tris-HCl buffer and centrifuged again. Membrane fractions in the supernatant were concentrated by centrifugation at $100{,}000g_{max}$ for 30 min at 4°C. The final pellets were resuspended in cold Tris-HCl buffer and stored at −80°C until used. Protein content was determined by the method of Lowry *et al.*[15] with bovine serum albumin as standard.

Radioligand Binding Assay

The radioligand (−)[^{125}I]iodocyanopindolol (ICYP; New England Nuclear, Boston, MA) was used for identifying β-adrenoceptors, as previously described.[16] The membranes were incubated at 37°C for 30 min with increasing concentrations of [^{125}I]ICYP (12.5–1600 pM). All values in binding experiments are the average of triplicates. Specific binding was defined as binding inhibited by 10 μM propranolol, and the equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}) were determined by Scatchard analysis.

Adenylate Cyclase Assay

Adenylate cyclase activity was determined in cardiac membranes (50–100 μg) by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cyclic AMP as described previously.[17] Isolation of [^{32}P]cyclic AMP formed during the reaction was accomplished by the method of Salomon *et al.*[18] When isoproterenol was used as a stimulant, 0.1 mM GTP was added to the mixture. Assays were performed in triplicate, and the results are expressed as picomoles of cyclic AMP per milligram of protein per 10 min.

Western Blot Analysis

Immunoblotting was performed as demonstrated in our previous report,[19] except that the membrane preparations mentioned previously were used as samples. The blots were incubated for 60 min at room temperature with specific antibodies recognizing $G_{s\alpha}$ (1:3000; 3A-160; Gramsch Laboratories, Schwabhausen, Germany; and 1:1000; RM/1; New England Nuclear), $G_{i\alpha}$ (1:300; Oncogene Research Product, Cambridge, MA), and $G_{\beta\gamma}$ (1:2000; Calbiochem-Novabiochem Corporation, San Diego, CA), and then washed three times for a 10-min duration with Tris-buffered saline-Tween buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM $NaHPO_4$, 1.5 mM KH_2PO_4, 0.1% Tween 20) to remove any nonspecifically bound antibodies. After being incubated with horseradish peroxidase–conjugated anti-rabbit antibody (1:6000 dilution; Bio-Rad Laboratories, Hercules, CA) for 60 min at room temperature, the blots were visualized with the

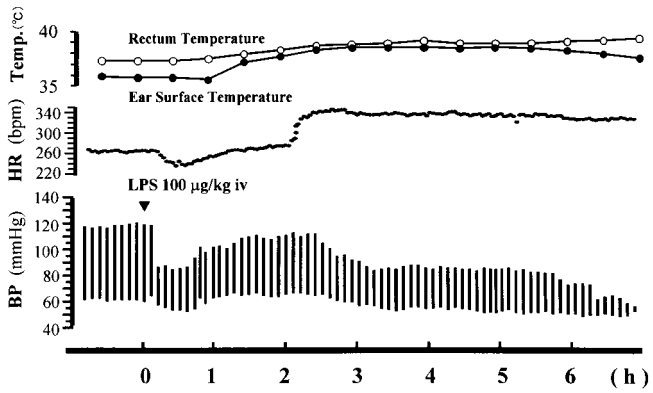

Fig. 1. Changes in heart rate (HR), blood pressure (BP), and body temperature (Temp.) in the rabbit after intravenous injection of 100 μg/kg lipopolysaccharide (LPS). The results shown are representative of four additional experiments. bpm = beats/min.

enhanced chemoluminescence detection system (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, United Kingdom), exposed to x-ray film for 10 min, and analyzed using the free software NIH Image (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Total RNA Extraction and Northern Blot Analysis

Total RNA was extracted from ventricular myocardium as described previously[19,20] by a guanidinium thiocyanate–phenol–chlorform method according to the protocol of Chomczinski and Sacchi.[21] Briefly, frozen ventricles were placed in ISOGEN (Nippon Gene, Tokyo, Japan) and homogenized with a polytron. Subsequently, 200 μl chloroform was added; the mixture was shaken vigorously for 15 s and was kept at room temperature for 5 min. The mixture was centrifuged at 12,000g_{max} for 15 min at 4°C; the aqueous phase was transferred into a fresh tube; 500 μl isopropanol was added; and the sample was centrifuged at 12,000g_{max} for 5 min at 4°C. Total RNA was resuspended in diethyl pyrocarbonate-treated water. The amount of RNA present was determined by ultraviolet absorption. The optical density (OD) ratio of $OD_{260\ nm}$:$OD_{280\ nm}$ was 1.97–2.14 in all specimens.

RNA, 30 μg/lane, was subjected to electrophoresis on 1.2% agarose–6.5% formaldehyde gels and then transferred to a Hybond-N^+ nylon membrane (Amersham). The membrane was prehybridized in prewarmed rate-enhanced hybridization buffer (Rapid-hyb buffer; Amersham) at 42°C for 60 min. The $G_{s\alpha}$ and $G_{i\alpha}$ oligonucleotide probes (New England Nuclear) were labeled with [α-^{32}P]dATP (Amersham) using an oligonucleotide 3′-end labeling system (New England Nuclear). After being hybridized in the buffer containing ^{32}P-labeled probe (10^7 cpm/ml) at 42°C for 120 min, the membrane was washed with 2% standard saline citrate–0.1% sodium dodecyl sulfate at room temperature and with 0.5% standard saline citrate–0.1% sodium dodecyl sulfate twice at 42°C. The G_α messenger RNA (mRNA) was quantitated by counting the radioactivity using a bioimaging analyzer system (Fujix BAS 2000; Fuji Photo Film, Tokyo, Japan).[20] To control for differences in RNA conditions, the membranes were sequentially probed for β-actin using an oligonucleotide probe (40-mer; New England Nuclear). The membranes were stripped of the G_α probe by soaking the membranes twice for 20 min in 0.1% SDS that had been brought to boil, and subsequent hybridization of the β-actin probe was performed as described previously. Thus, each membrane was sequentially probed for the mRNA of G_α and then normalized using the mRNA of the constitutively expressed protein β-actin.

Statistical Analysis

Data are expressed as mean ± SD. Statistical assessment of the data was made by Student *t* test for unpaired data or one-way analysis of variance followed by the Scheffé multiple comparison test to locate differences between groups. The analyses were carried out using StatView software (Abacus Concepts, Berkeley, CA). A significant difference was assumed to exist if the *P* value was less than 0.05.

Results

Hemodynamic Changes after Lipopolysaccharide Administration

After recovery from anesthesia, baseline hemodynamics in the rabbits remained constant for 60 min. Basal HR and BP of animals before injection of lipopolysaccharide were 259 ± 7 beats/min and 113 ± 8/66 ± 5 mmHg (n = 5). Figure 1 illustrates a typical example of changes in HR, BP, and body temperature after injection of

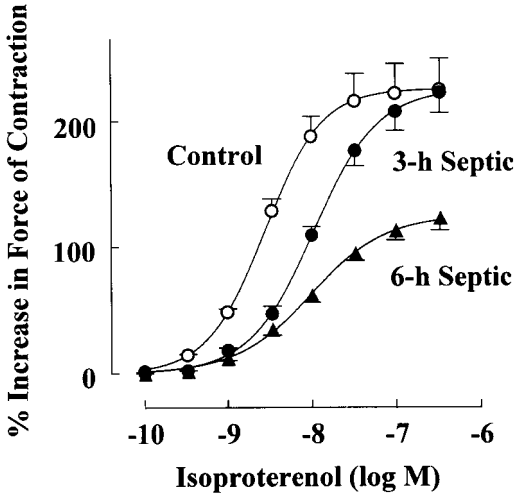

Fig. 2. Concentration-response curves for the positive inotropic effect of isoproterenol in papillary muscles from control (open circles), 3-h septic (filled circles), and 6-h septic (triangles) rabbits. Each symbol shows the net increase in force of contraction expressed as a percentage of basal values recorded before the addition of isoproterenol. Mean of data from five muscles are presented, with SD shown by vertical lines.

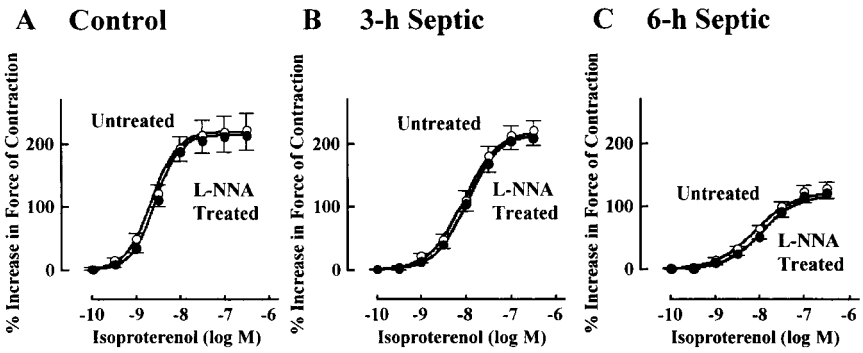

Fig. 3. Influence of N^G-nitro-L-arginine (L-NNA) treatment on the positive inotropic effect of isoproterenol in papillary muscles from (*A*) control, (*B*) 3-h septic, and (*C*) 6-h septic rabbits. The concentration-response curves for isoproterenol in the absence (open circles) and presence (filled circles) of 100 μM L-NNA are shown. L-NNA was added to the bath at least 30 min before isoproterenol application. Mean of data from five muscles are presented, with SD shown by vertical lines.

100 μg/kg lipopolysaccharide intravenously. Administration of lipopolysaccharide caused a progressive fall in BP over a 6-h period, after a delay of approximately 2.5 h with a transient decrease at 5 min (84 ± 6/56 ± 5 mmHg), to 81 ± 7/52 ± 4 mmHg at the nadir. At the 7-h timepoint, BP was undetectable. Heart rate was significantly reduced to 226 ± 10 beats/min within 5 min after lipopolysaccharide injection and gradually returned to baseline by 2 h, followed by a maintained elevation over a 7-h period (322 ± 18 beats/min). The core body temperature showed a gradual elevation from 37.0 ± 0.2 to 39.1 ± 0.4°C after lipopolysaccharide injection. However, the difference between core and surface temperatures remained essentially constant (< 0.5°C) over the full time course.

Positive Inotropic Responses to Isoproterenol and Other Agents

When right ventricular papillary muscles isolated from rabbits at 3 and 6 h after induction of sepsis by lipopolysaccharide were stimulated electrically at 1 Hz, the values for the basal force of contraction (304 ± 49 and 303 ± 38 mg, n = 20) were similar to those obtained in control muscles (315 ± 37 mg, n = 20). As shown in figure 2, however, the positive inotropic effect of isoproterenol was significantly affected by sepsis. Thus, the concentration–response curve for isoproterenol was shifted 2.8-fold to the right (assessed by EC_{50} value; 7.9 ± 0.9 *vs.* 2.8 ± 0.5 nM; n = 5 for each; $P < 0.001$) with no change in the maximum response in muscles at 3 h of sepsis. Furthermore, an overall shift in both sensitivity (EC_{50}: 9.3 ± 0.6 nM; $P < 0.001$) and maximum response (128 ± 23 *vs.* 224 ± 56%; $P < 0.01$) was evident in muscles at 6 h of sepsis. Treatment with 100 μM L-NNA did not cause any change in the positive inotropic effect of isoproterenol regardless of whether muscles were isolated from control or septic rabbits (fig. 3). In contrast to the response to isoproterenol, the positive inotropic responses to colforsin daropate, a water-soluble forskolin derivative which directly activates adenylate cyclase,[22] and DBcAMP were identical between control and 6-h septic groups (fig. 4).

β-Adrenoceptor Binding

The specific binding of [^{125}I]ICYP to myocardial membranes from control and septic rabbits showed a saturable process of high affinity (figs. 5A and 5B). Scatchard analysis of the data revealed that [^{125}I]ICYP bound to a single population of binding sites in both control and septic myocardial membranes (figs. 5C and 5D). The B_{max} values for [^{125}I]ICYP obtained in myocardial membranes from rabbits 3 and 6 h after induction of sepsis (139 ± 20 and 162 ± 37 fmol/mg protein, n = 5 for each) were not significantly different from that in controls (152 ± 50 fmol/mg protein, n = 5). The K_d values

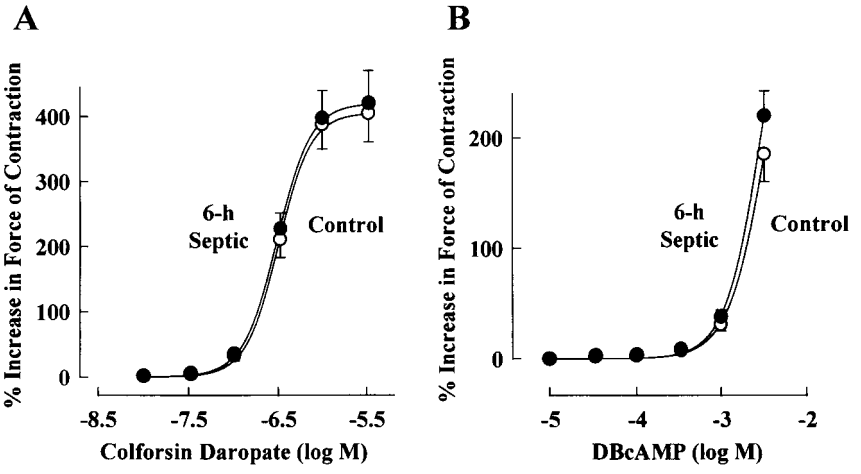

Fig. 4. Concentration–response curves for the positive inotropic effects of (*A*) colforsin daropate and (*B*) dibutylic cyclic adenosine monophosphate (DBcAMP) in papillary muscles from control (open circles) and 6-h septic (filled circles) rabbits. Mean of data from five muscles are presented, with SD shown by vertical lines.

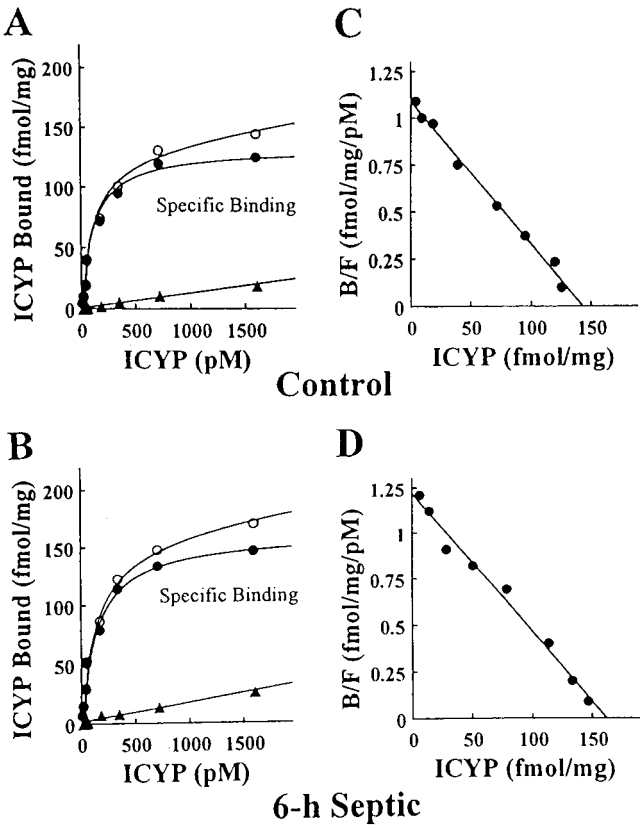

Fig. 5. Binding of (−)[^{125}I]iodocyanopindolol ([^{125}I]ICYP) to myocardial membranes from (*A* and *C*) control and (*B* and *D*) 6-h septic rabbits. (*A* and *B*) Saturation isotherm and (*C* and *D*) Scatchard plots were obtained from a representative experiment performed in triplicate. Similar results were obtained with four additional experiments.

were also comparable among the three groups (control, 52 ± 24 nM; 3-h sepsis, 48 ± 14 nM; 6-h sepsis, 51 ± 24 nM).

Adenylate Cyclase Activity

Basal adenylate cyclase activity, which was measured without using any activator, in myocardial membranes prepared from animals at 6 h of sepsis was not significantly different from that in controls (543 ± 358 *vs.* 582 ± 407 pmol cyclic AMP/mg protein/10 min, n = 5 for each group). In the presence of 0.1 mM GTP, the adenylate cyclase response to isoproterenol was significantly attenuated in septic myocardial membranes. Thus, the overall concentration–response curve for isoproterenol-stimulated adenylate cyclase activity was substantially shifted downward by induction of sepsis (fig. 6A). Adenylate cyclase activity stimulated with 100 μM 5′-guanylyl imidodiphosphate (GppNHp), a nonhydrolyzable GTP analog, was also markedly reduced in septic myocardial membranes (fig. 6B). However, adenylate cyclase activity stimulated with 10 μM colforsin daropate was similar in two groups of membranes (fig. 6B).

Expression of G Protein and mRNA

The relative levels of $G_{s\alpha}$ protein were determined in ventricular myocardium taken from control and septic rabbits by Western blot analysis with the use of two different antisera, 3A-160 (which recognizes the third loop of $G_{s\alpha}$) and RM/1 (which recognizes the C-terminus of $G_{s\alpha}$). Both of the two antisera consistently identified a single band that migrated with an apparent molecular weight of 45 kilodaltons (kDa; fig. 7). In the experiments shown in figure 7, the amount of $G_{s\alpha}$ protein decreased markedly at 3 and 6 h after induction of sepsis compared with control ($P < 0.001$). When 3A-160 was used as $G_{s\alpha}$ antibody, the relative levels were diminished to 56 ± 6 and 48 ± 5% (n = 4) of the control level in 3- and 6-h septic ventricles (fig. 7A). When RM/1 was used as $G_{s\alpha}$ antibody, the levels were 68 ± 12 and 41 ± 17% of control (n = 4) in 3- and 6-h sepsis (fig. 7B).

Immunoblots were also obtained using the $G_{i\alpha}$ antibody, and the specific binding of this antibody was detected as a single band at 40-kDa apparent molecular weight (fig. 8A). Quantitative analysis of immunoblots showed that the levels of $G_{i\alpha}$ protein were substantially

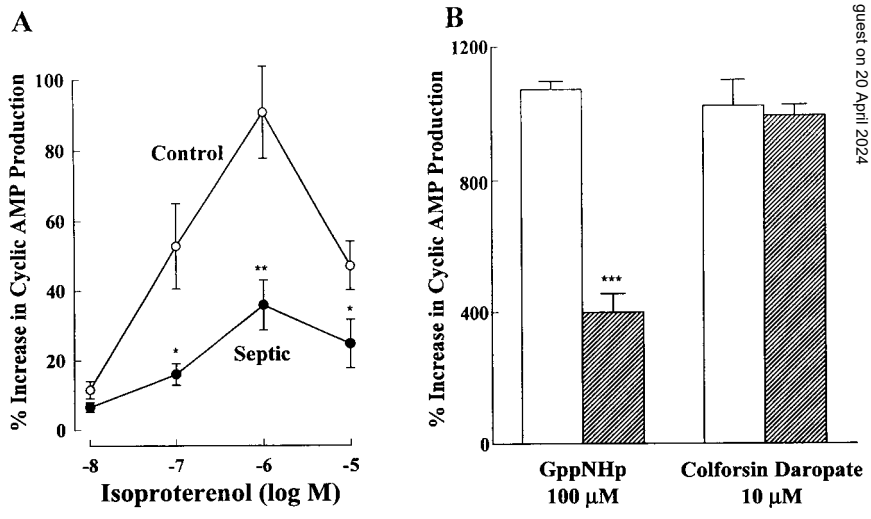

Fig. 6. Adenylate cyclase activity in myocardial membranes prepared from control and 6-h septic rabbits. (*A*) Concentration–response curve for isoproterenol-stimulated adenylate cyclase activity. Guanosine triphosphate, 0.1 mM, was present throughout. (*B*) Adenylate cyclase activity stimulated with 100 μM 5′-guanylyl imidodiphosphate (GppNHp) and 10 μM colforsin daropate. Open and hatched columns indicate the responses of control and septic myocardial membranes, respectively. Each value represents the net increase in adenylate cyclase activity expressed as a percentage of the corresponding activity in the (*B*) absence and (*A*) presence of guanosine triphosphate. Mean of data from (*A*) six and (*B*) five preparations are presented, with SD shown by vertical lines. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* the corresponding control values. AMP = adenosine monophosphate.

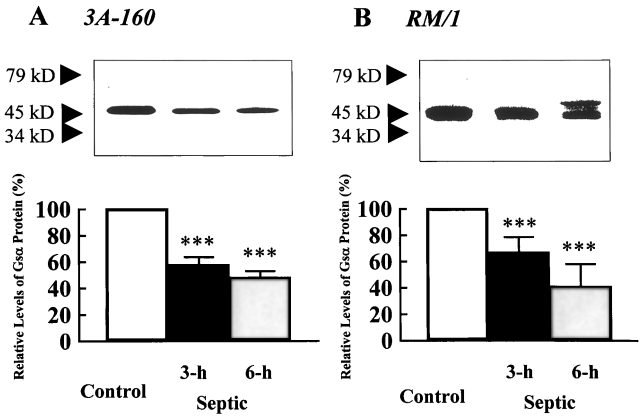

Fig. 7. Immunoblot analysis of $G_{s\alpha}$ in ventricular myocardium from control, 3-h septic, and 6-h septic rabbits. Two different antisera, (*A*) 3A-160 and (*B*) RM/1, were used to identify $G_{s\alpha}$. (*Upper traces*) Representative Western blot indicating marked decreases in expression of 45-kDa band in sepsis. (*Lower traces*) Summary comparing the immunostained band for $G_{s\alpha}$ in the three groups of ventricular myocardium. Densitometric results are expressed as percent of the band obtained with control in each experiment. Mean of data from four experiments are presented, with SD shown by vertical lines. ***Significant difference from control ($P < 0.001$).

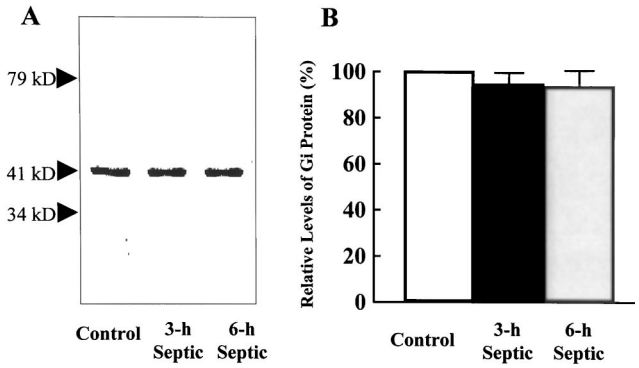

Fig. 8. Immunoblot analysis of $G_{i\alpha}$ in ventricular myocardium from control, 3-h septic, and 6-h septic rabbits. (*A*) Representative Western blot indicating no change in expression of 40-kDa band in sepsis. (*B*) Summary comparing the immunostained band for $G_{i\alpha}$ in the three groups of ventricular myocardium. Densitometric results are expressed as percentage of the band obtained with control in each experiment. Mean of data from four experiments are presented, with SD shown by vertical lines.

unchanged from the control level in 3- and 6-h septic ventricles (95 ± 5 and 93 ± 7% of control, n = 4; fig. 8B).

The antibody that specifically reacts with $G_{\beta\gamma}$ heterotrimeric complex of G proteins identified one single band with a molecular mass of about 35 kDa in rabbit myocardium (data not shown). No change in the protein expression level of $G_{\beta\gamma}$ was observed in 3- and 6-h sepsis (102 ± 11 and 100 ± 10% of control, n = 4).

As illustrated in figure 9A, $G_{s\alpha}$ mRNA was detected as a single band of 1.8 kilobases (kb) that was consistent with the size previously reported for this mRNA.[23] Since the bioimaging analysis of Northern blots indicated that β-actin mRNA was unaffected by sepsis, this mRNA was used as an internal standard. When normalized to β-actin mRNA levels, there was a significant decrease in the steady-state levels of $G_{s\alpha}$ mRNA in ventricular myocardium from 3- and 6-h septic animals (53 ± 5 and 46 ± 4% of control, n = 4; $P < 0.001$; fig. 9B). Northern blot analysis using the $G_{i\alpha}$ probe detected two major mRNA species of approximately 2.2 and 3.4 kb in rabbit myocardium (fig. 10A), which were referred to as $G_{i\text{-}2\alpha}$ mRNA and $G_{i\text{-}3\alpha}$ mRNA.[19] The relative mRNA levels of $G_{i\text{-}2\alpha}$ and $G_{i\text{-}3\alpha}$ were unchanged at 3 h (94 ± 5 and 93 ± 6% of control, n = 4), while being slightly but significantly decreased at 6 h after induction of sepsis (93 ± 5 and 90 ± 7% of control, n = 4; $P < 0.05$; fig. 10B).

Discussion

In the current study, treatment of rabbits with a moderate dose of 100 μg/kg lipopolysaccharide resulted in immediate and sustained reductions in BP during the period of observation (about 7 h following injection of lipopolysaccharide). The changes in HR were not straightforwardly a reflex response to the reduced BP. This dissociation between the changes in BP and HR may, to some extent, be related to a febrile factor or induction of endogenous products causing a direct action on HR. There was only a small difference between the surface and core body temperatures throughout the 7-h observation period, characterizing warm extremities. Thus, the model of endotoxemia studied here could be considered to represent the early stages of endotoxemia which do not follow cooling of the extremities associated with elevated peripheral resistance. In such early stages of septic shock, cardiac output is observed to be increased as peripheral resistance decreases.[24] However, it is likely that this increase in cardiac output is the result of the decrease in afterload (*i.e.*, widespread peripheral vasodilation) rather than increased cardiac function. We found that the basal force of contraction of electrically stimulated ventricular papillary muscles from rabbits 3 and 6 h after induction of sepsis by lipopolysaccharide was the same as that obtained in control animals. Although myocardial contractility is known to decline with profound progression of septic shock,[24] our data imply that the basal contractile force of myocardium was not impaired in this experimental model.

Despite the apparently preserved basal contractile force, the positive inotropic effect of β-adrenoceptor stimulation with isoproterenol was significantly impaired in papillary muscles from lipopolysaccharide-treated rabbits. Thus, the reduced sensitivity to isoproterenol was found at 3 h, and the decrease in the maximum response was also evident at 6 h of sepsis. It has been shown that nitric oxide donors can inhibit the positive inotropic effect of isoproterenol in human atrial muscles[25] and in rat ventricular myocytes.[26] Sun *et al.*[27] have reported that the inotropic response to isoproter-

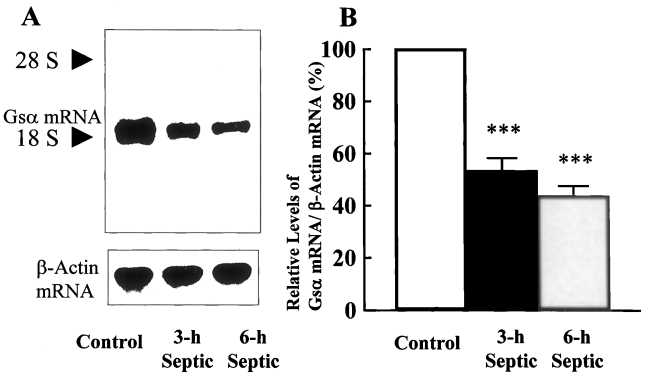

Fig. 9. Northern blot analysis of $G_{s\alpha}$ messenger RNA (mRNA) expression in ventricular myocardium from control, 3-h septic, and 6-h septic rabbits. (*A*) Representative autograph of Northern blot analysis of $G_{s\alpha}$ mRNA and β-actin mRNA expression. The positive signals localized at the molecular size appropriate for the specific mRNA with minimal background. The locations of the 28-S and 18-S ribosomal RNA are indicated. (*B*) Summary of quantification of the steady-state level of $G_{s\alpha}$ mRNA which was normalized using that of β-actin mRNA. Mean of data from four experiments are presented, with SD shown by vertical lines. ***Significant difference from control ($P < 0.001$).

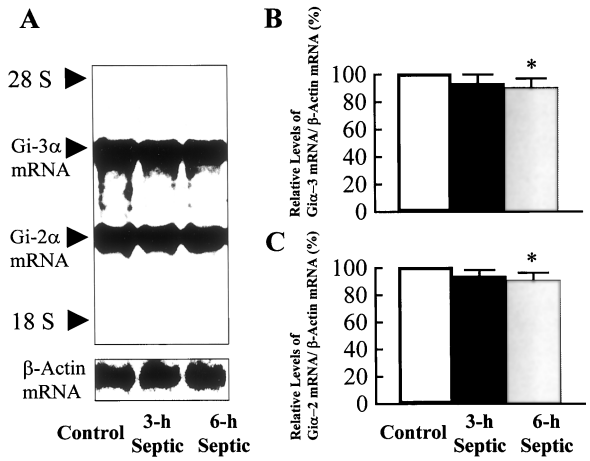

Fig. 10. Northern blot analysis of $G_{i\text{-}2\alpha}$ messenger RNA (mRNA) and $G_{i\text{-}3\alpha}$ expression in ventricular myocardium from control, 3-h septic, and 6-h septic rabbits. (*A*) Representative autograph of Northern blot analysis of $G_{i\text{-}2\alpha}$ mRNA, $G_{i\text{-}3\alpha}$ mRNA, and β-actin mRNA expression. The positive signals localized at the molecular size appropriate for the specific mRNA with minimal background. The locations of the 28-S and 18-S ribosomal RNA are indicated. (*B*, *C*) Summary of quantification of the steady-state mRNA levels of $G_{i\text{-}2\alpha}$ and $G_{i\text{-}3\alpha}$, which were normalized using that of β-actin. Mean of data from four experiments are presented, with SD shown by vertical lines. *Significant difference from control ($P < 0.05$).

enol is attenuated in isolated hearts from rats treated with lipopolysaccharide and the depression was prevented by pretreatment with dexamethasone or in the presence of L-NNA. Furthermore, the nitric oxide synthase inhibitor-sensitive reduction in the positive inotropic effect of isoproterenol has been demonstrated in rat ventricular myocytes exposed to endotoxin-activated macrophages.[28] Yasuda and Lew[29] have shown that the lipopolysaccharide-induced depression of the contractile response of cardiac myocytes to isoproterenol results from a nitric oxide–mediated decrease in myofilament responsiveness to Ca^{2+}. These works suggest that the diminished inotropic responsiveness to β-adrenoceptor stimulation may result from excess nitric oxide generated by inducible nitric oxide synthase (iNOS). However, we found that the inhibition of iNOS by L-NNA did not alter the positive inotropic response to isoproterenol in muscles from either control or septic animals, indicating a minor role of overproduction of nitric oxide by iNOS in the impaired inotropic effect of β-adrenoceptor stimulation in this experimental model. This is in good agreement with the results of other investigators who have shown that induction of iNOS activity alone has no effect on the inotropic responsiveness of rat ventricular myocytes to isoproterenol.[30] It is possible that the protocols that have implicated iNOS in mediating the diminished cardiac or isolated myocyte contractile response to β-adrenoceptor stimulation in animals injected with lipopolysaccharide or infused with specific recombinant cytokines involve induction of multiple cytokine and other autacoid signaling pathways.

In contrast to the effect of isoproterenol, the positive inotropic effects of colforsin daropate, a direct adenylate cyclase activator,[22] and DBcAMP were unchanged in muscles from rabbits 6 h following induction of sepsis. Furthermore, adenylate cyclase activity stimulated directly with colforsin daropate was similar in myocardial membranes from control and septic rabbits. These results suggest that a potential defect in the inotropic responsiveness to β-adrenoceptor stimulation may reside at the level before adenylate cyclase. Previous work has shown a 25% reduction in the density of β-adrenoceptors, which are identified by the use of the radioligand [^{3}H]CGP 12177, in myocytes harvested from rats 4 h after a bolus injection of 10 mg/kg *E. coli* endotoxin.[13] In the current study, however, we found that neither the density of β-adrenoceptors nor the affinity for the antagonist [^{125}I]ICYP was changed in myocardial membranes from rabbits 3 and 6 h following lipopolysaccharide treatment.

There is an important difference between the two models. In our septic model, the changes in plasma catecholamines were insignificant, at least within 6 h after lipopolysaccharide treatment (unpublished results). Conversely, plasma catecholamine levels are extremely elevated in their model,[13] a feature that may have contributed to changes in myocardial β-adrenoceptor expression. A marked elevation of plasma catecholamines may be induced by the high dose of endotoxin. In accordance with our current finding, Joe *et al.*[31] have reported no change in either β-adrenoceptor number or affinity in rat cardiomyocytes cultured in lipopolysaccharide-activated macrophage-conditioned medium where elevation of cyclic AMP in response to isoproterenol was markedly attenuated. We thus conclude that the level of myocardial β-adrenoceptors may

not be a key component of the decreased inotropic responsiveness to β-adrenoceptor stimulation.

Bernardin *et al.*[32] recently demonstrated that the number of β-adrenoceptors is significantly reduced and adenylate cyclase stimulation is heterogenously desensitized in peripheral blood mononuclear cells freshly isolated from septic patients. Although these results are not reconciled with our present data, whether the changes observed in lymphocytes can extrapolate to the heart of septic patients remains an open question. Furthermore, the possibility cannot be entirely ruled out that catecholamine therapy may have influenced, in part, β-adrenoceptor density and adenylate cyclase responses in lymphocytes of septic patients.

The principal finding of this work is that expression of the 45-kDa form of $G_{s\alpha}$ was markedly reduced in myocardium at 3 and 6 h following induction of sepsis. The decrease in expression of $G_{s\alpha}$ was paralleled by a similar decrease in its mRNA level. This suggests that control of $G_{s\alpha}$ protein expression in sepsis occurs, at least in part, at the level of gene expression. To our knowledge, the current study is the first demonstration that cardiac $G_{s\alpha}$ can be regulated at the mRNA level in sepsis. It was found that myocardial adenylate cyclase activity with GppNHp was significantly diminished at 6 h of sepsis. The cardiac protein and mRNA levels of $G_{i\text{-}2\alpha}$ and $G_{i\text{-}3\alpha}$, which are highly and moderately expressed in the heart, respectively,[19] were marginally affected by sepsis. In addition, no change in the $G_{\beta\gamma}$ protein level was found in septic myocardium. It is thus most likely that the effect of GppNHp on myocardial adenylate cyclase seen in sepsis depends on the reduced amount of $G_{s\alpha}$ protein. Furthermore, the decrease in $G_{s\alpha}$ expression may partly explain the attenuated response of myocardial adenylate cyclase to isoproterenol in sepsis. However, it is perhaps now worth considering the effects of the septic state on the inotropic responsiveness to isoproterenol in papillary muscles. Thus, the decrease in the sensitivity to isoproterenol was essentially progressive during the development of sepsis, and the maximum inotropic response to isoproterenol was decreased only at 6 h of sepsis. This cannot be explained simply on the basis of a slight difference in the extent of decreased expression of cardiac $G_{s\alpha}$ between 3 and 6 h of sepsis. In this regard, the attenuated inotropic responsiveness to isoproterenol at 6 h of sepsis may be related to a loss of functional activity of $G_{s\alpha}$ in addition to changes in its protein content. However, experimental evidence to support this speculation awaits further study.

The positive inotropic response to stimulation of myocardial β-adrenoceptors was significantly impaired in the early stage of sepsis as a result of injection of the moderate dose of lipopolysaccharide in rabbits. The lack of effect of L-NNA on the impaired inotropic response excludes involvement of increased nitric oxide formation. Our data demonstrate that profound changes in adenylate cyclase activity stimulated with isoproterenol and GppNHp and the levels of $G_{s\alpha}$ protein and mRNA occurred in this model. Thus, the primary defect in the impaired inotropic responsiveness to β-adrenoceptor stimulation in septic hearts appears to reside at the level of gene expression of $G_{s\alpha}$. Several lines of evidence suggest that proinflammatory cytokines, including tumor necrosis factor-α and interleukin-1β, are synthesized and released during endotoxin shock.[33–35] Whether one or more of these cytokines can mediate the sepsis-induced changes in the β-adrenoceptor–linked signal transduction pathway observed in this study remains the subject of ongoing investigation.

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