2005

Ischemic Preconditioning Protects Adult Rat Cardiomyocytes Against Necrosis but not Apoptosis, via Activation of PKG

Marc J. Caligtan
Virginia Commonwealth University

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ISCHEMIC PRECONDITIONING PROTECTS ADULT RAT CARDIOMYOCYTES
AGAINST NECROSIS BUT NOT APOPTOSIS VIA ACTIVATION OF PKG

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology at Virginia Commonwealth University.

by

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Bachelor of Science, University of Virginia, 2002

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Virginia Commonwealth University
Richmond, Virginia
May, 2005
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>adPKG -1α</td>
<td>adenoviral construct of PKG -1α</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<tr>
<td>CHD</td>
<td>coronary heart disease</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>GTP</td>
<td>guanyl triphosphate</td>
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<tr>
<td>hrs</td>
<td>hours</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<td>IPC</td>
<td>ischemic preconditioning</td>
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<td>KATP</td>
<td>ATP sensitive potassium channel</td>
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<tr>
<td>Kir</td>
<td>inward rectifying potassium channel</td>
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<tr>
<td>LNAME</td>
<td>Nω-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>min</td>
<td>Minutes</td>
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<tr>
<td>nNOS</td>
<td>neural nitric oxide synthase</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>PC</td>
<td>preconditioning</td>
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<td>PKG</td>
<td>cGMP dependent protien kinase</td>
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<td>RO</td>
<td>reoxygenation</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SEM</td>
<td>standard error measurement</td>
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<tr>
<td>SI</td>
<td>simulated ischemia</td>
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<tr>
<td>Sur</td>
<td>sulfonyl urea binding unit</td>
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<tr>
<td>SWOP</td>
<td>second window of protection</td>
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<tr>
<td>5-HD</td>
<td>5-hydroxy decanoate</td>
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Abstract

Ischemic Preconditioning Protects Adult Rat Cardiomyocytes Against Necrosis but not Apoptosis, via Activation of PKG

By Marc Caligtan, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology at Virginia Commonwealth University.

Virginia Commonwealth University, 2005

Major Director: Rakesh C. Kukreja, Ph.D
Professor of Internal Medicine, Physiology and Emergency Medicine

The role of cyclic guanosine monophosphate (cGMP) dependent protein kinase (PKG) in necrotic and apoptotic pathways of many cell types is well established; however its role in the ischemic preconditioning (IPC) of cardiomyocytes is not clearly defined. In the current study, we assessed the hypothesis that PKG protects against cell death following ischemia/reperfusion injury in myocytes subjected to IPC.

Freshly isolated adult rat ventricular myocytes were subjected to IPC by incubating in ischemic buffer for 30 minutes (min) followed by incubation in normal medium for 30 min. Prolonged simulated ischemia (SI) was created by incubating myocytes in the ischemic buffer for 90 min and reoxygenation (RO) for 120 min in the normal medium. Necrosis was determined by trypan blue exclusion and apoptosis was
assessed by TUNEL assay. IPC reduced necrosis as shown by significant decrease in
trypan blue positive cells as compared to virgin non-preconditioned myocytes subjected
to SI and RO alone (p<.01). Similarly, the number of TUNEL positive myocytes
following SI and 18 hrs of RO were significantly reduced in the IPC group. Treatment
with PKG inhibitor, KT5832 (2μM) completely abolished the protection against necrosis
by IPC. However, KT5832 failed to abolish the protective affect of IPC against
apoptosis. Furthermore, myocytes infected with an adenoviral construct of PKG-1α
(1x10^4 particles/cell) significantly reduced the number of trypan blue and TUNEL
positive cells. These results suggest that the PKG signaling pathway plays an essential
role in the preconditioning of myocytes against necrosis following SI / RO injury.
Furthermore, while the overexpression of PKG protects myocytes against necrosis, as
well as apoptosis, IPC may not induce a sufficient level of PKG during 18 hours of RO to
induce protection against apoptosis.
CHAPTER 1

Introduction

According to the American Heart Association in 2002, 38% of all deaths in the United States were caused by Coronary Heart Disease (CHD) and 7.1 million Americans suffered myocardial infarction (MI). It is therefore important to find effective forms of prevention or treatment for such a common disease. While many patients may benefit from conventional pharmaceuticals and life style modifications others need an alternative means of treatment. One possibility that remains is the preconditioning of the myocardium in order to prevent cellular death as a result of MI.

Ischemic preconditioning (IPC) is the phenomenon whereby a sublethal period of ischemia and reperfusion initiate a cascade of events that eventually protects the heart against further ischemic injury. This was first demonstrated by Murry et al., 1986. Their study showed that a series of shorter circumflex occlusions and reperfusions preconditioned the canine heart against a subsequent longer period of ischemia. The preconditioned group demonstrated a 75% smaller infarct size than the control group subjected to the longer period of ischemia alone (22).

The immediate protection conferred by IPC is now commonly referred to as the classic or early form and lasts between 1-4 hrs (8, 23). Other studies have shown that protection returns within 24h after IPC and is referred to as the second window (SWOP).
or the late phase of preconditioning (21). The SWOP is longer but the fortification against cell death is less pronounced than its classical counterpart. Since 1986, research in the field of IPC has focused on its underlying mechanisms which are quite complex and may involve several parallel pathways. Research has also shown that several pharmacological agents such as sidenafil (Viagra), acetylcholine, and bradykinin mimic IPC (26, 28, 48). The mechanism of IPC involves the up regulation of nitric oxide synthase (NOS) which consequently increases the production of NO (Fig. 1). Subsequently, NO activates guanylyl cyclase which increases the conversion of guanylyl triphophate (GTP) into cGMP. Increased levels of cGMP further activate PKG, which may phosphorylate $K_{ATP}$ channels resulting in cardioprotection. The purpose of this study was to specifically identify the role of PKG in the protection against necrosis and apoptosis following IPC and to test whether the overexpression of PKG induces tolerance against SI and reoxygenation (RO).

In the literature, the process of IPC has been divided into three specific components; triggers, mediators, and effectors. The distinction among all three elements is temporal and relative to the final period of ischemia (Fig. 2). The trigger is the initiator of molecular signaling that responds to the first bout of ischemia. Mediators are molecular signaling events that occur during and after the longer bout of ischemia. Lastly, effectors are the signaling events that directly cause protection against cell death.
Figure 1: Proposed mechanism of ischemic and pharmacological preconditioning
Figure 2: Temporal Representation of Triggers, Mediators and End Effectors
Signaling Cascade

Ischemia causes physiologic changes within the heart including the release of adenosine, bradykinin, norepinephrine, and opioids. Attachment of these endogenous molecules to their respective cell surface receptors initiates the IPC response. Other triggering substances include NO and ROS.

Nitric Oxide / Nitric Oxide Synthase

NO is an endogenous molecule that regulates vascular tone, heart rate, and contractility. It is produced by the conversion of L-arginine to citrulline by the enzyme nitric oxide synthase (NOS). There are three types of NOS isozymes that originate in the heart; endothelial NOS (eNOS), inducible NOS (iNOS), and neural NOS (nNOS). Cardiomyocytes express both iNOS and eNOS. The two isozymes differ, in that eNOS is constitutively active whereas iNOS is not. The role of NO in the classical form of IPC is somewhat controversial. Some studies have shown that NO plays a major role in classic PC. Others suggest that endogenous NO does not induce early cardioprotection (CP). In contrast, the role of NO in initiating and mediating the SWOP is more clearly defined. Several studies demonstrated that inhibiting NOS with N-nitro-L-arginine methyl ester (L-NAME) blocked delayed protection against myocardial stunning and infarction. Bolli et al. further purposed that NO played a bifunctional role in IPC. They suggested that eNOS initiated the protective response in early PC and iNOS mediated the SWOP. Subsequent measurements of NOS activity showed that NO production followed a biphasic pattern which confirmed the aforementioned hypothesis. Furthermore, Kukreja et al. showed that the non-toxic
endotoxin monophosphoryl lipid A (MLA), a pharmaceutical trigger of the SWOP, failed to protect in iNOS knockout mice. This study was the first to demonstrate the importance of iNOS in delayed pharmacological preconditioning using iNOS knockout mice (45).

**cGMP Activated Protein Kinase (PKG)**

PKG is a serine/threonine kinase that is activated by cGMP. PKG consists of two types. Type 1 is a soluble homodimer and it is found mostly in smooth muscle, endothelial cells, and platelets of the cardiovascular system. PKG type 1 has two isoforms α and β. The α form is more sensitive to activation by cGMP. Abundant amounts of PKG1α are found in the human aorta, heart, kidneys, and adrenal glands. PKG1β is found in the human uterus (42). Type II is membrane bound and it is predominantly found in the intestine, kidney, and brain.

PKG mediates vascular smooth muscle tone and growth. It also governs endothelial cell mobility and permeability and platelet function (5). PKG is thought to mediate these processes through regulation of cytoplasmic Ca²⁺ levels. It has been demonstrated that homozygous deletion of the PKG 1 gene abolished the NO/cGMP relaxation of smooth muscle. The knockout mice also had vascular and intestinal dysfunction and died at an early age (32). PKG II knockout mice lived normal lifespans but were affected by increased longitudinal bone growth, increased intestinal chloride secretion, and altered renin secretion (31). The PKG pathway has been shown to be integral in preventing apoptosis in neural cells (41). Interestingly, the adenoviral overexpression of PKG was found to have an antiproliferative and pro-apoptotic effect in
vascular smooth muscle (9) however, the effect of apoptosis in cardiomyocytes remains unclear.

PKG also plays an important role in the cardiovascular system. Initially, PKG was found to phosphorylate and subsequently activate the $K_{\text{ATP}}$ channel in isolated rat cardiomyocytes (12, 18). Also, it was shown that NO induced cardioprotection through the opening of mito$K_{\text{ATP}}$ channels was PKG dependent (46). PKG is also known to be integral in both bradykinin and sildenafil mediated cardioprotection (26, 28).

**ATP sensitive Potassium Channels**

The $K_{\text{ATP}}$ channel is a potassium channel that is inhibited by physiological levels of ATP (49). Cardiomyocytes have both sarcolemmal and mitochondrial $K_{\text{ATP}}$ channels (mito$K_{\text{ATP}}$) (50). Both types are composed of an inward rectifying channel ($K_{ir}$) and a sulfonyl urea binding protein ($S_{ur}$). These channels differ however in their response to various pharmacological agents. The mito$K_{\text{ATP}}$ channel is more sensitive to the blocker, 5-hydroxydecanoate (5-HD) and the opener of the channel diazoxide (13). It has been shown that the opening of the mito$K_{\text{ATP}}$ channel is mostly responsible for the IPC response (20, 36).

**$K_{\text{ATP}}$ as end effectors**

In the late nineties some studies suggested that the opening of $K_{\text{ATP}}$ channels was the end effector of IPC. Holmuhamedov et. al., 1999 showed that the influx of potassium into the mitochondrial matrix changed the relative osmotic gradient rendering the
mitochondria more resistant to Ca\(^{2+}\) entry (16). Garlid suggested that the influx of potassium caused the mitochondria to swell which in turn disrupted the translocation of adenosine diphosphate (ADP) into the intermembrane space. The subsequent lack of ADP resulted in the phosphorylation of creatine which in effect created a more efficient means of energy transfer to other organelles (11). Additionally, the opening of K\(_{\text{ATP}}\) channels may have an energy sparing effect. Opening of K\(_{\text{ATP}}\) channels was shown to decrease glycogen depletion and lactate accumulation (17). Other studies suggested that the opening of the mitoK\(_{\text{ATP}}\) channel may function as both trigger and mediator. The research in this area focused on timing of 5-HD infusion. It has been shown that blocking the mitoK\(_{\text{ATP}}\) channel during the initial period of ischemia blocked cardioprotection, however the majority of the studies have found that administration of 5-HD during the second longer phase of ischemia attenuated cardioprotection, suggesting the role of mitoK\(_{\text{ATP}}\) channels as a mediator of cardioprotection (30, 43). It was then shown by several investigators that the opening of the mitoK\(_{\text{ATP}}\) channel resulted in the production of reactive oxygen species (ROS). ROS then serves as second messenger activating signaling molecules including PKC and p38 MAP kinase leading to the activation of the end effector (27). Furthermore, it was found that the PC effect elicited by bradykinin, opioids, and acetylcholine was dependent on the opening of K\(_{\text{ATP}}\) channels and the subsequent generation of ROS (27, 48).

**Necrosis & Apoptosis**

The two most common types of cell death that occur as a result of ischemic injury are necrosis and apoptosis (19). Cell death is most clearly defined as the point at which
the cell becomes unable to recover its normal function and morphology. Necrosis and apoptosis differ greatly in their inherent cause and resulting effect. Necrosis occurs as a result of exogenous injury and results in cell swelling and lysis. Morphologically, it is characterized by the disruption of cellular organelles, accumulation of ribosomes on the nucleus, and irregular aggregation of chromatin within the nucleus. Apoptosis differs from necrosis because it is programmed cell death and it results in cell condensation and cell fragmentation. Apoptosis is characterized by crescent shaped chromatin, apoptotic bodies, and DNA fragmentation.

The intracellular pathway leading to apoptosis involves the releases of cytochrome c from the mitochondria. Cytochrome c activates a caspase cascade which leads to protein cleavage and DNA fragmentation eventually causing cell death. Bax promotes apoptosis by facilitating the release of cytochrome c from the mitochondria. Conversely, Bcl-2 is an anti-apoptotic protein which deactivates BAX, subsequently preventing the release of cytochrome c and activation of the caspase cascade.

Goals of the Study

The goal of the current study was to establish the role of PKG in IPC against both necrosis and apoptosis. The second objective of the study was to determine if the direct overexpression of PKG-1α induced protection against necrosis and apoptosis. The role of PKG in IPC was investigated by using the PKG inhibitor KT5832. Given that PKG is known to participate in several forms of pharmacologically induced cardioprotection (26, 28), we assumed that KT5832 would abrogate the protective effect of IPC. Furthermore,
we investigated the role of PKG in protection against ischemia/reperfusion injury through the adenoviral overexpression of PKG-1α.
CHAPTER 2

Methods & Materials

Isolation of Ventricular Myocytes

Adult Wistar rats were purchased from Harlan (Indianapolis, IN). Ventricular myocytes were isolated using a modified enzymatic technique (34, 44). In short, the rat was anesthetized with pentobarbital sodium (100mg/kg, i.p.) and the heart was quickly removed from the chest. The aortic opening was cannulated onto the Langendorff perfusion system within three min. and the heart was retrogradely perfused (37°C) for approximately 15 min with Ca\(^{2+}\) free bicarbonate based buffer containing (m mol/L): 120 NaCl, 5.4 KCl, 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 5.6 glucose, 20 NaHCO\(_3\), 10 BDM (2,3-butanedione monoxime) and 5 taurine, which was continuously gassed with 95% O\(_2\) + 5%CO\(_2\). The heart was then subjected to enzymatic digestion by adding collagenase type II (Worthington, 0.5mg/ml) and protease type XIV (0.02 mg/ml) to the perfusion buffer for 45 min. The heart was then removed from the Langendorff system and the atrium was separated from the ventricle. The digested ventricular tissue was minced into smaller fragments and gently aspirated with a transfer pipette to facilitate cell dissociation. The cell pellet was resuspended in a three step Ca\(^{2+}\) restoration procedure (i.e. 125, 250, 500 \(\mu\)mol/L Ca+2). The freshly isolated cardiomyocytes were then suspended in Medium 199 containing 2mmol/L L-carnitine, 5mmol/L creatine, 5mmol/L Taurine, 5mmol/L glucose, .1\(\mu\)mol/L insulin, 10% fetal bovine serum (FBS) and 0.1% penicillin-streptomycin (PS).
for 18 hrs. The cells were then plated onto 35mm cell culture dishes, which were pre-coated with 20 μg/ml mouse laminin in PBS + 1% PS for 1 hour. The cardiomyocytes were cultured in the presence of 5% CO₂ for at least 18 hrs in a humidified incubator at 37°C, which allowed myocytes to attach to the dish surface prior to experimental protocol.

**Experimental Protocol**

The detailed experimental protocol is diagrammed in (Fig. 3). The cultured myocytes were incubated under 37°C and 5% CO₂ for one hour with or without the PKG inhibitor, KT 5832 (2μmol/L) by adding the drug directly to the cell medium. In an alternate experimental group, cells were infected with a PKG 1α adenoviral construct 24 hrs prior to SI and RO at a concentration of 1x10⁴ particles per cell (Fig. 4). Ischemic preconditioning was induced by replacing the cell medium with an “ischemic buffer” which contained (in mmol/L): 118 NaCl, 24 NaHCO₃, 1.0 NaH₂PO₄, 2.5 CaCl₂-2H₂O, 1.2 MgCl₂, 20 sodium lactate, 16 KCl, 10 2-deoxyglucose (pH adjusted to 6.2) similar to a previously published method(34). Afterwards, cells were incubated under hypoxic conditions at 37°C by adjusting the tri-gas incubator to 1% O₂ and 5% CO₂ for 30 min. IPC was concluded by replacing the ischemic buffer with normal medium under normoxic conditions for 30 min. Following IPC cells were subjected to longer periods of ischemia for 90 min. and RO which lasted either 120 min (for assessing necrosis) or 18 hours (for detecting apoptosis).
Figure 3: Experimental Protocol showing times of administration of ischemic preconditioning (IPC), PKG blocker KT 5832 and the composition of ischemic buffer.

**Ischemic Buffer (mM/L):**
- 118 NaCl
- 24 NaHCO₃
- 1.0 NaH₂PO₄
- 2.5 CaCl₂·2H₂O
- 1.2 MgCl₂
- 20 sodium lactate
- 16 KCl
- 10 2-deoxyglucose
- (pH adjusted to 6.2)

- Cells were infected by adenoviral construct of PKG1α (1x10^4 particle/cell)

Figure 4: Experimental protocol showing the time of administration of the adenoviral construct of PKG 1α (AD-PKG 1α).
Evaluation of Cell Viability

Cell viability was assessed by trypan blue exclusion assay. At the end of protocol, 40 μL of 0.4% trypan blue (Sigma-Adrich) was added into the culture dish. After ~5min of equilibration, stained cells were counted under the microscope.

TUNEL Staining and Measurement of Mitochondrial Membrane Potential.

Apoptosis was analyzed by terminal deoxynucleotidyl transferase mediated nick labeling (TUNEL) staining, using a kit purchased from BD Biosciences, which detects nuclear fragmentation by a fluorescence assay. In brief, after SI and 18 hrs of RO, the cells were fixed in two chambered slides by 4% formaldehyde/PBS at 4°C for 25 min and subjected to TUNEL assay according to the manufacturer's protocol. The slides were then counter-stained with Vectashield mounting medium with DAPI (4’6-diamidino-2-phenylindole, a DNA inatrcalating dye for visualizing nuclei in fixed cells, catalogue # H-1200, Vector Laboratories).

Western Blots for BAX and Bcl-2

Total soluble protein was extracted from the cells with Reporter Lysis Buffer (Promega). The homogenate was centrifuged at 10,000 g for 5 min under 4°C and the supernatant was recovered. 25 μg of protein from each sample was separated by 12% acylamide gels and transferred to a nitrocellulose membrane, and then blocked with 5% non-fat dry milk in TBST (10 mmol/L Tris-HCL, ph 7.4, 100 mmol NaCl, and .1% Tween 20) for 60 min. The membrane was then incubated with rabbit polyclonal primary antibody at a dilution of 1:1000 for BAX and Bcl-2 (Santa Cruz) for 120 min before
being washed and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000, Amersham) for 1 hr. The blot was developed using a chemiluminescent system. The optical density for each blot band was scanned and quantified using a densitometric system (Bioquant 98).

**Data Analysis and Statistics**

Data were presented as means ± SEM. The differences between control and experimental groups were analyzed with one-way ANOVA followed by Student-Newman-Keuls post hoc tests. P<0.05 was considered statistically significant.
CHAPTER 3

Results

Effect of Ischemic Preconditioning on Cardiomyocyte Necrosis

The method for cell preparations yielded at least 70% of the myocytes with rod shaped morphology which was similar to previously reported studies (34, 44). Figure 5 shows a typical preparation of isolated adult rat cardiomyocytes used in the present study. IPC resulted in the decrease in trypan blue positive cardiomyocytes, i.e. from 22.3±2.40% of total counted cells in the SI group to 11.2±0.83% in the IPC group. Pretreatment with the PKG inhibitor KT5832 abrogated IPC protection and resulted in similar amounts of trypan blue positive cells between IPC versus SI groups, i.e. 25.32±2.73% and 26.65±0.61% respectively. (Fig. 6, 7)

Effect of Ischemic Preconditioning on Cardiomyocytes Apoptosis

As shown in (Fig. 8, 9) apoptotic cell death became evident following 90 min of ischemia and 18 hrs of RO, i.e. 29.91±3.54% of total counted myocytes. The number of TUNEL positive cells were reduced to 16.42±0.76% following IPC. Pretreatment of cells with KT5832 did not block IPC induced protection against apoptosis. In groups
Figure 5: Representative image of freshly isolated rat cardiomyocytes.
Figure 6: Effect of ischemic preconditioning on necrosis. Bar diagram showing quantitative data from four separate experiments.
Figure 7: Representative images of cardiomyocytes stained with trypan blue. A) Myocytes subjected to 90 min of SI and 120 min of RO alone. Cell necrosis is clearly evident by the increased number of trypan blue-positive myocytes. B) Myocytes subjected to IPC prior to SI and RO. Showing less trypan blue-positive myocytes compared with untreated myocytes in A). C) Myocytes pretreated with KT 5832 prior to SI and RO. D) Myocytes pretreated with KT 5832 prior to IPC. Note that there is no significant difference between C) and D).
Figure 8: Effect of ischemic preconditioning on apoptosis. Bar diagram showing quantitative data from four separate experiments.
Figure 9: Representative images of cardiomyoctes subjected to TUNEL assay. A) Myocytes subjected to 90 min of SI and 120 min of RO alone. Apoptosis is clearly evident by the increased number of TUNEL positive nuclei indicated by the presence of the fluorescent green stain. B) Myocytes subjected to IPC prior to SI and RO. Note that there are less TUNEL positive myocytes compared to SI alone. C) Myocytes pretreated with KT 5832 prior to SI and RO. D) Myocytes pretreated with KT5832 prior to IPC. Note that there are significantly less TUNEL positive cells in both groups subjected to IPC compared to cells subjected to SI and RO alone.
pretreated with KT 5832, IPC resulted in the decrease of TUNEL positive cardiomyocytes, i.e. from 26.93±2.03% in the SI group to 11.22±1.33% in the IPC group.

We also examined the effect of IPC on the expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax in cardiomyocytes. As shown in (Fig. 10A) IPC enhanced the expression of Bcl-2. The induction of Bcl-2 occurred after 90 min of SI and 120 of RO. The Bcl-2 / Bax ratio was partially attenuated by the pretreatment of cells with KT 5832 (Fig. 10B).

**Effect of PKG overexpression on Cardiomyocyte Necrosis & Apoptosis**

To further evaluate the role of PKG in cardioprotection, we used the adenoviral construct of PKG-1α to overexpress the protein in the cardiomyocytes: As shown in (Fig. 11A), adenoviral construct PKG-1α increased the levels of the PKG-1α as compared to control. The overexpression of PKG-1α resulted in the decrease of trypan blue positive cardiomyocytes, i.e. from 36.07±3.12% of total counted myocytes in the untreated control group to 20.67±1.00% in cells infected with the PKG-1α construct (Fig. 11 B,C). Similarly, the overexpression of PKG-1α also reduced the number of TUNEL positive myocytes from 15.30±0.70% in the untreated control to 6.30±1.10% in the group infected with the adenovirus (Fig. 12).
Figure 10: Effect of IPC on the expression of Bcl-2 & Bax. A) Representative gel showing Bcl-2, Bax, and β-actin expression following IPC and KT 5832 administration. B) Bar diagram showing the quantitative change in Bcl-2/Bax ratio.
Figure 11: Overexpression of PKG 1α protects myocytes against necrosis. A) A representative gel showing PKG 1α in myocytes infected with adenovirus. B) Bar diagrams showing cell viability determined by trypan blue staining. C) Representative picture of trypan blue staining. Note that the blue stained cells represent myocytes affected by necrosis.
Figure 12: Effect of the overexpression of PKG 1α on apoptosis. A) Bar diagram shown quantitative data that assessed cell viability through TUNEL staining. B,C) non infected control C,D) Adenoviral PKG 1α infected cells. B,D) TUNEL positive myocyte nuclei (stained in green fluorescent color). C,E) Total nuclei (4,6-diamidino-2-phenylindole staining). A significant number of myocytes underwent apoptosis whereas infection with adenovirus reduced TUNEL positive nuclei.
Chapter 4
Discussion

Brief periods of ischemia have been found to elicit physiological changes in the heart that protect the myocardium against longer subsequent periods of ischemia. The complete cellular pathway that confers this type of protection has been the subject of many studies, but the specific sequence of events and the end effector is still unknown. Insights into the pathway that lead to CP have profound clinical implications. Mainly IPC is of interest because it may lead to new pharmacological treatments for heart disease. The current investigation is relevant in that it clarifies the role of PKG within the IPC cascade.

It has been shown through patch clamp studies on isolated myocytes that the NO-cGMP-PKG pathway contributes to the phosphorylation and activation of $K_{ATP}$ channels (14). The opening of the mito$K_{ATP}$ channels has been shown to trigger CP through the generation of ROS (25, 30). Other studies have shown that pharmacological agents such as sildenafil and bradykinin may also induce CP through the NO-cGMP-PKG-mito$K_{ATP}$ pathway.
Kukreja and colleagues have shown that Sildenafil induced a powerful cardioprotective effect in vivo (26). Sildenafil preconditioned mice cardiac myocytes against apoptosis and necrosis through a NO dependent signaling pathway (10). These results suggested that the increased concentration of cGMP is integral in the PC cascade. PKG is known to be a cellular target of cGMP in the putative PC cascade. Sildenafil inhibits the degradation of cGMP, subsequently increasing its concentration allowing for a more robust activation of PKG.

The primary objective of this study was to determine the role of PKG in IPC induced protection against both necrosis and apoptosis in the isolated cardiomyocyte model. A second goal was to show that the direct overexpression of PKG-1α induced protection against necrosis and apoptosis in cardiomyocytes. Our experimental model examined the effect of IPC on the isolated myocyte which allowed for conjecture regarding the resulting endogenous processes which is untainted by other cell types, their products, or any other hemodynamic factors. Evaluation of these results, however should take into account the possibility that the isolation procedure, which induces physical and environmental stress, may itself serve as a PC stimulus (33).

**IPC Induced Protection against Necrosis**

Trypan blue exclusion assay measured the osmotic fragility of individual cells. Necrosis involves the swelling and lysis of cells allowing for trypan blue uptake. Similar to other studies, when subjecting the cells to IPC prior to a longer period of ischemia, cell viability was significantly increased (39). Specifically, IPC decreased the number of
trypan blue positive cells by 51% relative to the untreated SI group. The results confirm the ability of myocytes to endogenously protect themselves through IPC.

Previous studies have shown that various PKG modulators influence ROS generation (29, 46). Oldenberg and colleagues demonstrated that the NO-cGMP-PKG-mitoKATP pathway triggered by bradykinin lead to CP, similarly Xu and colleagues have shown this pathway to be triggered by an exogenous supply of NO supplied by S-nitroso-N-acetylpenicillamine (SNAP). These experiments suggested that PKG is involved in the production of ROS which were also associated with cardioprotection. The experiments however only provided circumstantial evidence that PKG is responsible for cardioprotection. In the present study, we established a direct link between IPC, PKG, and CP against necrosis. We have shown that pretreatment with the PKG inhibitor KT5832 abrogated IPC’s protective effect against necrosis suggesting an essential role of PKG in CP.

**IPC induced protection against Apoptosis**

In addition to necrosis, apoptosis is known to contribute to cell death in ischemic injury (2,15). Apoptosis is programmed cell death that results in a variety of morphological and functional changes. A distinguishing feature of apoptosis is the fragmentation of nuclear DNA. The TUNEL assay attaches fluorescein-dUTP to fragmented DNA pieces allowing for the quantitative analysis of cells affected by apoptosis.
There are conflicting reports as to PKG's role in apoptosis. The PKG pathway has been shown to play an essential role in preventing apoptosis in neural cells (41). A previous report by Shimojo et al. revealed that the NO-cGMP pathway induced apoptosis in neonatal cardiomyocytes (37). A subsequent study by Akao and colleagues asserted that the opening of mitoKATP channels inhibited apoptosis induced by oxidative stress (1). These results appear to be incongruent given that the NO-cGMP-PKG pathway triggers the opening of mitoKATP channels. The contrasting results may be explained by the dose of SNAP (0.1mM) used by Shimojo and colleagues. The cardioprotective effect against necrosis was shown to be elicited by 20 μM dose of SNAP (46), which suggests that the NO signaling pathway can have both deleterious and protective effects. However, the therapeutic capacity is highly dependent on the dose of NO and cell type (35).

The current study has shown that IPC significantly decreased the number of TUNEL positive cells as compared to cells subjected to SI alone which indicates that myocytes posses the endogenous ability to protect themselves against apoptosis. IPC's protective effect against apoptosis was not diminished by KT5832 suggesting that PKG was not solely responsible for IPC induced protection against apoptosis. The anti-apoptotic protein, Bcl-2 was up regulated during IPC while the pro apoptotic Bax protein level was not significantly changed. Administration of KT 5832 diminished the IPC induced increase of Bcl-2. PKG was involved in the IPC induced alteration of the Bax /Bcl-2 ratio which favored anti-apoptosis. Although protein analysis seems to implicate PKG in IPC mediated protection against apoptosis, the induced change of the Bax / Bcl-2 ratio may not have been strong enough to protect the cells against apoptosis eighteen
hours post ischemia. This hypothesis may explain the inability of KT 5832 to abrogate IPC induced protection against apoptosis. In contrast, the overexpression of PKG-1α by adenoviral gene transfer reduced necrosis as well as apoptosis as demonstrated by significantly less trypan blue and TUNEL positive stained cells.

Conclusion

Both necrosis and apoptosis proceed through vastly different cellular signaling pathways, therefore it might be logical to conclude that the pathways governing IPC against each form of cell death may also follow vastly different pathways. In the present study, we have shown that PKG was involved in IPC against necrosis but not apoptosis. The overexpression of PKG protects myocytes against both necrosis and apoptosis. Our results further suggest that PKG can protect against apoptosis by altering the Bcl-2/Bax ratio. Further studies are needed to understand the downstream signaling pathway by which PKG exerts protective effects against necrosis and apoptosis in the myocytes.
List of References


Vita

Marc Jason Caligian was born on October 6, 1979 in Washington, D.C. Marc graduated from Gonzaga College High School in 1997. He received his Bachelors of Science degree in Kinesiology with a concentration in Sports Medicine from the University of Virginia in May of 2002. He will attain his Masters of Science in Physiology from the Medical College of Virginia in August of 2005.