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G PROTEIN AND ADENYLATE CYCLASE COMPLEX-MEDIATED SIGNAL TRANSDUCTION IN THE RAT HEART DURING SEPSIS

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ABSTRACT—Changes in the protein level of various subunits of GTP-binding protein and the activity of adenylate cyclase in the rat heart during different phases of sepsis were studied. Sepsis was induced by cecal ligation and puncture (CLP). Experiments were divided into three groups: control, early sepsis, and late sepsis. Early and late sepsis refers to those animals sacrificed at 9 and 18 h, respectively, after CLP. The protein levels of various subunits of GTP-binding protein were determined by Western blot analysis. The activity of adenylate cyclase was measured based on the rate of formation of cAMP from [α-^{32}P]ATP. The results show that protein levels of Gαs and Gβ remained stable during the early and the late phases of sepsis. The protein levels of Gαi-2 and Gαi-3 remained relatively unaltered during the early phase of sepsis, but they were increased by 46.5% ($P < 0.05$) and 61.3% ($P < 0.01$), respectively, during the late phase of sepsis. The basal adenylate cyclase activity remained unchanged during the early phase while it was decreased by 25.7% ($P < 0.05$) during the late phase of sepsis. The isoproterenol-stimulated adenylate cyclase activity was unchanged during early sepsis while it was decreased by 44.6% ($P < 0.01$) during late sepsis. These data demonstrate that during the late hypodynamic phase of sepsis, myocardial Gαi-2 and Gαi-3 protein levels were increased and the increases were coupled with a reduction in adenylate cyclase activity. Because GTP-binding proteins mediate sympathetic control of cardiac function, the present findings may have a pathophysiological significance in contributing to the understanding of the pathogenesis of cardiac dysfunction during the late stage of sepsis.

KEYWORDS—GTP-binding proteins, signal transduction, septic shock, myocardial dysfunction, heart failure

INTRODUCTION

Sympathetic control of cardiac function is mediated through GTP-binding proteins. GTP-binding proteins are composed of a large family of highly homologous proteins that couple more than 100 different receptors with different effector enzymes (1–3). β-adrenergic receptor (βAR; β_1AR and β_2AR) and α-adrenergic receptor (αAR; α_1AR) are coupled to Gαs and Gq subunits, respectively, whereas α_2AR is coupled to Gαi subunit of GTP-binding proteins (1, 4–6). There is direct evidence that organ-specific alterations in GTP-binding protein expression contribute to the pathogenesis of heart diseases in human and animal models. Changes in Gαs and Gαi protein levels and the gene transcripts encoding them are found to be associated with heart failure, ischemic cardiomyopathy, hypertrophy, and hypertension (7–13). In shock and sepsis, Gαi levels are found to be elevated in the heart of catecholamine-refractory septic shock patients (14) and in the neonatal rat heart myocytes upon exposure with the plasma of noradrenaline-treated septic shock patients (15). Because the available data in the literature regarding changes in myocardial GTP-binding proteins are limited to the hypodynamic phase and because sepsis is a two-phase process in which patients initially go through a hyperdynamic phase and, subsequently, the hypodynamic phase, it is important that an animal model capable of exhibiting a biphasic septic syndrome be adapted for the investigation of the role of GTP-binding proteins in the pathogenesis of heart dysfunction in septic shock. Furthermore, because previous work from this laboratory has revealed that βAR and αAR undergo a biphasic expression in the rat heart during the two distinct cardiodynamic phases of sepsis (16–18), the present work dealing with altered G protein/adenylate cyclase system was undertaken to clarify changes in postreceptor events in an attempt to understand the pathogenesis of myocardial dysfunction during the progression of sepsis.

MATERIALS AND METHODS

Animal model

All animal experiments in this study were performed with the approval of the Animal Care Committee of Saint Louis University School of Medicine and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats weighing from 270–320 g were used. All animals were fasted overnight with free access to water before the induction of sepsis. Sepsis was induced by cecal ligation and puncture (CLP) as described by Wichterman et al. (19) with minor modifications (20). Under halothane anesthesia, a laparotomy was performed (the size of the incision was 2.5 cm), and the cecum was ligated with a 3-0 silk ligature and punctured twice with an 18-gauge needle. The cecum was then returned to the peritoneal cavity and the abdomen was closed in two layers. Control rats were sham operated (a laparotomy was performed and the cecum was manipulated but neither ligated nor punctured). It should be mentioned that the values of the biochemical and the molecular biological indexes (GTP-binding protein levels and adenylate cyclase activities) that we reported in this study were indistinguishable between the sham-CLP and the nonsurgical controls. All animals were resuscitated subcutaneously with 4 mL of 0.9% NaC1/100 g body weight at the completion of surgery and also at 7 h postsurgery. Animals were fasted but had free access to water after operative procedure. Experiments were divided into three groups: control, early sepsis, and late sepsis. Early and late sepsis refers to those animals sacrificed at 9 and 18 h, respectively, after CLP. Previous experi-

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ments have indicated that myocardium was in the hyperdynamic state during the early phase, whereas it was in the hypodynamic state during the late phase of sepsis (18). The mortality rates were 0% for control, 4% for early sepsis, and 27% for late sepsis.

Preparation of cardiac sarcolemmal membranes

Rat heart sarcolemmal membranes were prepared according to a procedure previously used in this laboratory (16, 17). Hearts removed from control and septic rats were weighed, freeze-clamped with aluminum clamps precooled in liquid nitrogen, and pulverized with a mortar and pestle precooled in liquid nitrogen. The powdered tissues were thawed and homogenized with Tekmar Tissumizer (Model SDT) in 5 vol of buffer A (50 mM $NaH_2PO_4/Na_4P_2O_7$, pH 7.4; 10 mM EDTA; 25 mM NaF; 1 μg/mL soybean trypsin inhibitor; 1 μg/mL aprotinin; 0.75 μg/mL pepstatin A; and 2 μg/mL leupeptin). The homogenates were centrifuged at 14,000 g for 20 min. The resulting pellets were suspended in buffer B (0.6 M NaCl, 50 mM $NaH_2PO_4/Na_4P_2O_7$, pH 7.4; 10 mM EDTA; and 25 mM NaF), rehomogenized, and recentrifuged at 14,000 g for 20 min. The pellets from the second centrifugation were suspended in buffer A, homogenized three times (each time for 30 s), and centrifuged at 2300 g for 15 min. The resulting pellets were resuspended, rehomogenized, and recentrifuged at 2300 g for 15 min. All homogenizations throughout were performed with Tekmar Tissumizer. The 2300-g supernatants were combined and centrifuged at 79,700 g for 40 min. The 79,700-g pellets were suspended in 1 M sucrose dissolved in buffer C (0.3 M NaCl, 50 mM $NaH_2PO_4/Na_4P_2O_7$, and 100 mM Tris-HCl, pH 7.4). Ten milliliters of this suspension were layered at the bottom of a discontinuous sucrose gradient consisting of 9 mL of 0.6 M sucrose (dissolved in buffer C) and 9 mL of 0.25 M sucrose (dissolved in 10 mM histidine, pH 7.4). The gradients were centrifuged at 254,000 g for 70 min. Fractions at 0.6:0.25 M sucrose interfaces were collected, diluted with buffer C, and then centrifuged at 162,600 g for 40 min. The final pellets were suspended in a buffer containing 0.25 M sucrose and 30 mM histidine (pH 7.4), stored at −80°C, and then used as sarcolemmal membranes. The entire procedure was performed at 4°C unless otherwise stated.

Determination of GTP-binding protein levels by Western blot analysis

Western blot analysis was performed according to the method of Ausubel et al. (21) with minor modification (22). Sarcolemmal membranes containing various amounts of protein (100 μg for Gαs, 100 μg for Gαi-2, 100 μg for Gαi-3, and 40 μg for β-subunit) were denatured and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10–20% polyacrylamide gradient gel). Using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell, proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were transferred to a polyvinylidene fluoride membrane (Bio-Rad) at 8 mA (constant current) for 14–16 h in transfer buffer (25 mM Tris base, pH 8.3; 192 mM glycine; 5% [vol/vol] methanol). Nonspecific binding sites were blocked with 10% (wt/vol) of nonfat dry milk in Tris-buffered saline (TBS: 20 mM Tris-HC1, pH 7.4; 137 mM NaC1) for 4 h at 4°C. Blots were washed three times (10 min each) with TBST (TBS containing 0.5% Tween-20) followed by incubation with a 1:1000 dilution of a polyclonal antibody recognizing Gαs, Gαi-1 and Gαi-2, Gαi-3, or Gβ subunit (Calbiochem-Novabiochem International, San Diego, CA) for 5 h at room temperature. Subsequently, the blots were washed three times (10 min each) with TBS and then incubated with a 1:2,000 dilution of an antirabbit immunoglobulin, peroxidase-linked species-specific whole antibody (Amersham Life Science) for 1 h at room temperature. The blots were then washed four times (10 min each) with TBST, followed by incubation with enhanced chemiluminescent (ECL) Western blotting detection reagent (Amersham Life Science), and finally exposed to Hyperfilm-ECL (Amersham Life Science) for 40 s. Autoradiographs were scanned with Hewlett-Packard ScanJet 4C Scanner, and the relative densities were quantified by Jandel Scientific Software program (Sigma Gel). It should be mentioned that the quantitation of immunoblots was performed by comparing each individual subunit of GTP-binding protein to the control only for that particular blot. For each subunit, six separate blots were performed. Each blot consisted of control, early septic, and late septic samples in equal amount of protein. Preliminary experiments have indicated that the control levels (six control samples prepared from six different animals and performed on the same blot) for each subunit were almost identical.

Assay of adenylate cyclase activity

Adenylate cyclase activity was determined by a method previously used in this laboratory (22) with modification. The reaction mixture in a final vol of 200 μL contained 50 mM Tris-HC1, pH 7.5; 5 mM $MgCl_2$; 5 mM creatine phosphate; 0.4 mg/mL creatine phosphokinase; 1 mM cAMP; 1 mM dithiothreitol; 1 mM [α-^{32}P]ATP (2×10^6 counts/min). The reaction was initiated by the addition of sarcolemmal membranes containing 100 μg of protein and was allowed to proceed for 8 min at 37°C. The reaction was terminated by the addition of 0.2 mL of a solution containing 2% sodium dodecyl sulfate; 40 mM ATP; 50 mM Tris-HCL, pH 7.5; 1.4 mM [2,8-^{3}H]cAMP (20,000 counts/min). The reaction mixture was then diluted with 0.6 mL of deionized water and was decanted into Dowex columns. The eluate from this and two subsequent 1-mL washes was discarded. Three milliliters of water were then added to each column and the eluate was collected in a test tube containing 0.2 mL of 1.5 M imidazole (pH 7.5). After mixing, the contents of each tube were poured into a second column containing neutral alumina pre-equilibrated with 0.1 M imidazole HC1 (pH 7.5). The eluate of the alumina column was collected directly into a scintillation vial and then counted by a liquid scintillation counter. It should be mentioned that [2,8-^{3}H]cAMP was included in the stop solution to determine the recovery of cAMP from the columns.

Other assays

(Na^+-K^+)-ATPase, Ca^{2+}-ATPase, and glucose-6-phosphatase activities were determined as described previously by us (22). The protein concentration of sarcolemmal membranes was determined by the method of Lowry et al. (23).

Statistical analysis

The statistical analysis of the data was performed using one-way ANOVA followed by Student-Newman-Keuls tests. A P value of less than 0.05 was accepted as statistically significant.

Materials

[α-^{32}P]ATP (650 Ci/mmol) and [2,8-^{3}H]adenosine-3′,5′-cyclic monophosphate (38 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc. Horseradish peroxidase-labeled second antibody conjugate (donkey anti-rabbit), ECL Western blotting detection reagent, and Hyperfilm were obtained from Amersham Life Science. ATP, cyclic AMP, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical Co. Purified primary antibodies, including rabbit anti-Gαs subunit, C-terminal (385-394); rabbit anti-Gαi-1 and Gαi-2 subunits, C-terminal (345-354); rabbit anti-Gαi-3 subunit, C-terminal (345-354); and rabbit anti-G-protein β subunit, internal (127–139) were supplied by Calbiochem-Novabiochem International. Other chemicals and reagents were of analytical grade.

RESULTS

Table 1 shows marker enzyme activities of heart sarcolemmal membranes isolated from control and septic rats. (Na^+-K^+)-ATPase served as marker for sarcolemmal membranes, while Ca^{2+}-ATPase and glucose-6-phosphatase served as markers for sarcoplasmic reticulum. In sarcolemmal membranes, (Na^+-K^+)-ATPase activities were enriched 17- to 19-fold whereas Ca^{2+}-ATPase and glucose-6-phosphatase activities were not enriched as compared with the homogenates within each experimental group. The pattern of changes in the enzymatic marker in the sarcolemmal membrane fraction was essentially identical among the three experimental groups: control, early sepsis, and late sepsis. These data indicate that sarcolemmal membrane preparations were highly purified and minimally contaminated with sarcoplasmic reticulum. Furthermore, the degrees of purity of sarcolemmal membranes and the extents of cross contamination with sarcoplasmic reticulum

TABLE 1. **Marker enzyme activities of cardiac sarcolemmal membranes isolated from control and septic rats**

	Control		Early sepsis		Late sepsis	
	Homo	SL	Homo	SL	Homo	SL
(Na^+ + K^+)-ATPase	1.5 ± 0.2	29.0 ± 1.4	1.7 ± 0.4	28.1 ± 0.9	1.4 ± 0.2	24.0 ± 1.7
Ca^{2+}-ATPase	2.4 ± 0.7	3.3 ± 0.4	2.1 ± 0.6	3.2 ± 0.3	2.7 ± 0.9	3.1 ± 0.2
Glucose-6-phosphatase	2.5 ± 0.6	2.4 ± 0.4	2.1 ± 0.3	3.1 ± 0.2	2.1 ± 0.3	3.3 ± 0.2

Values are Mean ± SE in μmol/mg/h. Each value represents a mean of six experiments. Homo, homogenate; SL, sarcolemmal membrane.

among the control, the early septic and the late septic groups were the same.

Preliminary experiments were conducted to correlate autoradiographic intensities of the immunoblots with various amounts of GTP-binding protein (Gαs, Gαi-2, Gαi-3, and Gβ) in the control rats before the full-scale Western blot analysis. The values of signal intensity obtained were proportional to the amounts of protein in the putative GTP-binding protein subspecies bands (data not shown). Based on these observations, appropriate concentrations of sarcolemmal membrane proteins within linear range were then selected (100 μg for Gαs, 100 μg for Gαi-2, 100 μg for Gαi-3, and 40 μg for β subunit) for Western blot analysis for the comparison between control and septic experiments.

Figure 1 shows the Western blot analysis of Gαs protein levels in rat heart sarcolemmal membranes during different phases of sepsis. Two putative bands for Gαs, with molecular weights of 45 and 42 kDa, were detected. This finding is consistent with that reported in the literature (24). Quantitative analysis of the data reveals that the protein levels for 45-kDa and 42-kDa forms of Gαs were unaltered during both early and late phases of sepsis. These findings demonstrate that Gαs protein levels remained unaffected in rat heart sarcolemmal membranes during the progression of sepsis.

Figures 2 and 3 depict changes in Gαi-2 and Gαi-3 protein levels in rat heart sarcolemmal membranes during the early and the late phases of sepsis. Western blot analysis reveals that Gαi-2 protein levels were not significantly affected (+17.3%; $P > 0.05$) during the early phase but were increased by 46.5% ($P < 0.05$) during the late phase of sepsis (Fig. 2). It should be noted that the anti-Gαi-2 antibody used in this study cross reacts with Gαi-l species. The fact that Gαi-l signal was not detected in our study indicates that Gαi-l was absent from rat heart sarcolemmal membranes (25). As shown in Figure 3,

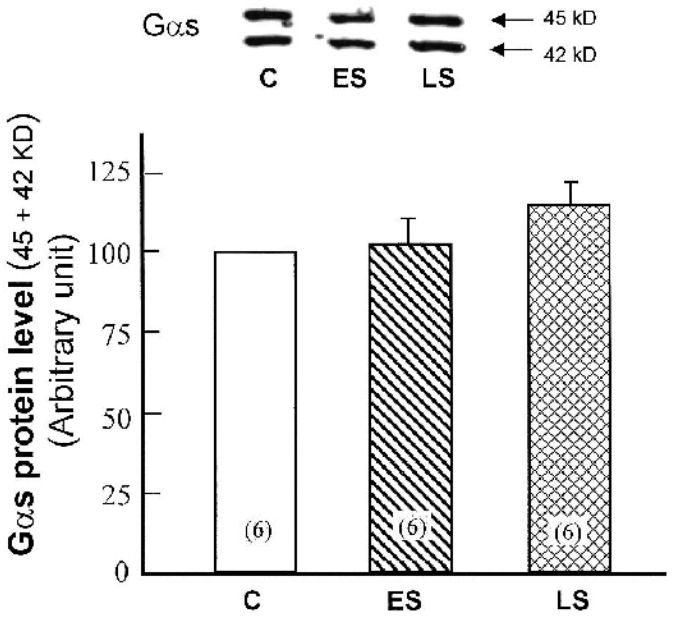

FIG. 1. **Western blot analysis of Gαs protein level in the sarcolemmal membranes of control and septic rat hearts.** Western blot analysis was performed as described under the Materials and Methods section using polyclonal antibody raised specifically against the Gαs subunits. Vertical bars indicate SEM. Number of experiments is shown in the parenthesis within each column. C, Control; ES, early sepsis; LS, late sepsis.

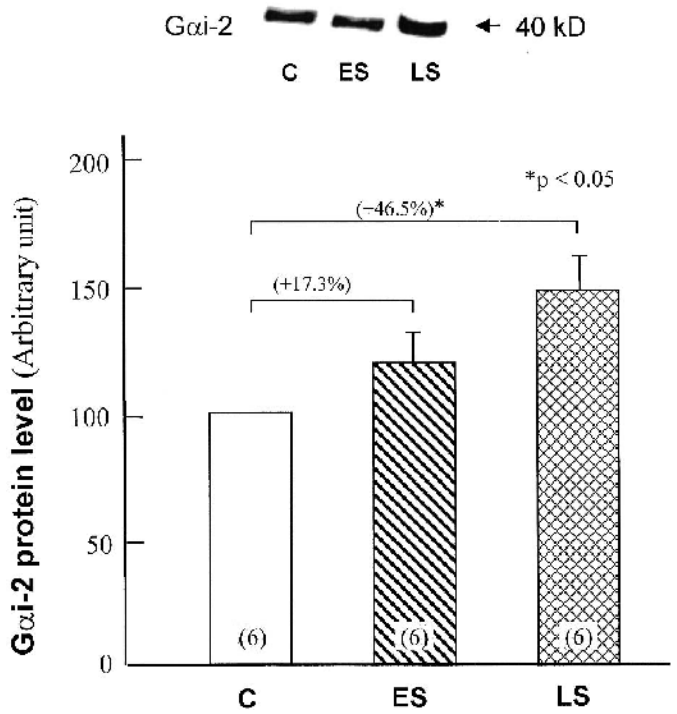

FIG. 2. **Western blot analysis of Gαi-2 protein level in the sarcolemmal membranes of control and septic rat hearts.** Western blot analysis was carried out as described under the Materials and Methods section using polyclonal antibodies raised specifically against the Gαi-1 and Gαi-2 subunits of G-protein. The arrow shows the molecular weight of Gαi-2 subunit. Vertical bars indicate SEM. Number of experiments is shown in the parenthesis within each column. C, Control; ES, early sepsis; LS, late sepsis.

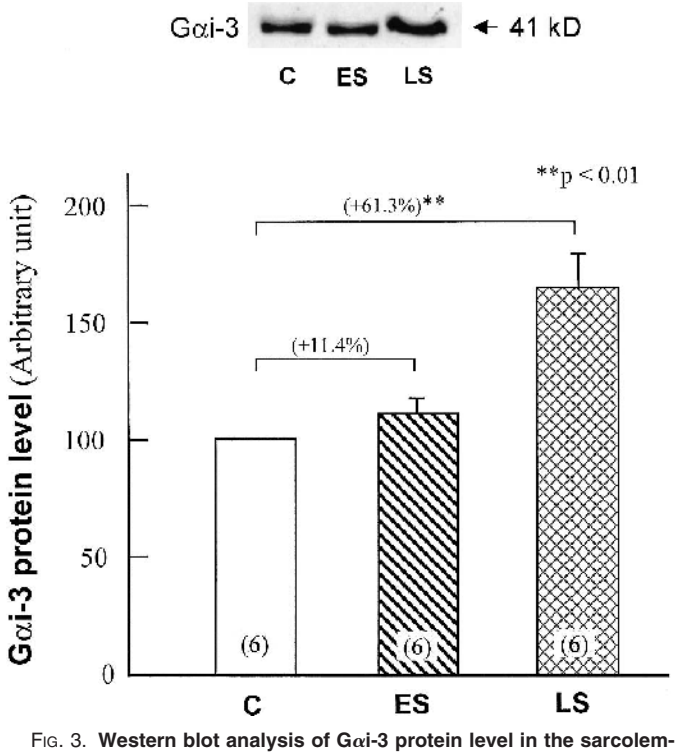

FIG. 3. **Western blot analysis of Gαi-3 protein level in the sarcolemmal membranes of control and septic rat hearts.** Western blot analysis was performed as described under the Materials and Methods section using polyclonal antibody raised specifically against the Gαi-3 subunit of G-protein. The arrow indicates the molecular weight of Gαi-3 subunit. Vertical bars indicated SEM. Number of experiments is shown in the parenthesis of each column. C, control; ES, early sepsis, LS, late sepsis.

Gαi-3 protein levels remained relatively stable (+11.4%, $P > 0.05$) during early sepsis whereas they were increased by 61.3% ($P < 0.01$) during late sepsis (Fig. 3). The data presented in Figures 2 and 3 demonstrate that Gαi-2 and Gαi-3 protein levels were increased during the late stage of sepsis.

Figure 4 shows the Western blot analysis of Gβ protein levels in rat heart sarcolemmal membranes during the progress of sepsis. Quantitative analysis of Western blot indicates that Gβ protein levels remained unaltered during the early and late phases of sepsis. It should be mentioned that the Gβ antibody used in this study cross reacts with both β1 and β2 subunits of GTP-binding protein. In our experiments, only β2 subunit with a molecular weight of 36 kDa was detected, indicating that β2 is the predominant subtype of Gβ expressed in the myocardium (26). These findings indicate that Gβ protein level was unaffected during the progression of sepsis.

Figure 5 depicts changes of adenylate cyclase activity in rat heart sarcolemmal membranes during the two cardiodynamically distinct phases of sepsis. Basal adenylate cyclase activity remained unchanged during the early phase whereas it was decreased by 25.7% ($P < 0.05$) during the late phase of sepsis (Fig. 5A). Similarly, the isoproterenol-stimulated adenylate cyclase activity was unchanged during early sepsis while it was decreased by 44.6% ($P < 0.01$) during late sepsis (Fig. 5B). These data indicate that the functional endpoint for α-subunit of GTP-binding protein, the adenylate cyclase activity, was decreased during the late hypodynamic phase of sepsis.

DISCUSSION

GTP-binding proteins mediate sympathetic control of cardiac function. The best characterized GTP-binding proteins consist of two major classes: the stimulatory component, Gs, which stimulates and the inhibitory component, Gi, which inhibits adenylate cyclase activity (27). Adenylate cyclase is a membrane-bond enzyme that catalyzes the hydrolysis of ATP to cAMP. cAMP, an intracellular messenger, activates protein kinase A and then initiates a series of enzymatic reactions leading to a phosphorylation cascade of multiple proteins that eventually regulate both the rate and the force of cardiac contraction (27). In the heart, catecholamines released into the synaptic cleft at sympathetic nerve terminals bind to either αAR or βAR on the cardiac sarcolemma. Stimulation of β_1AR, β_2AR, and α_1AR activates Gαs and leads to increased cAMP production through adenylate cyclase activation, whereas stimulation of α_2AR activates Gαi and leads to decreased cAMP production through adenylate cyclase inactivation (1, 27). An increase or a decrease in Gαs and/or Gαi levels coupled with activation or inactivation of adenylate cyclase, thus function as a modulating system regulating myocardial contractility. The present study in cardiac sarcolemma reveals that during late sepsis, the Gαi-2 and Gαi-3 protein levels were increased and the increases were coupled with a decrease in adenylate cyclase activity, may have a pathophysiological significance in contributing to the understanding of the pathogenesis of cardiac dysfunction during the late stage of sepsis.

The exact mechanisms responsible for the increase in Gαi-2 and Gαi-3 protein levels coupled with a decrease in adenylate cyclase activity were not completely understood. It has been reported that in the rat, a prolonged infusion of isoprenaline decreased myocardial βAR number and increased heart Gαi content (28, 29). In rat heart muscle cells, exposure to noradrenaline decreased βAR density, increased Gαi-2 and Gαi-3 protein levels, and reduced adenylate cyclase sensitivity (30). The noradrenaline-induced increase in Gi protein α-subunits and adenylate cyclase desensitization were blocked by a βAR antagonist (30). In human end-stage heart failure, myocardial βAR density was decreased and the decrease in βAR density was accompanied by an increase in Gαi protein content (7, 12, 31). These reports demonstrate that a decreased βAR density in response to an enhanced sympathetic drive is an important causative factor leading to the increased Gαi protein level and the decreased adenylate cyclase sensitivity.

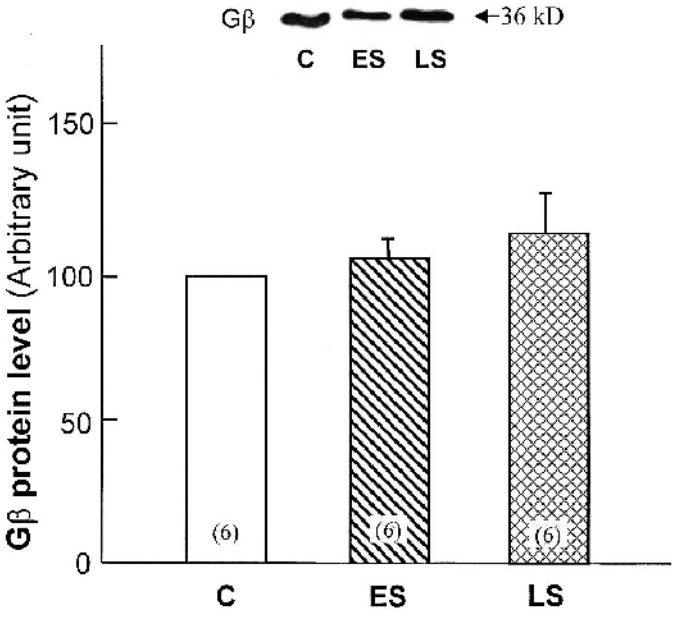

FIG. 4. **Western blot analysis of Gβ protein level in the sarcolemmal membranes of control and septic rat hearts.** Western blot analysis was carried out as described under the Materials and Methods section using polyclonal antibody raised specifically against the β subunit of G-protein. The arrow shows the molecular weight of Gβ subunit. Vertical bars indicate SEM. Number of experiments is shown in the parenthesis within each column. C, Control; ES, early sepsis; LS, late sepsis.

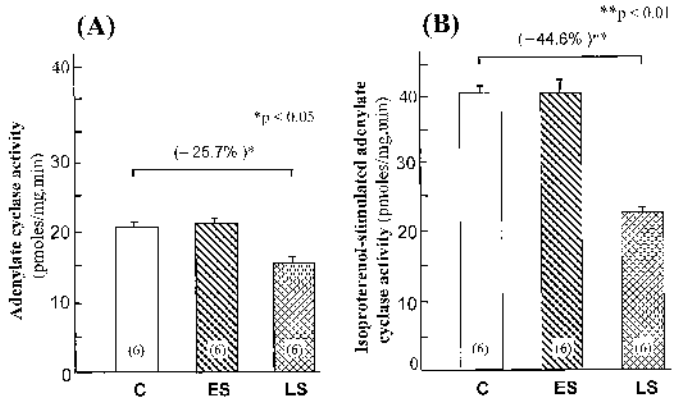

FIG. 5. **Changes in adenylate cyclase activities in the rat heart during different phases of sepsis.** Adenylate cyclase activities were assayed as described under the Materials and Methods section except that for isoproterenol stimulated adenylate cyclase (B), 1 mM of isoproterenol was present in the reaction mixture. Vertical bars indicate SEM. Number of experiments is shown in the parenthesis within each column. C, Control; ES, early sepsis; LS, late sepsis.

The fact that myocardial βAR and αAR densities have been found to greatly reduced in response to the elevated circulating catecholamines during the late phase of sepsis (16–18), it is conceivable that the observed increase in the Gαi-2 and Gαi-3 protein levels and the decrease in the adenylate cyclase activity are the results of underexpression of βAR and αAR. In addition to the altered βAR and αAR dynamics, changes in adenosine A1 receptor may contribute to the increase in Gαi protein level and the decrease in adenylate cyclase activity because adenosine A1 receptor is known to exist in cardiac myocytes which coupled to Gαi and cyclase inhibition (32). Further study is required to clarify role of adenosine A1 receptor on the altered Gαi and cyclase inhibition in the heart during sepsis.

Because receptors and their coupling proteins belong to different groups of genes that are controlled by distinct mechanisms of regulation (33), it is possible that the altered expression of various subspecies of GTP-binding protein is a result of modification of their gene transcripts, instead of a secondary response to the altered receptor dynamics. In heart failure, plasma catecholamine levels were elevated and the elevated plasma catecholamine was correlated with an increase in the myocardial Gαi-2 mRNA abundance and an unchanged Gαs mRNA level (7, 12). It is of interest to note that a consensus sequence of a "cAMP response element" has been described in the promoter region of the Gαi-2 gene (34), whereas it is lacking in the Gαs gene (35). These findings suggest that an increased sympathetic drive via the cAMP cascade would lead to an increased transcription rate of the Gαi-2, but not that of the Gαs genes. Whether a similar transcriptional regulatory mechanism is responsible for the increase in Gαi-2 and Gαi-3, but not the Gαs, protein levels in the rat heart during the late stage of sepsis, remains to be investigated.

It is of interest to note that lack of change in Gβ subunit of GTP-binding protein in the rat heart during sepsis is strikingly similar to that reported in the liver (22). Furthermore, Gβ as well as Gαs are found to be quite stable under other pathological conditions (7, 12, 22). Because chronic infusion of isoprenaline in rats leads to increases in Gαi-2 and Gαi-3 mRNA levels whereas Gαs and Gβ mRNA levels remain unchanged (7), these seem to suggest that Gβ and Gαs subunits constitute the major components of the GTP-binding protein associated signal transduction pathway that is irresponsive to the external stimuli.

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