Long-acting phosphodiesterase-5 inhibitor, tadalafil, induces sustained cardioprotection against lethal ischemic injury

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Ahmad N, Wang Y, Ali AK, Ashraf M. Long-acting phosphodiesterase-5 inhibitor, tadalafil, induces sustained cardioprotection against lethal ischemic injury. Am J Physiol Heart Circ Physiol 297: H387–H391, 2009. First published May 8, 2009; doi:10.1152/ajpheart.00169.2009.—The ability of pharmacological preconditioning mimetics to confer long-lasting and sustained cardioprotection may be a logical criterion to develop a drug that can be used clinically for cardioprotection. We propose here that the use of long-acting phosphodiesterase-5 inhibitor, tadalafil, may confer sustained cardioprotection against ischemia. Tadalafil (5 mg/kg) was administered orally to male C57B/6J mice (n = 6 in each treatment subgroup at each time point studied). Hearts were isolated and subjected to 40 min of ischemia and 30 min of reperfusion on Langendorff’s apparatus at 1, 12, 24, 36, 48, 60, 72, and 108 h after tadalafil administration. In 1- to 48-h subgroups, tadalafil was given once at 0 h only. In 60- and 72-h subgroups, tadalafil was given twice at 0 and 36 h. Similarly, in the 108-h subgroup, tadalafil was administered at 0, 36, and 72 h. In the same subgroups, wortmannin (15 µg/kg ip), an inhibitor of phosphatidylinositol 3-kinase 3-kinase or 5-hydroxydecanoic acid (5 mg/kg ip), an inhibitor of mitochondrial ATP-sensitive K+ channels, was given together with tadalafil, and the hearts were subjected to ischemia-reperfusion at 36 h to determine whether the effect of tadalafil on ischemia-reperfusion injury was abolished. As a result, tadalafil treatment reduced left ventricular end-diastolic pressure and increased left ventricular developed pressure as well as reduced lactate dehydrogenase release. This protection remained till 36–40 h, and thereafter it vanished. The readministration of tadalafil at 36 and 72 h restored the protection till 108 h. Tadalafil treatment accelerated Akt phosphorylation in cardiac tissue and decreased myocyte apoptosis. The administration of wortmannin abolished the beneficial effects of tadalafil on hemodynamic parameters and myocyte apoptosis, with significantly reduced Akt phosphorylation. 5-Hydroxydecanoic acid also abolished the antiapoptotic effect of tadalafil. It is concluded that tadalafil treatment induces the long-term protection of ischemic myocardium via phosphatidylinositol 3-kinase/Akt signaling pathway.

Akt: preconditioning; sustained cardiac protection

THE EFFECTIVENESS OF MYOCARDIAL protection after ischemic preconditioning (IPC) is well documented (12, 23). The fundamental concept of IPC is that a brief period of ischemia and reperfusion delimits myocardial cellular damage during a subsequent event of prolonged lethal ischemia. The effects of IPC have been reproduced by using preconditioning mimetics such as mitochondrial ATP-sensitive K+ (mitoKATP) channel openers (23). Unfortunately, the protective effects of IPC are short-lived. The search for pharmacological agents that can keep the heart in a sustainable state of protection will be a right step in the right direction. BMS-191095, a specific KATP channel opener, given at an interval of 20 h, kept the heart in protective state (23). The emerging concept of chronic intermittent preconditioning in which the heart can be constantly protected by chronic exposure to morphine (15) could be extended to the clinic by using promising pharmacological agents that can provide long-lasting protective effects to the heart before surgical procedures.

Phosphodiesterase inhibitors (PDIs) have important vascular and myocardial protective effects and have shown therapeutic usefulness in the clinical settings for treatment of patients with heart failure, pulmonary hypertension, and coronary artery disease (2, 7). PDIs selectively antagonize phosphodiesterase 5 (PDE5), which is found in high abundance in a variety of cells in humans (9), including canine and mouse cardiomyocytes (4, 18). The pharmacodynamics of PDIs in general and sildenafil in particular were consistent with the tissue distribution profile of PDE-5 and its isoform PDE-3. PDIs prevent the breakdown of nitric oxide (NO)-driven cGMP, primarily in vascular smooth muscle cells, and act as potent vasodilators. In vitro, PDIs have shown protection of adult cardiomyocytes during ischemia-reperfusion (I/R) injury with a possible role for cGMP-dependent protein kinase 1α (4). Sildenafil has also been shown to produce a marked preconditioning-like effect in the intact rabbit hearts (14). It appears that the protection incurred by sildenafil lasts for a limited period. Kukreja and colleagues (3) have also demonstrated that the potent effect of sildenafil-induced cardioprotection was due to the early translocation of protein kinase C (PKC) from cytosol to the membrane (3). These data provided direct evidence of an essential role of PKC in sildenafil-induced cardioprotection.

Since one of the PDE-5 inhibitors, tadalafil, has long-lasting effects, we reasoned that tadalafil can be used for longer-term protective effects. The present study was designed to demonstrate the persistent cardioprotective effects of tadalafil using an in situ model of myocardial infarction.

MATERIALS AND METHODS

The current study conforms to the guidelines established by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996), and the protocol was approved by the Institutional Animal Care and Use Committee. Adult male mice (C57B/6J) were obtained from Harlan. Tadalafil (Lilly), wortmannin (Wort) and 5-hydroxydecanoic acid (5-HD; Sigma), and Akt kit (Cell Signaling Technology) were purchased for use in the study.

Experimental Protocol

The experimental design has been summarized in Fig. 1. A total of 80 adult male mice C57B/6J mice, each weighing 25–30 g, were used in this study. Animals were randomized into five groups and multiple subgroups (n = 6 in each subgroup), which were harvested at the time points shown in Fig. 1. Group 1 consisted of ischemic control with no drug treatment; group 2, tadalafil treatment at 0 h. Hearts were isolated at 1, 12, 24, 36, and 48 h and subjected to I/R. Group 3...
consisted of tadalafil treatment at 0 and 36 h. Hearts were isolated at 60 and 72 h for I/R. Group 4 was given tadalafil three times at 0, 36, and 72 h. Hearts were isolated at 108 h for I/R. Group 5 was given tadalafil/Wort (15 mg/kg ip) (5), an inhibitor of phosphatidylinositol 3-kinase (PI3K), or tadalafil/5-HD ip, a specific inhibitor of mitoKATP channel. Tadalafil tablets were crushed in water and given to animals orally via gauge needle. Hearts were subjected to Langendorff perfusion at various time indicated. After an equilibration of 25 min, ischemia was induced for 40 min by shutting off the oxygenated buffer, followed by reperfusion for 30 min. Four hearts in each group were used for phosphorylated (p)Akt measurement by Western blot analysis.

Heart Preparation for Langendorff Perfusion

The animals 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 pH 7.4 Krebs-Henseleit buffer (in mM) consisting of 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25 NaHCO3, and glucose (23). The buffer was saturated with 95% O2-5% CO2 at 37°C for 25 min. The heart was perfused at a constant pressure of 80 mmHg. A homemade water-filled balloon was inserted into left ventricle (LV) through left atrium and adjusted to a LV end-diastolic pressure (LVEDP) of 5–8 mmHg during the initial equilibration. The distal end of the catheter was connected to a DigiMed heart performance analyzer (model 210, version 1.01, Micro-Med) by way of a pressure transducer (Case, Lakewood). Coronary flow (CF) was measured by a transit time flowmeter (Transonic Systems). The heart was paced at 350 beats/min except during ischemia. The pacing was reinitiated after 3 min of reperfusion for 30 min in all groups. The index of myocardial function was determined as previously described (22).

Measurement of Lactate Dehydrogenase

Lactate dehydrogenase (LDH), an indicator of myocardial tissue injury, was determined in the coronary effluent by a coupled enzymespectrometric technique (DU Series 500 Spectrophotometer, Beckman Instruments) using LDH assays kit (MBL) as per the manufacturer's instructions. LDH was measured at 3, 5, 10, 20, and 30 min after reperfusion. The accumulated amount was obtained by integrating the area underneath the individual time-course curve for 30 min of reperfusion, and the mean value was calculated form six animals per group.

Western Blot Analysis for Akt Phosphorylation

Pretreated hearts (LVs) were harvested, weighed, and stored at −70°C until used for protein extraction. Each heart tissue sample was homogenized for six bursts (15 s each) at 4°C with a Polytron-PT homogenizer using 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 4-(2-aminoethyl)benzenesulfonyl fluoride, and a cocktail of protease inhibitors. After sonication for 5 s, the tissue lysate was centrifuged at 14,000 rpm for 5 min at 4°C. The protein contents were determined using Protein Assay Reagent kit (Pierce, Rockford, IL). Equal amounts of protein samples (30 μg/μl) were mixed with equal volume of sample buffer containing 2% SDS, 100 mM Tris, 0.2%
bromophenol blue, 20% glycerol, and 200 mM dithiotheritol, boiled for 15 min, and electrophoresed on 10% polyacrylamide gels (Precast Gels, ISC Bioexpress). The electrophoresed proteins were transferred from the gel to nitrocellulose membranes (Bio-Rad) and immunodetected for total Akt and pAkt using anti-Akt and anti-pAkt antibodies (each 1:1,000 dilution) and horseradish-conjugated anti-rabbit secondary antibody (BD Pharmingen) as described earlier (13). The blots were developed with LumiGlo developing solutions for a visualization of the immune reaction. The amount of total and pAkt was quantified by densitometry (13).

Terminal dUTP Nick End Labeling Assay

For apoptosis, additional hearts were reperfused for 2 h after ischemia to render deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL)-positive nuclei visible. TUNEL was performed on 5-μm-thick deparaffinized histological sections, using MEBSTAIN Apoptosis Kit II (Medical and Biological Laboratories). The sections were made from the mid-LV of the heart and were stained with fluorescent TUNEL using the method described previously (20). The sections were stained with 4,6-diamidino-2-phenylindole to visualize nuclei and photographed with an Olympus BX41 microscope (Olympus America, Melville, NY) equipped with a digital camera.

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 as statistically significant.

RESULTS

Hemodynamic and Biochemical Assessment of LV Function

Preischemic LV functional parameters. The mean values of LV developed pressure (LVDP), LVEDP, and CF were not significantly different between the groups during the equilibration period. Similarly, there was also no significant difference in the body or heart weight between the groups.

Postischemic LV functional parameters. Cardiac function was assessed after each heart was subjected to ischemia (40 min) and reperfusion (30 min). LVEDP, LVDP, and CF were recorded and compared with the ischemic control group (Fig. 2, A–C). Heart function was significantly reduced after I/R. CF was also decreased, whereas LVEDP was increased after I/R (Fig. 2).

Protective effects of tadalafil on postischemic injury. Following tadalafil pretreatment, hearts examined at 1, 12, 24, 36, and 48 h showed significantly increased LVDP and CF till 40 h after I/R compared with the control. On the other hand, LVEDP was decreased in tadalafil-treated hearts compared with the ischemic control animals. LDH release was greatly reduced from hearts treated at different time intervals compared with hearts from the ischemic control group (Fig. 2D). The cardioprotective effects of tadalafil persisted until 36–40 h after pretreatment (Fig. 2). However, by 48 h, the cardioprotective effects of tadalafil were dissipated as indicated by the reduced LVDP and CF and the elevated LVEDP and LDH release compared with those levels in the ischemic control group. Interestingly, the readministration of tadalafil at 36 h restored cardioprotection until 72 h. The readministration of tadalafil at 0, 36, and 72 h extended protection until 108 h.

Antiapoptotic Effect of Tadalafil on Ischemic Injury Through Akt Signaling Pathway

The mitoK\(_{\text{ATP}}\) channel is an important modulator of various events in a cell, especially those involved in cell-death signal-

Fig. 3. Representative photomicrographs showing terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL)-positive nuclei in various treatment groups. A: normal heart tissue. B: TUNEL-positive nuclei (green, white arrow) in I/R control group. C: tadalafil-treated group at 36 h (T36). TUNEL-positive nuclei were significantly decreased in tadalafil-treated hearts compared with those in the I/R group. D: tadalafil-treated group at 48 h (T48) showing reappearance of apoptosis. E: tadalafil + Wort treatment group. F: tadalafil + 5-HD treatment group. Apoptosis is similar to I/R group (B). All nuclei were stained blue by 4,6-diamidino-2-phenylindole, and heart tissue was stained with α-sarcomeric actin (red) for cardiomyocytes. Magnification, ×400; n = 4 animals for each group. Bars = 100 μm. G: quantitative estimate values of TUNEL-positive nuclei in data are means ± SE. *\( P < 0.05 \) vs. I/R Control; n = 4 animals for each group.
ing pathways. The activation of these channels by Akt prevents
the release of cytochrome-c and Ca\textsuperscript{2+} influx into the cell and
increase ATP synthesis. In the mice treated with tadalafil at
different times, a significant reduction in the number of
TUNEL-positive nuclei was observed compared with that in
the ischemic control group (Fig. 3). In line with these results,
apoptosis was significantly increased in tadalafil-treated hearts
after the administration of either Wort or 5-HD (Fig. 3, E and
F). The data further support the concept that the PI3K/Akt
pathway plays a critical role in cardioprotection induced by
tadalafil.

Phosphorylation of Akt by Tadalafil

Western blot analysis revealed that the phosphorylation of
Akt was increased in tadalafil-treated hearts at different time
points (Fig. 4, A and B). A significant increase in Akt phos-
phorylation was observed at 24 and 36 h (group 2), 60 and 72 h
(group 3), and 108 h (group 4). The administration of Wort, a
specific PI3K inhibitor, downregulated Akt phosphorylation.
Similarly, the administration of 5-HD, which acts at the mito-
chondrial level, significantly reduced Akt phosphorylation
(Fig. 4).

DISCUSSION

The major findings of this study are that the heart is pro-
tected against ischemia with long-acting tadalafil for 40 h and
that protection continues till 108 h if the heart is treated again
with the drug at 36 and 72 h. The protection appears to be
mediated by PI3K/Akt and mitoK\textsubscript{ATP} channel pathways.
Among the various pharmacological strategies for cardiac
protection, the use of clinically relevant agents is more appeal-
ing. PDE-5 inhibitors have a potential use in cardiovascular
diseases (7, 9). This class of drugs has myocardial and vascular
protective effects. Moreover, these drugs are free of serious
side effects except under conditions of hypotension in patients
caused by the combined use of \textalpha-blockers and nitrates as
reviewed by Gross et al. (6). Sildenafil has been shown to be
cardioprotective in rabbits after myocardial infarction (14).
There was an improved postischemic recovery of ventricular
function, a reduced incidence of ventricular fibrillation, and a
decreased myocardial infarction. Sesti et al. (19) also reported
that tadalafil reduced infarct size in rats following coronary
artery ligation. In the present study, we have demonstrated that
tadalafil was significantly cytoprotective and resultantly pre-
served the LV contractile functions. Moreover, we have shown
that 5-HD, a mitoK\textsubscript{ATP} channel blocker, or the inhibition
of PI3K/Akt signaling by Wort abrogated the effects of tadalafil,
thus suggesting the protective role of mitoK\textsubscript{ATP} channels via
PI3K signaling.

Activation of PI3K Signaling Attenuated Cell Death

We hypothesized that activation of PI3K signaling pathway
upstream of mitoK\textsubscript{ATP} channel resulted in decreased cardiac
cell death and apoptosis. Akt activation not only reduces the
number of apoptotic cells but also substantially delimits
infarct size and improves cardiac function (11, 20). Western
blot analysis and TUNEL assay clearly indicated that
tadalafil activated PI3K/Akt pathway for its antiapoptotic
activity. The administration of Wort, which is a specific
inhibitor of PI3K, abolished Akt activation when given with
tadalafil. Similarly, 5-HD, when given together with tadalafil,
also abolished the beneficial effects of tadalafil at 36 h by
blocking the activation of mitoK\textsubscript{ATP} channels, which are down-
stream targets of Akt. These results support the concept that a
downstream target of PI3K system is the mitoK\textsubscript{ATP} channel.

Apoptosis

There is growing evidence that cardiomyocytes undergo
apoptosis primarily during reperfusion (25). Preconditioning
activates PI3K and suppresses caspase-3 and DNA fragmenta-
tion, thus reducing apoptosis (8, 16). These studies highlight
the role of caspase-3 as an important signaling component of
apoptosis and cell death. Although apoptosis in the ischemic

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Fig. 4. Western blot analysis for Akt protein. A, top: time-dependent phos-
phorylation of Akt (pAkt; groups 1–4). A, bottom: quantitative measurement
of pAkt. Data are means ± SE; n = 4 animals for each group. B: effect of Wort
and 5-HD on phosphorylation of Akt. Data are means ± SE; n = 6 animals for
each group. *P < 0.05 vs. control. \textbeta-Actin was used as equal-loading control.
myocardium is controversial, reperfusion is known to accelerate cardiomyocyte death in the ischemic myocardium. TUNEL-positive cells were significantly increased in ischemic hearts treated with tadalafil at various time intervals compared with ischemic control group. The increased number of TUNEL-positive cells was also observed in the tadalafil + Wort-treated group, thus suggesting that PI3K pathway is prosurvival. In the tadalafil + 5-HD group, an increased number of apoptotic nuclei were observed, which supports the concept that Akt is upstream of the mitoKATP channels (1). Another study by Kukreja’s group (17) using mice reported that the protection by sildenafil was mediated by the upregulation of inducible and endothelial NO synthases.

This is the first report to show that PDE-5 inhibitor tadalafil, which has been clinically approved for erectile dysfunction, can provide long-lasting protection against ischemia compared with the other known pharmacological preconditioning agents. The selective activation of mitoKATP channels seems to play an important role in cardioprotection against ischemia via PI3K and Akt phosphorylation. Although the cardioprotective effect of tadalafil is abolished with the mitoKATP channel inhibition, it is also likely that protection may also be mediated by alternate pathways. Thus cross talk between PI3K/Akt and downstream targets, such as glycogen synthase 3 (21), or other prosurvival kinases remains to be investigated. The concept of sustained cardioprotection by long-acting PDE5A inhibitor tadalafil is novel and can be exploited under the clinical settings.

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