Chronic preconditioning: a novel approach for cardiac protection

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Wang Y, Ahmad N, Wang B, Ashraf M. Chronic preconditioning: a novel approach for cardiac protection. Am J Physiol Heart Circ Physiol 292: H2300–H2305, 2007. First published January 5, 2007; doi:10.1152/ajpheart.01163.2006.—Ischemic preconditioning is the most powerful protective mechanism known against lethal ischemia. Unfortunately, the protection lasts for only a few hours. Here we tested the hypothesis that the heart can be kept in a preconditioned state for constant protection against ischemia. In this study we chose BMS-191095 (BMS), a highly selective opener of mitochondrial ATP-sensitive K⁺ (mitoK<sub>ATP</sub>) channels. BMS (1 mg/kg ip) was administered to rats every 24 h until 96 h. In other groups, BMS plus wortmannin (WTN, 15 µg/kg ip), an inhibitor of the phosphatidylinositol 3-kinase (PI3-K), or BMS plus 5-hydroxydecanoic acid (5-HD, 5 mg/kg ip), an inhibitor of mitoK<sub>ATP</sub>, or BMS plus N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) (30 µg/kg ip), an inhibitor of nitric oxide (NO) syntheyse, were administered to rats. Rats were then subjected to 30-min left anterior descending coronary artery occlusion and 120-min reperfusion. Cardiac function, infarct size, pathological changes, and apoptosis were assessed at the end of treatments. Saline-treated hearts displayed marked contractile dysfunction and underwent pathological changes. BMS-treated rats showed significant improvement in cardiac function, and infarct size was significantly reduced in BMS-treated hearts. However, protection by BMS was abolished by 5-HD, WTN, or l-NAME. These data demonstrate that hearts can be chronically preconditioned and retain their ability to remain resistant against lethal ischemia and that this protection is mediated by activation of mitoK<sub>ATP</sub> via NO and PI3-K/Akt signaling pathways.

mitochondrial ATP-sensitive K⁺ channels; BMS-191095; heart function

CARDIAC ISCHEMIA-REPERFUSION contributes to heart injury, morbidity, and mortality in a variety of cardiac diseases. Ischemic and pharmacological preconditioning (PC) provides protection against ischemic injury. Ischemic PC (IPC) refers to a phenomenon in which a brief period of ischemia protects the heart against a subsequent longer period of lethal ischemia and protects myocardium against reversible and irreversible injury (3, 6, 16). This protection has been found to have a biphasic window: an early phase, which persists for 1–3 h after IPC, and a delayed phase reappearing after 24 h (late phase) (5, 25). Although acute IPC has been extensively studied, little is known about the extended cardioprotective effect of PC. Various pharmacological agents have been used to mimic the effect of PC. Pharmacological openers of mitochondrial ATP-sensitive K⁺ (mitoK<sub>ATP</sub>) channels are cardioprotective in numerous experimental models of ischemia-reperfusion (7). mitoK<sub>ATP</sub> channel has been previously proposed as an end-effector of IPC (32), in addition to being also an excellent trigger for both acute and late PC (25, 29). We and others reported that the specific mitoK<sub>ATP</sub> channel openers diazoxide and BMS-191095 (BMS) increased ischemic tolerance in rat hearts (7, 8, 32). BMS is a selective mitoK<sub>ATP</sub> channel opener and does not possess peripheral vasodilator or cardiac action potential shortening activity. To make the PC phenomenon clinically more attractive, efforts have been made to keep the heart in a preconditioned state for a longer period of time with opioids that are known to precondition the heart (18, 19). Thus prolonged and extended protection by PC pathways is here referred to as chronic PC. Chronic PC is a relatively new approach in which PC mimetics are administered intermittently to maintain cardioprotection for a longer period of time. Since mitoK<sub>ATP</sub> channel is an effective end effector of IPC, it is very likely that its intermittent activation by specific openers can keep the heart tolerant against ischemia. Therefore, we hypothesize that chronic PC by intermittent administration of the powerful mitoK<sub>ATP</sub> channel opener BMS provides constant and prolonged protection against lethal ischemia.

MATERIALS AND METHODS

All procedures on animals were approved by the University of Cincinnati Animal Care and Use Committee and were carried out in accordance with guidelines of National Institutes of Health.

Chemicals

BMS was a gift from Bristol-Myers Squibb. Diazoxide, wortmannin (WTN), and 5-hydroxydecanoic acid (5-HD) were purchased from Sigma Chemical. WTN was dissolved in DMSO before being added into the perfusion buffer. The final concentration of DMSO was <0.1%. Other reagents were dissolved in PBS. DMSO alone in this concentration had no effects on hemodynamics (31).

Surgical Preparation for Coronary Artery Ligation

The left anterior descending coronary artery (LAD) was ligated to produce infarction, as previously described (28). In brief, rats were anesthetized with pentobarbital sodium (40 mg/kg ip). A midline cervical skin incision was performed for intubation. The animals were mechanically ventilated with a Harvard model 683 respirator (Harvard Apparatus, South Natick, MA). The heart was exposed by left side thoracotomy, and LAD was dissected free above the first diagonal branch and ligated for 30 min, followed by 120 min of reperfusion. Body temperature was carefully monitored with a rectal probe (Cole-Parmer Instrument, Vernon Hill, IL) and maintained at 37°C throughout the surgical procedure.

Experimental Protocol

Eight-wk-old Fischer 344 adult male rats were used in these experiments. Rats were randomly divided into eight groups and underwent LAD occlusion for 30 min and 2 h of reperfusion, as shown in Fig. 1. In group 1, saline was administered (0.1 ml ip) at 0 h, 24 h, 48 h, 72 h, and 96 h, and hearts were then subjected to LAD occlusion...
and reperfusion. In group 2, animals received BMS (1 mg/kg ip) at 0 h and 24 h, followed by LAD occlusion and reperfusion. Group 3 was given BMS at 0 h, 24 h, and 48 h. Group 4 was given BMS at 0 h, 24 h, 48 h, and 72 h. Group 6 was similar to group 5, except that 5-HD (5 mg/kg ip), a specific blocker of mitoKATP channel, was given with BMS at each interval. Group 7 was also similar to group 5, except that WTN (15 μg/kg ip), an inhibitor of Akt, was given together with BMS at different intervals. In group 8, Nω-nitro-l-arginine methyl ester (l-NAME; 30 μg/kg ip), a general inhibitor of nitric oxide (NO) synthase (NOS), was given together with BMS. The proper doses for BMS, WTN, and L-NAME have been published previously (1, 27). Effect of WTN or L-NAME alone on infarction was also determined (1, 27).

**Assessment of Heart Function**

After anesthesia, the right carotid artery was cannulated with a microtip pressure transducer catheter (SPR-869, Millar Instruments) connected to a pressure-volume conductance unit (MPCU-200). Pressure-volume analysis provided analog outputs of the time-varying ventricular pressure and volume signals for data acquisition. The inferior vena cava was exposed, and inferior vena cava occlusion was performed by external compression, as described previously by Wang et al. (30). Hemodynamic parameter analysis was carried out using Millar’s PVAN software (version 3.2).

**Determination of Cell and Tissue Injury**

**Measurement of myocardial infarction.** After left thoracotomy, animals were injected with 3 M potassium chloride directly into the left ventricle, and the heart was excised. The left ventricle was sliced parallel to the atrioventricular groove into 2- to 3-mm-thick sections, and the sections were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution prepared in phosphate buffer, pH 7.4, for 30 min at 37°C (25). In viable normal myocardium, TTC is converted by dehydrogenase enzyme to a red formazan pigment that stains tissue
dark red. The infarcted myocardium that does not take up TTC stain due to loss of dehydrogenase enzyme remains pale in color. Slices were fixed in the 10% formalin and were digitally imaged by using an Olympus microscope, and infarct size was determined by using MetaMorph software (Molecular Devices, Downington, PA).

Terminal dUTP nick-end labeling assay for apoptosis. Terminal dUTP nick-end labeling (TUNEL) assay was performed on 6-μm-thick deparaffinized histological sections with a MEBSTAIN Apoptosis Kit II (Medical and Biological Laboratories). Sections were stained with 4-6-diamidino-2-phenylindole to visualize nuclei and later were photographed with an Olympus BX41 microscope (Olympus America, Melville, NY) equipped with a digital camera. Each apoptotic nucleus was carefully identified at high magnification (×1,000) and counted.

Statistical Analysis
Pressure-volume relationship was analyzed by one-way analysis of variance (ANOVA, StatView 4.0) for repeated measures. Individual variables, such as apoptotic myocytes, were tested by one-way ANOVA, followed by Bonferroni/Dunn test or unpaired t-test. P value <0.05 was considered significant. All data are presented as means ± SE.

RESULTS
Effect of PC on Infarct Size
The infarct size was found to be 48.7 ± 4.8% in ischemic control animals (Fig. 2, A and B) and was significantly decreased with the treatment of BMS to 30.5 ± 3.2%, 26.4 ± 2.8%, 33.8 ± 2.5%, and 21.7 ± 2.4% [in groups 2, 3, 4, and 5, respectively, as compared with control group (P < 0.05)]. Interestingly, hearts given BMS five times (group 5) at intermittent intervals were more tolerant to ischemia-reperfusion injury, resulting in marked reduction in infarct size compared with groups 2, 3, and 4 (P < 0.05) (Fig. 2B). However, administration of 5-HD, WTN, or l-NAME with BMS abolished the protection, and infarct was similar to control ischemic hearts (Fig. 2, A and B). Pretreatment of the hearts with these inhibitors did not cause any additional infarction (data not given).

Hemodynamic Effects of PC
Hemodynamic data are shown in Fig. 3. At the end of reperfusion, heart function was significantly decreased in the ischemic control group, as indicated by first derivative of left ventricular (LV) pressure increase (+dP/dt) (6,184.6 ± 326.4 mmHg), first derivative of LV pressure decrease (−dP/dt) (5,742.7 ± 371.5 mmHg), and LV ejection fraction (EF) (18.6 ± 4.4%), when compared with the BMS-treated group. A significant increase in LV +dP/dt, LV −dP/dt, and LV EF was observed in BMS treatment groups as compared with the control group. In the 96-h BMS treatment group, the cardiac function was better preserved than in other BMS-pretreated groups. However, the chronic protective effects of BMS were abolished by 5-HD, WTN, or l-NAME when given together with BMS (Fig. 3).

Antiapoptotic Effect of PC on Ischemia-Reperfusion Injury
Apoptosis in BMS pretreatment groups at 24, 48, 72, or 96 h was significantly reduced compared with ischemia-reperfusion control group (Fig. 4). Among BMS-pretreated groups, the 96-h treatment group showed the lowest number of TUNEL-positive nuclei as compared with other BMS-treated groups (P < 0.05). However, the protective effect of BMS on apoptosis was abolished with 5-HD, WTN, or l-NAME given together with BMS.

DISCUSSION
IPC has a powerful cardioprotective effect against ischemia, which remains unmatched by other therapeutic agents (3).
Although the cardiac protection by acute PC (18) is short lived, its diverse signaling pathways have allowed us to develop several PC mimetics to combat ischemic disease. These pharmacological mimetics of IPC could be further tested for their prolonged effects. A more viable and attractive strategy in cardioprotection is to put the heart in a constant preconditioned state so that it can resist any sudden lethal ischemic episode. A recent report supports the concept that hearts given opioids for 5 days before isolation and perfusion displayed significant improvement in postischemic contractile function (19). The results of our study are in full agreement with the conclusions of Peart and Gross (19) that the heart can be maintained in a protective state for a longer time duration with preconditioning agents.

MitoK\textsubscript{ATP} channel plays a pivotal role in PC-induced protection. The importance of this channel is quite evident owing to the fact that it is localized in mitochondria, a lifeline cell organelle that occupies one-third of the cell volume. This channel is also believed to be the end effector of protection by PC (32). This study took advantage of the fact that pharmacological openers of this channel produce both immediate and late protection. BMS is a specific opener of mitoK\textsubscript{ATP}, does not effect vasodilator or action potential shortening, and protects effectively against ischemic injury in the heart (8) and brain (13). We administered BMS intermittently at an interval of 24 h until 96 h. The heart was constantly preconditioned against the lethal ischemia. The cardiac function was well preserved at all time in these hearts. It could be argued that the prolonged protective effect of BMS is due to its accumulation within mitochondrial membranes for a longer duration, thus inducing PC effects. The half-life of BMS in rats is 3 h (21), and loss of effect after 12 h (in our unpublished data) strongly supports this view. BMS treatment for 5 days significantly decreased TUNEL-positive nuclei in rats (Fig. 4).

Fig. 4. Representative photomicrographs of terminal dUTP nick-end labeling (TUNEL)-positive nuclei in various treatment groups. A: normal control; 4-6-diamidino-2-phenylindole (DAPI)-stained nuclei (blue; arrow). B: TUNEL-positive nuclei (green; arrow) in ischemia and reperfusion control group; sections stained with α-sarcomeric actin, DAPI, and TUNEL. C: same area as B, except all nuclei were stained by DAPI. D: same section as B, except green fluorescence shows TUNEL-positive nuclei (arrow). E: intermittent PC after 96 h (G5). TUNEL-positive nuclei were significantly decreased in BMS-treated hearts as compared with ischemic control group. Rats treated with BMS at different intervals were also significantly protected from apoptosis (data not shown). F: occurrence of apoptosis after treatment with BMS + 5-HD. Treatment with WTN or l-NAME produced results similar to ischemic control (data not shown). Magnification, ×400; n = 4 for each group; bars, 20 μm. G: semiquantitative estimate of TUNEL-positive nuclei in various groups. Values are means ± SE. *P < 0.05 vs. ischemia-reperfusion control. †P < 0.05 vs. G2–G4.
suggests that BMS is not trapped within the mitochondrial membranes.

Apoptosis was significantly reduced in BMS-pretreated hearts for 96 h as compared with the 24-h treated group, suggesting that the effect of chronic PC is more robust than that of acute PC. A specific inhibitor of mitoK<sub>ATP</sub> channels, 5-HD, completely abrogated the effects of BMS-induced cardiac protection. These observation clearly depict that mitoK<sub>ATP</sub> channel plays an important role in chronic PC.

Recent reports suggest that the heart can be put in preconditioned state with intermittent administration of opioids even after their withdrawal (18, 19). In the latter report (19), chronic morphine exposure gave stronger and sustained protection for 48 h after withdrawal of the drug. These observation are consistent with our results in which BMS was given intermittently at an interval of 24 h up to 96 h, resulting in significant protection as compared with one-time BMS-pretreated group (Figs. 2–4).

Signaling pathway for this unique phenomenon of constant protection is not completely known. The blockade of protection by mitoK<sub>ATP</sub> channel antagonists and inhibitors of NOS and Akt suggests the involvement of mitoK<sub>ATP</sub> channel, NO, and Akt signaling in this protection. However, it is not clear from this study whether mitoK<sub>ATP</sub> channel remains open constantly or can be activated upon oxidative stimulus. It appears that the latter option is more likely because inducible NOS (iNOS) and Akt are known to be upregulated during the later phase of PC. The release of NO and Akt translocation to mitochondria could be important factors in the channel-mediated protection (1, 10, 20).

Infarct size is a standard marker of cell injury following permanent ischemia-reperfusion (9). A majority of cells die in the infarcted myocardium due to increased sarcolemmal permeability, ATP depletion, and loss of Ca<sup>2+</sup> homeostasis (33), whereas many cells at the infarct border zone undergo apoptosis. PC effectively reduces both necrosis and apoptosis within the infarcted heart. The cardioprotective effects of Akt have been mainly attributed to the reduction of myocardial apoptosis and have a pivotal role in vascular homeostasis and angiogenesis (14, 24). The antiapoptotic activity of Akt is mediated through the activation of phosphatidylinositol 3-kinase (PI3-K) (6). There is strong support to the hypothesis that BMS activates PI3-K/Akt pathway during late PC (1). Previous studies have shown mitochondria as the downstream target of Akt activation, which causes phosphorylation of various proteins in the mitochondria (2). The eventual outcome is a reduced release of both apoptosis-inducing factor and cytochrome c, thus alleviating cell apoptosis (34).

The participation of PI3-K/Akt in opening of mitoK<sub>ATP</sub> channels is highly attractive. Activation of PI3-K/Akt has multiple cardiovascular functions, including cell migration and growth, homeostasis, angiogenesis (24), and protection against ischemia-reperfusion injury (12). Akt is activated downstream of PI3-K during IPC and, in turn, phosphorylates a number of downstream targets relevant to cell survival, including the proapoptotic Bcl-2 family member Bad, NOS, and nuclear factor-κB (24, 26). Phosphorylation of Bad by Akt inhibits its proapoptotic function and promotes cell survival (11). Akt, which is phosphorylated by BMS in the cytosol, is translocated to mitochondria (1). This is in agreement with the work of Sale et al. (22), who reported that Akt phosphorylation in mitochondria causes activation of various proteins, such as ATP synthase and GSX-3β within the mitochondria, which are eventually cardioprotective as they aid in the reduction of apoptosis. Thus we postulate that activation of the PI3-K signaling pathway upstream of mitoK<sub>ATP</sub> channel results in decreased cardiac cell death and apoptosis.

NO regulates many physiological and pathological processes controlling the cardiovascular system (15). Modulation of mitoK<sub>ATP</sub> channels occurs via enhanced levels of cGMP. The cGMP-dependent protein kinase in turn may phosphorylate mitoK<sub>ATP</sub> channels and prime the channels to provide cardiac protection (4). In addition, there is a possibility for the involvement of NO in cardiac protection mediated by pharmacological PC with BMS. NO being upstream of PKC and the mitoK<sub>ATP</sub> channels acts as a free radical, combining with superoxide to generate peroxynitrite, that could activate PKC, leading to opening of the mitoK<sub>ATP</sub> channels (35). NO produced from cytosolic and mitochondrial NOS plays an important role as a trigger of both early and delayed PC (17) and is known to potentiate mitoK<sub>ATP</sub> opening (3). Furthermore, L-NAME, the nonselective inhibitor of NOS, blocked the protective effect of BMS. The results of this study are consistent with other reports that NO enhances mitoK<sub>ATP</sub> channel opening (23), and they are further supported by the observations that diazoxide did not provide any protection in iNOS knockout mice, thus suggesting that opening of mitoK<sub>ATP</sub> channels was iNOS dependent (27). All these studies support our view that NO is an important trigger of mitoK<sub>ATP</sub> channel activation during chronic PC.

Conclusion

Chronic or intermittent PC is a clinically relevant approach by which the heart could be protected against ischemia for a longer period via activation of mitoK<sub>ATP</sub> channel, Akt translocation, and NO signaling pathways.

GRANTS

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