Level of G protein–Coupled Receptor Kinase-2 Determines Myocardial Ischemia/Reperfusion Injury via Pro- and Anti-Apoptotic Mechanisms

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Rationale: Activation of prosurvival kinases and subsequent nitric oxide (NO) production by certain G protein–coupled receptors (GPCRs) protects myocardium in ischemia/reperfusion injury (I/R) models. GPCR signaling pathways are regulated by GPCR kinases (GRKs), and GRK2 has been shown to be a critical molecule in normal and pathological cardiac function.

Objective: A loss of cardiac GRK2 activity is known to arrest progression of heart failure (HF), at least in part by normalization of cardiac β-adrenergic receptor (βAR) signaling. Chronic HF studies have been performed with GRK2 knockout mice, as well as expression of the βARKct, a peptide inhibitor of GRK2 activity. This study was conducted to examine the role of GRK2 and its activity during acute myocardial ischemic injury using an I/R model.

Methods and Results: We demonstrate, using cardiac-specific GRK2 and βARKct-expressing transgenic mice, a deleterious effect of GRK2 on in vivo myocardial I/R injury with βARKct imparting cardioprotection. Post-I/R infarct size was greater in GRK2-overexpressing mice (45.0±2.8% versus 31.3±2.3% in controls) and significantly smaller in βARKct mice (16.8±1.3%, P<0.05). Importantly, in vivo apoptosis was found to be consistent with these reciprocal effects on post-I/R myocardial injury when levels of GRK2 activity were altered. Moreover, these results were reflected by higher Akt activation and induction of NO production via βARKct, and these antiapoptotic/survival effects could be recapitulated in vitro. Interestingly, selective antagonism of β2ARs abolished βARKct-mediated cardioprotection, suggesting that enhanced GRK2 activity on this GPCR is deleterious to cardiac myocyte survival.

Conclusion: The novel effect of reducing acute ischemic myocardial injury via increased Akt activity adds significantly to the therapeutic potential of GRK2 inhibition with the βARKct not only in chronic HF but also potentially in acute ischemic injury conditions. (Circ Res. 2010;107:00-00.)

Key Words: acute myocardial ischemia • ischemia/reperfusion injury • cardioprotection • G protein–coupled receptor kinase-2 • βARKct • myocyte apoptosis

Morbidity and mortality of myocardial infarction (MI) remains significant with resulting left ventricular (LV) systolic function presenting as a major determinant of clinical outcome. Protecting the myocardium against ischemia/reperfusion (I/R) injury has become a major target of therapeutic investigation.1,2 Included in this approach is limiting myocyte death, which is robustly induced by ischemic/hypoxic conditions.3 Limiting cell death and infarct size after MI through pharmacological or molecular intervention may significantly reduce LV dysfunction and prevent the development of chronic heart failure (HF). Apoptosis, necrosis, and autophagy have been proposed as mechanisms for the significant amount of cell death occurring in reperfused ischemic myocardium4 and is thought to contribute significantly to HF progression.5–6 Protective signaling pathways involving activation of phosphatidylinositol-3 kinase (PI3K) and its target...
Akt/protein kinase B mediate cytoprotective effects via phosphorylation of a number of proteins and induction of nitric oxide (NO) production, altogether resulting in limitation of apoptosis and improved myocyte survival.

A variety of agents used in clinical management of patients with MI, such as β-adrenergic receptor (βAR) blockers, adenosine, and opioids are thought to impart some cardioprotection via manipulation of G protein–coupled receptor (GPCR) signaling. Importantly, these GPCR ligands have been shown to reduce myocardial apoptotic cell death in animal models of ischemic injury supporting their use as cardioprotective agents. βARs are especially interesting in this regard because β1- and β2-ARs, expressed in myocardium, have differential effects on myocyte survival because β1-ARs mediate the proapoptotic effects of norepinephrine, whereas β2-ARs have been shown to promote antiapoptotic signaling via a Gi-mediated mechanism. βARs, and their regulation via GPCR kinases (GRKs), are important therapeutic targets in the management of chronic myocardial disease states such as HF. Of interest to the present study, we have shown that upregulation of GRK2, a biochemical hallmark of failing myocardium, leads to chronic desensitization of βARs and a loss of inotropic reserve.

It has been postulated that increased GRK2 activity in the injured/stressed heart is protective to fight against overstimulation of βARs caused by sympathetic nervous system hyperactivity, however, we have shown that inhibition of GRK2 actually protects the heart against the development of HF. This work has primarily been done using a peptide inhibitor of GRK2, called the βARKct, which is comprised of the carboxyl-terminal 194 amino acids of GRK2 that competes with endogenous GRK2 for membrane binding and activation against GPCR substrates through association with the disoriented G protein βγ-subunits (Gβγ). Most recently we have shown that gene transfer of an adeno-associated virus (AAV) containing the βARKct peptide can rescue LV dysfunction chronically in a rat model of HF causing improved cardiac performance and reversal of LV remodeling. Similarly, cardiac-specific knockout of the GRK2 gene after development of LV dysfunction in mice improved cardiac function and morphology. These studies solidly data suggesting that GRK2 is pathological in the heart and its inhibition or lowering of expression is therapeutic.

The present study was designed to determine whether GRK2 upregulation, which has been shown to occur early after ischemic cardiac injury, is beneficial or maladaptive in a model of acute ischemic injury. We have subjected mice with cardiac-specific overexpression of GRK2 or expression of the βARKct to I/R injury and explored specific mechanisms related to myocyte death and survival. Our results clearly show that even in the acute setting, increased GRK2 activity is deleterious to myocytes, and we present novel data demonstrating that inhibition of GRK2 activity specifically toward β2-ARs can result in cardioprotection.

**Methods**

Experimental Animals

Cardiac-specific transgenic mice with GRK2 overexpression or expression of βARKct have been described previously. All animal procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Determination of Myocardial Apoptosis

In vivo myocardial apoptosis was determined using TUNEL staining and caspase-3 activity assay as described previously with minor modifications (see the Online Data Supplement for details).

Neonatal Rat Ventricular Myocyte Isolation and In Vitro Models of I/R Injury or Oxidative Stress

Neonatal rat ventricular myocytes (NRVMs) from 1- to 2-day-old rats were isolated as described previously. Gene delivery of GRK2 or βARKct was carried out in cultured NRVMs using adenoviral vectors. After 48 hours, adenoviral-treated NRVMs were subjected to simulated I/R (medium inducing ischemia-like condition for 45 minutes and reperfusion of 24 hours) as described previously or exposed to oxidative stress (H2O2, 150 μmol/L for up to 16 hours). Some myocytes were treated with tumor necrosis factor (TNFα) (Sigma) or pretreated with inhibitors of PI3K (LY294002, Tocris) or NO synthase (NOS) (Nω-nitro-l-arginine methylester [L-NAME]; Sigma).

Measurement of NO Content In Vivo and In Vitro

Total NO content was measured in the LV area at risk using quantification with a chemiluminescence detector (Sievers 280i NO Analyzer) as described previously (see the Online Data Supplement).
NO production in cultured myocytes was measured as the stable metabolite nitrite by Griess method according to the protocol of the manufacturer (R&D Systems).

Western Blot Analysis
Western blotting was performed as described previously.20 Immunoblots were performed for LV protein levels of phosphoSer473-Akt, phosphoThr1177-eNOS (Cell Signaling), GRK2 (Santa Cruz Biotechnology), and GAPDH (Chemicon). All antibodies were used at 1:1000 dilution.

Statistical Analysis
All values are presented as means±SEM. Statistical significance of multiple treatments was determined using GraphPad Prism Software Version 5.9 (San Diego, Calif) by Student’s t test or ANOVA followed by either Newman–Keuls or Bonferroni post hoc test when appropriate. For all tests, a probability value of <0.05 was considered significant.

Results
Increased GRK2 Levels and Activity Are Deleterious to Postischemic Myocardium
LV infarct size resulting from temporary coronary artery ligation and 24 hours of reperfusion was measured in transgenic mice with altered cardiac GRK2 expression and activity, and significant differences were found (Figure 1). In control nontransgenic (NLC) mice, LV infarct size was calculated to be 31.3±2.2% of the area at risk (Figure 1B). The NLC group contains combined nontransgenic littermates from GRK2 and βARKct breeding pairs because we found no differences between these control mice (data not shown). In cardiac GRK2-overexpressing transgenic mice, there was enhanced injury as the LV infarct size significantly increased to 43.1±2.8% (P<0.05 compared with NLC and βARKct; Figure 1B). On the other hand, βARKct expression specifically in myocytes led to cardioprotection as the LV infarct size was significantly reduced to 16.8±1.3% (P<0.05 compared with GRK2 and NLC; Figure 1B). The area at risk within the LV was similar between all groups (Figure 1C).

These results demonstrate that GRK2 overexpression is deleterious to the reperfused myocardium in acute I/R injury and that its inhibition via the βARKct can impart a cardioprotective phenotype.

GRK2 Activity in the Postischemic Heart Promotes Apoptosis
Myocardial apoptosis is major factor contributing to I/R injury.4 Accordingly, we assessed programmed cell death using two independent methods. First, TUNEL staining was used on tissue sections following ischemia and 3 hours of reperfusion and apoptosis assessed in the ischemic border zone surrounding the infarcted area. As shown in Figure 2A, TUNEL-positive nuclei were ubiquitous showing enhanced apoptotic cell death in GRK2-overexpressing myocardium compared with NLC hearts. Interestingly, apoptosis was found to be significantly less in βARKct-expressing post-I/R myocardium. Quantification of these findings are shown in Figure 2B where GRK2 overexpression led to a ~2-fold increase in TUNEL-positive cells compared with post-I/R NLC hearts (437.3±43.0 nuclei per high-power field versus 257.1±30.7 nuclei per high-power field; P<0.05). In contrast, βARKct expression had >50% fewer TUNEL-positive cells compared with NLC heart after I/R injury (97.3±10.3 nuclei per high-power field; P<0.05 versus NLC and GRK2 mice).

Independently, we performed caspase-3 activity measurement in cardiac extracts from the various post-I/R mouse hearts, which serves as a marker of DNA cleavage induced by this enzyme (Figure 2C). Similar to the TUNEL results, I/R injury in NLC mice induced significant caspase-3 activity (2.9±0.4 pmol/mg protein per hour) compared with Sham mice (1.1±0.2 pmol/mg protein per hour, P<0.05) and GRK2 overexpression significantly enhanced this activity (4.5±0.5 pmol/mg protein per hour; P<0.05 versus NLC). As with TUNEL, βARKct expression led to significantly less myocardial caspase-3 activity after I/R (1.3±0.3 pmol/mg protein per hour; P<0.05 versus NLC and GRK2) and actually was not statistically different from sham values (Figure 2C). Overall, these results demonstrate a robust effect
of the βARKct to limit myocardial apoptosis acutely after ischemic injury.

Cardioprotective Induction of Nitric Oxide Synthesis is Increased by GRK2 Inhibition

One end point of cardioprotective signaling known to be regulated by adrenergic signaling is NOS activity and NO production. Indeed, we found myocardial synthesis of NO to increase in the heart after I/R, with βARKct expression significantly augmenting this response (almost doubling), whereas, reciprocally, GRK2 overexpression negated a significant increase in post-I/R NO production in myocardium (Figure 3A). This novel result mirrors the findings depicted in Figure 1 and represents a downstream effect of survival

Figure 2. Post-I/R apoptosis in hearts overexpressing GRK2 or βARKct. A, Representative photographs of TUNEL-stained tissue sections from post-I/R NLC, βARKct, and GRK2 mice including sham-negative control sections. Scale bar, 50 μm. TUNEL (green), apoptotic nuclei, and DAPI (blue), total nuclei. B, TUNEL-positive nuclei quantification represented as number per high-power field (HPF). C, Caspase-3 activity represented as picomoles of substrate per milligram of protein per hour (pmol/mg prot/h). *P<0.05 vs NLC; †P<0.05 vs βARKct; ‡P<0.05 vs sham (n=6 to 9/group).

Figure 3. Production of NO is induced by facilitated adrenergic signaling. A, Reciprocal effects of GRK2 overexpression and its inhibition by βARKct were found in measurements of myocardial NO content in the myocardial area at risk (AR) after 30 minutes of ischemia and 3 hours of reperfusion. *P<0.01 vs control (CTR); †P<0.01 vs control (n=8 to 22/group). B, Synthesis of NO under basal conditions and stimulated by TNFα (300 ng/mL) and norepinephrine (NE) (1 μmol/L) in NRVMs overexpressing either GRK2 or expressing βARKct. GFP-infected cells were used as a control and βARKct-expressing cells showed significantly induced NO production in the latter and this effect was abolished by blocking of NO synthases by L-NAME. *P<0.001 vs GFP (n=3, experiments done in duplicate).
signaling resulting in reduced apoptosis and implying that NOS regulation is a major GRK2-dependent pathological mechanisms in the ischemic heart. To further investigate this finding, we used isolated NRVMs with adenoviral-induced overexpression (see the Online Data Supplement) of GRK2, as well as expression of the ARKct peptide, and measured basal and stimulated NO release (Figure 3B). GRK2 inhibition via ARKct led to a 60% higher NO release basally (versus GRK2 and control), as well as after induction of NO synthesis via TNF-α together with the catecholamine, norepinephrine by 3-fold, and control (green fluorescent protein [GFP]-infected), and GRK2-overexpressing cells lacked this increase in NO (Figure 3B). Importantly, the ARKct-mediated enhancement of NO production was eliminated when cells were pretreated with L-NAME, an inhibitor of NOS, although this inhibitor had no effect in GRK2-overexpressing myocytes (Figure 3B). Catecholamine stimulation alone did not increase NO production, consistent with previous reports.25

Cardiomyocyte Apoptosis Following I/R or Oxidative Stress In Vitro Is Reduced by ARKct Expression

To determine whether the differential effects on cell survival found in hearts after I/R could be reproduced in NRVMs, we subjected these cells to simulated I/R conditions or oxidative stress via H2O2 treatment (see the Online Data Supplement). Using a GFP-adenovirus as a control, NRVMs overexpressed GRK2 to a similar level (3- to 4-fold) seen in GRK2 transgenic mice.15 Following introduction of these stress conditions, myocytes underwent TUNEL staining (Figure 4A). Apoptosis was significantly enhanced by both I/R or H2O2 exposure; however, ARKct expression led to significantly less TUNEL staining compared with GFP or GRK2, although there was an upward trend. This difference from the in vivo results was also apparent in NO production (Figure 3B), implying either
a compensating mechanism only in NRVMs or this could be attributable to the higher density of β2ARs potentially causing less desensitization effects in these cells. Alternatively, the apoptotic index in NRVMs under our experimental conditions was maximal even without GRK2 overexpression. Importantly, these results do support the robust in vivo data demonstrating the myocyte protective effect after ischemic and oxidative stress induced by the βARKct. Furthermore, disruption of signaling through PI3K, via its inhibitor LY294002, or inhibition of NOS significantly decreased the beneficial effect of βARKct (Figure 4A).

Mechanism of βARKct Cardioprotection Involves Akt Activation and Downstream Activation of NOS

Previous studies have shown that prosurvival signaling in ischemic hearts is mediated by Akt phosphorylation downstream of PI3K activation, which can occur following GPCR stimulation. According, we measured Akt phosphorylation as a marker of activation of this kinase in GRK2-overexpressing and βARKct overexpressing NRVMs exposed to oxidative stress (150 μmol/L H2O2). We found a significant increase in Akt phosphorylation after H2O2 treatment in control and βARKct groups, however, GRK2 overexpression significantly blunted Akt phosphorylation to only 44 ± 9.3% compared with control myocytes (Figure 4B and 4C). Importantly, analysis of Akt phosphorylation in vivo using cardiac extracts from post-I/R GRK2, βARKct and NLC hearts revealed that βARKct expression leads to enhanced Akt activation whereas GRK2 overexpression leads to decreased Akt phosphorylation (Figure 5A). Thus, it appears that a loss or gain in activity of GRK2 can have profound reciprocal effects on Akt activation both in vitro and in vivo in myocytes exposed to acute ischemic injury and the altered activity of this prosurvival kinase could explain the above differences in apoptosis. Interestingly, a recent report showed that GRK2 can interact directly with Akt and inhibit its activity, and our present data in myocytes are consistent with this result.

One effector known to be downstream of the GRK2-Akt interaction is NOS, and activation of endothelial (e)NOS, as well as inducible NOS, are known to have cytoprotective, as well as antiapoptotic, effects. Parallel to the increase in Akt phosphorylation, we found a significant 47% higher eNOS activation determined by assessing its phosphorylation in post-I/R βARKct hearts compared with NLC (Figure 5B). These data are consistent with a prosurvival pathway in myocytes via GRK2 inhibition allowing for increased signaling through the Akt-NOS signaling axis. This is consistent with downstream inhibition of Caspase-3 activity and apo-
ptotic cell death. Consistent with this mechanism, the induction of NO production was elevated by catecholamine stimulation in NRVMs expressing βARKct compared with controls, and this effect was abrogated in GRK2-infected cells (Figure 3B).

Enhanced GRK2-Mediated I/R Injury and βARKct Cardioprotection Can Be Reversed by Manipulation of β2AR Signaling

GPCR targets of myocardial GRK2 activity are the βARs. β1- and β2AR subtypes have opposing actions on myocyte apoptosis with β2ARs, promoting cell survival.9,10 To investigate whether βARKct-mediated cardioprotection may involve enhanced signaling through β2ARs (via a lack of desensitization), we treated βARKct mice with the selective β2AR antagonist ICI 118,551 and subjected them to I/R injury. As shown in Figure 6A and 6B, βARKct mice treated with ICI 118,551 no longer had reduced infarct sizes and myocardial injury was the same as in NLC mice. As in the above data (Figure 1) the area at risk after I/R was the same in both groups (Figure 6C). In a similar fashion, we treated NLC and GRK2-overexpressing transgenic mice with the selective β2AR agonist fenoterol before subjecting them to I/R injury. Interestingly, when treated with fenoterol, LV infarct size in GRK2 mice was no longer increased compared with NLC injury (Figure 6D through 6F). These results suggest that the β2AR signaling is playing a significant role in how GRK2 influences post-I/R injury because, importantly, antagonizing β2AR signaling reversed the prosurvival events mediated by GRK2 inhibition.

Discussion

In this study, we have found that GRK2 is a key pathological regulator of the myocardial response to acute ischemic injury. Using transgenic mice with a gain or loss of function of this enzyme in cardiac myocytes we have found a profound reciprocal effect on I/R induced myocardial injury and resultant apoptotic response. Importantly, our findings include that in the setting of I/R, inhibition of GRK2 can impart cardioprotection with significant myocardial salvage through survival signaling resulting from increased NO production downstream of Akt activation.

These novel findings using the βARKct add acute ischemic cardioprotection to the beneficial effects of GRK2 inhibition already shown in chronic HF models. Studies using viral-mediated gene delivery of the βARKct to myocardium has led to prevention and even reversal of HF,16,19,27 including a study demonstrating reversal of contractile dysfunction in failing human ventricular myocytes.28 Most recently, we have shown that βARKct delivery to a failing rat heart using AAV6-based gene therapy can chronically alter the disease, leading to improved cardiac function and reversal of pathological LV remodeling.16 Interestingly, especially in light of our present results, βARKct gene delivery to rabbit hearts has led to acute functional improvement globally in hearts undergoing ischemic insult either by cardioplegic arrest29 or permanent coronary artery ligation and MI.30 Of note, our present study adds important mechanistic insight into how βARKct expression not only can improve contractile function of ischemic myocytes but also leads to prosurvival signaling to prevent I/R-induced cell death, leading to an increased amount of viable myocardium. It appears
that βARKct expression leads to increased activation of the prosurvival kinase Akt and downstream effectors after acute I/R injury. Moreover, this cell survival signaling pathway may be induced by the β2-AR, which would be less desensitized with GRK2 inhibition.

Data with the βARKct are significant from a therapeutic point of view, but our results with clinically significant myocardial GRK2 overexpression (3- to 4-fold) is the same found in failing human myocardium.31 are critical in proving GRK2 as a pathological target in the heart. GRK2 is increased to this level in several animal models of cardiac injury18,32,33 and appears to be among the first molecular changes after myocardial ischemia.19,34 Interestingly, we found in this study that elevated myocyte GRK2 activity leads to increased cell death after I/R injury through an increased apoptotic index. Moreover, our data are consistent with a lack of Akt activation, which GRK2 has recently been shown to interact with and inhibit.26 A previous study focusing solely on cardiac contractile function and βAR responsiveness also found that this level of GRK2 overexpression leads to an intolerance to ex vivo ischemia.35 Moreover, in a recent study using cardiac-specific GRK2 gene deletion, we have found that induced loss of GRK2 two weeks after a MI significantly prevents all subsequent death.17 Thus, our data clearly show enhanced GRK2 to be pathological in myocardium, even acutely, resulting, in part, from an inhibition of Akt activity.

Interestingly, Ad-GRK2–infected cultured neonatal cardiomyocytes did not show significantly enhanced apoptotic indices after oxidative stress probably because of compensating mechanisms, for instance, the higher expression of protective β1-AR in neonatal myocytes, as well as less sensitivity to catecholamines, compared with adult cardiac myocytes.36 Nonetheless, βARKct expression clearly prevents ischemic apoptosis of myocytes and it appears that this novel cardioprotective effect of βARKct is mediated via enhanced Akt activation. In light of the findings that GRK2 can interact with and inhibit Akt, it might be rather intuitive to find increased Akt with βARKct expression, however, this has not been previously documented and our results demonstrate this finding for the first time in the heart. The Akt/eNOS signaling axis, which has been shown to play a critical role in postinjury production of NO and protection from cell death,37 also appears to be a direct effector of βARKct-mediated cardioprotection. Because the cardioprotective role of NO in I/R injury is clearly established,38 as well as a novel role for its metabolite nitrite,39 our data permit the conclusion that indeed, the antiapoptotic effect of GRK2 inhibition involves this important downstream effector.

Another novel aspect of our study is the finding that the reciprocal effects of GRK2 and βARKct in cardiac injury after I/R could be reversed in a β2-AR-dependent manner with a β2-AR agonist protecting against GRK2-induced injury and a β2-AR antagonist negating the cardioprotective effects of βARKct. These results could point toward differential regulation of βAR subtypes by GRK2, or even if the affinity for GRK2-mediated desensitization of the two receptors is equal (which is believed to be the case), β2-ARs could be more tightly coupled to cell survival signaling pathways. Clearly, β1-ARs and β2-ARs are coupled to different intracellular pathways leading to either Gs stimulation in the case of the β1-AR or dual coupling to Gs and Gi for the β2-AR.12,40 Downstream of the β2-AR, protein kinase A activation40,41 and Ca2+/calmodulin-dependent kinase II activation42,43 are known stimulators of myocyte apoptosis. On the other hand, β2-AR coupling to Gi leads to cell survival and reduced apoptosis.10,44 As shown in our present study, the βARKct-mediated protective phenotype can be reversed by blocking β2-AR with the addition of ICI 118,551, implying a β2-AR-Gi–dependent mechanism in play following I/R that is enhanced when GRK2 is inhibited. Similarly, stimulating β1-AR with the selective agonist fenoterol prevented the enhanced post-I/R injury with GRK2 overexpression. These results further promote the idea that β2-AR signaling (via Akt/eNOS) is cardioprotective in acute myocardial injury and demonstrates that GRK2 is a key regulator of this pathway in the heart (see Figure 7).
In contrast to our findings, a previous study using an ex vivo perfusion system found that ischemic preconditioning–induced cardioprotection (IPC) was not enhanced by the expression of βARKct. Importantly, significant differences between this model and our present study (ex vivo versus in vivo) and protocols (global ischemia versus I/R through a single vessel) may explain this discrepancy. However, consistent with our results was the finding that inhibition of Gi signaling with pertussis toxin could blunt IPC-induced protection ex vivo, and hearts lacking the β2AR had a blunted response to IPC. These data are in agreement with our observations where the β2AR-Gi signaling is protective and desensitization evoked by GRK2 activity leads to deleterious outcome in I/R.

Mechanistically, a caveat that cannot be ignored is that the effects of βARKct expression in the I/R injury model may be attributable to the sequestration of G_{q/y} independent of GRK2 activation. Certainly, G_{q/y} inhibition is a potential effect of the βARKct. However, our data with enhanced Akt activation and reversal of βARKct-mediated cardioprotection by β2AR blockade argue that inhibition of GRK2 activity appears to play a major mechanistic role in our present results. Therefore, overall, our results represent a novel extension to the beneficial effects of GRK2 inhibition in myocardium showing that beyond prevention of chronic HF, βARKct expression can impart novel β2AR-mediated cardioprotection.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Upregulation of G protein–coupled receptor kinase (GRK2) in the injured myocardium is pathological during chronic heart failure (HF).
- βARKct expression can rescue several models of HF by improving contractile function.
- G protein–coupled signaling protects against acute myocardial ischemic injury.

What New Information Does This Article Contribute?

- GRK2 activity negatively impacts myocyte survival in acute ischemic injury in the heart.
- βARKct is a novel cardioprotective agent that inhibits GRK2.
- We provide the first evidence that the PI3K-Akt-NOS signaling axis is downstream of GRK2 activity in the ischemic heart and that it profoundly affects myocyte survival after an ischemic insult.
- GRK2-related survival and apoptotic signaling in the ischemic heart appears to be primarily attributable to its activity on myocardial β2ARs.

Myocardial ischemic injury results in myocyte cell death, and protection can be afforded by Akt activation, which leads to prosurvival signaling. Akt activation is downstream of several agents that act through G protein–coupled receptors. We investigated the role of the GRK2 in acute myocardial ischemic injury because this kinase has been implicated as being pathological in HF. In fact, inhibition of GRK2 by the βARKct peptide rescues several models of HF. Our results show that GRK2 is pathological in the acutely injured heart because an increase in its activity increases myocyte death following ischemia/reperfusion. We report for the first time that Akt activity and subsequent activation of the cardioprotective NO synthase–NO signaling are decreased with GRK2 and that βARKct expression enhances this prosurvival signaling axis. Most of the GRK2-mediated myocardial ischemic injury appears to be mediated by β2ARs. Overall, this study suggests that βARKct and GRK2 inhibition may be therapeutic not only during chronic HF but also in acute conditions that negatively affect the myocardium.
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MATERIALS AND METHODS

In vivo Ischemia-Reperfusion (I/R) Injury Model

Surgical induction of I/R injury was performed as previously described.\(^1\) Briefly, mice (8-10 weeks old) were anesthetized with 2% isoflurane inhalation. The heart was exposed and exteriorized through a left thoracotomy at the level of the fifth intercostal space. A slipknot was made around the left anterior descending coronary artery (LAD) 1-2 mm from its origin with a 6-0 silk suture. After the slip knot was tied, the heart was immediately placed back into the intrathoracic space followed by closure of muscle and the skin. Sham-operated animals were subjected to the same surgical procedures except that the suture was passed under the LAD but was not tied. Following 30 min of ischemia, the slipknot was released and the myocardium was reperfused for up to 24h depending on experimental endpoint. Confirmation of transgene expression was assessed in all mice via Western blotting of cardiac extracts.\(^2\)

Infarct size after I/R injury was determined as previously described.\(^1,3\) Briefly, at the end of a 24 hr reperfusion period, mice were anesthetized and the LAD was re-ligated at the previous ligation site and 2% Evans blue dye was injected. The dye was circulated uniformly and distributed in the heart to areas perfused by the open coronary arteries. The heart was quickly excised and immediately frozen, sliced and the sections were then incubated in a 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) solution and then digitally photographed. The area not at risk (ANAR, Evan’s blue–stained area), and the area at risk (AR, including both TTC-stained and TTC-negative staining area [infarcted tissue, I]) were measured using the computer-based image analyzer SigmaScan Pro 5.0 (SPSS Science, Chicago, IL). Infarct
size was expressed as a percentage of the AR (I/AR) and the AR was expressed as the percentage of total LV (AR/(AR+ANAR)).

**Determination of Myocardial Apoptosis**

Myocardial apoptosis was determined as previously described with minor modifications.\(^1,3\) Terminal deoxynucleotidyl-transferase mediated dUTP nick-end labeling (TUNEL) staining and caspase-3 activity assay were performed. For TUNEL staining, at the end of 3 hours of reperfusion, mice were anesthetized and their hearts were removed and fixed in 4% paraformaldehyde. The hearts were then embedded in paraffin and cut into 6µm thickness sections and treated as instructed in the *In Situ* Cell Death Detection kit (Roche, 11 684 795 910, Indianapolis, IN, USA). Slides were covered with a glass cover slide applied with mounting media containing DAPI. The entire population was visualized under a fluorescence microscope with the DAPI filter (330-380 nm); the same population was also examined with a FITC filter (465-495 nm) and apoptotic cells with green fluorescence were counted under a high power field. More than 5 fields in >3 different sections/animals in the infarction border zone were examined.

Cardiac caspase-3 activity was measured as previously described\(^1,3\) by using a caspase colorimetric assay kit following the manufacturer’s instructions (Chemicon, Temecula, CA). After 3 hrs reperfusion, mice were anesthetized, hearts removed and myocardial tissue homogenized then centrifuged for 5 mins at 10,000 x g at 4°C. Supernatants were collected, and protein concentrations were measured by the bicinchoninic acid method (Pierce Chemical, Rockford, IL). To each well of a 96-well plate, supernatant containing 200 µg of protein was loaded and incubated with 25 µg Ac-DEVD-pNA as colorimetric-specific substrate at 37°C for 1.5 hours. The pNA was cleaved from DEVD by the caspase-3 and this
free pNA was quantified using a SpectraMax-Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 405 nm. Changes in caspase activity in post-I/R tissue samples (or Sham) were calculated against the mean value of caspase activity in the sham tissue and expressed as pmol pNA/mg protein/hour (pmol/mg prot/h). Activities were measured in the area at risk as well as in the area not at risk (non-ischemic).

**Neonatal Rat Ventricular Myocyte (NRVM) Isolation, Adenoviral infection and Exposure to In Vitro I/R or Oxidative Stress**

Ventricular cardiomyocytes from 1-2-day-old rat hearts were isolated as previously described. NRVMs were cultured in Ham’s F-10 supplemented with penicillin/streptomycin (100 U/ml) and 5% fetal calf serum at 37°C in a 5% CO₂ humidified atmosphere. Cells were infected with adenoviral vectors containing cDNAs for GFP (control), βARKct or GRK2 using a multiplicity of infection of 1000 viral particles per cell (20 infectious units per cell). Efficiency of adenoviral gene transfer was monitored 24 hrs later by GFP fluorescence. After 48 hrs, infected NRVMs were subjected to *in vitro* I/R (medium inducing ischemia-like condition for 45 min and reperfusion of 24 hrs) or exposed to oxidative stress (H₂O₂, 100 µM for 16 hrs) as previously described. Afterward, NRVMs were stained for TUNEL according to the manufacturer’s protocol (Roche, Indianapolis, IN, USA). Apoptotic index was calculated as the percentage of TUNEL positive cells over total nuclei (DAPI).

**Measurement of NO concentration after I/R in vivo**

Total NO content in cardiac tissue was measured in an additional experiment (n=8-20/group) previously described. Tissue samples from the AAR were rinsed in PBS, homogenized in
deionized water (1:10 wt/vol), and centrifuged at 14000G for 10 minutes. The nitric oxide concentration in the supernatant was quantified by a chemiluminescence detector (Sievers 280i NO Analyzer).

**Nitrite/NO production of rat neonatal cardiac myocytes**

Myocytes were isolated and infected with adenoviral vectors as stated above. Subsequently, TNFα (300ng/ml) and Norepinephrine (1µM) were added at the indicated times and NO production was evaluated measuring content of the stable metabolite nitrite in the medium. This was achieved using the Griess reaction protocol, i.e. mixing equal amounts of supernatant medium with Griess reagent and measuring the absorbance at 543nm. Concentrations were calculated according to a standard curve provided by the manufacturer (R&D systems).

**Western Blot Analysis**

Western blotting was performed as previously described. Immunoblots were performed for LV protein levels of phosphoSer473-Akt (Cell Signaling), GRK2 (SantaCruz Biotechnologies) and GAPDH (Chemicon). All antibodies were used at 1:1000 dilution.

**Statistical Analysis**

All values are presented as mean±SEM. Statistical significance of multiple treatments was determined by Student t-test or one-way analysis of variance followed by the Bonferroni post-hoc test when appropriate. For all tests, a $P$ value $<$0.05 was considered significant.


