Ischemic Inactivation of G Protein–Coupled Receptor Kinase and Altered Desensitization of Canine Cardiac β-Adrenergic Receptors

Xichun Yu, MD; Min Zhang, MD; Kimberly Kyker, MS; Eugene Patterson, PhD; Jeffrey L. Benovic, PhD; David C. Kem, MD

Background—G protein–coupled receptor kinases (GRKs) modulate myocardial β-adrenergic receptor (βAR) signaling. We examined whether GRK activity was altered 6, 24, and 96 hours after left anterior descending coronary artery ligation (LAD CAL) in the dog.

Methods and Results—GRK activity was measured in arrhythmogenic subepicardial border zone (EBZ) tissue overlying the infarct and from nonischemic remote-site (RS) subepicardial tissue from the same animal. GRK activity in the ischemic EBZ was 15% of RS (P=0.03, n=6) 24 hours after CAL and appeared to start as early as 6 hours through 96 hours. GRK activity and immunoblot data demonstrated a marked decrease of GRK2 but not GRK5 at 24 hours. EBZ tissue exhibited high-affinity binding for (−)-isoproterenol (Kᵢ of 0.076±0.026 nmol/L (SEM)) at 24 hours, which was not significantly different from control tissue from nonoperated animals (1.2±0.8 nmol/L, P>0.05, n=6). A significantly lower Kᵢ of 13.8±2.8 nmol/L (P<0.001, n=6) was observed for RS taken from the ischemic animals. This was reflected by a 4-fold increase in the EC₅₀ of isoproterenol-stimulated adenylyl cyclase activity from 18 nmol/L in EBZ tissue to 73 nmol/L in RS (P<0.05, n=4).

Conclusions—There is a selective decrease in GRK2 activity and a loss of the ability of the arrhythmia-prone EBZ tissue to desensitize to β-adrenergic stimulation 24 hours after CAL. This correlates temporally with a second (late) peak in sudden cardiac death previously observed between 6 and 24 hours in dog and rat models of myocardial infarction.

Key Words: proteins ■ kinases ■ receptors, adrenergic, beta ■ death, sudden ■ myocardial infarction

Time-dependent alterations in sensitivity to agonists of the β-adrenergic receptor–G-protein–adenylyl cyclase (BAR-G,AC) transduction pathway occur during myocardial infarction, congestive heart failure, and acute and chronic myocardial ischemia.1–10 β-Adrenergic receptor kinase 1 (βARK1; alternative nomenclature, G protein–coupled receptor kinase, GRK2), GRK5, and cAMP-dependent protein kinase A (PKA) regulate βAR sensitivity by phosphorylation of their target receptors.11–13 PKA phosphorylation of the βAR is independent of receptor occupancy. By contrast, GRK-mediated phosphorylation is dependent on agonist binding to the receptor. Ligand-dependent phosphorylation of the βAR initiates uncoupling from the G-protein complex, resulting in reduced agonist affinity and diminished myocardial βAR sensitivity.

βAR sensitivity increases 30 minutes after coronary artery ligation (CAL).7,8 This is associated with a transient increase in βAR B₅₀, elevated catecholamines, and increased AC activity. By 1 hour, a decrease in βAR B₅₀ and G, activity is associated with a diminished AC responsiveness.1–6 These changes have been described up to 6 hours3 and at 120 hours14 after CAL. There are few studies of βAR transduction sensitivity in animal models of myocardial infarction during the subacute period extending from 6 to 24 hours after CAL. This period roughly coincides with an observed second peak for susceptibility and development of βAR-sensitive ventricular arrhythmias and sudden cardiac death in animal models.15–19 There is evidence that the epicardial border zone (EBZ) overlying the infarct is especially sensitive to arrhythmogenic delayed or early afterdepolarizations by β-adrenergic agonists 24 hours after CAL.20 EBZ tissue is an important substrate for development of fatal ventricular tachyarrhythmias in this experimental model.17,18,21 The present study has examined whether ischemia alters GRK activity.
Table 1. Na⁺,K⁺-ATPase Activity in RS (control) and EBZ Tissue of Five 24-Hour CAL Dogs

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>RS</th>
<th>P</th>
<th>EBZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ATPase activity</td>
<td>1.13±0.223</td>
<td>NS</td>
<td>0.99±0.0721</td>
</tr>
<tr>
<td>Total ATPase activity with 1.1 mM ouabain</td>
<td>1.02±0.215</td>
<td>0.83±0.055</td>
<td></td>
</tr>
<tr>
<td>Specific Na⁺,K⁺-ATPase activity</td>
<td>0.11±0.011</td>
<td>NS</td>
<td>0.16±0.0312</td>
</tr>
</tbody>
</table>

Values are μmol P · mg protein⁻¹ · h⁻¹.

and other indices of β-adrenergic sensitivity in EBZ tissue during this second high-risk period.

Methods

Ischemic Dog Model
Thirty male dogs (30 to 45 lb) had a 2-step ligation of the LAD below the first branch during sodium pentobarbital (30 mg/kg IV) anesthesia. Three remained under anesthesia for 6 hours, and their hearts were removed. Twenty-seven dogs were allowed to awaken and were given nalbuphine 0.15 mg/kg for postoperative analgesia. Of these 27, 3 died during the 12- to 24-hour period, and 4 others were excluded from study by ECG criteria. Seventeen were reanesthetized at 24 hours and 3 at 96 hours after CAL. A thoracotomy was performed, and the heart was removed and immersed in and perfused with ice-cold Tyrode’s buffer via the coronary artery. The outer 1.5- to 2.0-mm subepicardial border zone (EBZ) tissue overlying the left ventricle 3.0 cm above the ligature. The tissues were rapidly divided into small aliquots and stored in liquid nitrogen.

Control Dog Model
RS- and EBZ-equivalent tissues were obtained from 4 unstressed control dogs. These hearts were removed within 10 minutes of anesthesia to minimize stress-induced catecholamines.

The animal protocols were approved by the Institutional Animal Resource Committee and conform to the Helsinki International Guidelines for animal experimentation.

βAR Binding Assays

Membrane Preparation
Samples (0.5 g) were minced with scissors and homogenized in 3 mL of buffer (50 mmol/L Tris-HCl, 5 mmol/L EDTA, pH 7.4). After homogenization, 3 mL of 0.5 mol/L KCl was added, and the homogenates were incubated on ice for 20 minutes to extract contractile proteins. After homogenization, 3 mL of 0.5 mol/L KCl was added, and the homogenates were incubated on ice for 20 minutes to extract contractile proteins. The tissue extract was centrifuged at 15000 g for 10 minutes, and the supernatant was collected and centrifuged at 45 000 g for 15 minutes, resuspended, and centrifuged in 2 mL homogenization buffer. Membrane protein concentration was measured by BCA assay (Pierce Inc) and frozen at −70°C.

Binding Saturation Isotherms
Binding saturation isotherms were used to determine βAR density. ¹²⁵I-labeled cyanopindolol (7.5 to 300 pmol/L; ¹²⁵I-CYP, Amersham Life Sciences, Inc) was incubated with 7.5 μg of membrane protein for 60 minutes in incubation buffer (50 mmol/L Tris-HCl [pH 7.4], 10 mmol/L MgCl₂, 5 mmol/L EDTA) at 37°C. The membranes were filtered through Whatman GF/C glass fiber filters with a 24-sample cell harvester (Brandel, Inc) and washed 2 times with iced buffer to separate bound from unbound ligand. Nonspecific binding (determined by concurrently adding 200 μmol/L isoproterenol) was <30% of total binding. The percentage of β-AR was determined by use of the specific β₂-antagonist ICI 118,551 (Research Biochemicals International) for displacement analysis. Binding isotherms were analyzed with nonlinear plotting software (GraphPad Prism, Intuitive Software of Science, Inc) to determine receptor sites (Bmax) and the equilibrium dissociation constant (Kd).

β-Adrenergic Competition Binding Assay
Membrane preparations were as described. ¹²⁵I-CYP (55 pmol/L) was added to 7.5 μg of tissue protein in the presence of increasing concentrations of isoproterenol (10⁻¹⁰ to 10⁻⁴ mol/L). A concurrent assay was prepared by addition of 100 μmol/L of guanosine 5’-O-thiotriphosphate (GTPγS) to the incubation mixture. After 1 hour, bound and free ¹²⁵I-CYP were separated as described above. The percentage of low- and high-affinity receptors and their Kᵢ values were derived from the saturation binding isotherms. Data were analyzed with software for nonlinear curve fitting for 1- or 2-site competition binding analysis (Prism).

GRK Activity
Frozen canine cardiac tissue (0.5 g) from each site was homogenized in 2.5 mL of ice-cold lysis buffer. Cytosol and membrane fractions were collected as described. Protein (50 μg) from each fraction and 250 pmol of urea-treated, dark-adapted rod outer segments were incubated in light for 30 minutes at 30°C in buffer (20 mmol/L Tris-HCl [pH 7.5], 1.8 mmol/L EDTA, 4.8 mmol/L MgCl₂, 84 μmol/L ATP, and 222 TBq/mmol [γ⁻³²P]ATP). GRK-dependent phosphorylation of rhodopsin was confirmed by heparin (1 μmol/L) inhibition and a lack of suppression by the PKA inhibitor PKI (1 μmol/L, Promega Inc). Specific GRK activity was identified in the presence of anti-βARK1 or anti-GRK5 antibody (10 μg/mL). The pellet samples were electrophoresed on 10% SDS-PAGE, followed by autoradiography and counting the rhodopsin bands. GRK-mediated phosphorylation was calculated as pmol ³²P uptake · min⁻¹ · mg membrane protein⁻¹.

Na/K-ATPase (Ouabain-Suppressible)
Membranes from RS and EBZ tissue were assayed by modification of the method described by Jones and Besch. Na⁺/K⁺-specific ATPase activity was calculated by subtracting the difference of ouabain-suppressed ATPase activity from total ATPase activity. Data were expressed as pmol free P · released · mg membrane protein⁻¹ · h⁻¹.

AC Activity
Isoproterenol-stimulated AC activity was measured by use of a modified assay from Salomon et al. Concentrations ranging from 10⁻³ to 10⁻⁵ mol/L isoproterenol were added to the assay mixtures in the presence of 7.5 mmol/L theophylline. Data were expressed as pmol · min⁻¹ · mg tissue⁻¹. Values were expressed as percent maximal stimulation with 10⁻⁴ mol/L isoproterenol to normalize the data from 4 different dogs.

Western Blot Analyses
Cytochrome c and membrane samples containing 100 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated overnight at 4°C with anti-GRK1 or anti-GRK5 antibody (10 μg/mL). Antibody binding was detected with enzyme-linked chemiluminescence detection reagents (Amersham). Quantification of immunoreactive proteins was performed by densitometric scanning and with Image QuaNT software (Molecular Dynamics).

Statistics
Normalized GRK activity and relative immunoblot data were analyzed with a 2-tailed nonparametric sign test. Other data are...
Results

The EBZ tissue is a 1.5- to 2-mm thin rim overlying the infarct zone. The viable tissue collected weighed 1.0 to 1.5 g. We therefore used tissue from different dogs for a given assay.

GRK Activity

There was no significant difference in GRK activity in RS- and EBZ-equivalent sites from normal control animals [RS cytosol 5.6±1.2, EBZ cytosol 7.4±2.2 (P=0.26, n=4) and RS membrane 6.5±1.6, EBZ membrane 5.5±1.2 (P=0.44, n=4) pmol [32P] × mg protein −1 × min −1]. EBZ-GRK activity was expressed as percent RS-GRK activity to compensate for different rhodopsin preparations used in the course of the study. GRK activity in the EBZ membrane and cytosol was significantly decreased compared with RS tissue at 24 hours (n=6, Figure 1). We performed a preliminary study to estimate the time course of GRK activity suppression. These data indicated that suppression might occur by 6 hours and last up to 96 hours. Owing to the small number, the nonparametric analysis did not establish the level of significance for 6 and 96 hours. Preincubation of the cytosol and membrane fractions with anti-GRK5 antibody failed to significantly diminish GRK activity, whereas anti-βARK1 or heparin diminished GRK activity by >70% (Figure 1, inset).

Measurement of Tissue Na,K-ATPase

There were no significant changes in either total or specific Na/K-ATPase activity between the tissues as an independent marker of membrane integrity (Table 1). There was no evidence that changes in βAR binding affinities, AC sensitivity, and GRK activity were related to a generalized loss of membrane integrity, and so membrane protein did not require correction.

Immunoblots

The GRK2 band at 80 kDa (Figure 2A) was significantly decreased in the EBZ and infarct compared with RS. Although there was an increase in GRK5 density in the EBZ compared with the RS, this did not reach significance by nonparametric statistical analysis (Figure 2B). Immunoblots for \( \text{G}_{\alpha} \) and \( \text{G}_{\beta} \) in EBZ, RS, and infarct tissue demonstrated no significant change in band mobility or density (Figure 3A and 3B).

Radioligand Binding Data

The B_{max} for βAR in the EBZ tissue was not significantly different from RS or normal tissue (Table 2). The K_{d} for the EBZ tissue was significantly different from both RS and normal tissue. The percentage of β_{2} AR did not differ at 24 hours (28.8±2.9% in RS and 29.0±2.6% in EBZ [SEM], P>0.05, n=6).

Figure 1. GRK activity of cytosol (Cyt) and pellet extracts from myocardial tissue 6, 24, and 96 hours after LAD ligation. Each bar is mean for normalized EBZ GRK activity, expressed as percent of RS (100%) activity. EBZ GRK activity was significantly decreased at 24 hours (n=6, \( P=0.03 \) vs RS, 2-tailed sign test). Preliminary studies (n=3) demonstrated a similar decrease in EBZ GRK activity at 6 and 96 hours. However, nonparametric test requires n=6 for significance. Inset. Relative contributions of GRK2 and GRK5 determined by preincubation of anti-GRK2 or anti-GRK5 antibody with cytosol and membrane (Mem) fractions. Anti-GRK2 but not anti-GRK5 markedly inhibited GRK activity. Heparin is a nonspecific inhibitor of GRK activity.

Figure 2. Representative immunoblots of (A) GRK2 (cytosol) and (B) GRK5 (membrane) from nonoperated normal animals and 24-hour CAL tissue. Lane 1, normal RS; lane 2, normal EBZ; lane 3, RS; lane 4, EBZ; lane 5, infarct; lane s, purified GRK2 (A) or GRK5 (B). Relative density of GRK for EBZ (n=6) is expressed as a percentage of RS (100%), and mean values are shown in bar graphs. GRK2 level in EBZ was significantly decreased by use of a nonparametric sign test (\( P=0.03, n=6 \)). Rise in GRK5 did not reach significance (NS).
**β-Adrenergic Binding Displacement Curves**

β-Adrenergic binding displacement curves were performed on EBZ and RS tissues from 6 animals and from EBZ and RS sites from 3 of the 4 normal animals (Table 2 and Figure 4). There was no difference in high-affinity $K_i$ between the EBZ and RS sites in the 3 control animals, so these were pooled for subsequent statistical analyses. There was no significant difference between the "high-affinity" $K_i$ for the control animal tissues and the EBZ. The $K_i$ for RS was significantly lower and shifted to the right compared with normal and EBZ tissues. The percentage of "higher-affinity" receptors for RS tissue was similar, but the $K_i$ was significantly larger, indicating that the RS receptors were in a lower-affinity state. When GTP$_g$S was added, all of the curves reverted to low-affinity $K_i$ states. The RS sites, already markedly shifted to the right, were uncoupled, as were the EBZ and normal tissues.

**AC Activity**

To determine whether loss of GRK activity in the EBZ tissue was associated with an altered βAR responsiveness, AC activity was measured in RS and EBZ membranes (Figure 5). The dose-response curve in the RS tissue at 24 hours was shifted to the right of that observed for EBZ tissue. The RS EC$_{50}$ for AC activity was increased 4-fold over EBZ tissue. Basal AC activity was unchanged between the RS and EBZ tissue (129±48 versus 106±12 pmol·min$^{-1}$·mg protein$^{-1}$, $P>0.05$, $n=3$). Forskolin-stimulated AC activity in EBZ tissue was also not significantly different at 24 hours from RS (748±264 versus 1335±388 pmol·min$^{-1}$·mg$^{-1}$, $P>0.05$, $n=3$).

**Discussion**

EBZ tissue is a major site for recurrent ventricular tachyarrhythmias leading to sudden cardiac death.$^{17-21}$ These occur within 2 discrete periods during the first 24 hours after induction of ischemia in experimental models of myocardial infarction.$^{16-19,28}$ The first peak occurs within minutes to $\leq 1$ hour after CAL and is associated with abnormal βAR signal transduction. The second peak occurs 6 to 24 hours after CAL. An increased sensitivity to catecholamine-induced triggered beats was observed in the EBZ of similarly prepared dogs 24 hours after CAL.$^{20}$ Because human studies indicate that the risk of sudden cardiac death is highest during the first 24 hours and diminishes thereafter,$^{28}$ it is imperative that molecular abnormalities be fully investigated throughout this time period.

We have identified alterations in GRK activity as early as 6 hours after CAL lasting through 96 hours. We have examined βAR signal transduction in EBZ tissue 24 hours after CAL. The EBZ is best delineated at this interval and temporally overlaps with the second peak of sudden cardiac death.
death in the canine model. Although \( \beta \)AR density and the \( \beta_1: \beta_2 \) ratio returned to parity at 24 hours, there was a significant difference in ligand affinity between the EBZ and RS tissues at 24 hours. The RS \( K_i \) values were uniformly shifted to the right, indicating a lower \( \beta \)AR affinity and providing protective desensitization to this tissue. The EBZ \( \beta \)AR remained in the high-affinity state and failed to desensitize appropriately despite the presence of \( \beta \)AR agonists.

We observed no significant difference in the levels of \( G_\alpha_i \) or \( G_\alpha_s \) by quantitative immunoblotting between EBZ and RS tissue at 24 hours. Despite no significant change in basal or forskolin-stimulated maximal AC activity, RS tissue had a 4-fold shift to the right in the isoproterenol dose-response curve, indicating that it had desensitized, whereas EBZ tissue did not.

GRK activity in EBZ was dramatically reduced 24 hours after LAD ligation compared with the RS tissue. No such decrease in activity was observed in equivalent tissues from control animals. Immunoblots with specific monoclonal antibodies demonstrated a marked decrease in the amount of GRK2 in the EBZ tissue. A recent report suggested that progressive N-terminal degradation of GRK2 by the proteasome pathway occurs in hepatic tissue and serves as another regulatory element of GRK activity.\(^{29}\) Two groups\(^{22,30}\) have presented data indicating that a reduction in \( \beta \)AR activity downregulates \( \beta \)ARK expression. The EBZ tissue used in our study lies in an area that is denervated by either CAL or latex embolization.\(^{31}\) Such tissue, distal to LAD embolization and comparable to our EBZ, had decreased tissue norepinephrine and demonstrated hypersensitivity to infused catecholamines.\(^{31}\) It is therefore possible that cardiac denervation may also alter GRK2 expression in the EBZ tissue. Inhibition of both GRK2 and GRK5 by activation of calmodulin might be one of several inhibitory mechanisms after ischemia.\(^{32,33}\) An ischemia-induced activation of PKC would inhibit GRK5 activity but would slowly activate GRK2.\(^{11–13}\) Although there was no diminution in GRK5 by immunoblot and its contribution to total GRK activity appeared to be minimal, post-translational inhibition of GRK5 activity may also contribute to the overall decrease in GRK activity.

Ungerer et al\(^{34}\) demonstrated a rise in GRK activity in left ventricular tissue 1 and 6 hours after global ischemia in Langendorf buffer–perfused rat hearts but did not measure \( \beta \)AR sensitivity or AC responsiveness after 2 hours. Chen et al\(^{35}\) examined the effect of in vitro global ischemia (6 minutes)/reperfusion (20 minutes) on myocardial function in transgenic mice overexpressing GRK2. The animals with elevated GRK2 had diminished function and recovery compared with the controls. Maurice et al\(^{36}\) reported increased GRK activity in failing rabbit ventricular tissue 3 weeks after...
LAD ligation. These studies differed in the following ways. (1) They were performed in animals with only brief periods of global ischemia.34,35 (2) Rodent rather than canine models were used.34–36 (3) The in vitro studies used buffer rather than fresh blood to perfuse the ischemic tissue.34,35 (4) The in vivo rabbit model tested failing tissue rather than any EBZ cardiac tissue that might remain after 3 weeks, and GRK activity would be expected to rise in the failing tissue.36

In preliminary studies, an apparent decrease in EBZ GRK activity compared with RS was observed as early as 6 hours after ligation (Figure 1). The timing of these changes in GRK activity from 6 to 96 hours overlaps the second peak in sudden cardiac death observed 6 to 24 hours after CAL in this canine model of human cardiac ischemia. These observations support the concept that changes in β-adrenergic sensitivity reflect dynamic alterations in most if not all components of the βAR-G<sub>S</sub>-AC transduction system in the heart. It would appear that alterations in βARK and GRK activity must also be included in this assessment.

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References
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