One Hour of Myocardial Ischemia Decreases the Activity of the Stimulatory Guanine-Nucleotide Regulatory Protein $G_s$

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The effect of 1 hour of myocardial ischemia on the function of the stimulatory guanine-nucleotide-binding protein $G_s$ was examined. This study follows our recent finding that myocardial ischemia increases the density of $\beta$-adrenoreceptors in a conscious canine model while having the opposite effect on the activity of adenylate cyclase. Coronary artery occlusion was induced in five conscious dogs and verified by measurement of blood flow using the Doppler and microsphere techniques. Alterations in the level and function of $G_s$ were examined in sarcolemmal membranes prepared from ischemic and nonischemic regions of the left ventricle. After 1 hour of coronary artery occlusion, the functional activity of sarcolemmal $G_s$, as determined by reconstitution with cAMP membranes, decreased by 27±7% in the ischemic zone. Cholera toxin labeling performed in parallel with the reconstitution studies demonstrated a similar decrease of 28±7%. This was associated with decreases in basal activity and decreases in adenylate cyclase activity stimulated by GTP, GTP plus isoproterenol, sodium fluoride, and forskolin. Thus, a defect distal to the $\beta$-adrenoreceptor occurs in the transduction of adrenergic signals to the heart as a consequence of 1 hour of ischemia. (Circulation Research 1989;65:1145-1150)

Several abnormalities in the autonomic control of cardiac function have been identified in experimental animals and in patients with myocardial ischemia. However, the biochemical mechanisms underlying the pathophysiology of myocardial ischemia are yet to be defined. The membrane-bound $\beta$-adrenoreceptor-adenylate cyclase system is of special interest because cyclic AMP (cAMP) not only is important in the regulation of myocardial contractility and metabolism but also is thought to be involved in pathological sequelae including arrhythmias.1 Whereas numerous studies have shown that $\beta$-adrenoreceptor density is increased in the ischemic myocardium,2-4 reports of altered adenylate cyclase activity and tissue cAMP content have been conflicting.1-9 We have recently shown that the increase in $\beta$-adrenoreceptor density in the ischemic myocardium in a conscious dog model is accompanied by a decrease in the activity of adenylate cyclase.6 Furthermore, the change in the activity of the enzyme was noted in the presence of stimuli acting through the $\beta$-adrenoreceptor or distal to it at the level of the stimulatory guanine-nucleotide-binding protein $G_s$ and the catalytic unit.6 These findings suggested that the depressed responsiveness of adenylate cyclase in ischemia could be ascribed to a deficiency in the activity of the $G_s$ protein. The results of the present study indicate that 1 hour of coronary artery occlusion in the conscious dog leads to a decrease in the level of $G_s$ activity in the ischemic zone.

Materials and Methods

Five adult dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg) and ventilated with a Harvard respirator (Harvard Apparatus, South Natick, Massachusetts). A left thoracotomy was performed through the fifth intercostal space using sterile surgical technique. A Doppler flow probe and hydraulic occluder were implanted around the left circumflex artery. Catheters were implanted in the descending thoracic aorta and left atrium, and a miniature pressure gauge was implanted in the left ventricle (LV). A pair of ultrasonic crystals was implanted on the pos-
Hemodynamics

All hemodynamics were measured continuously in the conscious state. The hemodynamic data were recorded on multichannel tape recorders and played back on a direct-writing oscillograph. Arterial pressure was measured from the aortic catheter with a strain gauge manometer (model P23ID, Statham, Oxnard, California). LV pressure was measured with a solid state miniature pressure gauge and calibrated in vitro against a mercury manometer and in vivo against an arterial and left atrial pressure manometer. A cardiotachometer triggered by the pressure pulse provided instantaneous and continuous records of heart rate. Regional posterior left ventricular wall thickening was measured with an ultrasonic dimension gauge. Regional myocardial blood flow was measured with tracer-labeled microspheres (15 μm in diameter, New England Nuclear, Boston, Massachusetts) by the reference withdrawal method. Every experiment included two injections of one of several isotopes (95Nb, 141Ce, 85Sr, or 45Sc) for each flow determination. Approximately 1–2 million microspheres were injected via the injection of microspheres and completed 2 minutes later.

On the day of the experiment, the coronary artery occluder was inflated after intramuscular administration of 0.2 mg/kg morphine sulfate, recording of baseline hemodynamics, and the baseline injection of radioactive microspheres. Occlusion was confirmed by the absence of the blood flow signal using the Doppler ultrasonic technique. After 45–50 minutes of coronary artery occlusion, radioactive microspheres were administered again. The dogs were anesthetized with 30 mg/kg sodium pentobarbital, and their hearts were excised and placed in iced saline at 1 hour after coronary artery occlusion. The distribution of the occluded and nonoccluded coronary arteries was used as a guide to divide the LV into potentially central ischemic and nonischemic zones. The ischemic zone comprised the central area of the left circumflex territory. The nonischemic zone comprised the central area of the left anterior descending territory.

Preparation of Myocardial Membranes

Membranes were prepared from ischemic and nonischemic regions of the LV as previously described. The crude membrane preparation was used for radioligand binding studies and adenylate cyclase assays. To verify that the tissue being prepared corresponded to ischemic and nonischemic regions, blood flow measurements were determined from the levels of microspheres present in the sediment of the first centrifugation. Purified sarcolemmal membranes were also prepared by removing the contractile proteins and separating the sarcolemma by centrifugation in a sucrose density gradient. This highly enriched sarcolemmal preparation (25-fold) was used in cholera toxin labeling and reconstitution studies. In two of the five dogs, sarcolemma were prepared from ischemic myocardium that had been separated into endocardial and epicardial layers.

β-Adrenoceptor Binding Studies

β-Adrenoceptor density was determined in 10 μg membranes using [125I]cyanopindolol (0.025–1.0 nM) in the presence and absence of 0.1 mM isoproterenol. Binding data was analyzed by the nonlinear interactive "Ligand" program of Munson and Rodbard.

Adenylate Cyclase Assay

The method of Salomon et al. was used to measure basal adenylate cyclase activity, as well as the activity obtained in the presence of 0.1 mM isoproterenol with 0.1 mM Gpp(NH)p, 10 mM sodium fluoride, and 0.1 mM forskolin. The assay buffer contained 2×106 cpm of [3H]cAMP used as a recovery marker. The reaction was initiated by the addition of membranes and terminated after 10 minutes with the addition of 100 μl 2% sodium dodecyl sulfate. cAMP was separated by sequential chromatography on Dowex 50 cation exchanger and on neutral alumina.

Gs Activity

Reconstitution experiments were performed as an evaluation of Gs functional activity. The following method has been published elsewhere. Three hundred micrograms of purified sarcolemma were solubilized in 100 μl of 2% cholate for 1 hour on ice. After centrifugation the adenylate cyclase was heat-inactivated for 10 minutes at 30°C. The extract was then serially diluted in 0.1% lubrol, and 15 μl Gs was mixed with 25 μl (60 μg) ccc membrane. Pretreatment of Gs was initiated by the addition of 20 μl buffer containing (mM) HEPES 125 (pH 8.0), MgCl2 50, ATP 2.5, GTP 500 (μM), EDTA 1, phosphocreatine 30, creatine phosphokinase 350 (units/ml), cAMP 2.5, and 20 μl sodium fluoride 40. After a
20-minute incubation at 30°C, the reconstituted adenylate cyclase activity was assessed as described above. Adenylate cyclase activity was plotted as a function of added sarcolemma. The activity of G, in reconstituting adenylate cyclase was measured as the slope of the curve where adenylate cyclase activity increases linearly in proportion to the amount of added sarcolemmal G,. The activity of reconstituted G, was linear from 0 to 300 ng sarcolemma.

**Cholera Toxin Labeling**

Differences in the level of G, from normal and ischemic ventricle were determined in experiments with [32P]nicotinamide adenine dinucleotide (NAD) and cholera toxin. The method has been previously described by our laboratory. Briefly, 10 µg purified sarcolemma were labeled for 90 minutes at 30°C in a buffer containing (mM) potassium phosphate 125, pH 7.4, phosphocreatine 20, thymidine 10, creatine phosphokinase 60 (units/ml), ATP 5, GTP 0.25, NAD phosphate 1.25, MgCl₂ 5, DTT-activated cholera toxin 200 (µg/ml), and [32P]NAD 5 (µM). Forty micrograms of membranes prepared from the G,-deficient S49 cys�� cells was also included as a source of ADP-ribosylation factor. Membranes were centrifuged and resuspended in electrophoresis sample buffer, and the labeled proteins were resolved on 13% polyacrylamide gels. Nonspecific labeling was determined in the absence of cholera toxin. The molar amount of incorporated label was calculated from the total counts contained in the labeled bands and the specific activity of the [32P]NAD.

**Analysis**

The concentrations of tissue and plasma catecholamines were determined by the radioenzymatic assay of Peuler and Johnson. Protein concentration was measured by the method of Lowry. Data were analyzed by paired t test and are expressed as mean±SEM.

**Results**

**Hemodynamics**

Baseline and pre- and post–coronary artery occlusion hemodynamics are reported in Table 1. One hour of coronary artery occlusion did not significantly affect LV systolic pressure, mean arterial pressure, LV dP/dt, or heart rate. LV end-diastolic pressure was increased. As is characteristic of the canine model of myocardial ischemia, the reduction in blood flow was much more marked in the endocardial than in the epicardial layers. With coronary artery occlusion, end-diastolic wall thickness decreased from 4.0±1.1 mm in the nonischemic zone to 0.1±0.4 mm in the ischemic zone; this occurrence indicated complete loss of systolic wall thickening.

**Table 1. Hemodynamics**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Coronary occlusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure</td>
<td>100±3.4</td>
<td>99±5.6</td>
</tr>
<tr>
<td>(mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV systolic pressure</td>
<td>126±6.2</td>
<td>123±9.2</td>
</tr>
<tr>
<td>(mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV dP/dt</td>
<td>3,977±324</td>
<td>3,491±268</td>
</tr>
<tr>
<td>(mm Hg/sec)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate</td>
<td>86±4.0</td>
<td>110±9.1</td>
</tr>
<tr>
<td>(beats/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic zone wall</td>
<td>4.0±1.1</td>
<td>0.1±0.4*</td>
</tr>
<tr>
<td>thickening (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood flow</td>
<td>1.00±0.13</td>
<td>0.13±0.08*</td>
</tr>
<tr>
<td>Endocardial (ml/min/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epicardial</td>
<td>0.88±0.06</td>
<td>0.43±0.31</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *p<0.05 different from control group.

**Receptor Binding and Adenylate Cyclase Activity**

The β-adrenoreceptor density in the ischemic LV (102±20 fmol/mg) was higher than that in the nonischemic LV (65±10 fmol/mg) (Table 2), whereas receptor affinity (K,) for [125I]cyanopindolol in the ischemic LV (0.14±0.02 nM) was similar to that in the nonischemic LV (0.19±0.03 nM). In contrast to the results from the receptor binding studies, the basal activity of adenylate cyclase or of that stimulated by GTP, isoproterenol, Gpp(NH)p, sodium fluoride, or forskolin was consistently lower in membranes prepared from ischemic LV than in those prepared from nonischemic LV. These results recapitulate our previously published finding of increased β-adrenoreceptor density and decreased adenylate cyclase activity in this same model. The combined results from the five dogs in the current investigation and the four intact dogs studied previously (with 1 hour of coronary artery occlusion) are also summarized in Table 2. Both the

**Table 2. Effect of 1 Hour of Myocardial Ischemia on β-Adrenergoreceptor Density and Adenylate Cyclase Activity**

<table>
<thead>
<tr>
<th>Number of dogs</th>
<th>Nonischemic</th>
<th>Ischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>65±10</td>
<td>102±20</td>
</tr>
<tr>
<td>9</td>
<td>72±8</td>
<td>106±13*</td>
</tr>
<tr>
<td>β-Adrenoceptor density (fmol/mg)</td>
<td>5</td>
<td>78±14</td>
</tr>
<tr>
<td>Isoproterenol (0.1 mM)</td>
<td>9</td>
<td>81±9</td>
</tr>
<tr>
<td>Gpp(NH)p (0.1 mM)</td>
<td>5</td>
<td>239±42</td>
</tr>
<tr>
<td>Sodium fluoride (10 mM)</td>
<td>9</td>
<td>218±25</td>
</tr>
<tr>
<td>Forskolin (0.1 mM)</td>
<td>5</td>
<td>474±88</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>418±52</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>365±52</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>333±32</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1,001±185</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>857±117</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *p<0.05 compared with values in nonischemic zone.
FIGURE 1. Left panel: Graph showing effect of myocardial ischemia on the activity of sarcolemmal Gs, as determined by reconstitution experiments. The bars show average values obtained from five dogs. The mean activity of binding protein Gs decreased by 27±7% (p<0.01) in the left ventricular (LV) ischemic zone. Right panel: Graph showing data from a representative experiment in which Gs activity is reconstituted over a range of solubilized sarcolemma concentrations. In the example shown in the right panel, Gs activity in the ischemic zone (☆) was decreased by 32% when compared with the nonischemic zone (○).

Increases in β-adrenergic receptor number and decreases in adenylate cyclase were significant in the ischemic zone, p<0.05, as compared with corresponding values in the nonischemic zone.

Reconstitution Studies

The activity of Gs was assessed by its ability to reconstitute fluoride-stimulated MgATP-dependent adenylate cyclase activity in membranes of the S49 cys lymphoma cell.21 There was essentially no measurable adenylate cyclase activity in either the cys membranes before reconstitution of Gs or in the heat-inactivated sarcolemmal extracts. The mean value for reconstituted adenylate cyclase activity in the ischemic zone was 223±39 fmol cAMP/10 min/ng and that for the nonischemic zone was 309±46 fmol cAMP/10 min/ng (p<0.01; Figure 1, left). A representative experiment is shown in Figure 1, right.

Gs activity reflects the presence or absence of ischemia; this finding is underscored by the results of the experiment illustrated in Figure 2, left. After 1 hour of coronary artery occlusion, the activity of Gs in the epicardium of the ischemic zone (261 fmol cAMP/10 min/ng) did not differ from that in the nonischemic region (262 fmol/10 min/ng), whereas a clear decrease in activity occurred in the endocardium of the ischemic zone (195.5 fmol/10 min/ng). Thus, the changes in the activity of Gs occurred to the major extent in the endocardium, the site experiencing the most intense ischemia during coronary artery occlusion.

Cholera Toxin Labeling

The cholera toxin labeling was used as a correlative assay. One hour of myocardial ischemia decreased the transmural level of Gs from 7.6±0.7 pmol/mg in the nonischemic zone to 5.3±0.3 pmol/mg in the ischemic zone. However, in the two dogs whose ischemic epicardial and endocardial levels of Gs were studied, a decrease in Gs content (25%) occurred only in the endocardium, but the level in the epicardium was unaffected (Figure 2, right). As demonstrated earlier with the reconstitution assay (Figure 2, left), the changes in the level of Gs occurred principally in the ischemic endocardium.
Discussion

This is the first report describing an effect of myocardial ischemia on a receptor-coupling protein. We have shown that 1 hour of myocardial ischemia affects the β-adrenoreceptor transduction system in sarcolemma by decreasing the capacity of Gs to couple to adenylate cyclase.

After the initial reports of Mukherjee et al., other studies have demonstrated that myocardial ischemia increases β-adrenoreceptor density in the ischemic myocardium. The mechanism responsible for the increase in receptor density has been proposed to involve the translocation of β-adrenoreceptors to the sarcolemma. It has been speculated that the increase in β-adrenoreceptor density accounts for the enhanced effects of catecholamines during myocardial ischemia. Our present and previous findings using the model of 1 hour of coronary artery occlusion do not concur with this hypothesis. The activity of adenylate cyclase was depressed whether the enzyme was stimulated directly with isoproterenol or Gpp(NH)p or, more directly, with forskolin. Our findings are in contrast with those who have found ischemia to enhance the isoproterenol-stimulated adenylate cyclase activity and to increase tissue levels of cAMP.

These inconsistencies concerning the coupling of β-adrenoreceptors to adenylate cyclase may be ascribed to differences in experimental protocols. For example, general anesthesia and recent thoracotomy markedly alter physiological responses, which, in turn, are likely to affect the autonomic milieu induced by coronary artery occlusion and myocardial ischemia. In addition, one of the major problems associated with experimentally induced coronary artery occlusion is the variability in the resulting amount of ischemia. This is especially important in dogs that have a large collateral circulation. We have dealt with these issues by examining the effects of coronary artery occlusion in conscious dogs in which myocardial ischemia was verified by decreases in LV function and blood flow specific to the ischemic zone. In
most of the previously reported studies, ischemic zones were not delineated by physiological function and blood flow measurements. Thus, the changes we observed in the β-adrenergic receptor transduction system after coronary artery occlusion are definitely occurring in ischemic zones.

G-protein abnormalities have been described in a number of clinical disorders. Specifically, alterations in Gs have been reported as a result of cholera infections and in pseudohypparathyroidism. Alterations in Gs may also be involved in abnormal adrenergic receptor signaling in a number of pathophysiological states associated with impaired cardiac performance. We have recently documented a 50% decrease in the apparent concentration and function of Gs in sarcolemma from a canine model of LV failure. In addition, loss of agonist-stimulated adenylyl cyclase activity due to a deficiency in Gs also occurs as a result of chronic exposure to catecholamines. Finally, as shown in the present study, alterations in Gs function can occur rapidly, even with 1 hour of coronary artery occlusion. The present data does not allow us to determine whether changes in Gs are occurring at the pretranslational or posttranslational level. Possibly, protein modification by a rapidly occurring process such as phosphorylation, or enhanced degradation, may be responsible for the observed alterations.

The activity of the catalyst itself, adenylyl cyclase, has not been adequately characterized. Presently, we are identifying conditions that will allow us to assay its activity when fully uncoupled from Gs. Finally, inhibitory guanine-nucleotide–binding protein Gs, as assessed by pertussis toxin labeling, was not increased in ischemic zones (authors’ unpublished data).

In conclusion, 1 hour of myocardial ischemia without reperfusion in the conscious dog results in the uncoupling of the β-adrenergic receptor transduction system by decreasing the activity of Gs. Studies involving shorter durations of ischemia in the presence and absence of reperfusion may further elucidate the mechanisms underlying these biochemical derangements.

References


Key Words • ischemia • GTP-binding protein • adenylyl cyclase • β-adrenergic receptor
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guanine-nucleotide regulatory protein Gs.

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_Circ Res._ 1989;65:1145-1150
doi: 10.1161/01.RES.65.4.1145

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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