Cardiac protection by mitoK$_{\text{ATP}}$ channels is dependent on Akt translocation from cytosol to mitochondria during late preconditioning

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Apoptosis and necrosis are generally responsible for myocyte death during ischemia and reperfusion. Necrosis is a rapidly occurring event of cell death that triggers a significant inflammatory response. Typically, cellular changes during necrosis include severe cellular and organelle swelling, denaturation and coagulation of cytoplasmic proteins, and breakdown of cell organelles. Also, there is a depletion of ATP that may be due to a lack of oxygen, a loss of calcium homeostasis, and defects in membrane permeability, which eventually leads to cell death (6). Apoptosis is a genetically controlled programmed cell death and is also responsible for cell injury in the ischemic myocardium. Simultaneous occurrence of both apoptosis and necrosis in myocardium determines the lethality of myocardial injury after ischemia and reperfusion (2, 14, 19). The cardioprotective effects of protein kinase B (Akt) have been mainly attributed to the reduction of myocardial apoptosis and have a pivotal role in vascular homeostasis and angiogenesis (21). The ant apoptotic activity of Akt is mediated through the activation of the phosphatidylinositol 3-kinase (PI3K) system (10). There is a strong support to the hypothesis that mitochondrial ATP-sensitive K$^+$ (mitoK$_{\text{ATP}}$) channel openers like BMS-191095 (BMS) and diazoxide (DE) activate PI3K/Akt pathway during late preconditioning. Bijur and Jope (3) have proposed mitochondria as the downstream target of Akt activation, which causes phosphorylation of various proteins in the mitochondria. The eventual outcome is a reduced release of apoptosis-inducing factor (AIF) and a reduced release of cytochrome c, thus causing less apoptosis. We postulate that the underlying mechanism of mitoK$_{\text{ATP}}$ channel activation in myocyte protection involves translocation of phosphorylated Akt from cytosol to mitochondria. To our knowledge, the present study is the first to elucidate the role of mitochondrial phosphorylated Akt in mitoK$_{\text{ATP}}$ channel-mediated protection.

MATERIALS AND METHODS

Adult male mice (C57 Black/6J) were obtained from Harlan Laboratory. The University of Cincinnati Animal Care and Use Committee approved the use of mice in these experiments. Chemicals. BMS was a gift from Bristol-Myers Squibb. DE, wortmannin (WTN), and 5-hydroxydecanoic acid (5-HD) were purchased from Sigma Chemical. The kit for Western blot analysis of phosphorylated and total Akt was purchased from Cell Signaling Technology. DE and WTN were 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 has no effects on hemodynamics (25).

Heart preparation for Langendorff perfusion. Mice weighing 25–30 g were anesthetized with pentobarbital sodium (40 mg/kg ip) and heparinized (5,000 U/kg) to protect the heart against microthrombi. The chest was opened at the sternum, and the heart was quickly removed and cannulated with a 20-gauge phalanged stainless steel cannula. The heart was perfused on a noncirculating Langendorff apparatus with Krebs-Henseleit (KH) buffer, pH 7.4, containing (in mmol/l) 118 NaCl, 4.7 KCl, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 2.5 CaCl$_2$, 25 NaHCO$_3$, and 11 glucose (1, 26). The buffer was saturated with 95% O$_2$–5% CO$_2$ at 37°C for 25 min. Heart was perfused at a constant pressure of 80 mmHg. A homemade water-filled balloon was inserted into the left ventricle through the left atrium and was adjusted to a left ventricular end-diastolic pressure, thereby equilibrating LVEDP of 5–8 mmHg during initial equilibration. Thereafter, the balloon volume was not changed. However, it is also possible that with constant balloon volume, hearts may suffer some phenomenon of no reflow. The distal end of the catheter was connected to a Digi-Med heart performance analyzer.

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(model 210, version 1.01, Micro-Med) by way of a pressure transducer (Case, Lakewood, CO). Heart was paced at 350 beats/min, except during ischemia. Pacing was reinitiated after 3 min of reperfusion for 120 min in all groups. At the end of the equilibration periods, hearts exhibiting systolic pressure <75 mmHg were discarded from the study. The index of myocardial function was determined as previously described (25).

**Experimental protocol.** The experimental protocol is summarized in Fig. 1. A total of 216 male adult mice were used in this study. Four hearts from each group were used to examine phosphorylation of cytosolic Akt by Western blot analysis. Twenty-four animals from hearts from each group were used to measure mitochondrial Akt. Animals were randomized into six groups as follows (in each group, eight animals were used to obtain hemodynamic data): 1) ischemic control, with no pharmacological treatment except 0.9% normal saline; 2) BMS-treated group; 3) DE-treated group; 4) BMS + WTN-treated group; 5) BMS + 5-HD-treated group; and 6) WTN-treated group. WTN (15 μg/kg) was given 15 min before preconditioning with or without BMS and 15 min before the heart was isolated (half-life of WTN is 2 h). All animals were given drugs intraperitoneally for 20 h before their hearts were subjected to ischemia-reperfusion. After equilibration of 25 min, ischemia was induced by shutting off perfusion buffer for 40 min, followed by 120 min of reperfusion.

**Measurement of lactate dehydrogenase.** Lactate dehydrogenase (LDH), an indicator of myocardial tissue injury, was determined in the coronary effluent by a coupled enzyme-spectrometric technique (DU Series 500 spectrophotometer, Beckman Instruments), using LDH assay kits (MBL) (25). LDH was measured at 3, 5, 10, 20, and 30 min of reperfusion. The accumulated amount was obtained by integrating the area underneath the individual time course curve for 30 min of reperfusion (26) (Fig. 2D).

**Protein extraction for cytosolic Akt Western blot analysis.** All pretreated hearts were taken out in the morning to minimize the effects of insulin. Later, they were weighed and immediately immersed in liquid nitrogen and then stored at −70°C until use. Each heart tissue sample was homogenized for six bursts of 15 s each at 4°C with a Polytron PT homogenizer (Germany) with the use of lysis buffer containing (in mM) 0.1 NaCl, 10 Tris (pH = 7.6), 1 EDTA, 2 Na pyrophosphate, 2 NaF, 2 β-glycerophosphate, 0.5 AEBSF, and a cocktail protease inhibitor tablet as described by Kis and colleagues (15a). After sonication for 5 s, tissue lysate was centrifuged at 14,000 rpm for 5 min at 4°C. Sonication and centrifugation were repeated again, as previously described, before the supernatant was stored at −70°C for further analysis. Protein content was determined with bovine serum albumin protein assay reagent kit (Pierce).

**Isolation of mitochondria.** All steps in the isolation of mitochondria were performed at 4°C in a cold room. After anesthesia was administered, the chest was opened and the heart was quickly removed and perfused on Langendorff apparatus with KH buffer to wash out the blood. Hearts were then transferred into solution containing 180 mM KCl, 10 mM EGTA, and 0.5% bovine serum albumin (KEA). Hearts were dissected to remove atria, large vessels, and fat. Only ventricles were weighed and processed. Ventricular tissue was minced and homogenized in 15 ml KEA solution. KEA medium (10 ml) was then added to the homogenate, which was homogenized again. The homogenate was centrifuged at 2,000 g for 10 min. The supernatant fraction was filtered and centrifuged at 8,000 g for 10 min. The pellet was washed with a solution containing 180 mM KCl and 0.1 mM EGTA (KE) and resuspended. This suspension was centrifuged again at 8,000 g for 10 min. This washing procedure was repeated, and the final mitochondrial pellet was resuspended in a small volume of KE medium. After isolation, the Lowry assay was performed to determine protein concentration for Western blot analysis. The purity of mitochondrial fraction was determined as previously described by Ashraf’s laboratory (27).

**Western blot analysis for Akt.** Western blot analysis was performed to determine the effect of drugs on total Akt compared with phosphorylated Akt. Equal amounts of protein samples (30 μg of protein) were mixed with an equal volume of sample buffer [containing 2% SDS, 100 mM Tris, 0.2% bromophenol blue, 20% glycerol, and 200 mM DTT]. The samples were then boiled for 15 min before loading into each well on 10% polyacrylamide gels (Precast Gels, ISC Bioexpress) and run at 100 V for 2 h. These electrophoresed proteins were transferred from the gel to the nitrocellulose membranes (Bio-Rad). Ponceau’s red staining confirmed equal loading and transfer of

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**Fig. 1.** Experimental groups and protocol. BMS, BMS-191095; DE, diazoxide; 5-HD, 5-hydroxydecanoic acid; WTN, wortmannin.
proteins. The membranes were incubated for 60 min with 5% dry milk
and Tris-buffered saline to block nonspecific binding sites. Mem-
branes were incubated at 4°C with anti-total Akt antibody and anti-
phosphorylated Akt antibodies each in 1:1,000 dilutions on a rocking
platform overnight. After being washed thoroughly, blots were incu-
bated with 1:10,000 dilution of horseradish-labeled anti-rabbit IgG for
2 h at room temperature. Later, blots were developed with LumiGlo
developing solutions. The amount of total and phosphorylated Akt
was quantified by using a computer program.

Terminal dUTP nick-end labeling analysis. Terminal dUTP nick-
end labeling (TUNEL) assay was performed on 5-μm-thick deparaf-
finized histological sections with a MEBSTAIN Apoptosis Kit II
(Medical and Biological Laboratories). TUNEL assay may not be
entirely specific for apoptosis but is generally believed to be a good
marker for cell apoptosis. The heart tissue was obtained after 2 h of
postischemia/reperfusion and processed as described previously by
Wang et al. (24). Sections were stained with diamidino-2-phenylin-
dole to visualize nuclei. Later, they were photographed with an
Olympus BX41 microscope (Olympus America, Melville, NY),
equipped with a digital camera. Each apoptotic nucleus was carefully
checked at high magnification (×1,000) and counted. However, it was
difficult to discern cell membrane breaks with light microscopy unless
coupled with membrane permeability tracers.

Statistical analysis. All values are expressed as means ± SE.
Group comparisons were analyzed by one-way ANOVA (StatView
4.0). All groups were analyzed simultaneously with a Bonferroni/
Dunn test. A difference of \( P < 0.05 \) was considered statistically
significant.

RESULTS

Hemodynamics of normal nonischemic hearts. Preischemic
baseline values of left ventricular function in various treatment
groups are summarized in Fig. 2, A–C. The mean values of left
ventricular developed pressure (LVDP), LVEDP, and coronary
flow (CF) were not significantly different between the groups
during equilibration. There was also no significant difference
in the body and heart weights between the groups.

Hemodynamic and biochemical effects of ischemia. Heart
function was significantly decreased after ischemia. At the end
of reperfusion, LVDP and CF were decreased, whereas
LVEDP was increased (Fig. 2, A–C). LDH leakage was also
significantly increased on reperfusion in ischemic control
Fig. 2. Effect of various interventions on left ventricular (LV) developed pressure (LVDP; A), LV end-diastolic pressure (LVEDP; B), coronary flow (C), and lactate dehydrogenase (LDH; D). Isch Con, ischemic-reperfusion control; Eq, equilibrium. Values are means ± SE; \( n = 8 \) animals for each group. BMS, BMS-190190; BMS + WTN; WTN and BMS given together; BMS + 5HD, 5-HD + BMS given together; WTN, WTN given alone. *\( P < 0.05 \) vs. DE group and ISCH CON; #\( P < 0.05 \) vs. BMS group and ISCH CON.

groups.
creased compared with the BMS- or DE-treated groups, indicating greater damage to the heart (Fig. 2D).

**Role of Akt in mitoK<sub>ATP</sub> channel-mediated late preconditioning.** To determine the relationship between Akt and mitoK<sub>ATP</sub> channels, 5-HD, a specific blocker of the mitoK<sub>ATP</sub> channel, was given to the mice together with BMS 24 h before ischemia. A decrease in LVDP and CF and an increase in LVEDP were observed in 5-HD + BMS-treated hearts compared with BMS- or DE-treated hearts (Fig. 2, A–C). LDH leakage was also significantly increased compared with BMS- or DE-treated hearts (Fig. 2D).

**Phosphorylation of Akt in cytosol by BMS and DE.** If BMS and DE can activate PI3K, one would expect activation of Akt, a kinase downstream of PI3K. Phosphorylation of PKB/Akt protein was examined by Western blot analysis 24 h after the drugs were given to the animals. Representative Western blots are illustrated in Fig. 3, A and B. Phosphorylation of Akt was increased in BMS- and DE-treated hearts. PI3K-specific inhibitor WTN caused downregulation of Akt because less phosphorylation was evident in both BMS + WTN or WTN-treated hearts (DE + WTN, data not shown). Similarly, 5-HD, which acts at the mitochondrial level, had no effect on Akt phosphorylation in cytosol because Akt is phosphorylated upstream of mitochondria. This data concluded that mitoK<sub>ATP</sub> channel openers activated Akt in the cytosol and that only WTN inhibited Akt-phosphorylation here. 5-HD had no effect on Akt phosphorylation in cytosol, because it acts on mitochondrial channels only.

**Phosphorylation of Akt in mitochondria by BMS and DE.** Mitochondrial phosphorylation of Akt was examined by Western blot analysis 24 h after the drugs were given to the animals. Representative Western blots of mitochondrial fractions are illustrated in Fig. 3, C and D. Phosphorylation of Akt was increased in mitochondria isolated from BMS- and DE-treated hearts. PI3K-specific inhibitor WTN caused downregulation of Akt because less phosphorylation was evident in both BMS + WTN or WTN-treated hearts. Similarly, 5-HD inhibited Akt translocation from cytosol to mitochondria, suggesting that activation of mitochondrial channels plays a critical role in mediating cardioprotective effects of Akt. Despite Akt being phosphorylated in the cytosol upstream of mitochondria, treatment with 5-HD resulted in the inhibition of Akt expression within the mitochondria. These data suggest that mitoK<sub>ATP</sub> channel openers, such as BMS or DE, activate Akt in the cytosol and that only WTN resulted in inhibition of Akt-phosphorylation in cytosol and mitochondria. On the other hand, BMS + 5-HD inhibited phosphorylated Akt translocation to mitochondria.

**Time-dependent Akt phosphorylation by BMS.** Akt phosphorylation by BMS was time dependent and peaked out at 24 h (Fig. 3E). The effect of BMS disappeared after 72 and 96 h.

**Antiapoptotic effect of BMS and DE on ischemic injury through Akt signaling pathway.** Mice treated with BMS (18 ± 1.2%, P < 0.05) or DE (22 ± 1.4%, P < 0.05) had a significant reduction in the number of TUNEL positive nuclei compared with ischemic control mice (47 ± 1.4%, P < 0.05) shown in Fig. 4. Apoptosis was also increased in WTN, 5-HD, BMS + WTN, or BMS + 5-HD groups. This data provide further support to the concept that the PI3K-Akt pathway plays a critical role in the protection mediated by the activation of mitoK<sub>ATP</sub> channels.

**DISCUSSION**

Akt and mitoK<sub>ATP</sub> channels in late preconditioning. We have previously demonstrated that activation of mitoK<sub>ATP</sub> channels elicits strong protection against Ca<sup>2+</sup> overload and ischemic injury (23, 25, 26). These conclusions are well supported by several recent studies (9–11, 26) that document mitoK<sub>ATP</sub> channel as the end effector in cardioprotection against ischemia during late preconditioning. Grover and colleagues (12) have shown that BMS selectively opens mitoK<sub>ATP</sub> channels without affecting sarcolemmal channels in vascular smooth muscle, heart, or pancreatic B cells. Moreover BMS is devoid of any vasodilator or proarrhythmic activities (20) and had no effect on cardiac function in the normal heart as demonstrated with Millar’s pressure-volume system (data not shown). This study demonstrates that the opening of the mitoK<sub>ATP</sub> channel by BMS or DE produces delayed cardioprotection 24 h after initial treatment. Because half-life of DE in humans is 72 h (5), it is still not clear how long BMS will last in the plasma of mice. It could also be argued that DE or BMS is trapped in mitochondrial membranes for a longer duration, and thus they induce the late cardiac protective effect. Our results also indicate that BMS elicits stronger cardioprotection than DE. BMS caused less increase in LVEDP and greater improvement in LVDP and CF compared with the DE group (not statistically significant). The participation of Akt/PKB in the opening these mitoK<sub>ATP</sub> channels is highly attractive. WTN has abrogated this protection when used alone or when WTN and BMS are used together. Our data suggest that Akt phosphorylation in the cytosol causes translocation of phosphorylated-Akt to mitochondria and that this translocation induces its cardioprotection against ischemic-reperfusion injury. Bijur and Jope (3) have also reported similar results, which have suggested that Akt phosphorylation in mitochondria causes activation of various proteins like ATP synthase and glycogen synthase kinase 3-β within the mitochondria, and they aid in the reduction of apoptosis. Recently, Nagoshi et al. (18) demonstrated that chronic Akt activation resulted in decreased functional recovery and increased injury after ischemia-reperfusion, which is not in agreement with our current study. However, this disparity might be due to experimental conditions used in the later study in which transgenic mice with cardiac-specific expression of activated Akt were used. Western blot analysis results suggest that blocking Akt translocation to mitochondria prevents Akt from phosphorylating those proteins and results in the loss of its cardiac protection ability, despite the fact that Akt was phosphorylated in the cytosol.

**Activation of PI3K/Akt signaling pathway attenuates cell death.** We postulated that the PI3K signaling pathway is upstream of mitoK<sub>ATP</sub> channel, the activation of which results in decreased cardiac cell death and apoptosis. It has been suggested that Akt may mediate its antiapoptotic effects via phosphorylation of BAD (7), induction of Bcl-2 family of proteins (17), inhibition of cytochrome c release from mitochondria (15), and phosphorylation and inactivation of the caspase family (8). There is substantial evidence that Akt activation not only reduces the number of apoptotic cells but also substantially reduced infarct size and even more dramatically improved the cardiac function (16, 22). Western blot and TUNEL analysis clearly indicate that BMS and DE activated PI3K/Akt pathway and reduced a number of apoptotic nuclei in
Fig. 3. Western blot analysis for Akt protein. A, top: Western blots showing total Akt protein bands at 60 kDa in cytosol in various treatment groups. β-Actin was used as equal loading control (Con). A, bottom: quantitative measurement of total Akt. Data are means ± SE; n = 4 animals for each group. au, Arbitrary units. No significant differences were observed in all groups. B, top: Western blots showing phosphorylated Akt protein bands at 60 kDa in cytosol in various treatment groups. B, bottom: quantitative measurement of phosphorylated Akt levels that were normalized with total Akt. Data are means ± SE; n = 4 animals for each group. *P < 0.05 vs. Con. C, top: Western blot showing total Akt protein band at 60 kDa in mitochondria in various treatment groups. C, bottom: quantitative measurement of total Akt level. Neg Con, negative Con. Data are means ± SE; n = 24 animals for each group. No significant differences were observed in all groups; *P < 0.05 vs. Con. D, top: Western blots showing phosphorylated Akt protein bands at 60 kDa in mitochondria in various treatment groups. D, bottom: quantitative measurement of phosphorylated Akt in mitochondria. Data are means ± SE; n = 24 animals for each group. *P < 0.05 vs. Con. E, top: Western blots show that time-dependent phosphorylated Akt (Phos-Akt) in the cytosol fraction was significantly increased in BMS-treated hearts at 1, 8, and 24 h. E, bottom: quantitative measurement of time-dependent phosphorylation of Akt. Data are means ± SE; n = 6 animals for each group. *P < 0.05 vs. Con.
the myocardium. WTN, a pharmacological inhibitor of PI3K, when given with BMS abolished the protection, and there were no differences between the BMS and the ischemic control groups. When given with BMS, 5-HD had no effect on Akt activation in cytosol but abolished the beneficial effects of mitoK<sub>ATP</sub> channels. However, given the results obtained by using PI3K inhibitor, these findings provide pharmacological evidence supporting the notion that Akt phosphorylation mediated the opening of mitoK<sub>ATP</sub> channels, which are downstream targets of Akt.

Activation of Akt only in the cytosol is insufficient to provide cardioprotection unless it is translocated to the mitochondria to phosphorylate various proteins within the mitochondria. Our Western blot analysis data show that activation

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Fig. 4. Representative photomicrographs of terminal dUTP nick-end labeling (TUNEL) positive nuclei in various treatment groups. A: normal (NL) Con. Diamidino-2-phenylindole (DAPI)-stained nuclei (arrow) is shown B: TUNEL-positive nuclei (arrow) in Isch Con group. C: BMS-treated group. Green fluorescence shows TUNEL-positive nuclei (arrow). TUNEL-positive nuclei were significantly decreased in BMS-treated heart compared with Isch Con group. D: same area as in C, except all nuclei were stained by DAPI. E: same section as in C, except double stained with α-sarcomeric actin and TUNEL. F: BMS + WTN. Apoptosis in WTN, 5-HD, BMS + WTN, or BMS + 5-HD groups is similar to Isch Con group, except DE group that shows significantly less apoptosis than Isch Con group (data not shown). Magnification ×400; n = 4 animals for each group. Bars = 10 μm. G: semiquantitative estimate values of TUNEL-positive nuclei in data are means ± SE. *P < 0.05 vs. Isch Con.
of mitoK<sub>ATP</sub> channels results in increased phosphorylation of Akt protein within the mitochondria, leading to increased cell survival by attenuating cell apoptosis. Second, the persistent Akt phosphorylation is due to the persistent activation of the mitoK<sub>ATP</sub> channel by DE or BMS. (Fig. 4, A–F). These are important findings, which have strong bearing on the Akt and mitoK<sub>ATP</sub> channel relationship.

There is sufficient evidence in this study that PI3K/Akt plays a significant role in the reduction of apoptosis through activation of mitoK<sub>ATP</sub> channels. In the BMS + 5-HD group, an increased number of apoptotic nuclei were seen that support the concept that Akt is upstream of mitoK<sub>ATP</sub> channels. Despite the fact that Akt is activated in the cytosol, it cannot mediate its antiapoptotic function through mitochondrial K<sub>ATP</sub> channels unless it is translocated to mitochondria and causes phosphorylation of proteins within the mitochondria.

In conclusion, activation of mitoK<sub>ATP</sub> channels plays an important role in cardiac protection against ischemia via PI3K and Akt phosphorylation. Our data demonstrate for the first time that translocation of phosphorylated Akt from cytosol to mitochondria during activation of mitoK<sub>ATP</sub> Channels is crucial in the cardiac protection against ischemic-reperfusion injury.

GRANTS

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