Cytoprotective Effect of Sodium Orthovanadate on Ischemia/Reperfusion-Induced Injury in the Rat Heart Involves Akt Activation and Inhibition of Fodrin Breakdown and Apoptosis

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ABSTRACT

In a rat model of myocardial ischemic infarction, sodium orthovanadate rescued cells from ischemia/reperfusion injuries. Rats underwent 30 min of myocardial ischemia by occluding the left coronary artery followed by 24 h of reperfusion. Post-treatment with orthovanadate reduced infarct size in a dose-dependent manner. Orthovanadate treatment also ameliorated contractile dysfunction of the left ventricle 72 h after reperfusion. The cytoprotective action of orthovanadate treatment was closely associated with inhibition of fodrin breakdown. Since orthovanadate is a potent inhibitor for protein tyrosine phosphatases, thereby activating tyrosine kinases and phosphatidylinositol 3-kinase (PI3K) pathways, we investigated activities of protein kinase B (Akt), a downstream target of PI3K in cardiomyocytes. Orthovanadate-induced cytoprotection was associated with partial restoration of reduced Akt activity following myocardial infarction. Restoration of Akt activity by orthovanadate treatment correlated positively with increased phosphorylation of glycogen synthase kinase-3β and Bad in cardiomyocytes. Furthermore, orthovanadate treatment inhibited caspase-3 activation induced by ischemia. Taken together, orthovanadate post-treatment rescued cardiomyocytes from ischemia/reperfusion injuries via Akt activation and inhibition of fodrin breakdown, thereby inhibiting apoptosis.

To restore coronary flow, percutaneous transluminal coronary angioplasty, percutaneous transluminal coronary recanalization, or stenting has been applied to patients with acute myocardial infarction (Stone et al., 1993; Michels and Yusuf, 1995). However, the prognosis is not always excellent even if coronary reperfusion is completely achieved. In some cases, reperfusion itself causes myocardial dysfunction, which has been recognized as ischemia/reperfusion injury based on findings from animal experiments (Braunwald and Kloner, 1985; Kloner, 1993). Ischemia-induced cardiac dysfunction reflects a combination of cell death and myocardial dysfunction. Myocardial cell death itself occurs through both apoptotic and necrotic cell death, although indistinct cell death is also seen based on morphological and biochemical features (Ohno et al., 1998; Matsui et al., 2001). Several lines of evidence suggest that the progressive loss of cardiomyocytes by apoptosis significantly contributes to the development of heart failure (for a recent review, see Haunstetter and Izumo, 1998).

The ability of trophic factors to promote survival has been partly attributed to PI3K/protein kinase B (Akt) signaling (Datta et al., 1999). Akt exerts antiapoptotic properties either by activating antiapoptotic targets or inactivating pro-apoptotic factors. Akt targets include the Bcl-2 family member Bad (Bcl-associated death promoter) (Datta et al., 1997), procaspase-9 (Cardone et al., 1998), glycogen synthase kinase (GSK)-3β (Pap and Cooper, 1998), and the transcription factors such as nuclear factor-κB (Romashkova and Makarov, 1999) and members of the Forkhead family (Brunet et al., 1999; Kawano et al., 2002). Orthovanadate (Na3VO4; OV) is a phosphate analog generally thought to bind as a transition state analog to phosphoryl transfer enzymes. Orthovanadate inhibits ATPases such as Na+/K+-ATPase and Ca2+/Mg2+-ATPase at a high concentration (millimolar) and phosphoprotein tyrosine phosphatases at a low concentration (nanomolar to micromolar range) (Simons, 1979). A recent study also showed that vanadate directly activates PI3K through an increased H2O2 production in the human prostate cancer cell line (Gao et al., 2002). The PI3K/Akt activation by vanadate...
accounted for the induction of hypoxia-inducible factor 1α and vascular endothelial growth factor in the cell line. Furthermore, like insulin-like growth factor-I, vanadate activates tyrosine kinases and increases intracellular tyrosine phosphorylation levels via inhibition of nonselective protein tyrosine phosphatases (Swarup et al., 1982), thereby activating both Akt and mitogen-activated protein kinase (Zhao et al., 1996; Wijkander et al., 1997).

We previously showed that orthovanadate treatment rescues neurons from delayed neuronal death in the CA1 region of the hippocampus after transient forebrain ischemia via activation of both PI3K/Akt and mitogen-activated protein kinase pathways (Kawano et al., 2001; Hasegawa et al., 2003). We therefore asked whether orthovanadate elicits cytoprotective activity in ischemic infarction of peripheral organs. Here we show for the first time that orthovanadate treatment rescues cardiomyocytes from ischemia/reperfusion injury in the rat heart. Furthermore, in addition to activation of Akt, we show that inhibition of fodrin breakdown promoted by orthovanadate is closely associated with its myocardial protective action.

Materials and Methods

Materials. Anti-fodrin breakdown products antibody was a gift of Dr. Y. Shirasaki (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) (Sato et al., 1999). The following reagents and antibodies were obtained from respective sources: sodium orthovanadate, 2,3,5-triphenyltetrazolium chloride (TTC), and Evans blue (Sigma-Aldrich, St Louis, MO); anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-GSK-3α/β antibody (Cell Signaling Technology Inc., Beverly, MA); anti-Akt antibody and anti-phospho-Akt antibody (Ser-437) (Upstate Biotechnology, Lake Placid, NY); anti-rabbit antibody (Amersham Biosciences Inc., Piscataway, NJ); anti-sheep antibody (Jackson ImmunoResearch Laboratories, West Grove, PA); anti-goat IgG (Chemicon International, Temecula, CA); and horseradish peroxidase-conjugated streptavidin (Zymed Laboratories, South San Francisco, CA). Other reagents were of the highest quality available (Wako Pure Chemicals, Osaka, Japan).

Animals. Male Sprague-Dawley rats (10–12 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Rats were housed under climate-controlled conditions with a 12-h light/dark cycle and provided with standard food and water ad libitum. An acclimation period of at least 1 week was provided before initiating the experimental protocol. All procedures for handling animals were approved by the Animal Experimentation Committee of Tohoku University Graduate School of Pharmaceutical Sciences.

Myocardial Ischemia and Reperfusion in Rats. To induce myocardial infarction, the myocardial ischemia/reperfusion model was established according to a method reported by Aihara et al. (2000) with minor modifications. Briefly, rats were anesthetized with 40 mg/kg sodium pentobarbital i.p. The trachea was intubated, and the animal was artificially ventilated with room air by an animal respirator (SN-480-7; Shinano, Tokyo, Japan) with a frequency of 54 strokes/min and a tidal volume of 1.5 ml/100 g, and the tail vein was injected for drug administration. Left thoracotomy at the fifth intercostal space and pericardiotomy were performed, a 6/0 braided silk suture was placed around the left anterior descending coronary artery, and the coronary artery was occluded by pulling on the suture. A standard limb lead II electrocardiogram was monitored with a cardiograph (PowerLab; AD Instruments, Otago, New Zealand). After thoracotomy of the left side, the proximal portion of the left anterior descending (LAD) artery was surgically occluded for 30 min with a suture (size 6/0 NESCO suture; Azwell Inc., Osaka, Japan). The onset of ischemia was confirmed by the development of cyanosis and typical elevation of the ST segment in the electrocardiogram. At 30 min after occlusion, the heart was reperfused by releasing the ligature, and the thoracotomy was closed.

Drug Administration. Sodium orthovanadate was dissolved in 0.9% saline. Vehicle (0.9% saline) or sodium orthovanadate was infused by means of a syringe infusion pump (0.005 ml/min/100 g of b.wt. over 20 min, i.v.) 10 min before reperfusion or 30 min after reperfusion for postocclusion treatment.

Assessment of Myocardial Infarct Size. The infarct size and ischemic risk area were determined by methods described previously (Watanabe et al., 1995). At 24-h reperfusion, rats were anesthetized, and their hearts were excised and quickly hung on a Langendorff apparatus. After the heart was perfused with phosphate-buffered saline to wash out blood from coronary vessels, the coronary artery was religated. Then 1.5 ml of Evans blue dye (1% w/v) was injected into the aorta and coronary arteries to demarcate the ischemic risk (nonstained) or nonrisk (stained) area of the heart. Heart tissue was sliced on a slicer (RBS-2; Zivic-Miller Laboratories, Ze-lienople, PA), and 1.5-mm thick transverse slices were incubated with 1% TTC solution (Sigma-Aldrich) for 10 min at 30°C. The viable myocardium was stained with brick red (Michael et al., 1995, 1999). Tissue samples were then fixed in 10% formalin solution for 4 to 6 h. The cumulative sizes of the left ventricle (LV), risk area (nonstained with Evans Blue), and infarct area (nonstained with TTC) of each slice were quantified by an image analyzer (Image Gauge Software; Fuji Film, Tokyo, Japan). The LV size and risk and infarct areas of four sections were multiplied by the slice thickness. Risk area and infarct size were defined as [total risk area]/[total LV] × 100% and [total infarct area]/[total ischemic risk area] × 100%, respectively (Michael et al., 1995, 1999; Hutter et al., 1996). Left ventricular pressure was monitored using a polyethylene catheter (PE-50; Clay Adams, Parsippany, NJ) inserted through the right carotid artery and advanced to the left ventricle. Left ventricular contractility ([dP/dt]max) and left ventricular developed pressure (LVPD) were measured with a carotid catheterometer (AP-601G; Niho Kohden, Tokyo, Japan). Arterial blood pressure was measured via a polyethylene catheter (PE-50) inserted into the right carotid artery with a pressure transducer (AP-601G; Niho Kohden).

Western Blot Analysis. At 12 h after reperfusion, the isolated LV was divided into the risk and nonrisk areas with a razor blade. LV tissue samples were then rapidly frozen in liquid nitrogen and stored at −80°C before use. For assays, each frozen sample was homogenized with a homogenizer in a solution (350 µl) containing 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 150 mM NaCl, 1 mM NaVO₃, 30 mM sodium pyrophosphate, 50 mM NaF, 50 µg/ml leupeptin, 25 µg/ml pepstatin A, 50 µg/ml trypsin inhibitor, 1 mM diithiothreitol, and 100 nM calcinic A. Insoluble materials were removed by a 10-min centrifugation at 15,000g. Protein concentrations of each supernatant fraction were determined by the Bradford method using bovine serum albumin as a standard. The supernatants were then boiled in Laemmli’s buffer at 100°C for 3 min. An equal amount of protein for each sample (100 µg of total protein) was separated on a 7.5 to 15% SDS-polyacrylamide gel electrophoresis gel and transferred onto polyvinylidene difluoro membranes (Millipore Corporation, Billerica, MA). The membranes were then incubated for 1 h in Tris-buffere d saline/Tween 20 supplemented with 4.5% nonfat dry milk (blocking solution) at room temperature. Blots were then incubated overnight with the following primary antibodies: anti-fodrin breakdown product antibody (1:1000 dilution), anti-Akt antibody (1:2000), anti-phospho-Akt antibody (1:1000), anti-phospho-GSK-3α/β antibody (1:1000), or anti-caspase-3 antibody (1:500) in blocking solution. The membranes were washed three times in washing buffer (0.02 mol/l Tris-HCl, pH 7.5, 0.15 mol/l NaCl, and 1% Tween 20) and incubated for 90 min with anti-rabbit, anti-goat, or anti-mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences Inc.). After washing, peroxidase-labeled protein was detected using the enhanced chemiluminescence detection system (Amersham Biosciences) and visualized on X-ray film (Fuji Film). The autoradiographs were analyzed by densitometry.
Statistical Analysis. All data are expressed as means ± S.E.M. Multiple comparisons between experimental groups were made by two-way analysis of variance followed by Dunnett’s test. \( P < 0.05 \) was considered significant.

Results

Effects of Orthovanadate on Ischemia/Reperfusion-Induced Myocardial Infarction. We first investigated whether orthovanadate has a protective action against myocardial ischemia/reperfusion injuries in rats. Rats were subjected to a 30-min LAD ligation followed by 24 h of reperfusion (Fig. 1). The area at risk was approximately 50 to 55% in the whole ventricle and did not differ between groups. The infarct size in the vehicle group was 76.7 ± 2.7%. Treatment with orthovanadate (75 and 100 \( \mu \)mol/kg) significantly reduced infarct size in a dose-dependent manner (60.1 ± 2.4% and 46 ± 2.7% in 75 and 100 \( \mu \)mol/kg, respectively). This observation indicates that orthovanadate has cytoprotective effect on ischemia/reperfusion-induced myocardial infarction. To define the clinical benefit in humans, orthovanadate (75 \( \mu \)mol/kg) was infused 30 min after reperfusion. As seen in treatment 10 min before reperfusion, post-treatment with orthovanadate also significantly reduced infarct size (Fig. 1).

Effects of Orthovanadate on Blood Pressure. Orthovanadate is known to increase intracellular calcium concentration, thereby constricting vascular smooth muscle and elevating blood pressure. The alteration in blood pressure affects myocardial infarction size in ischemia/reperfusion. To examine the effects of orthovanadate on blood pressure, we measured blood pressure during its administration (Fig. 2). Intravenous administration of orthovanadate (50 and 75 \( \mu \)mol/kg) did not significantly affect blood pressure (2.13 ± 0.826 and 4.88 ± 2.0 mm Hg) but caused a significant elevation of blood pressure at a dosage of 100 \( \mu \)mol/kg (by 21.63 ± 7.94 mm Hg). Orthovanadate treatment did not affect heart rate (data not shown).

Effects of Orthovanadate on Left Ventricular Function (LVF). Since treatment with orthovanadate at 75 \( \mu \)mol/kg ameliorated the infarct size following ischemia/reperfusion and had no adverse effect on blood pressure, we evaluated left ventricular function (LVF) following treatment at that dosage of orthovanadate. To measure the direct effect of orthovanadate on the heart, we infused orthovanadate (75 \( \mu \)mol/kg) in sham-operated animals. Orthovanadate treatment did not affect LVDP and LV \( \Delta P/\Delta t \) (Fig. 3). Rats were subjected to 30 min of LAD ligation followed by 72 h of reperfusion, and LVF was measured. At 72 h after ischemia/reperfusion, LVDP was significantly higher in the orthovanadate group than that seen in the vehicle group (92.84 ± 9.30 vs. 76.90 ± 5.54 mm Hg/s) (Fig. 3A). LV \( \Delta P/\Delta t \) showed a marked depression in the vehicle group (3633.4 ± 326.52 mm Hg) compared with the sham-operated group (Fig. 3B), and

![Fig. 1. Effects of postocclusion treatment with orthovanadate on ischemia/reperfusion-induced myocardial infarction in rats. A, representative images of heart slices from vehicle or orthovanadate-treated group (100 \( \mu \)mol/kg). B, quantitation of effects of treatment with orthovanadate. Orthovanadate at the indicated dose or vehicle (saline) was intravenously infused (3 ml/kg/h, 50 and 100 \( \mu \)mol/kg over 20 min or 75 \( \mu \)mol/kg over 30 min) beginning 10 min before reperfusion or infused with 75 \( \mu \)mol/kg beginning 30 min after reperfusion. The area at risk was not significantly different among the groups. Treatment at 75 or 100 \( \mu \)mol/kg 10 min before reperfusion or infused with 75 \( \mu \)mol/kg 30 min after reperfusion significantly reduced myocardial infarct size. Each bar represents the mean ± S.E.M. for four experiments. * \( P < 0.05 \) and ** \( P < 0.01 \) versus the vehicle group.](https://jpet.aspetjournals.org/content/jpet/192/4/1251.full.pdf)

![Fig. 2. Effects of orthovanadate treatment on blood pressure. The drug at each dose or vehicle was intravenously infused (3 ml/kg/h, 50 and 100 \( \mu \)mol/kg over 20 min or 75 \( \mu \)mol/kg over 30 min). Administration of orthovanadate at 100 \( \mu \)mol/kg increased blood pressure. Changes in heart rate were not observed in any group. Each bar represents the mean ± S.E.M. for four experiments.](https://jpet.aspetjournals.org/content/jpet/192/4/1251.full.pdf)
the depression was significantly restored in the orthovanadate treatment group (5136.4 ± 601.12 mm Hg). The LV
\[ \text{dP/dt} \] also showed an elevation in the vehicle group
\[ (3277 ± 302.03 \text{ mm Hg}) \], whereas orthovanadate treatment
significantly restored the elevated LV \[ \text{dP/dt} \] (\(-4780 ± 136.09 \text{ mm Hg}\)) (Fig. 3B). These results demonstrate that
orthovanadate treatment significantly rescued left ventricular function 72 h after reperfusion.

Effect of Orthovanadate on Fodrin Breakdown. Fodrin is a major cytoskeletal component and a target of
calpains and caspase-3. Fodrin breakdown is associated with
cell injury and subsequent apoptosis. Calpains and caspase-3 cleave the 240-kDa fodrin into smaller fragments of 150 and
120 kDa, respectively. To define the cytoprotective mechanisms of orthovanadate, we tested its effect on ischemia/reperfusion-induced fodrin breakdown. The 150-kDa fodrin breakdown product was assessed by Western blotting analysis using a specific antibody recognizing only the 150-kDa breakdown product (Fig. 4). Treatment with orthovanadate (75 μmol/kg) significantly inhibited the breakdown of fodrin 12 h after ischemia/reperfusion compared with the vehicle group (627 ± 138.7% versus 1420 ± 113.3%, approximately 55% inhibition).

Effects of Orthovanadate on Akt Signaling. We previously showed that orthovanadate activated the PI3K/Akt pathway in neurons, thereby eliciting a neuroprotective action in brain ischemia (Kawano et al., 2001). To determine whether the PI3K/Akt pathway functions in the cytoprotective effect of orthovanadate on myocardial ischemia, we assessed Akt activity and phosphorylation of its downstream targets using immunoblotting analysis with phospho-specific antibodies (Fig. 5). To evaluate Akt activity after myocardial ischemia, we measured phosphorylation of Akt at Ser-473, which is required for Akt activation. As seen in forebrain ischemia, myocardial ischemia resulted in dephosphorylation of Akt-Ser-473 12 h after ischemia without changing Akt protein levels (Fig. 5A). Treatment with orthovanadate (75 μmol/kg) significantly inhibited dephosphorylation of Akt (Fig. 5A). To assess Akt activity in vivo, we measured phosphorylation of GSK-3β and Bad, which are in vivo substrates...
for Akt. Consistent with marked reduction of Akt phosphorylation, myocardial ischemia caused a significant decrease in GSK-3β phosphorylation without altering its protein levels. Treatment with orthovanadate (75 μmol/kg) significantly restored GSK-3β phosphorylation up to control levels (Fig. 5B). Although Bad phosphorylation did not change following ischemia, its phosphorylation was significantly elevated in the orthovanadate treatment group. These results indicate that orthovanadate treatment activates Akt signaling pathways, thereby inhibiting apoptotic signaling through phosphorylation of GSK-3β and Bad.

Effect of Orthovanadate on Cleavage of Caspase-3.
Cardiomyocyte apoptosis has been reported in a variety of cardiovascular diseases, including myocardial ischemia/reperfusion (MacLellan and Schneider, 1997). Caspase-3, a key molecule in apoptotic signaling, is cleaved in response to activation by caspase-8 or caspase-9. We asked whether orthovanadate inhibits apoptotic signaling via inhibition of caspase-3 activation. Treatment with orthovanadate (75 μmol/kg) significantly blocked myocardial ischemia-induced cleavage of caspase-3 12h after ischemia/reperfusion (91.1 ± 18.7% in the orthovanadate group versus 158 ± 16.6% in vehicle groups) (Fig. 6). These experiments support our hypothesis that orthovanadate-mediated activation of Akt signaling inhibits myocardial apoptosis.

Discussion
This study demonstrates that orthovanadate treatment protects cells from myocardial infarction induced by ischemia/reperfusion with concomitant recovery of LV +dP/dt, −dP/dt, and LVDP. We previously reported that pretreatment with orthovanadate rescues neurons from ischemic injury in the gerbil hippocampus (Kawano et al., 2001). In the present study, treatment with orthovanadate protected cardiomyocytes against ischemia/reperfusion injury, even when administered 20 min after occlusion or 30 min after reperfusion. The observation that post-treatment with orthovanadate significantly rescues decreased Akt activity following ischemia is particularly important in the clinical context of treating heart attacks in humans. The preservation of Akt activity possibly accounts for the orthovanadate-induced cytoprotective action in cardiomyocytes as well as neurons, as previously described (Kawano et al., 2001).

Fodrin breakdown is associated with the loss of membrane integrity, thereby leading to necrosis in the myocardium. Consistent with our observation, loss of fodrin (spectrin) from sarcolemmal membranes has been documented in ischemic myocardial infarction (Armstrong et al., 2001). Calpain activation following ischemia/reperfusion in the rat heart has also been reported by Kakkar et al., (2001). Although the precise mechanism underlying orthovanadate-induced inhi-
In ischemia/reperfusion injury, mitochondria-initiated apoptosis of cardiac cells may also contribute to cell death. This intrinsic pathway of apoptosis is initiated by the release of cytochrome-c from the intermembrane space of mitochondria into the cytoplasm through mechanisms not entirely understood. In the cytoplasm, cytochrome-c interacts with apoptotic protease-activating factor-1, which recruits procaspase-9 and forms a macromolecular complex called the apoptosome. During this process, caspase-9 is cleaved into active subunits. Active caspase-9, in turn, cleaves downstream caspases, such as caspase-3 and caspase-7. Here we have shown that cleavage of caspase-3 occurs in the myocardial infarction. Our data clearly demonstrate that orthovanadate treatment inhibits ischemia-induced caspase-3 activation in addition to inhibition of calpain-mediated fodrin breakdown (Fig. 7). Accumulating evidence suggests that cross-talk between calpain and caspase is involved in ischemia-induced apoptosis, particularly in neurons (Neumar et al., 2003; Rami, 2003). In this context, our observation that inhibition of fodrin breakdown and caspase-3 activation can rescue ischemia/reperfusion-induced myocardial infarction as well as ischemic brain injury is critical.

In summary, here we show that orthovanadate protects cardiomyocytes against ischemia/reperfusion injury in the rat heart. Orthovanadate-induced cardiomyocyte protection was mainly elicited by Akt activation and/or preservation. Further studies are needed to define the mechanisms underlying cross-talk between calpain and caspase-3 activation and rescue of myocardial dysfunction by orthovanadate treatment. The peripheral- and postadministration of orthovanadate are possible candidates for heart attack therapy in addition to surgical treatment.
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References


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