Pharmacological inhibition of PTEN limits myocardial infarct size and improves left ventricular function postinfarction

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Keyes KT, Xu J, Long B, Zhang C, Hu Z, Ye Y. Pharmacological inhibition of PTEN limits myocardial infarct size and improves left ventricular function postinfarction. Am J Physiol Heart Circ Physiol 298: H1198–H1208, 2010. First published January 22, 2010; doi:10.1152/ajpheart.00915.2009.—Phosphoinositide 3-kinase (PI3K) mediates myocardium protective signaling through phosphorylation of phosphatidylinositol (PtdIns) to produce PtdIns(3,4,5)P3. Lipid phosphatase and tensin homolog on chromosome 10 (PTEN) antagonizes PI3K activity by dephosphorylating PtdIns(3,4,5)P3; therefore, the inhibition of PTEN enhances PI3K/Akt signaling and could prevent myocardium from ischemia-reperfusion (I/R) injury. Here we studied 1) whether the pharmacological inhibition of PTEN by bisperoxovanadium molecules [BpV(HOpic)] attenuates simulated I/R (SIR) injury in vitro and 2) whether the administration of BpV(HOpic) either before or after ischemia limits myocardial infarct size (IS) and ameliorates cardiodynamics caused by infarction. First, adult rat cardiomyocytes were treated with or without BpV(HOpic) and then exposure to SIR. Second, anesthetized rats received BpV(HOpic) either before or after ischemia. IS was assessed at 4 h reperfusion, and left ventricular function was evaluated by echocardiography at 28 days postreperfusion. As a result, BpV(HOpic) decreased cell death, improved 3-[4,5-yl]-2,5-diphenyltetrazolium bromide (MTT) viability, and reduced apoptosis in cells exposed to SIR. These protective effects of BpV(HOpic) are associated with increased phospho-Akt and the repression of caspase-3 activity. Second, the administration of BpV(HOpic) significantly reduced IS and suppressed caspase-3 activity following I/R injury and consequently improved cardiac function at 28 days postinfarction. These beneficial effects of BpV(HOpic) are attributed to increases in myocardial levels of phosphorylation of Akt/enhylisoid nitric oxide synthase (eNOS), ERK1/2, and calcium-dependent nitric oxide synthase activity. In conclusion, the pharmacological inhibition of PTEN protects against I/R injury through the upregulation of PI3K/Akt/eNOS/ERK prosurvival pathway, suggesting a new therapeutic strategy to combat I/R injury. Akt; extracellular signal-regulated kinase 1/2; lipid phosphatase and tensin homolog on chromosome ten.

PHOSPHOINOSITIDE 3- KINASE (PI3K) has a key role in cardioprotection against ischemia-reperfusion (I/R) injury (2, 3, 8, 9, 17). PI3K phosphorylates phosphatidylinositol at position 3 of the inositol ring to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3), which recruits and activates a downstream cascade of prosurvival kinases, including Akt (9, 21), with a subsequent activation of endothelial nitric oxide synthase (eNOS) (12). Lipid phosphatase and tensin homolog on chromosome 10 (PTEN) is a dual protein-lipid phosphatase that degrades PIP3 to an inactive form phosphatidylinositol 4,5-bisphosphate (PIP2) (14, 16, 24, 30) and so inhibits Akt activation. PTEN is constitutively active and is the major downregulator of the prooncogenic PI3K/Akt (31). The overexpression of PTEN increases apoptosis in neonatal cardiomyocytes, whereas the inactivation of PTEN activates the Akt prosurvival pathway, reduces apoptosis, and increases survival (21, 27, 28, 33). PTEN activity is reduced after preconditioning with cycles of brief I/R and restored when the protective effect of preconditioning decays (6). PTEN upregulation may attenuate the infarct size (IS)-limiting effects of prolonged (>1 wk) pretreatment with atorvastatin (19). Moreover, myocardial levels of PTEN are increased in diabetic rats compared with nondiabetic rats, leading to a reduced ability to phosphorylate Akt and hence blunting the protective effects of ischemic preconditioning (20). In contrast, a partial deletion of PTEN reduces the threshold of protection by ischemic preconditioning (29). Therefore, PTEN inhibition may be beneficial in protecting the heart against I/R injury (21).

Targeting PTEN inhibition by a pharmacological approach may represent a window of opportunity to apply therapy against ischemia injury in the clinical setting. Currently, we do not have highly specific PTEN inhibitors. However, protein tyrosine phosphatase inhibitors such as bisperoxovanadium molecules (BpV) inhibit PTEN at very low concentrations (up to 100-fold lower than necessary for protein tyrosine phosphatase inhibition) (27). Here we studied whether the pharmacological inhibition of PTEN by BpV protects cardiomyocytes against simulated ischemia (SI) and I/R injury. In the next step, we assessed whether BpV, administered either before ischemia (BpV-BI) or just before reperfusion (BpV-BR), reduces myocardial IS in vivo.

METHODS

Simulated I/R Injury in Adult Rat Cardiomyocytes

The experimental designs and animal care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee.

The male Sprague-Dawley rats were housed at controlled room temperature (24.5–25.0°C). Cardiomyocytes from Sprague-Dawley rats were isolated using methods previously described with minor modification (10, 11). Myocytes were plated on a laminin-coated plate at a density of $2 \times 10^{4}$ cells/cm2 in culture media and incubated at 37°C in 5% CO2–95% room air for 12 h before experiments. Myocytes from one animal were used per experiment. Cardiac myocytes from rats were incubated with 0, 5, 10, 15, or 20 μM of BpV for 2 h. After treatment, myocytes were subjected to I/R incubation in normoxic...
conditions for 4 h (no simulated I/R (NSIR)), 2) 2 h incubation in normoxic conditions followed by 2 h of SI, or 3) 2 h SI followed by 2 h reoxygenation (SIR). Hypoxia (SI) was induced by adding a layer of mineral oil over a thin film of hypoxic media (prebubbled with N₂ gas) covering the cells for 2 h. Reoxygenation was performed by removing the mineral oil and replacing the medium with fresh medium and incubating for an additional 2 h. After 4 h, we used the cells exposed to NSIR and SIR for assessing cell death, viability, and apoptosis. We used cells treated without or with BpV (15 μmol) and exposed to NSIR, SI, and SIR to assess PTEN and caspase-3 activity.

Cell death was measured by counting trypan blue-stained cells and expressing the values as a percentage of the total cells counted. The test is based on the ability of live cells to exclude trypan blue. Second, cell viability was estimated by 3-(4,5-yl)-2,5-diphenyltetrazolium bromide (MTT) cell respiration assay. At the end of hypoxia/reoxygenation treatment (SIR) or incubation at normoxic conditions (NSIR) (after 4 h), the cells were treated with MTT (0.5 mg/ml) for an additional 4 h at 37°C. The attached cells were lysed in 2-isopropanol (after 4 h), the cells were treated with MTT (0.5 mg/ml) for an additional 4 h at 37°C. The attached cells were lysed in 2-isopropanol containing 0.04 M HCl, and the amount of metabolized MTT was determined using a microplate reader. Finally, terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) was performed using a TUNEL kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Surgical Protocol and Determination of Area at Risk and IS

Protocol 1. Sprague-Dawley rats received an intravenous injection of 0.4 ml pure H₂O (control), BpV (0.5 mg/kg), BpV (1.0 mg/kg), or BpV (1.5 mg/kg) 1 h before ischemia. Treated animals were subjected to 30 min ischemia and 4 h reperfusion (I^30m/R^4h), as described in detail (35).

Protocol 2. Rats were subjected to I^30m/R^4h. Rats received H₂O or BpV, the same as Protocol 1 5 min before reperfusion.

Protocol 3. One hour before ischemia, rats received 1) intravenous injection of 0.4 ml pure H₂O (control), 2) 0.4 ml 1% dimethyl sulfoxide in H₂O, 3) BpV (1.0 mg/kg) dissolved in 0.4 ml pure H₂O, 4) N^6-nitro-l-arginine methyl ester [l-NNAME, 30 mg/kg, a nitric oxide synthase (NOS) inhibitor] and BpV (1.0 mg/kg) dissolved (BpV-BI + l-NNAME), and 5) wortmannin (0.6 mg/kg) and BpV (1.0 mg/kg) (BpV-BI + wortmannin). Treated animals were subjected to I^30m/R^24h.

Protocol 4. Sprague-Dawley rats were subjected to I^30m/R^24h. Rats received intravenous 0.4 ml pure H₂O or BpV (1.0 mg/kg) 5 min before reperfusion (BpV-BR).

Surgery. Briefly, anesthesia was induced by an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg). The animals were intubated and connected to an animal ventilator (model 683, Harvard Apparatus, South Natick, MA) and ventilated using inspired oxygen fraction of 30%. The left carotid artery was cannulated for measuring blood pressure and heart rate (HR). The chest was opened, and the left coronary artery was encircled with a suture and occluded with a snare for 30 min. Ischemia was verified by regional dysfunction and discoloration of the ischemic zone. Isoflurane (1–2.0% titrated to effect) was added to maintain anesthesia. At 30 min of ischemia, the snare was released and myocardial reperfusion was verified by a change in the color of the myocardium. Subcutaneous 0.1 mg/kg buprenorphine was administered, the chest was closed, and the rats were recovered from anesthesia. HR and mean blood pressure (MBP) were noted at baseline (after cannulation of the carotid artery, before opening the chest), before coronary artery occlusion, at 25 min of coronary artery occlusion, and at 25 min after reperfusion (35). After reperfusion, the rats were reaneathedized, the coronary artery was reoccluded, 0.5 ml of 3% Evans blue dye was injected into the right ventricle, and the rats were euthanized under deep anesthesia. Hearts were excised, and the left ventricle was sliced transversely. Slices were weighed and incubated for 10 min at 37°C in 1% buffered (pH 7.4) 2.3,5-triphenyltetrazolium chloride (TTC), fixed in 10% formaldehyde, and photographed to identify the area at risk (AR) (uncolored by the blue dye), the IS (unstained by TTC), and the nonischemic zones (colored by blue dye). The AR and IS in each slice were determined by planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice, and the results were summed to obtain the weights of the myocardial AR and IS (35).

Echocardiography

Rats receiving BpV (1.0 mg/kg) either before ischemia (BpV-BI) or before reperfusion (BpV-BR) and control animals underwent 30

![Cell death (Trypan Blue)](image)

![Cell viability (MTT)](image)

![Apoptosis](image)

Fig. 1. A: cell death as assessed by the percentage of trypan blue-positive cells. Overall, there were significant differences among treatment groups [P < 0.001 for both cells not exposed (NSIR) or exposed (SIR) to simulated ischemia-reperfusion]. *P < 0.003 vs. bisperoxovanadium molecules (BpV) (0 μM) NSIR; #P < 0.006 vs. BpV (0 μM) SIR. B: cell viability [3-(4,5-yl)-2,5-diphenyltetrazolium bromide (MTT)]. Overall, there were significant differences among treatment groups (P < 0.001 for both NSIR and SIR). *P < 0.004 vs. BpV (0 μM) NSIR; #P < 0.001 vs. BpV (0 μM) SIR. C: apoptosis. Overall, there were significant differences among treatment groups (P < 0.001 for both NSIR and SIR). *P < 0.003 vs. BpV (0 μmol) NSIR; #P < 0.001 vs. BpV (0 μmol) SIR. There were 4 samples in each group.
min ischemia. The snare was released, the chest was closed as described in Surgery, and the rats were recovered from anesthesia. Sham-operated animals that underwent the open-chest procedure without being subjected to the coronary artery ligation served as baseline controls. Transthoracic echocardiography was performed 28 days after the induction of infarction (n = 7 to 8/group). Animals were anesthetized with ketamine-xylazine as described in Surgery in the I/R injury model. Echocardiography was performed using an HP Sonos 5500 echocardiograph. Two-dimensional and M-mode images in the parasternal short-axis view at the midpapillary level were obtained using a 15-MHz linear transducer. Key cardiac parameters, such as left ventricle internal diameters at end diastole (LVIDd) and systole (LVIDs), left ventricular fractional shortening (FS), and calculated left ventricle internal diameters at end diastole (LVIDd) and systole (LVIDs), left ventricular fractional shortening (FS), and calculated ejection fraction by the Teich method (32), were measured.

Western Blot Analysis

Animals received water (control) or BpV (1.0 mg/kg) either before ischemia or before reperfusion and underwent 30 min coronary artery occlusion followed by 4 h reperfusion, as described in Surgical Protocol and Determination of Area at Risk and IS. Hearts from sham-operated rats not exposed to I/R served as controls. Significant changes in enzyme expression and activity occur over time after ischemia and reperfusion. We decided to assess the protein expression and activity at 4 h of reperfusion, the same time used for assessing IS in protocols 1 and 2. Myocardial tissue from the ischemic border zone was homogenized in radioimmunoprecipitation assay buffer. Equal amounts of proteins were subjected to Western blot analysis [PTEN,

for 15 min. The

Table 1. Body weight, area at risk, and infarct size

<table>
<thead>
<tr>
<th>Protocol</th>
<th>N</th>
<th>Body Weight, g</th>
<th>Area at Risk, %LV</th>
<th>Infarct Size, %LV</th>
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<tr>
<td>Control</td>
<td>6</td>
<td>261 ± 3</td>
<td>28.9 ± 0.6</td>
<td>12.6 ± 0.9</td>
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<tr>
<td>BpV-BI (0.5 mg/kg)</td>
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<td>260 ± 3</td>
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<tr>
<td>BpV-BI (1.0 mg/kg)</td>
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<td>260 ± 2</td>
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<td>5.6 ± 0.5</td>
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<tr>
<td>BpV-BI + wortmannin</td>
<td>6</td>
<td>261 ± 3</td>
<td>30.3 ± 1.0</td>
<td>15.0 ± 1.5</td>
</tr>
<tr>
<td>BpV-BI + l-NNAME</td>
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<td>261 ± 3</td>
<td>27.8 ± 0.8</td>
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<tr>
<td>P value</td>
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<tr>
<td>Control</td>
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<td>29.7 ± 1.0</td>
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<tr>
<td>BpV-BR (0.5 mg/kg)</td>
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<td>261 ± 3</td>
<td>26.6 ± 1.1</td>
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<td>BpV-BR (1.0 mg/kg)</td>
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<td>261 ± 3</td>
<td>26.6 ± 1.1</td>
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<tr>
<td>P value</td>
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<td>0.760</td>
<td>0.053</td>
<td>&lt;0.001</td>
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</table>

Values are means ± SE; N, number of animals. LV, left ventricle; BpV-BI, bisperoxovanadium molecules (BpV) before ischemia; BpV-BR, BpV before reperfusion; l-NNAME, Nω-nitro-L-arginine methyl ester. *P < 0.002 vs. control.

Nitric Oxide Synthase Activity

Myocardial tissues from the previously ischemic zone were homogenized in a buffer containing 1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotonin, 1 μM leupeptin, 2 mM diithiothreitol, and 10 mM Tris-HCl (pH 8.0) on ice for 30 min and centrifuged at 15,000 g for 15 min. The protein concentration was determined using Lowry protein assay. To
determine the activity of caspase-3, assays were performed by incubating 200 μg protein of cell lysate in 100 μl of reaction buffer containing 5 μl of caspase-3 substrate (4 mM Asp-Glu-Val-Asp-p-nitroanilide) in 96-well plates. The reaction buffer contained 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 137 mM N-acetyl-L-cysteine, and 10% glycerol. The samples were incubated in the dark, and caspase-3 activity was evaluated using a spectrophotometer at 405 nm.

**Fig. 3.** A: myocardial infarct size (IS) in rats receiving BpV before ischemia (BpV-BI). Rats were subjected to ischemia for 30 min and reperfusion for 4 h (I30m/R4h). *P < 0.001 vs. control; #P < 0.001 vs. BpV-BI (1.0 mg/kg). AR, area at risk. B: mean blood pressure (MBP) at baseline, before coronary occlusion (Preocl), at 25 min of occlusion (Occl), and at 25 min of reperfusion (Postoccl) of rats treated with BpV-BI. C: heart rate [HR, in beats/min (bpm)] at baseline, Preocl, at 25 min of Occl, and at 25 min of Postoccl. Overall there were significant differences among the treatment groups (P = 0.010). HR also significantly changed over time (P < 0.001). P < 0.004 for the treatment × time interaction. D: HR of rats treated with BpV-BR. Overall, there were not significant differences among the treatment groups (P = 0.098). HR significantly changed over time (P < 0.001). P < 0.001 for the treatment × time interaction.
Immunoprecipitation. For the determination of Bax activation, we used anti-Bax 6A7 antibodies that recognize the conformational change of Bax. The samples were homogenized in immunoprecipitation buffer containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium orthovanadate, 50 mM glycerophosphate, and 1% protease inhibitor cocktail and were centrifuged at 10,000 g for 15 min at 4°C. The supernatant fraction was transferred to a fresh centrifuge tube. 6A7 Bax antibody was added at concentrations of 40 nM, and immunoprecipitation was performed overnight on a rotator. Immunoprecipitates were collected by incubating with protein G-Sepharose for 2 h, followed by pulse centrifugation (5 s in the microcentrifuge at 14,000 rpm). The beads were washed two times with wash buffer A, containing 10 mM HEPES (pH 7.4), 150 mM NaCl, and 2% 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS); three times with wash buffer B, containing 10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.2% CHAPS; and then an additional three times with wash buffer C, containing 100 mM Tris-HCl (pH 8) and 100 mM NaCl. Immunoprecipitates were released from the beads in SDS loading buffer and analyzed by Western blot analysis with the Bax 2D2 antibody.

Statistical Analysis

Data are presented as means ± SE. Analysis of variance (ANOVA) with Sidak correction for multiple comparisons was applied to compare the different groups. The differences in HR, MBP, and the echocardiographic parameters were compared using two-way repeated-measures ANOVA with Holm-Sidak multiple comparison procedures. Values of $P < 0.05$ were considered statistically significant.

Materials

BpV(0,001) was purchased from Enzo life science (Famingdale, NY). Anti-PTEN antibody, anti-total Akt antibody, anti-p-Akt antibody, MTT proliferation kit, and TUNEL apoptosis assay kit were from R&D Systems. Malachite green assay kit and Caspase-3 kit were from Cayman Chemicals (Ann Arbor, MI). Wortmannin and L-NAME were purchased from Sigma.

RESULT

Suppressing PTEN by BpV in Primary Cultured Cardiomyocytes Attenuated SIR Injury

We examined the effect of BpV on cell death and viability in primary cultures of rat cardiac myocytes using trypan blue staining, MTT assay. Cardiomyocytes were incubated with BpV (0, 5, 10, 15, or 20 μM) with or without exposure to SIR. Since primary cultured cardiomyocytes develop apoptosis and only survive ~8–10 days, we first examined the influence of BpV on cardiomyocytes without SIR (NSIR). BpV at 10, 15, and 20 μM decreased the percentage of trypan-positive cells compared with BpV at 0 μM (Fig. 1A). Cell death was induced by SIR and attenuated by BpV. The maximal effect was achieved at 15 μM ($P < 0.001$ vs. all other groups). However, the effect of BpV at 20 μM was smaller than that of 15 μM. As expected, the MTT viability score was also significantly improved by BpV in both NSIR and SIR (Fig. 1B). The maximal protective effect of BpV was achieved at 15 μM.

To determine the effect of BpV on cardiomyocytes apoptosis, we performed TUNEL assay. In the NSIR groups, BpV at 10, 15, and 20 μM decreased apoptosis compared with that in the control group (Fig. 1C). Among cells exposed to SIR, BpV at 5, 10, and 15 μM dramatically decreased apoptosis. The maximal effect was seen with 15 μM. Thus BpV improved cell viability attenuated cardiomyocyte injury and death caused by SIR.

BpV Decreases PTEN and Caspase-3 Activity in Cardiomyocytes

In groups without BpV treatment, PTEN activity slightly decreased by SIR and SIR compared with NSIR (Fig. 2A). BpV significantly attenuated PTEN activity during SI (214 ± 4 mol) and SIR (248 ± 4 μmol) compared with NSIR (326 ± 5 mol) and SIR (358 ± 5 μmol).
3.6 μmol). SI and SIR caused a significant increase in caspase-3 activity (Fig. 2B). BpV attenuated caspase-3 activity induced by SI or SIR.

Suppression of PTEN by BpV Limited Myocardial IS and Improved Heart Function

Animals received BpV before I<sup>30m</sup> and R<sup>4h</sup> (BpV-BI). A total of 40 rats were included (3 animal dead, Table 1, protocol 1). Body weight and the size of the ischemic AR were comparable among groups. IS was reduced by BpV-BI (Fig. 3A), and the maximum effect was achieved with BpV (1.0 mg/kg). There was no significant interaction between treatment and the time for MBP (Fig. 3B). HR was different from the control group when 1.5 mg/kg of BpV was given at preocclusion (Fig. 3C). Animals received BpV or vehicle 5 min before R<sup>4h</sup> (BpV-BR). A total of 31 rats were included (Table 1, protocol 2). Body weight and the size of the ischemic AR were comparable among groups. IS was reduced by BpV-BR, and the maximum effect was achieved with BpV (1.0 mg/kg) (Fig. 3D). There were small, yet statistically significant, differences in MBP among groups (Fig. 3E). HR significantly changed over time (P = 0.001) (Fig. 3F).

Suppression of PTEN by BpV Improved Heart Function Postinfarction

Animals received BpV (1.0 mg/kg) either 60 min before coronary artery occlusion (BpV-BI) or 5 min before reperfusion (BpV-BR), and an echocardiographic evaluation of the left ventricular dimension and systolic function was performed 28 days after surgery. There were eight rats in the sham-operated group, seven in the control group, seven in the BpV-BI group, and eight in the BpV-BR group. HR in the control group was 217.6 ± 12.8 beats/min. HR in the BpV-BI (227.1 ± 10.2 beats/min; P = 0.882) and BpV-BR (242.9 ± 6.2 beats/min; P = 0.227) were not significantly different from the HR in the control group. There were no

Fig. 5. Left ventricular internal diameter in diastole (LVIDd; A) and systole (LVIDs; B) and left ventricular fractional shortening (FS; C) and left ventricular ejection fraction (LVEF; D) in rats treated with vehicle (control) or BpV (1.0 mg/kg) either before ischemia (BpV-BI) or before reperfusion (BpV-BR). A: LVIDd: P = 0.242 for the treatment effect; P < 0.001 for the time effect; P = 0.058 for the treatment × time interaction. B: LVIDs: P = 0.097 for the group effect; P < 0.001 for the time effect; P = 0.128 for the treatment × time interaction. C: FS: P = 0.003 for the treatment effect; P = 0.003 for the time effect; P = 0.458 for the treatment × time interaction. D: LVEF: P = 0.003 for the group effect; P = 0.003 for the time effect; P = 0.349 for the treatment × time interaction. *P < 0.005 vs. control; #P < 0.04 vs. sham. There were 4 animals in each group.
Fig. 6. In vivo study. Samples of immunoblots (A) and densitometric analyses of myocardial levels of total Akt (B), Thr-308 phospho (p)-Akt (C), Ser-473 p-Akt (D), total endothelial nitric oxide synthase (eNOS; E), Ser-1177 p-eNOS (F), total ERK-1/2 (G), and p-ERK-1/2 (H) in the postischemic border zone. Rats were subjected to 30 min coronary artery occlusion and 4 h reperfusion and received either vehicle or BpV (1.0 mg/kg) either before ischemia (BpV-BI) or before reperfusion (BpV-BR). Sham-operated rats, not exposed to ischemia-reperfusion (I/R), served as baseline controls (Cont). There were 4 animals in each group. *P < 0.001 vs. sham-operated controls; #P < 0.001 vs. controls rats subjected to I/R.
statistically significant effects of BpV treatment ($P = 0.126$) or time ($P = 0.107$) on HR during the echocardiographic imaging.

LVIDd and LVIDs increased by I/R injury in the control group compared with the sham-operated group. BpV slightly reduced the increase of LVIDd but significantly reduced the increase of LVIDs after I/R injury ($P < 0.001$) (Fig. 5, A and B). FS and left ventricular ejection fraction were significantly lower in the control group than in the sham-operated group ($P < 0.001$) (Fig. 5, C and D). Both BpV-BI ($P < 0.001$) and BpV-BR ($P < 0.001$) attenuated the decrease in FS. These results indicate the important role for BpV in improving cardiac function postinfarct.

**Effects of BpV on the Expression of Akt, eNOS, and ERK-1/2**

Certain survival protein kinase such as PI3K/Akt/ERK-1/2 recruited at the time of myocardial reperfusion play an important role in mediating the cardioprotective effects against I/R injury.

To test whether PI3K/Akt cascade is involved in the cardioprotective effect of BpV, we performed immunoblotting to determine the expression levels of these proteins. As the maximal protective effect was achieved with BpV at a dose of 1.0 mg/kg, we proceeded with immunoblotting and ELISA using this dose. I/R had no significant effect on total Akt, total eNOS, or total ERK-1/2 levels (Fig. 6). Myocardial levels of Thr-308 p-Akt, Ser-473 p-Akt, Ser-1177 p-eNOS, and p-ERK-1/2 were slightly increased at the onset of reperfusion. BpV-BI and BpV-BR increased myocardial levels of Thr-308 and Ser-473 p-Akt, Ser-1177 p-eNOS, and p-ERK-1/2 (Fig. 6). Our data suggest that the survival protein kinase was strongly stimulated upon pharmacological inhibition of PTEN.

**Effects of BpV on PTEN and NOS Activity**

BpV suppressed PTEN activity in cultured cardiomyocytes (Fig. 2). We accessed the efficiency of this compound in the myocardium during I/R injury. I/R did not have a significant effect on PTEN activity (Fig. 7A). BpV-BI and BpV-BR
significantly inhibited PTEN activity in the ischemic border zone. In rats that received BpV (1.0 mg/kg) or H2O 1 h before 30 min ischemia, PTEN activity was determined at reperfusion at 4, 12, or 24 h (Fig. 7B). PTEN activity was significantly lower in the BpV group at 30 min of ischemia and 4 and 12 h of reperfusion. At 24 h of reperfusion, PTEN activity in the control and BpV-BI group was comparable, suggesting that PTEN inhibition by a single injection of BpV lasted <24 h. BpV-BI and BpV-BR augmented cNOS activity (Fig. 7C) but had no effect on calcium-independent NOS activity (Fig. 7D). The maximum augmentation of cNOS activity was seen when BpV was administered before ischemia (P = 0.005 vs. BpV-BR) (Fig. 7A).

**PTEN Inhibition by BpV Prevents I/R Injury by Inhibiting Caspase-3 Activity and Attenuates the Conformational Change of Bax**

Caspase-3 has been implicated to be involved in apoptotic cell death in I/R injury (4). In this experiment, caspase-3 activity was induced by I/R injury and attenuated by BpV-BI and BpV-BR (Fig. 8A). Bax, a proapoptotic member of the Bcl-2 protein family, has been reported to activate caspase-3 in injured muscle (15). To support our observation, we measured the activity of Bax. Immunoprecipitation was performed with anti-Bax 6A7 that recognizes the conformational changed BAX protein (34). The administration of either BpV-BI or BpV-BR attenuated BAX activation (Fig. 8B). These findings indicate that BpV exerts its antiapoptotic effects in the heart by inhibiting Bax conformational change and caspase-3 activity.

**DISCUSSION**

Both the PI3K/Akt cascade and the MAPK family, including ERK-1/2, are members of the survival kinases. PTEN is a potent negative regulator of PI3K activity in many cell types (31), and thus the inhibition of PTEN is a potential target for the induction of protection against I/R injury. In the present study, we show that PTEN inhibition with BpV protected cardiomyocytes against SIR injury, limited myocardial IS, and improved left ventricular function postinfarction. Moreover, the transient inhibition of PTEN activated the pro-survival pathways with the augmentation of cNOS activity and increased Akt and eNOS phosphorylation.

In the in vitro experiment, we observed that the maximal protective effect was achieved with BpV at a concentration of 15 μM. At a higher concentration (20 μM), we found less protection (Fig. 1). Similarly, in the in vivo model, we found that at the maximal IS limitation with BpV at a dose of 1.0 mg/kg and at a higher dose (1.5 mg/kg), IS tended to be higher than in the BpV (1.0 mg/kg) groups (Fig. 3). BpV is a relatively specific inhibitor of PTEN at low concentrations (27). It might be that with higher concentrations, the activity of PI3K/Akt signaling is dramatically increased to an uncontrolled high level. The functional consequence of this sudden change could rapidly modify energy metabolism of cells, resulting in an augmented oxygen and glucose consumption. The myocardium normally responds to this change by altering the substrate metabolism to increase energy efficiency. Myocytes under the ischemic condition may not function very well and prevent this adaptive response, therefore, leading to further injury. This highlights the need for developing specific PTEN inhibitors to achieve safe and reversible manipulation of the PTEN function.

To investigate the specific effects of PTEN inhibition on I/R, cardiac morphology and postinfarction function should be part of the phenotypic analysis at both basal and post-I/R. In our study, IS measured at 4 h and 24 h after reperfusion was significantly smaller in the BpV-treated groups than in the control group (Figs. 3 and 4). Echocardiography showed the attenuation of left ventricular dilatation and the deterioration of left ventricular systolic function when BpV was administered (Fig. 5). It was reported that the chronic inhibition of PTEN caused cardiac hypertrophy and contractile dysfunction (29). The prolonged inhibition of PTEN with the subsequent uncontrolled activation of Akt and the prosurvival kinases may induce malignancies, myocardial hypertrophy, and fibrosis (7, 13, 18, 21–23, 28). In our current study, the transient inhibition of PTEN by BpV did not lead to contractile dysfunction as demonstrated by echocardiography. Therefore, the transient inhibition of PTEN immediately before ischemia or reperfusion is a plausible target for minimizing the damage of I/R injury without contractile dysfunction consequences. These will offer us a promising tool for pharmacological manipulation of PTEN to study the significance of PTEN in ischemic heart disease.

Two recent studies have shown that the genetic inactivation of PTEN protects against I/R injury in an isolated heart model. Siddall et al. (29) showed that the threshold of ischemic preconditioning was significantly reduced in the PTEN+/− mice. Ruan et al. (26) reported that PTEN inactivation improved heart function and limited IS compared with that in the
control. PTEN inactivation led to decreased apoptosis and greater ERK and BCL-2 activities following I/R injury. Ruan et al. (26) found that the myocardial levels of p-ERK-1/2 were increased after I/R in the PTEN knockdown mice compared with the wild-type mice. Our findings are in agreement, since BpV increased the myocardial levels of p-ERK-1/2 after I/R (Fig. 6). Activating prosurvival kinase cascades of PI3K/AKT/eNOS and ERK-1/2 at the time of reperfusion have been demonstrated to confer protection against reperfusion-induced injury (8). ERK-1/2 is a member of the prosurvival kinases and downstream of PI3K (1, 8, 9, 25). PTEN inhibition enhances PI3K activity and, therefore, subsequently changes in downstream signaling. ENOS is considered to be essential for mediating protection by ischemic preconditioning, especially late ischemic preconditioning (5, 9). Here we show that the administration of either BpV-BI or BpV-BR augmented eNOS phosphorylation. Moreover, L-NAME, a nonspecific NOS inhibitor, abrogated the IS-limiting effect of BpV. Our data would suggest that PI3K/Akt/eNOS/ERK signaling mediates the cardioprotective effect of PTEN inhibition by BpV. Although the salvage survival signaling PI3K/Akt is stimulated at the onset of reperfusion, however, without pharmacological manipulation, there is less PI3K/Akt activity to constitutively protect the heart from reperfusion.

Collectively, these results support the notion that PTEN inhibition could have therapeutic implications in the survival of the myocardium following I/R injury. It is a great opportunity to investigate cardioprotection of PTEN inhibition on both acute I/R injury and long-term remodeling. Additional studies, however, will be required to fully delineate the biochemical mechanisms for PTEN-mediated pathway in ischemic disease.

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DISCLOSURES
No conflicts of interest are declared by the author(s).

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