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. (*Circulation*. 2000;102:2535-2540.)

**Key Words:** proteins ▪ kinases ▪ receptors, adrenergic, β ▪ death, sudden ▪ myocardial infarction

*Time-dependent alterations in sensitivity to agonists of the β-adrenergic receptor—G-protein–adenyl cyclase (βAR-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 Bmax, elevated catecholamines, and increased AC activity. By 1 hour, a decrease in βAR Bmax 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...*
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 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 infarct was shaved by scalp dissection. A longitudinal strip of tissue was stained with triphenyl tetrazolium chloride (0.1%) to determine the degree of viable and nonviable tissue. Remote-site (RS) control tissue was obtained from a superior portion of the lateral 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

Tissue 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. The tissue extract was centrifuged at 1500 g for 10 minutes, and the supernatant was collected and centrifuged at 45 000g for 15 minutes, resuspended, and centrifuged twice 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 βAR 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 total receptor sites (Bmax) and the equilibrium dissociation constant (Ki).

### 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

<table>
<thead>
<tr>
<th>Tissue Type</th>
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Values are μmol P · mg protein−1 · h−1.

## 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 ouabain-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 pelleted 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 10−6 to 10−9 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

Cytosol 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-GRK2 (monoclonal C5/1) or anti-GRK5 (monoclonal A16/17) antibody (R. Lefkowitz, MD, Duke University, Durham, NC, and Upstate Biotechnology), followed by incubation with peroxidase-linked anti-mouse IgG (Upstate Biotechnology). For Gαs and Gαo, protein immunoblots were carried out on 40 μg of membrane protein with rabbit polyclonal antibodies (Gαs, Santa Cruz Biotechnology; Gαo, NEN) and peroxidase-linked anti-rabbit IgG (1:3000; Amersham). 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

TABLE 1. Na⁺,K⁺-ATPase Activity in RS (control) and EBZ Tissue of Five 24-Hour CAL Dogs

<table>
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<tr>
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Were analyzed with software for nonlinear curve fitting for 1- or 2-site competition binding analysis (Prism).
expressed as mean±SEM. Normally distributed data were examined with a 1-way ANOVA. Bonferroni’s multiple comparison test was used for post-test analysis. Data for the competition binding and dose-response curves were analyzed with a 2-way ANOVA. Significance was ascribed to a value of \( P < 0.05 \).

**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 \( ^{32} \text{P} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1} \)]. 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-\( \beta\)ARK1 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 \( \beta\)AR 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 \( G_a \) and \( G_s \) in EBZ, RS, and infarct tissue demonstrated no significant change in band mobility or density (Figure 3A and 3B).

**Radioligand Binding Data**

The \( B_{\text{max}} \) for \( \beta\)AR 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 \( \beta_2\)AR did not differ at 24 hours (28.8±2.9% in RS and 29.0±2.6% in EBZ [SEM], \( P > 0.05 \), \( n = 6 \)).
**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$_{gS}$ 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 ≥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

**TABLE 2. Binding Data Summary for 24-Hour CAL (RS and EBZ) and Normal Animals**

<table>
<thead>
<tr>
<th></th>
<th>RS</th>
<th>EBZ</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_{max}$, fmol/mg</td>
<td>38.2±2.8</td>
<td>33.6±4.0</td>
<td>34.9±1.9</td>
</tr>
<tr>
<td>$K_i$, pmol/L</td>
<td>43.1±1.6</td>
<td>36.0±1.9</td>
<td>47.1±3.6</td>
</tr>
<tr>
<td></td>
<td>No GTP$_{gS}$</td>
<td>+GTP$_{gS}$</td>
<td>No GTP$_{gS}$</td>
</tr>
<tr>
<td>% High affinity</td>
<td>39.5±5.5</td>
<td>0</td>
<td>38.3±3.7</td>
</tr>
<tr>
<td>% Low affinity</td>
<td>60.5±5.5</td>
<td>100</td>
<td>61.7±3.7</td>
</tr>
<tr>
<td>$K_{in}$, nmol/L</td>
<td>13.8±2.8</td>
<td>0</td>
<td>0.076±0.026†</td>
</tr>
<tr>
<td>$K_{out}$, μmol/L</td>
<td>0.73±0.16</td>
<td>0.38±0.09</td>
<td>0.37±0.06</td>
</tr>
</tbody>
</table>

*Values are mean±SEM, n=6. All parameters were calculated by Prism. $K_{in}$ and $K_{out}$ indicate affinity values at high- and low-affinity states, respectively.

†Significantly different from the normal value ($P<0.05$).

‡Significantly different from the RS value ($P<0.001$).
death in the canine model. Although βAR density and the
β1:β2 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 Kᵢ values were uniformly
shifted to the right, indicating a lower βAR affinity and
providing protective desensitization to this tissue. The EBZ
βAR remained in the high-affinity state and failed to desen-
sitize appropriately despite the presence of βAR agonists.

We observed no significant difference in the levels of Gᵢₐ or Gₛₐ 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 an-
tibodies 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 protea-
some pathway occurs in hepatic tissue and serves as another
regulatory element of GRK activity.²⁹ Two groups²²,³⁰ have
presented data indicating that a reduction in βAR activity
downregulates βARK 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 catechol-
amines.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 contribu-
tion 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 al³⁴ 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
βAR 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 com-
pared with the controls. Maurice et al.³⁶ reported increased
GRK activity in failing rabbit ventricular tissue 3 weeks after

![Figure 4](image_url)

**Figure 4.** Composite competition curves (n=6, each point performed in triplicate) of isoproterenol with specific ¹²⁵I-CYP binding to
myocardial membranes from 24-hour RS (left), EBZ (center), and pooled normal tissue (right). Each displayed similar proportions of
receptors showing high-affinity binding. There was a highly significant difference (P<0.001, n=6) between high-affinity Kᵢ for RS and
EBZ. There was no significant difference between Kᵢ for normal (NL) and EBZ tissues.

![Figure 5](image_url)

**Figure 5.** Isoproterenol-AC activity dose-response curve in EBZ and RS tissue obtained 24 hours after LAD ligation. Data are
shown for 1 of 4 experiments. Each point is mean of duplicate
samples. RS dose-response curve was shifted to right. Mean
EC₅₀ value for RS (inset) was significantly different from EBZ
(73±22 vs 18.0±8.9 nmol/L [SEM]) (P<0.05, n=4).
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 tissue that might remain after 3 weeks, and GRK activity support the concept that changes in β-adrenergic sensitivity reflect dynamic alterations in most if not all components of the βAR-Gs·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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