Prodeath Signaling of G Protein–Coupled Receptor Kinase 2 in Cardiac Myocytes After Ischemic Stress Occurs Via Extracellular Signal–Regulated Kinase-Dependent Heat Shock Protein 90–Mediated Mitochondrial Targeting

Mai Chen,* Priscila Y. Sato,* J. Kurt Chuprun,* Raymond J. Peroutka, Nicholas J. Otis, Jessica Ibetti, Shi Pan, Shey-Shing Sheu, Erhe Gao, Walter J. Koch

**Rationale:** G protein–coupled receptor kinase 2 (GRK2) is abundantly expressed in the heart, and its expression and activity are increased in injured or stressed myocardium. This upregulation has been shown to be pathological. GRK2 can promote cell death in ischemic myocytes, and its inhibition by a peptide comprising the last 194 amino acids of GRK2 (known as carboxyl-terminus of β-adrenergic receptor kinase [bARKct]) is cardioprotective.

**Objective:** The aim of this study was to elucidate the signaling mechanism that accounts for the prodeath signaling seen in the presence of elevated GRK2 and the cardioprotection afforded by the carboxyl-terminus of β-adrenergic receptor kinase.

**Methods and Results:** Using in vivo mouse models of ischemic injury and also cultured myocytes, we found that GRK2 localizes to mitochondria, providing novel insight into GRK2-dependent pathophysiological signaling mechanisms. Mitochondrial localization of GRK2 in cardiomyocytes was enhanced after ischemic and oxidative stress, events that induced prodeath signaling. Localization of GRK2 to mitochondria was dependent on phosphorylation at residue Ser670 within its extreme carboxyl-terminus by extracellular signal–regulated kinases, resulting in enhanced GRK2 binding to heat shock protein 90, which chaperoned GRK2 to mitochondria. Mechanistic studies in vivo and in vitro showed that extracellular signal–regulated kinase regulation of the C-tail of GRK2 was an absolute requirement for stress-induced, mitochondrial-dependent prodeath signaling, and blocking this led to cardioprotection. Elevated mitochondrial GRK2 also caused increased Ca^2+–induced opening of the mitochondrial permeability transition pore, a key step in cellular injury.

**Conclusions:** We identify GRK2 as a prodeath kinase in the heart, acting in a novel manner through mitochondrial localization via extracellular signal–regulated kinase regulation. (Circ Res. 2013;112:1121-1134.)

Key Words: G protein–coupled receptor kinase 2 • heat shock protein 90 • mitochondria • myocyte apoptosis • necrosis • oxidative stress

G protein–coupled receptor (GPCR) kinase-2 (GRK2) is the most studied member of the GRK family of serine/threonine kinases that phosphorylate activated receptors leading to a cessation of signaling, a process known as desensitization. GRK2 is ubiquitously expressed but is the most abundant GRK isoform in the heart and, over the past 2 decades, this kinase has been shown to be a critical regulator of cardiac function, especially in disease, in which it is upregulated, resulting in increased levels in the failing human heart. In addition to classically serving as kinases regulating GPCR coupling, it is becoming increasingly apparent that GRKs play multifaceted roles in cells because several nonreceptor-binding partners have been uncovered. For example, GRK2 has been shown to interact with tubulin, clathrin, Akt, heat shock protein 90 (Hsp90), and 1xβα. In addition, there are various nonreceptor proteins typically found associated with the plasma membrane that are known to regulate GRK2 localization and activity. Thus, a fresh look at the emerging evidence suggests that GRK2 is a potential nodal protein, intersecting multiple signaling pathways within the cell.
A large GRK2 interactome is emerging, and GRK2 can undergo dynamic regulation via phosphorylation. Protein kinase C (PKC)\(^{10}\) and c-Src\(^{11}\) both have been shown to phosphorylate GRK2 under conditions of cellular stress. Moreover, GRK2 has been shown to be phosphorylated by extracellular signal–regulated kinase (ERK) mitogen-activated protein kinase (MAPK) at the specific residue Ser670.\(^{12}\) Recently, Ser670 also was identified as a site for phosphorylation of GRK2 by phosphoinositide-3-kinase.\(^{13}\) In addition to phosphorylation-dependent regulation of GRK2, an area suggesting novel roles for GRKs beyond GPCR desensitization is novel subcellular localization of these kinases. For example, we have recently described that GRK5 is targeted to the nucleus of cardiomyocytes, where it acts as a novel kinase for class II histone deacetylases, and this activity of GRK5 in the nuclear context contributes to cell survival after oxidative stress in myocytes.\(^{14}\) Most recently, GRK2 was detected in mitochondria of endothelial cells and human embryonic kidney (HEK) cells, and it had an effect on mitochondrial biogenesis.\(^{15}\)

GRK2 is not targeted to the nucleus of cells; however, in this study, we found that it localizes to heart mitochondria and we have elucidated the signal transduction mechanism that targets GRK2 to mitochondria after oxidative and ischemic stress. We previously reported that GRK2 is a prodeath kinase in the heart, promoting ischemic injury, whereas its inhibition by the peptide inhibitor, carboxyl-terminal of β-adrenergic receptor kinase (βARKct), comprising the carboxyl-terminal domain of bovine GRK2, confers cardioprotection.\(^{16-18}\) Specifically, the novel results presented in this study indicate that mitochondrial targeting of GRK2 in myocytes after ischemic injury promotes prodeath signaling because mitochondrial accumulation of GRK2 in myocytes increases after oxidative stress. Preventing this mitochondrial targeting, which can be accomplished with βARKct expression, prevents death signaling and confers cardioprotection. Mechanistically, we found that this novel subcellular localization that occurs after oxidative stress is dependent on ERK-mediated phosphorylation of GRK2 at Ser670, and that the subsequent movement to mitochondria is dependent on binding of phosphorylated GRK2 to Hsp90, a known mitochondrial chaperone. Thus, the data reported herein reveal that mitochondrial-targeted GRK2 is essential for prodeath signaling, occurring after oxidative stress in myocytes, and assigns a novel role for this GRK based on specific ERK regulation and its unique cellular localization.

**Methods**

**Experimental Animals**

Cardiac-specific transgenic (TG) mice with GRK2 or βARKct overexpression have been described previously,\(^{19}\) and for all in vivo experiments nontransgenic littermate control mice were used. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University School of Medicine.

**In Vivo Ischemia/Reperfusion Injury Model**

Surgical ischemia/reperfusion (I/R) injury was performed on 8- to 10-week-old mice as previously described.\(^{20}\) All I/R (30 minutes of ischemia and 30 minutes of reperfusion) procedures were controlled by a sham procedure without coronary ligation. We did not measure area at risk or infarct size in this study, but area at risk of the left ventricular is predicted to be 40% to 50%, and infarct of 30% to 40% of area at risk would be expected. I/R procedures were performed on βARKct TG mice and non-TG littermate control mice. At the end of 30 minutes of reperfusion, the mice were euthanized and the hearts were removed, frozen, and saved for biochemical analysis. The numbers of mice used in a particular experiment are provided in the Figure legends. Confirmation of transgene expression was assessed in all mice via Western blotting of cardiac extracts.

**Cell Culture**

Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured in Ham'sF10 as previously described.\(^{21}\) H9c2 cells, HEK 293 cells, and HeLa cells were cultured in DMEM supplemented with 10% bovine calf serum and penicillin-streptomycin in a humidified incubator with 5% CO\(_2\) at 37°C.

**Adenovirus, Plasmids and Transfection**

Flag-tagged βARKct (GRK2 amino acids 495–689) was created by polymerase chain reaction amplification using full-length bovine GRK2 in the pRK5 vector as a template and the appropriate primer to introduce an N-terminal flag tag. Inserts were subcloned into the pRK5 vector by standard molecular biology techniques.

Using the Quick-change kit from Stratagene 2 flag-tagged βARKct mutants in plasmid pRK5 were created. Serine 670 was mutated to either alanine (S670A) or aspartic acid (S670D). Similar mutations were made in the full length GRK2 cDNA and adenovirus was prepared following cloning into the appropriate AdEasy vectors according to the manufacturer’s instructions (Agilent Technologies). Mutations were confirmed by sequencing. Transient transfections were conducted by using 5 μg plasmid DNA and 10 μg Lipofectamine 2000 (Invitrogen) per 100-mm dish of either HEK293 or HeLa cells. Experiments were conducted on cells 48 hours after transfection. Adenoviral-mediated infections of neonatal rat ventricular myocytes were performed using adenovirus–green fluorescent protein, adenov–GRK2, adeno–GRK2S670A, and adeno–βARKct at multiplicity of infection of 10 on the day after infection, and cells were used for experiments 48 hours later. Dr S. Kornbluth at Duke University provided flag-tagged Hsp90, and the glutathione S-transferase-Hsp90 construct was kindly provided by Dr A. Chadli at the Medical College of Georgia.

**Electron Microscopy**

Immunogold electron microscopy was performed as previously described.\(^{22}\) Briefly, nontransgenic littermate control or GRK2-TG mice at 5 weeks of age were anesthetized with isoflurane inhalation, and heparin was injected at 50 U per mouse. Hearts were excised and retrogradely perfused with a solution containing 118 mol/L NaCl, 4.8 mmol/L KCl, 25 mmol/L HEPES, 1.25 mmol/L KHPO\(_4\), 1.25 mmol/L MgSO\(_4\), and 11 mmol/L glucose (pH, 7.4). Mice were perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH, 7.4), and their hearts were dissected. For immuno-electron microscopy, dissected

**Nonstandard Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>βARKct</td>
<td>carboxyl-terminal of β-adrenergic receptor kinase</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal–regulated kinase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein–coupled receptor</td>
</tr>
<tr>
<td>GRK2</td>
<td>G protein–coupled receptor kinase 2</td>
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<tr>
<td>Hsp90</td>
<td>heat shock protein 90</td>
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<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>NRVM</td>
<td>neonatal rat ventricular myocyte</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>S670A</td>
<td>serine to alanine mutation at amino acid 670 in GRK2</td>
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<td>S670D</td>
<td>serine to aspartic acid mutation at amino acid 670 in GRK2</td>
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mice hearts were further fixed in freshly prepared 3% paraformaldehyde in 0.1 mol/L phosphate buffer containing 0.1% glutaraldehyde and 4% sucrose (pH 7.4). Tissues were washed, dehydrated, embedded in Lowicryl K4M (Polysciences), and polymerized under ultraviolet light (360 nm) at −35°C. Ultrathin sections were cut and mounted on Formvar-Carbon-coated nickel grids. After incubation with primary antibodies at 4°C overnight, gold-conjugated secondary antibodies (15 nm Protein A Gold; Cell Microscopy Center, University Medical Center Utrecht; 18 nm Colloidal Gold-AbiPure Goat Anti-Rabbit IgG [H+L]; Jackson Immunoresearch Laboratories) were applied and stained with uranyl acetate and lead citrate by standard methods. Control staining was performed using heat-inactivated GRK2 antibody. Stained grids were examined under Philips CM-12 electron microscope (FEI) and photographed with a 4kx2.7k digital camera (Gatan).

**Immunofluorescence**

HEK cells seeded onto glass cover slips were cotransfected with plasmids, directing expression of mitochondrial-targeted green fluorescent protein and GRK2. Forty-eight hours after transfection, the cells were fixed in 4% paraformaldehyde for 15 minutes, followed by permeabilization for 5 minutes with 0.1% triton in Dulbecco's phosphate-buffered saline. Cells were briefly washed with Dulbecco's phosphate-buffered saline several times and then blocked 90 minutes using Dulbecco's phosphate-buffered saline containing 1% bovine serum albumin, 0.05% Tween 20. Anti-GRK2 antibody (sc-562; Santa Cruz) was added at 1:200 dilution in blocking buffer and applied for 90 minutes with gentle rocking, which was followed by brief washing and addition of anti-rabbit alexa 594 for 90 minutes. Cells were washed, cover slips were mounted on glass slides, and images were acquired using an Olympus Fluoview FV500 confocal laser scanning microscope.

**Mitochondria Isolation From Mouse Hearts and Cells**

Mitochondria were prepared from mouse hearts or cells as previously described. 2 Briefly, hearts were minced and resuspended in mannitol sucrose hepes (MSH) buffer (210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L HEPES, pH 7.5) supplemented with 1 mmol/L EDTA, homogenized with a glass/glass dounce homogenizer, and centrifuged at 600 g for 10 minutes at 4°C. The supernatant was removed, resuspended at 600 g for 10 minutes, removed, and centrifuged again at 5500 g for 20 minutes at 4°C. The mitochondrial pellet was resuspended in fresh MSH buffer without EDTA and centrifuged again at 5500 g for 20 minutes. The mitochondrial supernatant was centrifuged for 20 minutes at 100,000 rpm for 1 hour at 4°C, and then centrifuged at 13,000 rpm for 10 minutes at 4°C. The clarified lysate was taken to be the mitochondrial extract and was used for Western blotting or immunoprecipitation. In some cases, the mitochondrial pellet was solubilized directly into Laemmli loading buffer. When mitochondria were made from cells, a 100-nm dish containing ≈5×10^6 cells was used, cells were washed once with Dulbecco's phosphate-buffered saline, scraped into MSH buffer, dounce-homogenized, and processed as described.

**Proteinase K Assay**

For each experiment, 3-male C57 mice at 12 to 14 weeks old were anesthetized with isoflurane, and the hearts were excised and homogenized in MSH buffer to prepare mitochondria. Mitochondrial exposure to proteinase K was performed with minor modification as previously described. 8 Briefly, 40 μg of mitochondrial protein was incubated on ice for 30 minutes in MSH buffer containing 20 μg/mL proteinase K (Life Technologies). Digestion was stopped by adding phenylmethane-sulfonyl fluoride to a final concentration of 2 mmol/L. The mitochondrial pellet was isolated by centrifugation and solubilized directly in Laemmli loading dye and analyzed for GRK2 by Western blot. Control antibody used for the outer membrane was voltage-dependent anion channel (Neuromab N152B23), and cytochrome c oxidase 5 (Abcam 110263) was used to examine integrity of the inner membrane.

**Immunoprecipitations**

GRK2 or Hsp90 was immunoprecipitated from heart radioimmunoprecipitation assay lysates, cells, or mitochondrial extracts using anti-GRK2 or anti-Hsp90 antibody from Santa Cruz (sc-562 and sc-13119 for GRK2 and Hsp90, respectively). Control immunoprecipitations were conducted using the same antibodies that were heat-inactivated before use as previously described. 21 Flag-tagged βARKct was immunoprecipitated using Flag M2-agarose conjugate or rabbit polyclonal Flag antibody (Sigma-Aldrich). For immunoprecipitations, 1 mg heart lysate was used and, for cells, clarified radioimmunoprecipitation assay extracts prepared from 5×10^6 cells were used. For mitochondrial IPs, detergent extracts of mitochondria obtained from a single heart or from 5×10^6 cells were used. Lysates were incubated with primary antibody for 2 hours, followed by incubation with protein A/G Plus agarose beads (Santa Cruz) for 90 minutes. Beads were washed 3 times with radioimmunoprecipitation assay and, proteins in the immunocomplex were eluted from the beads by the addition of Laemmli loading buffer and then heated for 5 minutes at 95°C before loading onto Tris-Glycine gels (Invitrogen).

**Immunoblotting and Densitometry**

After SDS-PAGE and transfer to nitrocellulose membranes, primary antibody incubations were performed overnight at 4°C. Fluorescent secondary antibodies were obtained from either Molecular Probes or Li-Cor. Membranes were scanned with the Odyssey infrared imaging system (Li-Cor), and the densities of target bands were quantified using the application software of the Odyssey infrared imaging system. Target band density was normalized to the appropriate loading control as described in the Figure legends. Primary antibodies used and sources were the following: anti-GRK2 from Santa Cruz and Millipore; anti-GAPDH from Millipore; anti-phospho-dependent anion channel from BD Biosciences; anti-Hsp90 from Santa Cruz, BD Biosciences, and Stressgen; anti-cleaved caspase-3 and anti-MAPK (phospho and total) from Cell Signaling; anti-histidine from Santa Cruz; anti-phosphoser670–GRK2 from Invitrogen; and anti-Flag from Sigma-Aldrich.

**In Vitro Binding Experiments**

HeLa cells were transfected either with a control plasmid or with flag-tagged Hsp90; after 48 hours, the mitochondrial fraction was prepared. Equal amounts of mitochondria were incubated with purified HIS-tagged GRK2 (Invitrogen) in binding buffer composed of phosphate-buffered saline with 0.2 mmol/L ATP and rotated for 2 hours at 4°C. The mitochondria were pelleted by centrifugation, washed with phosphate-buffered saline, and resuspended directly in Laemmli loading buffer. Western blots were conducted for flag-Hsp90, HIS-GRK2, and voltage-dependent anion channel. In another set of experiments, wild-type and mutant βARKct were transfected into HEK cells and immunoprecipitated from whole-cell lysates using flag antibody. The washed beads containing βARKct were incubated with glutathione S-transferase-Hsp90 in binding buffer composed of phosphate-buffered saline with 1 mmol/L dithiothreitol, 0.5% Teflon-Potter, and 0.5% Triton X-100 rpm for 2 hours at 4°C, and then the beads were pelleted and washed in the same buffer. The samples were electrophoresed and transferred to membranes for immunoblotting. Western blotting with antilglutathione S-transferase antibody was conducted to determine the amount of Hsp90 bound to βARKct.

**Mitochondrial Ca2+ Uptake Assay**

Fluorometric Ca2+ uptake experiments were performed as previously described. 24 Briefly, 10- to 12-week-old male mice were euthanized via CO2 inhalation. For each experiment, 3 mice per group were age-matched and processed together for the isolation of mitochondria, which provided enough protein to run the uptake assay in triplicate each time. Hearts were excised and placed in a beaker containing buffer A (225 mmol/L mannitol, 70 mmol/L sucrose, 1 mmol/L EGTA, 10 mmol/L HEPES, pH 7.2). Hearts were washed to remove residual blood, and then the atria was removed and frozen for Western blot to confirm transgene overexpression. Ventricles were minced into pieces, put in a beaker with 5 mg protease type XIV (Sigma) in buffer A, and stirred for 7 minutes, and then bovine serum albumin was added at 0.02 g/mL final concentration. The tissue was homogenized with a Teflon-Potter and spun at 1000g for 3 minutes. Supernatant was filtered through a 100-μm mesh, and pellet was rehomogenized in a Teflon-Potter in buffer A, spun at 1000g for 3 minutes, and filtered through a 100-μm mesh. Pellet was discarded and supernatants were
spun at 10733g for 10 minutes. Pellets were combined, resuspended in buffer B (225 mmol/L mannitol, 70 mmol/L sucrose, 10 mmol/L HEPES, pH 7.2), and spun at 10733g for 10 minutes. Pellet was resuspended in buffer B, and protein content was determined via bicinechonic acid protein assay. To begin the assay, which was conducted at room temperature, respiration buffer (120 mmol/L KCl, 70 mmol/L mannitol, 25 mmol/L sucrose, 20 mmol/L HEPES, 5 mmol/L KH2PO4, 0.5 mmol/L EGTA) containing 0.1 μmol/L calcium green 5 N (Invitrogen) was added to a quartz cuvette, and a zero baseline was established. Mitochondria were added at 0.73 mg/mL, and then 30 μmol/L free calcium was added every 2 minutes. Measurements (excitation-emission at 503–535 nm) were obtained using a Cary Eclipse Fluorescent Spectrophotometer.

Statistics
Data are expressed as mean±SE. Statistical significance was determined by an unpaired t test or ANOVA and Bonferroni test for multiple comparisons using GraphPad Prism software version 5.9. P<0.05 was considered significant.

Results

GRK2 Localizes to Mitochondria and After Myocardial Ischemic and Oxidative Stress There Is Increased Mitochondrial GRK2 Translocation
Recent data from our laboratory have shown GRK2 to be prodeath in the heart after ischemic injury; in pursuit of mechanisms for this prodeath signaling, we found GRK2 to be present in the mitochondrial fraction of neonatal rat ventricular myocyte and hearts (Figure 1A and 1B). Importantly, we confirmed this localization using anti-GRK2 and immunogold electron microscopy of cardiac sections where GRK2 is present within mitochondria (Figure 1C). This was an unexpected finding because GRK2 has been thought to be primarily cytosolic, especially in the heart where it regulates several GPCRs, including β-adrenergic receptors that are crucial regulators of cardiac function.1,2 We also conducted immunofluorescence experiments in HEK cells, looking at the possible...
colocalization of GRK2 with a mitochondrial-targeted green fluorescent protein marker protein. The merged confocal image in Online Figure I indicates colocalization. Finally, to further explore the localization of GRK2 in mitochondria, we treated isolated mouse heart mitochondria with proteinase K and, after Western blot, we found that GRK2 was nearly completely digested; as an outer membrane positive control, we found that voltage-dependent anion channel also was decreased after treatment, as would be expected. Cytochrome C oxidase 5, a marker for the inner membrane, was not altered, indicating that GRK2 was primarily associated at the outer membrane, at least basally under control conditions (Online Figure II). Although the functional role of GRK2 in mitochondria is not understood, we found that the mitochondria prepared from the hearts of transgenic mice overexpressing GRK2 specifically in the heart had an increased sensitivity to Ca2+-induced opening of the mitochondrial permeability transition pore, a key step in oxidative stress–mediated cell injury (Figure 1D). As shown in Figure 1D, the Ca2+ retention capacity, up to the point at which further addition of a Ca2+ pulse results in precipitous Ca2+ release because of opening of the mitochondrial permeability transition pore, was decreased by 16.8% in GRK2 transgenic mice (362.9±20.6 for control and 288.9±16.29 nmol Ca2+/mg protein for TG; n=3 separate experiments for both groups). The full pathophysiological role for this negative relationship will be further investigated, but as we began to explore whether the unique subcellular localization of this GRK2 was related to its prodeath signaling in myocytes, we found, interestingly, more GRK2 within cardiac mitochondrial preparations after I/R injury in mice (Figure 1A). Furthermore, oxidative stress in cultured myocytes after chelerythrine treatment also led to increased mitochondrial GRK2 localization (Figure 1B). Importantly, chelerythrine has been shown to induce apoptosis in myocytes through the generation of reactive oxygen species (ROS), and chelerythrine induces apoptosis despite PKC downregulation, suggesting that PKC inhibition is not the mechanism of chelerythrine-induced apoptosis.25 We confirmed that treatment of cardiomyocytes with 10 μmol/L chelerythrine for 30 minutes led to increased cleaved caspase-3 formation (Figure 1E). The chelerythrine-induced mitochondrial localization of GRK2 was inhibited by cellular pretreatment with the antioxidant N-acetyl-cysteine, providing further evidence that apoptosis was dependent on ROS generation (Figure 1B). N-acetyl-cysteine also blocked chelerythrine-mediated apoptotic signaling as measured by cleaved caspase-3 levels (Figure 1E).

ROS-Mediated Mitochondrial Localization of GRK2 Is Dependent on Hsp90 Binding

The localization of GRK2 to mitochondria was surprising, given that it has no targeting consensus sequence, although it should be noted that ≈50% of all mitochondrial proteins do not have mitochondrial target sequences. Therefore, we hypothesized that an interaction with an intermediate protein could be involved in this mechanism. A potential candidate emerged from known GRK2-interacting proteins. GRK2 has been shown to associate with Hsp90,8 and this Hsp is known to be associated with mitochondria.25 Importantly, recent data have shown that mitochondrial association of other kinases, such as Akt11 and PKCe,26,28 is dependent on Hsp90. We found a parallel increase in mitochondrial-localized Hsp90 and GRK2 in mouse hearts after ischemic injury (Figure 2A) and in cultured myocytes after chelerythrine treatment (Figure 2B). Previously in the human leukemia-60 cell line GRK2 and Hsp90 have been shown to coimmunoprecipitate, and the interaction was blocked by geldanamycin, a known Hsp90 inhibitor.8 To explore whether this interaction occurs in myocytes after ischemic or oxidative stress, we treated myocytes with chelerythrine without and with pretreatment with geldanamycin. As shown in Figure 2C, we found decreased chelerythrine-stimulated association of both Hsp90 and GRK2 with mitochondria using geldanamycin. We found that GRK2 and Hsp90 associate in whole-cell lysates prepared from myocytes (Figure 3A, lane 1) and coimmunoprecipitation between these 2 proteins also occurs specifically in mitochondrial preparations (Figure 3A, lane 2). We also found that GRK2 and Hsp90 can interact and bind directly using a cell free system. We transfected cells either with a control plasmid or with flag-tagged Hsp90, prepared the mitochondrial fraction from each, and then incubated equal amounts of mitochondria with purified 6x HIS-tagged GRK2. In mitochondria prepared from Hsp90-transfected cells, significantly more GRK2 was present compared with control mitochondria (Figure 3B). Finally, arguing in favor of a physiological relevance for this interaction, we found that more GRK2 was associated with Hsp90 (via coimmunoprecipitations) in mouse hearts exposed to I/R (30 minutes of ischemia, 30 minutes or reperfusion) injury and in myocytes when oxidative stress was increased by treatment with chelerythrine (Figure 3C and 3D).

ERK Phosphorylation of GRK2 Is Essential for Oxidative Stress–Induced Mitochondrial Localization and Apoptosis

In addition to ROS being necessary for enhanced localization of GRK2 to mitochondria after stress (in vivo studies), we explored other downstream mediators of ischemic and oxidative stress using chelerythrine-stimulated myocytes. We focused initial attention on activation of ERK (p42/44 MAPK), because this has been shown to be activated by chelerythrine,29 and because ERK can phosphorylate GRK2 specifically at residue Ser670 within its carboxyl terminus.13 When myocytes were treated for 20 minutes with 10 μmol/L chelerythrine, we observed ≈1.5-fold increase in the amount of phosphorylated (ie, activated) ERK (measured as both p-ERK1 and p-ERK2; Figure 4A), whereas in cells pretreated for 20 minutes with the MEK (the upstream ERK-activating kinase) inhibitor PD98059, chelerythrine-activated ERK was significantly inhibited (Figure 4A). Therefore, this pathway downstream of chelerythrine-mediated stress is active in myocytes. Importantly, a site-specific antibody that recognizes GRK2 when phosphorylated at Ser670 is available, and we examined whether chelerythrine leads to the phosphorylation of GRK2 at this site in myocytes. These experiments were conducted by immunoprecipitating GRK2 from myocytes and then blotting for pSer670-GRK2. As shown in Figure 4B, the amount of pSer670-GRK2 was increased >2-fold after a 20-minute treatment of cells with chelerythrine, and this was significantly decreased in the presence of PD95089.
Similarly, we found that the chelerythrine activation of ERK and the concomitant phosphorylation of GRK2 at Ser670 were blocked by pretreatment of cells with the antioxidant N–acetyl-cysteine (Figure 4C).

Based on these experiments, we hypothesized that phosphorylation of GRK2 at Ser670 might regulate stress-induced translocation of GRK2 to mitochondria and cell death. When cardiomyocytes were pretreated with PD98059, we found that the amount of GRK2 that was localized to mitochondria after treatment with chelerythrine, which normally is increased, was significantly decreased, and the proportion of GRK2 that was in the phospho-Ser670 state within the mitochondrial fraction was also decreased compared with cells in which ERK was not inhibited (Figure 4D). To strengthen our findings, we used a different cellular model to examine the prodeath capacity of GRK2. Accordingly, we found that cells overexpressing the S670A-GRK2 mutant had less H2O2-induced death as determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. As shown in Online Figure III, myocytes were infected with either wild-type GRK2 or the S670A-GRK2 mutant and then treated with 5 μmol/L geldanamycin (GA) for 2 hours and then stimulated for 30 minutes with 10 μmol/L chelerythrine. Mitochondrial fractions were then prepared, and immunoblotting was conducted for GRK2 and Hsp90 and normalized to VDAC levels. *P<0.05 (n=3).

Interestingly, we found an ERK dependence on the Hsp90 binding of GRK2, because PD98059 treatment decreased the amount of wild-type GRK2 bound (Figure 4E), which parallels the PD98059-induced reduction of GRK2 in mitochondrial fractions after chelerythrine (Figure 4D). The directed phosphorylation of GRK2 at Ser670 seems to mechanistically target binding to Hsp90 before mitochondrial localization, because a mutant GRK2 in which Ser670 was replaced by the nonphosphorylated Ala (GRK2-S670A) displayed significantly less Hsp90 binding in myocytes after oxidative stress compared with wild-type GRK2 (Figure 5A). Thus, nonphosphorylated GRK2 would not be expected to localize to mitochondria after oxidative stress, which is consistent with less death seen with GRK2-S670A overexpression.

Ser670 resides within the βARKct; thus, we have a powerful molecular tool to further explore the mechanistic role of ERK-mediated GRK2 recruitment to mitochondria. This is even more intriguing because myocyte expression of βARKct leads to cardioprotection in vivo after ischemic injury with significantly decreased myocardial apoptotic signaling. Accordingly, using wild-type βARKct as the standard for prosurvival signaling in...
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In stressed myocytes, we constructed 2 βARKct mutants, βARKct-S670A and βARKct-S670D, the latter as a potential phosphomimetic mutant of this GRK2 peptide, and studied these in transiently transfected HEK cells. Importantly, we found that wild-type βARKct (flag-tagged) could be immunoprecipitated with Hsp90 in cell extracts, showing that the carboxyl-terminus of GRK2 directs binding (Figure 5B). Furthermore, as with full-length GRK2, chelerythrine treatment resulted in enhanced phosphorylation of the βARKct at Ser670 (Figure 5C). However, and as would be predicted, the βARKct-S670A mutant showed no signal with the pSer670 antibody (Figure 5C), a result that also documents the specificity of this antibody (the βARKct contains 9 serine residues).

Next, we used an in vitro binding assay to examine whether the phosphorylation of Ser670 influences the Hsp90 and βARKct interaction. Flag-tagged βARKct, βARKct-S670A, or βARKct-S670D was immunoprecipitated from whole-cell extracts, and then purified glutathione S-transferase-Hsp90 was added to these samples. As shown in the Western blot in Figure 5D, βARKct-S670D displayed the highest level of Hsp90 binding, whereas βARKct-S670A was lower compared with wild-type βARKct binding. These results support the hypothesis that phosphorylation at this site places the carboxyl-terminus of GRK2 in a configuration that is structurally favorable for binding with Hsp90. Moreover, we found that wild-type βARKct, like GRK2, robustly localizes to mitochondria, and this is increased after chelerythrine treatment; however, βARKct-S670A mitochondrial localization after oxidative stress is significantly impaired (Figure 5E). This strongly argues for the importance of ERK phosphorylation at this site on GRK2 to direct Hsp90-dependent mitochondrial translocation. To solidify this mechanism, we found that expression of wild-type βARKct inhibits the stress-induced mitochondrial localization of endogenous GRK2, whereas expression of...
βARKct-S670A did not have this effect (Figure 5F). The major interpretation of this result is that βARKct competes with GRK2 for Hsp90 binding, and because βARKct-S670A binds less favorably to Hsp90, the inhibition of GRK2 translocation is lost, allowing for the mitochondrial-dependent prodeath activity of the kinase.

Figure 4. Chelerythrine-activated extracellular signal–regulated kinase (ERK) phosphorylates G protein–coupled receptor kinase 2 (GRK2) at serine 670, regulating its heat shock protein 90 (Hsp-90)–dependent mitochondrial translocation. A, Neonatal rat ventricular myocytes (NRVMs) were stimulated for 30 minutes with 10 μmol/L chelerythrine in the absence or presence of PD98059 (30 minutes pretreatment, 50 μmol/L), and whole-cell lysates were analyzed by immunoblotting to detect phospho-ERK and total-ERK. A representative immunoblot is shown along with quantification of the results, which are expressed as fold increase over basal. *P<0.05 (n=3). B, The amount of chelerythrine-induced phosphorylation of Ser670 within GRK2 was analyzed by immunoprecipitating GRK2 from NRVM whole-cell lysates followed by immunoblotting for both p-Ser670 and total GRK2. Pretreatment and stimulation conditions were the same as in (A). The p-Ser670 signal at each condition was normalized to the amount of GRK2 immunoprecipitated. *P<0.05 (n=3). C, NRVMs were stimulated for 30 minutes with 10 μmol/L chelerythrine in the absence or presence of N-acetyl-cysteine (NAC; 5 mmol/L, 40 minutes of pretreatment). Whole-cell lysates were immunoblotted for ERK activation (p-ERK) and phosphorylation status of GRK2 Ser670. *P<0.05 NAC vs control (n=3). D, Mitochondrial fractions were prepared from chelerythrine-treated NRVMs in the absence and presence of pretreatment with the ERK inhibitor, PD98059. Immunoblotting was used to determine the amount of p-Ser670 and total GRK2 in mitochondria. Voltage-dependent anion channel is shown as a loading control, *P<0.05 PD98059 vs DMSO-treated control myocytes (n=3). E, After treatment of NRVMs with chelerythrine, in the presence or absence of pretreatment with PD98059, Hsp90 was immunoprecipitated from whole-cell lysates followed by immunoblotting for Hsp90 and GRK2. A representative immunoblot from 3 separate experiments is shown on the left, with quantitation showing PD98059 pretreatment decreased the amount of GRK2 interacting with Hsp90 shown on right. *P<0.05, PD98059 vs control.
βARKct-Mediated Cardioprotection In Vivo
Is Mediated Via Less Mitochondrial GRK2
Localization in Response to ERK Phosphorylation

We have shown that βARKct expression prevents GRK2 mitochondrial localization after oxidative stress in HEK cells, and that this mechanism also robustly occurs in cardiomyocytes (Figure 6A). Consistent with our findings in isolated myocytes, this is also the case in the ischemic heart in vivo, because we have found less GRK2 associated with purified cardiac mitochondria after I/R injury in βARKct transgenic mice compared with nontransgenic mice (Figure 6B). βARKct-mediated cardioprotection in ischemic mouse hearts was associated with lower apoptotic signaling, and we found that mechanistically this is attributable to inhibition of GRK2 mitochondrial localization, because we found that the lack of increased mitochondrial GRK2 accumulation after βARKct expression in myocytes exposed to chelerythrine results in significantly lower cleaved caspase-3 protein levels (Figure 6C). To bolster the hypothesis that less apoptotic signaling in βARKct-expressing myocytes is attributable to inhibiting GRK2 movement to mitochondria, we found that the βARKct-S670A mutant does not inhibit chelerythrine-mediated caspase-3 activation (Figure 6D), which is a parallel finding showing that this mutant βARKct does not block GRK2 mitochondrial translocation (Figure 5F).

We also have positively linked ERK-mediated phosphorylation of GRK2 to prodeath signaling and pathophysiology after ischemic stress, because we found that in cardioprotective βARKct transgenic mice after I/R injury, there is not only less mitochondrial GRK2 (Figure 6B) but also significantly less p-Ser670 in the mitochondrial GRK2, despite equal post-I/R cardiac ERK activation (Figure 7A). Interestingly, Ser670 of the βARKct transgenic peptide expressed in βARKct transgenic mice was robustly phosphorylated after I/R (Figure 7A), suggesting that after ERK activation this peptide binds Hsp90 more strongly and prevents GRK2 from inducing mitochondrial-mediated death. Of further importance to our hypothesis, these results were reproduced in cultured myocytes treated with chelerythrine (Figure 7B).

Discussion

Classically, GRKs phosphorylate agonist-occupied 7-transmembrane spanning receptors, known as GPCRs, and initiate an uncoupling process known as desensitization. The desensitization
mechanism includes targeted binding of β-arrestins to GRK-phosphorylated receptors, which blocks G protein activation. However, β-arrestins also direct G protein–independent signaling by recruiting signaling molecules themselves, opening a new area of GPCR signaling that is in addition to the desensitization process. Similarly, evidence is mounting that GRKs can regulate important cellular processes beyond membrane-bound GPCRs, a finding that has gained traction largely as the result of previous notions are being challenged and, as an example, GRK2/GRK3 have been found to be primarily cytosolic enzymes that associate with the sarcolemmal membrane after agonist occupancy of a GPCR, whereas GRK5/GRK6 are more avidly associated with the membrane at all times. However, these previous notions are being challenged and, as an example, GRK5 and GRK6 have a functional nuclear localization signal within their catalytic domains, and GRK5 has been shown to reside in the nucleus of myocytes. This nuclear localization of myocardial GRK5 has uncovered a novel role for this GRK as a class II histone deacetylase kinase, and its enhanced activity in the nucleus can promote maladaptive myocyte hypertrophy and heart failure.

Although we do not fully understand why GRK2 is associated with mitochondria under basal conditions, we have found that increased mitochondrial GRK2 promotes mitochondrial permeability transition pore opening to a lower threshold of Ca^2+, which in itself demonstrates GRK2 promoting an altered mitochondrial phenotype. We have identified in this report that GRK2 has crucial partners that direct its stress-induced translocation to mitochondria, including ERK MAPK and Hsp90. Of note, without phosphorylation of GRK2 after...
ROS formation by ERK, specifically at Ser670, there is no mitochondrial localization leading to cell death because this modification of GRK2 is required for it to bind to Hsp90, which directs its recruitment to the primary prodeath organelle within myocytes. Hsp90 is one of several Hsps that function as molecular chaperones involved in the assurance of correct target protein folding and assembly and, interestingly, Hsp90 seems to be more discriminating in terms of client interaction. Evidence suggests a preference for interactions with protein kinases, and kinases including Pim-1, Akt, and PKCε bind to Hsp90. Previously, an interaction between Hsp90 and GRK2 was identified with a potential role in the maturation of this kinase. However, our data uncover a second functional outcome of this interaction, and that is mitochondrial targeting, which is consistent with Hsp90 being identified as an important mitochondrial protein in a proteomic analysis of mouse mitochondria.

Because most mitochondrial proteins are nuclear-encoded and synthesized in the cytosol, the finding that molecular chaperones Hsp90 and Hsp70 deliver immature proteins to the mitochondrial import receptor Tom70 was a key discovery that seems to have broader implications in cellular functions, especially in cardiomyocytes after ischemic stress and this previously unappreciated mechanistic role for GRK2. Interestingly, Hsp90 also can play a critical role in cell survival by binding to components of the permeability transition pore and antagonizing its opening, thus preserving organelle integrity and inhibiting the onset of cell death. Our data suggest that in myocytes there seems to be a more complex relationship between Hsp90 and mitochondrial-mediated death, because the ROS-induced increased localization of GRK2 to mitochondria is associated with increased death signaling. Future studies that are beyond the scope of the present study will examine whether GRK2 specifically antagonizes the prosurvival effects of Hsp90 in mitochondria, because this could occur through a direct inhibition or perhaps GRK2 can antagonize the antideath effects of other known Hsp90 binding partners in the heart.

The mitochondrial uptake of several cardioprotective proteins, including connexin, Akt, and PKCε, has been shown to entail Hsp90-mediated import. Interestingly, a previous study in liver endothelial cells has shown that when GRK2 is elevated it binds to and inhibits Akt in the cytoplasm, suggesting the potential for this interaction to take place at the level of Hsp90 binding or perhaps at mitochondria. Certainly, our mitochondrial-dependent prodeath signaling ascribed to GRK2 in this study is consistent with potential antagonism.
of the prosurvival effects of Akt, and although we did not specifically find a direct interaction between these 2 kinases in mitochondrial fractions of myocytes, it is possible that the prodeath effects of GRK2 in ischemic myocytes opposes any benefit of Akt within mitochondria. Interestingly, in cardio-myocytes, Akt also was shown to phosphorylate mitochondrial hexokinase, a mitochondrial outer membrane protein, and to protect mitochondria from oxidant or calcium-induced stress.\(^{46}\) Further studies will be conducted to identify any potential substrates for GRK2 within mitochondria.

In addition to Hsp90 being required for mitochondrial localization of GRK2, we found that an absolute requirement was the ERK-mediated phosphorylation of GRK2 at Ser670 because the phosphorylation status at this site was a key determinant of Hsp90 binding and subsequent mitochondrial accumulation. This ascribes a specific and critical role for the regulation of GRK2 by this MAPK within cardiomyocytes and death signaling. Previous studies have shown that phosphorylation of GRK2 by ERK at Ser670 is involved in protein stability and promoted degradation of this kinase.\(^{12,47}\) Similarly, this seems to be the case for the homologous GRK3 after ERK phosphorylation.\(^{48}\) A more recent study has shown that Ser670 phosphorylation by the cell-cycle kinase, CDK2, may be more crucial for GRK2 degradation within its context as a cell-cycle regulator.\(^{49}\) Furthermore, in neuronal cells, phosphorylation of GRK2 at Ser670 by phosphoinositide-3-kinase promoted degradation.\(^{50}\) Thus, although Ser670 may be the key target to promote GRK2 degradation, differential regulation seems to occur via specific kinases, and our data would suggest that ERK-mediated Ser670 phosphorylation targets GRK2 to mitochondria. Importantly, targeting of GRK2 to mitochondria after this phosphorylation event would result in a subsequent loss of cytoplasmic GRK2 consistent with degradation; however, without knowledge of the unique cellular localization of GRK2 uncovered in our report, this aspect of GRK2 regulation was not studied. A number of studies indicate that activation of the reperfusion injury salvage kinase pathway at the time of injury is cardioprotective.\(^{50}\) ERK1/2 is one of the kinases involved in this pathway; in these studies, the beneficial effects of ERK were identified based on the finding that administration of growth factors or other agents at the time of reperfusion resulted in protection through phosphorylation and inhibition of proapoptotic proteins. In contrast to the signals triggered by administration of growth factors, ischemic stress activates a different repertoire of signaling molecules, and the final cellular response derives from the integration of beneficial and prodeath signals. In our study, we show ERK activation being involved in the prodeath mechanism of GRK2; however, this finding should not be interpreted as negating other protective aspects of ERK in accordance with the reperfusion injury salvage kinase hypothesis.

A recent study demonstrated mitochondrial localization of GRK2 in HEK and endothelial cells, and mitochondria with decreased GRK2 had less ATP production, suggesting that increased GRK2 is beneficial.\(^{51}\) Using GRK2 truncation mutants, it was reported that the amino-terminus of GRK2 associated with mitochondria, but that the carboxyl-terminus did not. We focused on the carboxyl-terminus because it contains the essential serine residue that, when phosphorylated, regulates its interaction with Hsp90. It is possible that multiple domains within GRK2 could regulate association with mitochondria, such as during basal or oxidative stress conditions; however, given the absence of a mitochondrial targeting sequence within GRK2, the lack of mechanistic detail regarding how GRK2 associates with mitochondria through the amino-terminus of the protein is a limitation of their study. Alternatively, cardiac mitochondrial targeting could be different. An important difference between our studies is that we show that increased GRK2 localized to cardiac mitochondria is a damaging phenotype and we provide mitochondrial Ca\(^{2+}\) uptake data to support this. Our data are also consistent with the physiological evidence that we and others have published showing that increased GRK2 levels in the heart are deleterious in the context of ischemic stress.

There are several potential limitations of our study. We measured I/R-induced (30 minutes of each) translocation of GRK2 to mitochondria and the signaling mechanisms involved, but we did not measure infarct size in a similarly treated cohort; therefore, we were not able to correlate myocardial injury with translocation of GRK2 to mitochondria. However, using the same I/R injury model, GRK2-TG mice had significantly greater 24-hour infarcts compared with non-TG littermate control mice.\(^{17}\) Furthermore, although the mechanism of cell death in mouse models of ischemic injury, such as I/R, remains controversial, it is likely that both apoptosis and necrosis are involved; however, in this study, we focused only on apoptotic signaling. We focused on apoptosis because a previous study\(^{17}\) reported that mice subjected to 30 minutes of ischemia and 3 hours of reperfusion had increased ventricular caspase-3 activity, which is indicative of apoptosis, and the cell death mechanisms in our study likely would be similar to those reported in that study. Accordingly, we treated neonatal rat ventricular myocytes with chelerythrine, which also increased caspase-3 activity, as a model to study the biochemical signaling pathways that would be activated during in vivo injury in the mouse heart. There could be differences in the relative contribution that apoptosis and necrosis play in adult compared with neonatal myocytes exposed to ischemic stress. For example, one study reported that isolated adult myocytes exposed to 6 hours of hypoxia and 6 hours of reperfusion had increased cytochrome C release and caspase activity compared with control cells,\(^{51}\) which supports apoptotic mechanisms in the adult cell. However, a different study showed changes in the level of proteins important in the assembly and formation of the apoptosis in adult compared with neonatal myocytes, supporting the view that adult myocytes are more resistant than neonatal myocytes to mitochondrial-driven apoptosis.\(^{52}\) Thus, the relationship between apoptosis and necrosis and the relative importance of each in neonatal and adult cell death during I/R injury continue to evolve, and future studies will help resolve these controversies.

Of potential therapeutic importance, our current data assign a new beneficial mechanism for the action of βARKct in the injured heart as sequences within this peptide direct phosphorylation-dependent binding to Hsp90, and the binding of this expressed and phosphorylated peptide blocks endogenous GRK2 binding to Hsp90 and subsequent mitochondrial localization preventing GRK2-mediated prodeath signaling. We have recently shown that βARKct expression in the infarcted heart significantly reduces cellular apoptosis; in this study, we
found that Ser670 is critical for βARKct-mediated cardioprotection, because we discovered that prodeath signaling after oxidative stress in myocytes was not blocked by a βARKct mutant that lacks the ERK phosphorylation site. This nonprotective βARKct mutant does not seem to prevent endogenous GRK2 from binding to Hsp90, thus GRK2 is free to translocate to the mitochondria and promote caspase activation.

In conclusion, our study is the first to report cellular association of GRK2 with mitochondria of cardiomyocytes, and that this localization increases on ischemic and oxidative stress, events that promote its prodeath signaling. These findings add significant mechanistic information for the maladaptive effect of GRK2 that occurs within the compromised myocardium. Importantly, we ascribe a novel regulatory feature to Ser670 within the carboxyl terminus of GRK2 in that after ROS-dependent ERK phosphorylation, there is increased binding to Hsp90 and subsequent translocation to mitochondria. Our results are clear that ERK phosphorylation and Hsp90-dependent mitochondrial localization of GRK2 in ischemic cardiomyocytes promote non-GPCR-mediated prodeath effects of this GRK2. This is an apparent key role for GRK2 in the pathogenesis of heart failure and increases the therapeutic importance of GRK2 inhibition as a strategy to combat heart disease.

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Disclosures

None.

References

Mitochondrial localization of GRK2 after extracellular signal–regulated kinase phosphorylation seems to promote its prodeath activity because mutation of GRK2 at Ser670 to Ala results in lower apoptosis 


Novelty and Significance

What Is Known?

- G-protein–coupled receptor kinases (GRKs) phosphorylate activated receptors, which turns off cellular signaling in a process known as desensitization.
- GRK2 is the predominant GRK isofrom in the heart. In human heart failure, the levels of GRK2 are elevated.
- Recent studies identified nonreceptor-binding partners for GRK2, suggesting that GRK2 regulates cellular signaling through mechanisms independent of receptor desensitization.
- A peptide comprising the last 194 amino acids of GRK2 (known as carboxyl-terminus of β-adrenergic receptor kinase) is cardioprotective in rodent models of cardiac injury, whereas GRK2 activity promotes cell death after myocardial ischemia.

What New Information Does This Article Contribute?

- Carboxyl-terminus of β-adrenergic receptor kinase–mediated cardioprotection occurs by competing with and inhibiting endogenous GRK2 binding to heat shock protein 90, thus decreasing the amount of GRK2 at mitochondria.
- Mitochondria with increased GRK2 have decreased calcium uptake capacity, which may mechanistically explain the damaging phenotype associated with increased GRK2 in myocytes.

The classical view of GRK2 as a kinase involved mainly in G-protein–coupled receptor signaling is quickly changing. Instead, the idea of a more dynamic GRK2 with multiple roles in regulating cell signaling is emerging. In this study, we show that GRK2 is present at the mitochondria within cardiomyocytes and also that its localization to this organelle is altered in response to ischemic injury. Oxidative stress after injury activates multiple signaling molecules, including extracellular signal–regulated kinase 1/2, resulting in the phosphorylation of GRK2 in the cytoplasm, leading to increased binding to heat shock protein 90, which chaperones GRK2 to mitochondria. We also show that mitochondria prepared from mice that overexpress GRK2 in the heart have worsened mitochondrial calcium handling. Furthermore, we show that mice having cardiac-specific expression of a peptide, known as carboxyl-terminus of β-adrenergic receptor kinase, fare better after ischemic injury, and this is mechanistically associated with decreased mitochondrial GRK2 translocation/localization. In summary, we elucidate a novel signaling mechanism in the heart in which GRK2 translocates to mitochondria after ischemic stress and acts as a prodeath kinase.
Prodeath Signaling of G Protein–Coupled Receptor Kinase 2 in Cardiac Myocytes After Ischemic Stress Occurs Via Extracellular Signal–Regulated Kinase-Dependent Heat Shock Protein 90–Mediated Mitochondrial Targeting

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SUPPLEMENTAL MATERIAL

Pro-Death Signaling of GRK2 in Cardiac Myocytes after Ischemic Stress Occurs via ERK-Dependent, Hsp90-Mediated Mitochondrial Targeting

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Detailed Materials and Methods

Experimental Animals
Cardiac-specific transgenic mice with GRK2 or β-ARKct overexpression have been described previously 1 and for all in vivo experiments non-transgenic littermate control (NLC) mice were used. All animal procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University.

Cell Culture
Rat neonatal ventricular myocytes were isolated and cultured in HamsF10 as previously described2. HEK 293 cells and HeLa cells were maintained in DMEM with 10% bovine calf serum, 1% penicillin-streptomycin in 5% CO2 at 37°C.

Plasmids and Transfection
Flag tagged-βARKct (GRK2 amino acids 495-689) was created by PCR amplification using full length bovine GRK2 in the pRK5 vector as a template and the appropriate primer to introduce an N-terminal flag tag and the insert was subcloned into the pRK5 vector by standard molecular biology techniques. This plasmid served as the template to create the flag-S670A and S670D mutations in βARKct and GRK2 using the Quick-change kit from Stratagene according to the manufacturer’s instructions. Mutations were confirmed by sequencing. The plasmid encoding for mitochondrial-localized EGFP was obtained from Clontech. Transient transfections were conducted using 5ug plasmid dna and 10ug Lipofectamine 2000 (Invitrogen) /100mm dish of either HEK293 or HeLa cells. Experiments were generally conducted on cells 48 hrs post transfection. Adenoviral-mediated infections of cardiomyocytes were performed using Adeno-GFP, Adeno-GRK2, Adeno-GRK2S670A, and Adeno-βARKct at an MOI of 10 on the day after isolation and cells generally used for experiments 48 hours later. We thank Dr. S. Kornbluth at
Duke University for flag-tagged Hsp90 and the Gst-Hsp90 construct was kindly provided by Dr. A. Chadli at the Medical College of Georgia.

In vivo Ischemia-Reperfusion (I/R) Injury Model
Surgical induction of I/R injury was performed as previously described 3. 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 30 min at which time the hearts were and removed flash-frozen in liquid nitrogen and saved for later biochemical analysis (signaling). Confirmation of transgene expression was assessed in all mice via Western blotting of cardiac extracts.

Electron Microscopy
Immunogold Electron Microscopy were performed as previously described 4. Briefly, mice at five weeks of age were anesthetized via isoflurane inhalation and heparin was injected at 50U/mouse. Hearts were excised and retrograde-perfused with a solution containing 118mM NaCl, 4.8mM KCl, 25mM HEPES, 1.25mM K2HPO4, 1.25MgSO4, 11mM Glucose (pH 7.4). Mice were perfusion-fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4), and hearts dissected. For immuno- electron microscopy, dissected mice hearts were further fixed in freshly prepared 3% paraformaldehyde in 0.1M phosphate buffer containing 0.1% glutaraldehyde and 4% sucrose (pH 7.4). Tissues were washed, dehydrated, embedded in Lowicryl K4M (Polysciences, Inc., Warrington, PA), and polymerized under UV light (360nm) at -35°C. Ultrathin sections were cut and mounted on Formvar-Carbon coated nickel grids. After incubation with primary antibodies at 4°C overnight, gold conjugated secondary antibodies (15nm Protein A Gold, Cell Microscopy Center, University Medical Center Utrecht, 35584 CX Utrecht, The Netherlands; 18nm Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG (H+L), Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were applied and stained with uranyl acetate and lead citrate by standard methods. Control staining was performed using heat-inactivated GRK2 antibody. Stained grids were examined under Philips CM-12 electron microscope (FEI; Eindhoven, The Netherlands) and photographed with a Gatan (4k x2.7k) digital camera (Gatan, Inc., Pleasanton, CA).

Mitochondria isolation from mouse hearts and cells
Mitochondria were prepared from mouse hearts or cells as previously described 5. Briefly, hearts were minced and resuspended in MSH buffer (210mM mannitol, 70mM sucrose, 5mM Hepes, pH 7.5) supplemented with 1mM EDTA, homogenized with a glass/glass dounce homogenizer and centrifuged at 600 x g for 10 min at 4°C. The supernatant was removed, respun at 600 x g for 10 min then removed and centrifuged again at 5500 x g for 20 min at 4°C. The mitochondrial pellet was resuspended in fresh MSH buffer without EDTA and centrifuged again at 5500 x g for 20 min. The washed mitochondrial pellet was resuspended in RIPA lysis buffer if being used for immunoprecipitation experiments, rotated for 1 hr at 4°C, then centrifuged at 13,000 rpm for 10 min at 4°C. The clarified lysate was taken to be the mitochondrial extract and was used for western blotting or immunoprecipitation. In some cases the mitochondrial pellet was solubilized directly into Laemmli loading buffer. When mitochondria were made from cells a 100mm dish containing about 5 x 10^6 cells was used, cells were washed once with DPBS, scraped into MSH buffer, dounce homogenized and processed as described above.
**Immunoprecipitations.**

GRK2 or Hsp90 was immunoprecipitated from heart RIPA lysates, cells, or mitochondrial extracts using anti-GRK2 or anti-Hsp90 antibody from Santa Cruz (cat # sc-562 and sc-13119 for GRK2 and Hsp90, respectively). Control immunoprecipitations were conducted using the same antibodies that were heat inactivated prior to use as previously described. Flag-tagged βARKct was immunoprecipitated using Flag M2-agarose conjugate or rabbit polyclonal Flag antibody (Sigma-Aldrich). Generally 1mg of heart lysate was used and for cells, clarified RIPA extracts prepared from 5x10⁶ cells was used. For mitochondrial IPs, detergent extracts of mitochondria obtained from a single heart or from 5x10⁶ cells was used. Lysates were incubated with primary antibody for 2 hrs followed by incubation with protein A/G Plus agarose beads (Santa Cruz) for 90 min. Beads were washed 3 times with RIPA and proteins in the immunocomplex were eluted from the beads by the addition of Laemmli loading buffer then heated for 5min at 95° C before loading onto Tris-Glycine gels (Invitrogen).

**Immunoblotting and densitometry.**

Following SDS-PAGE and transfer to nitrocellulose membranes, primary antibody incubations were performed overnight at 4° C. Fluorescent secondary antibodies were obtained from either Molecular Probes or Li-Cor. Membranes were scanned with the Odyssey infrared imaging system (Li-Cor) and the densities of target bands were quantified using the application software of the Odyssey infrared imaging system. Target band density was normalized to the appropriate loading control as described in Figure legends. Primary antibodies used and sources were as follows: anti-GRK2 from Santa Cruz and Millipore, anti-GAPDH from Millipore, anti-VDAC from BD Biosciences, anti-Hsp90 from Santa Cruz, BD Biosciences and Stressgen, anti-cleaved caspase 3 and anti-MAPK (phospho & total) from Cell Signaling, anti-HIS from Santa Cruz, anti-phosphoSer670–GRK2 from Invitrogen, and anti-Flag from Sigma-Aldrich.

**In-vitro Binding Experiments**

We also used a broken cell preparation to study whether mitochondria with increased Hsp90 would bind more GRK2. Hela cells were transfected with either a control plasmid or with flag-tagged Hsp90 and after 48 hrs the mitochondrial fraction was prepared. Equal amounts of mitochondria were incubated with purified his-tagged GRK2 (Invitrogen) in binding buffer composed of PBS with 0.2mM ATP and rotated for 2hr at 4° C. The mitochondria were then pellet by centrifugation and washed with PBS and resuspended directly in Laemmli loading buffer. Western blots were conducted for flag-Hsp90, His-GRK2, and VDAC. In another set of experiments, wild-type and mutant βARKct were transfected into HEK cells and immunoprecipitated from whole cell lysates using flag antibody. The washed beads containing βARKct were incubated with gst-Hsp90 in binding buffer composed of PBS with 1mM DTT and 0.5% Tween20, rotated for 2 hrs at 4° C then the beads were pelleted and washed in the same buffer. The samples were electrophoresed and transferred to membranes for immunoblotting. Western blotting with anti-gst antibody was conducted to determine the amount of Hsp90 bound to βARKct.

**Mitochondrial Ca²⁺ Uptake Assay**

Fluorometric Ca²⁺ uptake experiments were performed as previously described. Briefly, ten to twelve weeks-old male mice were euthanized via CO₂ inhalation. Three mice per group were age-matched and processed together for the isolation of mitochondria. Hearts were excised and placed in a beaker containing Buffer A (225mM Mannitol, 70mM Sucrose, 1mM EGTA, 10mM HEPES, pH 7.2). Hearts were washed to remove residual blood and then the atria was removed and frozen for Western blot to confirm transgene overexpression. Ventricle were minced into pieces, put in a beaker with 5mg Protease Type XIV (Sigma) in Buffer A and stirred for 7 min
and then BSA was added at 0.02g/mL final concentration. The tissue was homogenized with a Teflon-Potter and spun at 1000g for 3 min. Supernatant was filtered through a 100μm mesh and pellet was re-homogenized in a Teflon-Potter in Buffer A, spun at 1000g for 3 min, and filtered through a 100μm mesh. Pellet was discarded and supernatants spun at 10733g for 10 min. Pellets were combined, resuspended in Buffer B (225mM Mannitol, 70mM Sucrose, 10mM HEPES, pH 7.2) and spun at 10733g for 10 min. Pellet was resuspended in Buffer B and protein content determined via BCA protein assay. To begin the assay which was conducted at room temperature, respiration buffer (120mM KCl, 70mM Mannitol, 25mM Sucrose, 20mM HEPES, 5mMKH2PO4, 0.5mM EGTA) containing 0.1μM Calcium Green 5N (Invitrogen) was added to a quartz cuvette and a zero baseline established. Mitochondria were added at 0.73mg/mL and then 30μM of free calcium was added every 2 min. Measurements (excitation-emission @ 503-535nm) were obtained using a Cary Eclipse Fluorescent Spectrophotometer.

**TUNEL Assay**

*Cell culture:*

H9C2 cells (ATCC) were cultured and maintained in DMEM and passaged before reaching 80% confluency per ATCC recommendations. For experiments cells were plated at 50,000 cells/well onto 22mm glass coverslips held in 6-well plates and maintained at 5% CO2 and 37°C. Forty-eight hours after plating the cells were infected with an adenovirus directing the expression of the wild type GRK2 (WT-GRK2) or the mutant GRK2 (S670A). After an additional 48 hr the cells were either left untreated or treated for 16 hr with 75μM H2O2. Cells on coverslips were fixed with 4% paraformaldehyde for 1h and TUNEL staining proceeded according to the manufacturer’s protocol (ROCHE Cat#12156792910 version 11). For each TUNEL experiment cells were also plated on regular culture dishes and harvested for western blotting to confirm equal levels of WT-GRK2 and S670A overexpression.

*Imaging and Quantification:*

Cells were imaged with a Nikon Eclipse Ti inverted microscope. Exposure time was set using the negative control slide and gamma was maintained constant. For quantification purposes images were obtained with a 10X Nikon objective and a random area on each coverslip was chosen and 9 non-overlapping images in a 3x3 square were acquired automatically by the microscope. All images were analyzed using NIS-Elements AR 4.00.03. Cells were counted by an observer who was blinded with respect to adenovirus and treatment. Approximately 7000 total cells at each condition were counted during three independent experiments. Matching specific groups to results was done only after counting was finished. Statistical analysis was carried out using the t-test in GraphPad, (*) p<0.05.

**Proteinase K Assay**

For each experiment three male C57 mice at 12-14 weeks old were anesthetized with isoflurane and the hearts excised and homogenized in MSH buffer to prepare mitochondria. Mitochondrial exposure to proteinase k was carried out with minor modification as previously described. Briefly, 40μg of mitochondrial protein was incubated on ice for 30 min in MSH buffer containing 20μg/mL proteinase k (Life Technologies). Digestion was stopped by adding PMSF to a final concentration of 2mM. The mitochondrial pellet was isolated by centrifugation and solubilized directly in Laemmli loading dye and analyzed for GRK2 by western blot. Control antibody used for the outer membrane was VDAC (Neuramob N152B/23) and COX5 (Abcam 110263) was used to examine integrity of the inner membrane.
**Immunofluorescence**

HEK cells seeded onto glass coverslips were co-transfected with plasmids directing expression of mitochondrial-targeted GFP and GRK2. 48 hr after transfection the cells were fixed in 4% paraformaldehyde for 15 min followed by permeabilization for 5 min with 0.1% triton in DPBS-. Cells were briefly washed with DPBS- several times then blocked 90 min using DPBS-containing 1% BSA, 0.05% Tween 20. Anti-GRK2 antibody (Santa Cruz, sc-562) was added at 1:200 dilution in blocking buffer and applied for 90 min with gentle rocking, which was followed by brief washing and addition of anti-rabbit alexa 594 for 90 min. Cells were washed and then coverslips were mounted on glass slides and images acquired using an Olympus Fluoview FV500 confocal laser scanning microscope.

**Statistics**

Data are expressed as mean ± SEM. Statistical significance was determined by an unpaired t-test or ANOVA and Bonferroni test for multiple comparisons using GraphPad Prism software version 5.9. A p value of <0.05 was considered significant.

**Supplemental References**


**Supplemental Online Figure I:**

GRK2 localization to mitochondria in HEK293 cells. HEK cells were co-transfected with plasmids encoding for expression of GRK2 (red) and mitochondrial-localized GFP (green) then fixed and immunostained using an anti-GRK2 antibody. Shown are representative confocal images with the merged image (yellow) indicating localization of GRK2 in mitochondria.
Proteinase k treatment of mitochondria decreases GRK2 level. 40μg of mouse heart mitochondria were exposed to 20μg/ml proteinase k for 20 min and then subjected to immunoblotting for GRK2, VDAC, and COX5. Two independent experiments were conducted and the western blot is show.
Expression of S670A GRK2 mutant shows decreased H2O2-induced apoptosis. (A) H9C2 cells were infected with adenovirus encoding for GRK2 or GRK2Ser670A and untreated (basal) or exposed to 75μM H2O2 for 16hr. Cells were fixed and stained for apoptotic cells using the TUNEL assay. The nuclear stain DAPI identified total cells and red indicated TUNEL positive cells, (Scale bar, 100μm). (B) Quantification of TUNEL. During 3 independent experiments a total of approximately 7000 cells were counted for each condition. Results are mean ± SE, n=3, *p<0.05 (C) Western blot confirms similar amount of GRK2 overexpression.