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

YOKO TAKADA[1], MASAMI HASHIMOTO[1], JIRO KASAHARA[1], KAZUYUKI AIHARA[2] AND KOHJI FUKUNAGA[1]

[1]Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (Y.T., M.H., J.K., K.F.) [2]Drug Research Department, Toa Eiyo LTD, Fukushima, Japan

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b). Correspondence should be addressed to Dr. Kohji Fukunaga, Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aramaki-Aoba Aoba-ku, Sendai 980-8578, Japan
E-mail: **fukunaga@mail.pharm.tohoku.ac.jp**

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d). ABBREVIATIONS**:** LV, left ventricle; LAD artery, left anterior descending artery; LVF, left ventricular function; GSK, glycogen synthase kinase; IGF-1, insulin like growth factor-1; TTC, triphenyltetrazolium chloride; FBDPs, fodrin breakdown products

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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 hours of reperfusion. Post-treatment with orthovanadate reduced infarct size in a dose-dependent manner. Orthovanadate treatment also ameliorated contractile dysfunction of the left ventricle 24 hours 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 GSK-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.

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Introduction

In order to restore coronary flow, percutaneous transluminal coronary angioplasty, percutaneous transluminal coronary recanalization or stenting has been applied to patients with acute myocardial infarction (Michels and Yusuf, 1995; Stone et al., 1993). 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 (Matsui et al., 2001; Ohno et al., 1998). Several lines of evidence suggest that progressive loss of cardiomyocytes by apoptosis significantly contributes to development of heart failure (for a recent review, Haunstetter and Izumo, 1998).

The ability of trophic factors to promote survival have 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 proapoptotic 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 (NF-κB) (Romashkova and Makarov, 1999) and members of the Forkhead family (Brunet et al., 1999; Kawano et al., 2002). Orthovanadate (Na_3VO_4) is a phosphate analog generally thought to bind as transition state analog to phosphoryl transfer enzymes. Orthovanadate inhibits ATPases such as Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase at the high concentration (mM) and phosphoprotein tyrosine phosphatases at the low concentration (nM-μM range) (Simons, 1979). Recent study also showed that vanadate directly activates PI3K through an increased H_2O_2 production in human prostate cancer cell line (Gao et al., 2002). The PI3K/Akt activation by vanadate accounted for induction of hypoxia-inducible factor 1α and vascular endothelial growth factor in the cell line. Furthermore, like insulin-like growth factor-I (IGF-1), vanadate activates tyrosine kinases and increases intracellular tyrosine

phosphorylation levels via inhibition of non-selective protein tyrosine phosphatases (PTPs) (Swarup et al., 1982), thereby activating both Akt and MAPK (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 MAPK pathways (Hasegawa et al., 2003; Kawano et al., 2001). We therefore asked whether orthovanadate elicits cytoprotective activity in ischemic infarction of peripheral organs. Here we show for the first time that orthovanadate 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.

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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-triphenyl tetrazolium chloride TTC and Evan's blue from Sigma (St Louis, MO. U.S.A.), anti-caspase-3 antibody (Santa Cruz, CA, USA), anti-phospho-GSK-3α/β antibody (Cell Signaling), anti-Akt antibody and anti-phosphor-Akt antibody (Ser-437) (Upstate Biotechnology, Lake Placid, NY, USA), anti-rabbit antibody (Amersham Biosciences, NJ, USA), anti-sheep antibody (Jackson Immuno Research, PA, USA), anti-goat IgG (Chemicon International, CA, USA), and horseradish peroxidase (HRP)-conjugated streptavidin (Zymed Laboratories, CA, USA). Other reagents were of the highest quality available (Wako, Osaka, Japan).

Animals

Male Sprague-Dawley rats (10 to 12 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). Rats were housed under climate-controlled conditions with a 12-hour 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 sodium pentobarbital 40 mg/kg, i.p.. The trachea was intubated, and the animal was artificially ventilated with room air by an animal respirator (SN-480-7, Shinano, Tokyo) 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 ECG was monitored with a cardiograph (Power lab, AD Instruments, Otago, NZ). After thoracotomy of the left side, the proximal

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portion of the left anterior descending (LAD) artery was surgically occluded for 30 min with a suture (size 6-0, Neskosuture, Azwell, Osaka, Japan). The onset of ischemia was confirmed by 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 B.W. over 20 min, i.v.) 10 min before reperfusion or 30 min after reperfusion for post-occlusion treatment.

Assessment of myocardial infarct size

The infarct size and ischemic risk area were determined by methods described previously (Watanabe et al., 1995). After 24-hours reperfusion, rats were anesthetized and their hearts excised and quickly hung on a Langendorff apparatus. After the heart was perfused with PBS to wash out blood from coronary vessels, the coronary artery was religated. Then 1.5 ml Evan's blue dye (1% w/v) was injected into the aorta and coronary arteries to demarcate the ischemic risk (nonstained) or non-risk (stained) area of the heart. Heart tissue was sliced on a slicer (RBS-2, Zivic Miller Lab, Zelienople, PA, USA) and 1.5-mm thick transverse slices were incubated with a 1% triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) solution 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 4-6 hours. 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] x 100% and [Total infarct area]/[Total ischemic risk area] x 100%, respectively (Michael et al. 1995, 1999; Hutter et al. 1996). Left ventricular pressure was monitored using a polyethylene catheter (PE-50, CRAY-ADAMS, Parsippany, NM, USA) inserted through the right carotid artery and advanced to the left ventricle. Left ventricular contractility (±dP/dt) and left ventricular developed pressure (LVDP) were measured with a cardiotachometer (AT-601G, Nihon Kohden, Tokyo, Japan). Arterial blood

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pressure was measured via a polyethylene catheter (PE50) inserted into the right carotid artery with a pressure transducer (AP-601G, NihonKohden, Tokyo, Japan).

Western blot analysis

At 12 hours after reperfusion, the LV isolated was divided into the risk and non-risk 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 mmol/L Tris-HCl (pH 7.5), 0.5% Triton X-100, 4 mmol/L EGTA, 10 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L Na_3VO_4, 30 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 50 μg/ml trypsin inhibitor, 1 mmol/L dithiothreitol (DTT) and 100 nmol/L caliculin A. Insoluble materials were removed by a 10-minute centrifugation at 15,000 x g. 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 7.5-15% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Co., Billerica, MA, USA). The membranes were then incubated for 1 hour in TTBS supplemented with 4.5% nonfat dry milk (blocking solution) at room temperature. Blots were then incubated overnight with primary antibodies: anti-fodrin breakdown product antibody (1:1000, dilution), an anti-Akt antibody (1:2000), an anti-phospho-Akt antibody (1:1000), an 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, 1% Tween20) and incubated for 90 min with anti-rabbit, anti-goat or anti-mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, United Kingdom). After washing, peroxidase-labeled proteins were detected using the ECL detection system (Amersham Biosciences) and visualized on X-ray Film (Fujifilm, Tokyo, Japan). 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 ANOVA followed by Dunnett's test. $P<0.05$ was considered significant.

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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 - 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 μmol/kg) significantly reduced infarct size in a dose-dependent manner, (60.1 ± 2.4% and 46 ± 2.7% in 75 and 100 μmol/kg, respectively). This observation indicates that orthovanadate has cytoprotective effect on ischemia/reperfusion-induced myocardial infarction. To define clinical benefit in man, orthovanadate (75 μmol/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 effects of orthovanadate on blood pressure, we measured blood pressure during its administration (Fig.2). Intravenous administration of orthovanadate (50 μmol/kg and 75 μmol/kg) did not significantly affect blood pressure (2.13 ± 0.826, 4.88 ± 2.0 mmHg), but caused a significant elevation of blood pressure at a dosage of 100 μmol/kg (by 21.63 ± 7.94 mmHg. Orthovanadate treatment did not affect heart rate (data not shown).

Effects of orthovanadate on left ventricular function (LVF)

Since treatment with orthovanadate at 75 μmol/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 of direct effect of orthovanadate on the heart, we infused orthovandate (75 μmol/kg) in sham-operated animals. Orthovanadate treatment did not affect LV diastolic pressure (LVDP) and LV ±dP/dt (Fig. 3). Rats were subjected to 30 min of LAD ligation followed by

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72 hours of reperfusion and LVF was measured. At 72 hours after ischemia/reperfusion, LVDP was significantly higher in the orthovanadate group than that seen in the vehicle group (92.84 ± 9.30 versus 76.90 ± 5.54 mmHg/s) (Fig.3A). LV +dP/dt showed a marked depression in the vehicle group (3633.4 ± 326.52 mmHg) compared to sham-operated group (Fig. 3B), and the depression significantly restored in the orthovanadate treatment group (5136.4 ± 601.12 mmHg). The LV –dP/dt also showed an elevation in the vehicle group (-3277 ± 302.03 mmHg), whereas orthovanadate treatment significantly restored the elevated LV -dP/dt (-4780 ± 136.09 mmHg) (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 breakdown of fodrin 12 h after ischemia/reperfusion compared to 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 (75μmol/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

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marked reduction of Akt phosphorylation, myocardial ischemia caused a significant decrease in GSK-3β phosphorylation without altering its protein levels. Treatment with orthovanadate (75μmol/kg) significantly restored GSK3β 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. (Fig.5C). 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.

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Discussion

This study demonstrates that orthovanadate treatment protects cells from myocardial infarction induced by ischemia/reperfusion with concomitant recovery of LV +dP/dt and -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. 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 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 inhibition of fodrin breakdown is unclear, the present study suggested that orthovanadate treatment prevents loss of membrane integrity though inhibition of fodrin breakdown in cardiomyocytes.

Like orthovanadate-induced neuroprotection (Fukunaga and Kawano 2003; Kawano et al., 2001), preservation of Akt activity by orthovanadate treatment is likely the primary basis of its cytoprotective action in myocardial infarction. IGF-1, an endogenous growth factor activating PI3K/Akt signaling, is known to suppress myocardial apoptosis and improve myocardial function in various experimental models of cardiomyopathy, including ischemia/reperfusion (Lee et al., 1999; Buerke et al 1995). Activated Akt is believed to suppress apoptosis though phosphorylation of several substrates including the Bcl-2 family member Bad (Datta et al., 1997), caspase 9 (Cardone et al., 1998), Forkhead transcription factors (Burgering and Kops, 2002) and GSK-3β (Pap and Cooper, 1998). In the present study, we defined downstream targets of Akt in cardiomyocytes. For example, orthovanadate rescue of Akt activity blocked GSK-3β dephosphorylation and promoted Bad

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phosphorylation. We previously did not find apparent dephosphorylation of Bad in brain ischemic infarction (unpublished observation). Although Bad dephosphorylation in the myocardium is not associated with decreased Akt activity following ischemia/reperfusion, its phosphorylation is, however, markedly potentiated in cardiomyocytes by orthovanadate treatment. By contrast, GSK-3β phosphorylation is closely correlated with decreased Akt activity following ischemia and, similar to its response to Akt activity, is potentiated by orthovanadate treatment. These results suggest that both Bad and GSK-3β are Akt targets and mediate its cytoprotective action in cardiomyocytes. In addition to inhibition of necrosis, orthovanadate inhibits apoptosis in cardiomyocytes following ischemic infarction.

In ischemia/reperfusion injury, mitochondria-initiated apoptosis of cardiac cells may also contribute to cell death. This intrinsic pathway of apoptosis is initiated by 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 (Apaf-1), which recruits pro-caspase-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 post-administration of orthovanadate is possible candidate for heart attack therapy in addition to surgical treatment.

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Footnote

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Figure legends

Fig.1. Effects of post-occlusion treatment with orthovanadate on ischemia/reperfusion-induced myocardial infarction in rats. (A) Representative images of heart slices from vehicle or orthovanadate-treated group (100 μmol/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、100μmol/kg over 20 min or 75μmol/kg over 30 min) beginning 10 min before reperfusion or infused 75 μmol/kg beginning 30 min after reperfusion. The area at risk was not significantly different among the groups. Treatment at 75 or 100μmol/kg 10 min before reperfusion or 75 μmol/kg 30 min after reperfusion significantly reduced myocardial infarct size. Each bar represents the mean ± S.E.M. for four experiments. *$P < 0.05$, **$P < 0.01$ vs. the vehicle group.

Fig.2. Effects of orthovanadate treatment on blood pressure. The drug at each dose or vehicle was intravenously infused (3 ml/kg/h, 50、100μmol/kg over 20 min or 75μmol/kg over 30 min). Administration of orthovanadate at 100 μmol/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.

Fig.3. Effects of orthovanadate treatment on LVDP and LV dP/dt 72 hours after ischemia/reperfusion. Orthovanadate (OV) 75μmol/kg or vehicle was intravenously infused (3 ml/kg/h, over 30 min) beginning 10min before reperfusion. (A) In sham-operated animals, treatment with orthovanadate alone did not affect LVDP over 1 hour after treatment. LVDP was significantly reduced 72 hours after ischemia/reperfusion injury and partly restored by treatment with orthovanadate. (B) In sham-operated animals, treatment with orthovanadate alone did not affect ±dP/dt over 1 hour after treatment. Treatment with orthovanadate improved ±dP/dt 72hours after ischemia/reperfusion. Heart rate was about 400/min without differences between the groups, and mean blood pressure was about100 mmHg in the sham-operated group or 80–90 mmHg in the vehicle and orthovanadate-treated group. Each bar represents the mean ± S.E.M. for six experiments (vehicle group) or five experiments (normal and orthovanadate group). *$P < 0.05$ vs. the IR + vehicle group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs the sham-operated group.

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Fig.4. Effect of orthovanadate treatment on fodrin breakdown 12 hours after ischemia/reperfusion. (Upper) Representative image of an immunoblot probed with a specific antibody recognizing a 150 kDa-fodrin breakdown product. (Lower) Quantitative analysis of the fodrin breakdown product (bdp) was performed by densitometric analysis of the blots. Data are expressed as percentage of value of sham-operated rats. Treatment with orthovanadate (OV) reduced fodrin breakdown 12hours after ischemia/reperfusion. Each bar represents the mean ± S.E.M. **P < 0.01 vs. the vehicle group.

Fig.5. Effect of orthovanadate treatment on Akt, GSK3□ and Bad phosphorylation 12 hours after ischemia/reperfusion. Representative images of immunoblots using antibodies against phosphorylated Akt (Ser-473) (A), GSK-3β (Ser-9) (B) or Bad (Ser-136) (C) are shown in the upper parts of each panel. Total amounts of these three proteins were unchanged following ischemia as shown in the lower lanes of each immunoblot using conventional antibodies. Quantitative analyses of phospho- Akt, GSK3β or Bad levels performed by densitometric analyses are shown in the lower half of each panel. Data are expressed as percentage of value of sham-operated rats. Treatment with orthovanadate (OV) increased Akt, GSK3β and Bad phosphorylation 12hours after ischemia/reperfusion. Each bar represents the mean ± S.E.M. $^{\#\#}$P<0.01 vs. the sham-operated animals; **P < 0.01 vs. the vehicle group.

Fig.6. Effect of orthovanadate treatment on caspase-3 cleavage 12 hours after ischemia/reperfusion. (Upper panel) Representative image of an immunoblot probed with a specific antibody recognizing a 17-kDa caspase-3 cleavage product. (Lower panel) Quantitative analysis of the relative value of caspase-3 cleavage was performed by densitometric analysis. Data are expressed as percentage of value of sham-operated rats. Treatment with orthovanadate (OV) reduced caspase-3 cleavage 12hours after ischemia/reperfusion. Each column represents the mean ± S.E.M. *P < 0.05 vs. the vehicle group.

Fig.7. Schematic representation of the specific cytoprotective actions of orthovanadate

on ischemia-reperfusion-induced myocardial infarction. Binding of trophic/survival factors to tyrosine kinase receptors activate Akt through PI3k and phosphatidylinositol-dependent kinase-1 (PDK1) activation. Ischemia/reperfusion caused an inactivation of Akt thereby promoting apoptosis pathways such as GSK-3β, Bad and caspase-3 cascades. Ishcemic insults also induced calpain activation by Ca^{2+} mobilization. Orhtovanadate used in the present study inhibits protein tyrosine phosphatases (PTPs) or promotes PI3K activity by production of H_2O_2, thereby promoting or preserving the decreased Akt activity following ischemia. Therefore, GSK-3β and Bad is preferentially phosphorylated by Akt and these phosphorylation in turn inhibits the apoptosis signaling including caspase-3 activation. Calpain-induced fodrin breakdown production (BDP) also inhibited by treatment with orthovanadate.

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Fig.1

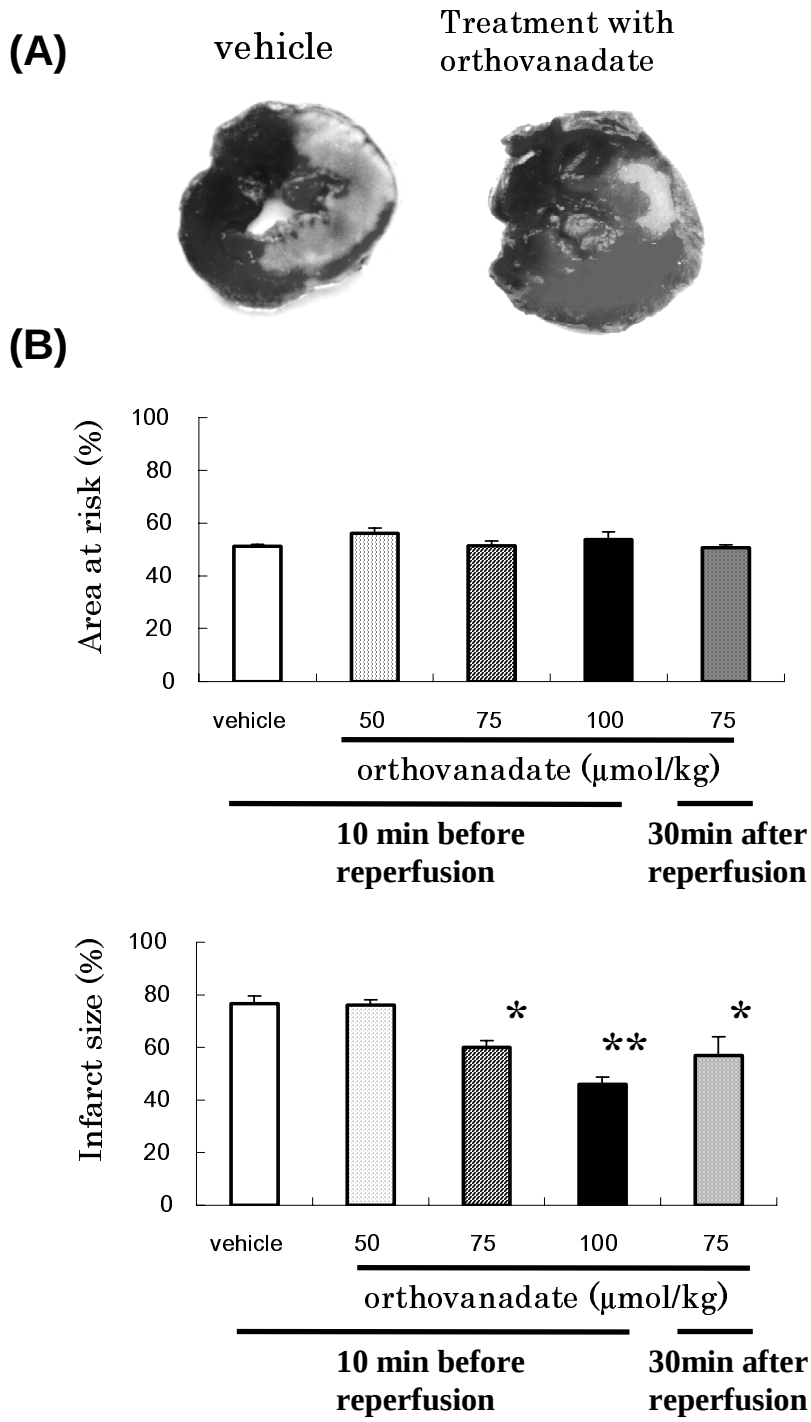

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Fig.2

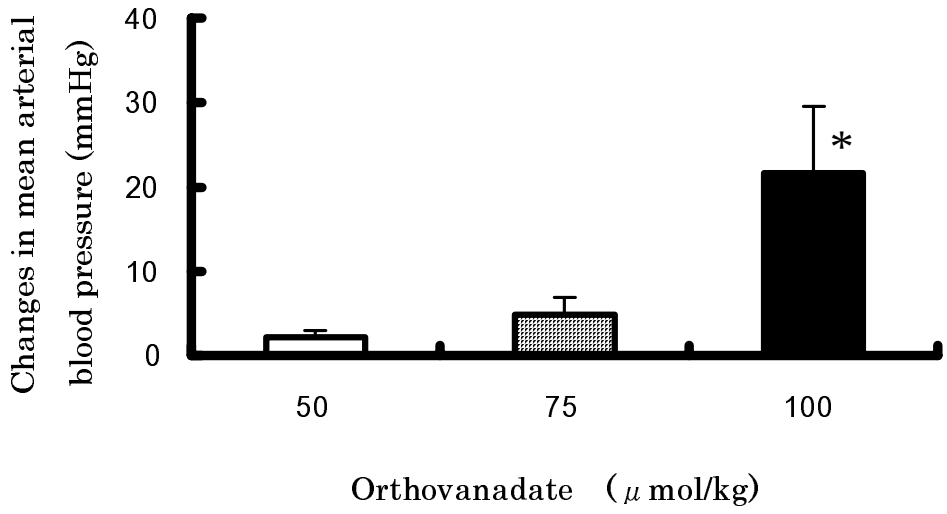

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Fig.3

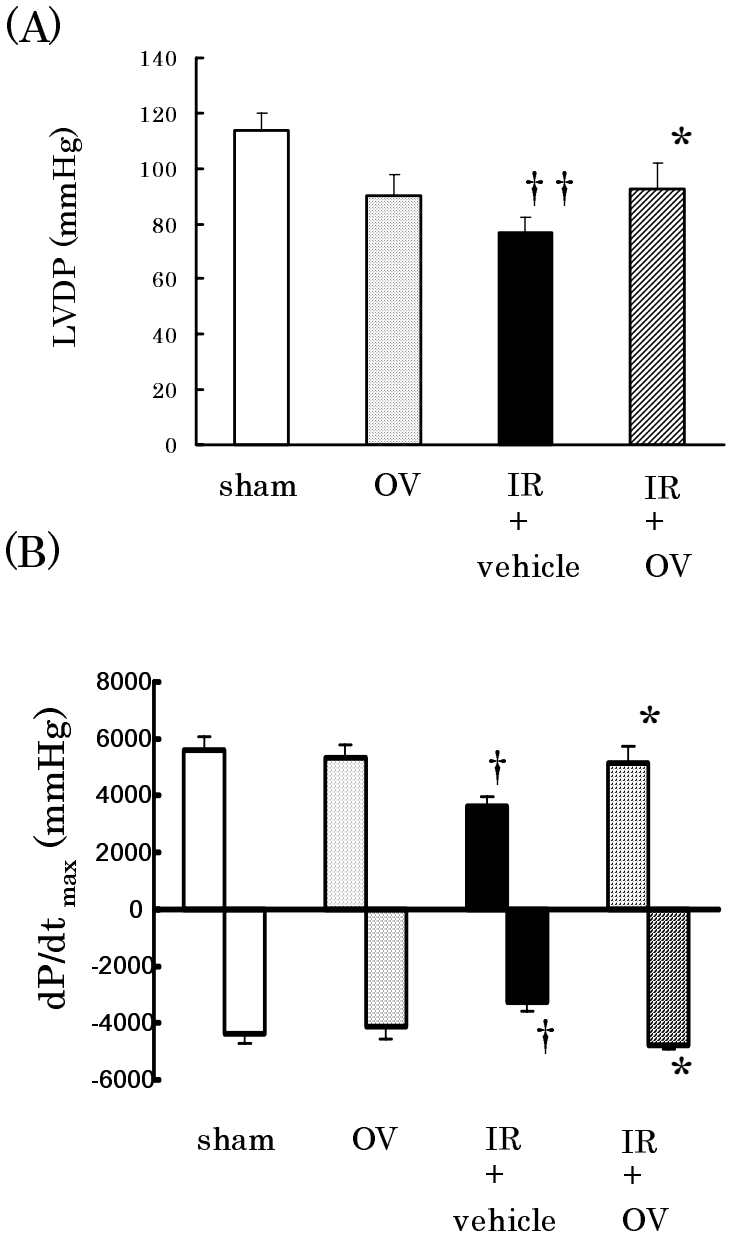

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Fig.4

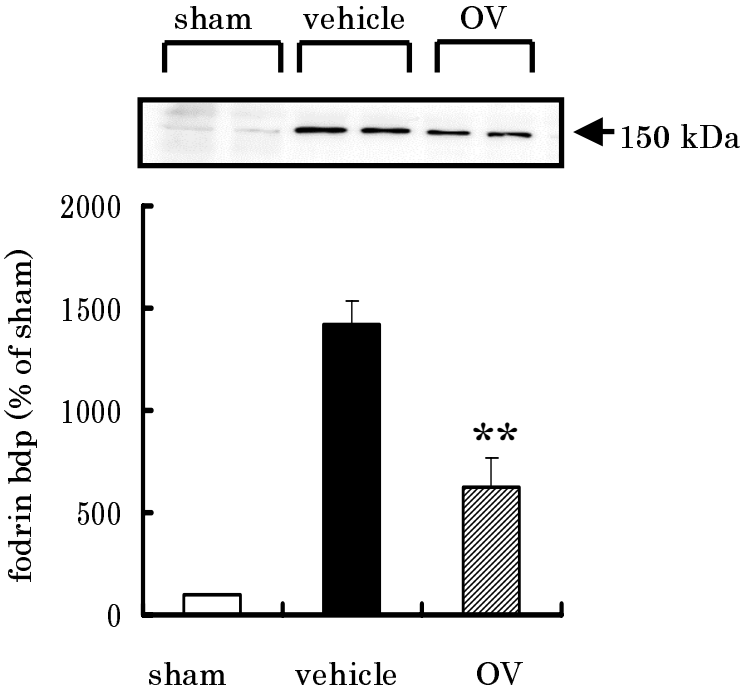

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Fig.5

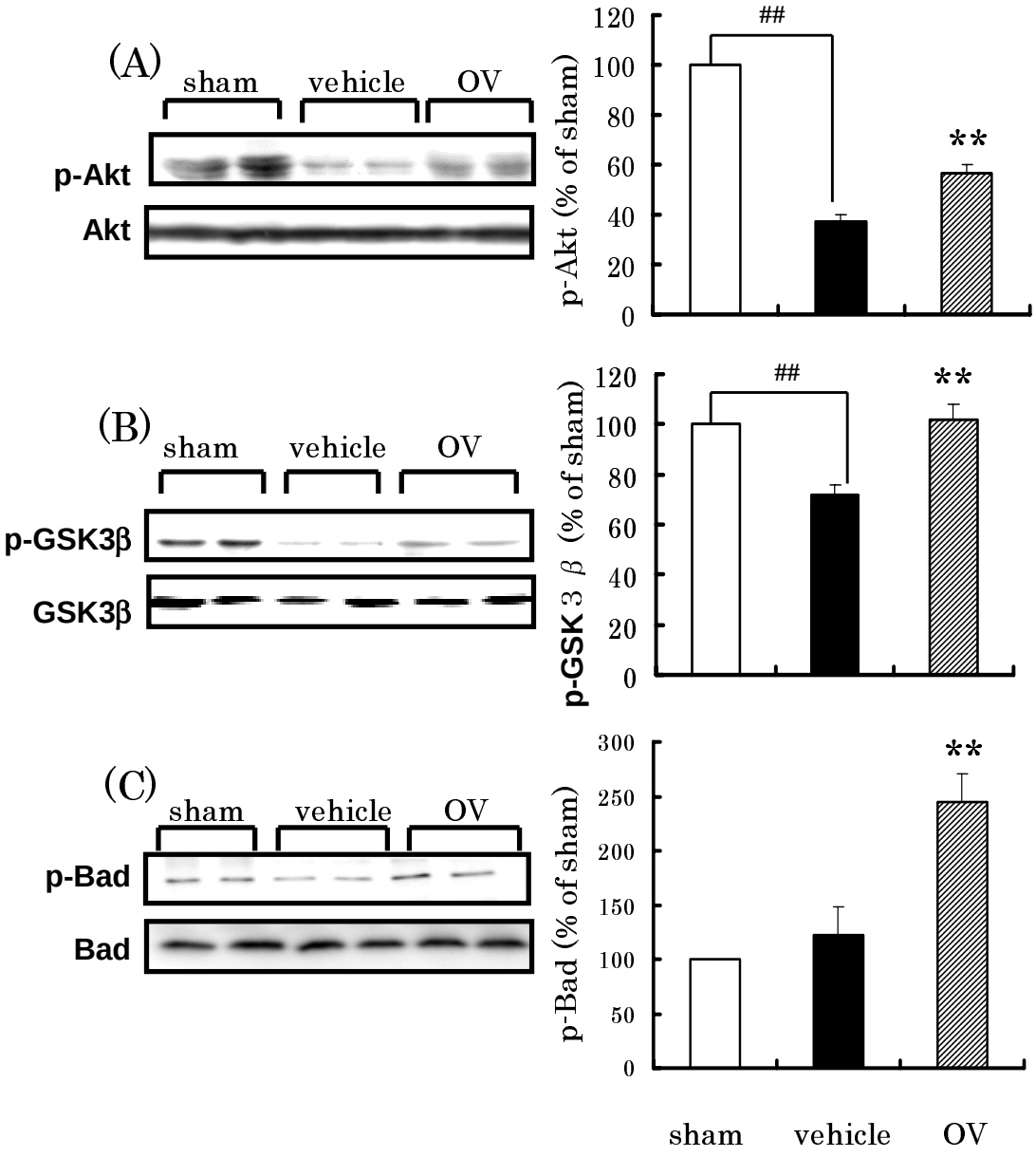

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Fig.6

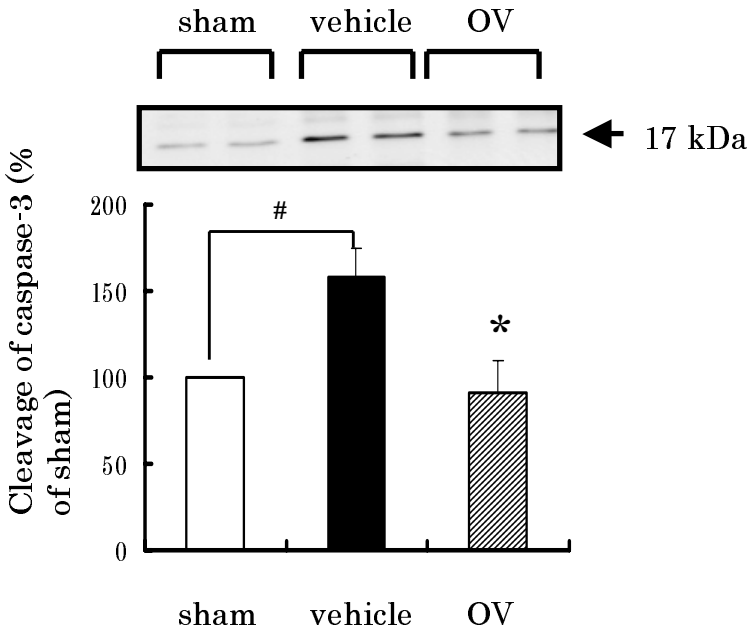

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Fig. 7