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A Novel and Orally Active Poly (ADP-Ribose) Polymerase-1 Inhibitor, 2-[Methoxycarbonyl(4-methoxyphenyl)methylsulfanyl]-1*H*-benzimidazole-4-carboxylic Acid Amide (KR-33889), Attenuates Injury in *in Vitro* Model of Cell Death and *in Vivo* Model of Cardiac Ischemia

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Running title: Cardioprotective effects of PARP-1 inhibitor KR-33889

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ABBREVIATIONS: PARP-1, poly(ADP-ribose) polymerase-1; KR-33889, 2-[Methoxycarbonyl (4-methoxyphenyl) methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide; KR-34285, 2-[Carboxy(4-methoxyphenyl)methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl tetrazolium bromide; LDH, lactate dehydrogenase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ANOVA, analysis of variance.

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ABSTRACT

Blocking of poly (ADP-ribose) polymerase-1 (PARP-1) has been expected to protect the heart from ischemia-reperfusion injury. We have recently identified a novel and orally active PARP-1 inhibitor, 2-[Methoxycarbonyl (4-methoxyphenyl)methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide (KR-33889), and its major metabolite, 2-[Carboxy(4-methoxyphenyl)methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide (KR-34285). KR-33889 potently inhibited PARP-1 activity with an IC_{50} value of 0.52 ± 0.10 μM. In H9c2 myocardial cells, KR-33889 (0.03-30 μM) showed a resistance to hydrogen peroxide (2 mM)-mediated oxidative insult and significantly attenuated activation of intracellular PARP-1. In anesthetized rats subjected to 30 min coronary occlusion and 3 h reperfusion, KR-33889 (0.3-3 mg/kg, i.v.) dose-dependently reduced myocardial infarct size. KR-34285, a major metabolite of KR-33889, exerted similar patterns to the parent compound with equi- or weaker potency in the same studies described in above. In separate experiments for therapeutic time window study, KR-33889 (3 mg/kg, i.v.) given at pre-ischemia, at reperfusion or in both, in rat models also significantly reduced the myocardial infarction compared with their respective vehicle-treated group. Furthermore, the oral administration of KR-33889 (1-10 mg/kg, p.o.) at 1 h before occlusion significantly reduced myocardial injury. The ability of KR-33889 to inhibit PARP in the rat model of ischemic heart was confirmed by immunohistochemical detection of poly (ADP-ribose) activation. These results indicate that

the novel PARP inhibitor KR-33889 exerts its cardioprotective effect in *in vitro* and *in vivo* studies of myocardial ischemia via potent PARP inhibition and also suggest that KR-33889 could be attractive therapeutic candidate with oral activity for several cardiovascular disorders, including myocardial infarction.

Introduction

Poly (adenosine 5'-diphosphate ribose) polymerase (PARP; EC 2.4.2.30, called also PARS and ADP-RT) is chromatin-bound enzyme which is constitutively expressed in most eukaryotic cells (Ikai and Ueda, 1983). The enzyme activity of PARP is significantly increased by DNA single-strand breaks and has been implicated in the regulation of a diverse array of cellular processes from DNA repair (Kraus and Lis, 2003) and genetic stability (Meyer-Ficca et al, 2005) to chromatin organization (Tulin et al, 2003), transcription (Kim et al, 2004), replication (Yang et al, 2004), and protein degradation (Erdelyi et al, 2005). Moderate activation of PARP facilitates the efficient repair of DNA damage. However, excessive activation of this enzyme produces extended chains of ADP-ribose on nuclear proteins and results in a substantial depletion of intracellular NAD^+ and subsequently ATP, which may ultimately cause cell death (Thies and Autor, 1991). Thus, PARP activation plays a pivotal role in the pathogenesis of several disease, including stroke, myocardial infarction, diabetes, shock, and allergy. The use of a PARP inhibitor has been considered to improve cardiac and vascular dysfunction with advanced aging by preventing NAD^+ and ATP depletion (Jagtap and Szabo, 2005) and to augment the activity of topoisomerase inhibitors in the treatment of cancer (Curtin, 2005). In fact, 3-aminobenzamide, nicotinamide, and several phenantridione and isoquinolinone derivatives have been reported as potent PARP inhibitors

that ameliorate ischemic myocardial damage (Wayman et al, 2001). Despite recent developments, a safe and orally active drug for clinical use against ischemic heart disease is presently not available. As an orally active PARP inhibitor would offer significant advantages in terms of safety and convenience with respect to intravenous injection over current clinical therapies, recent research has focused on the identification of small molecule PARP inhibitors with good oral bioavailability and pharmacokinetic properties.

In search for a new class of PARP inhibitors, we have recently identified 2-[methoxycarbonyl(4-methoxyphenyl)methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide (KR-33889, Fig. 1) as a novel, potent and orally active PARP inhibitor, by applying the rational discovery strategies, such as structure-based drug design and conventional structure-activity relationship to improve potencies. Thus, the present study was designed to investigate (i) the PARP-inhibiting activity of KR-33889 and its major metabolite, 2-[carboxy(4-methoxyphenyl)methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide (KR-34285), (ii) whether those compounds possess a resistance to hydrogen peroxide-mediated oxidative insult in H9c2 myocardial cells, and (iii) whether they protect the heart from ischemia-reperfusion injury. Finally, (iv) whether the oral administration of KR-33889 preserves the cardioprotective properties was evaluated.

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Methods

Evaluation of PARP-1 Inhibitory Activity. The converting biotinylated NAD-based colorimetric assays were performed in clear 384-well plates as previously reported (Lee et al, 2005b). Briefly, 12.5 μl of PARP cocktail followed by 5 μl of the inhibitors at various concentrations in PARP assay buffer were added into histone-precoated 384-well microplates. The ADP-ribosylation was initiated by adding 0.5 unit of PARP enzyme per well and incubated for 1 h at room temperature. To detect the extent of ribosylation by PARP-1 in the reaction mixture, plates were followed by the addition of streptavidin-linked peroxidase (Strep-HRP; Trevigen Inc., Gaithersburg, MD, USA) and incubated at 37°C for 30 min. After washing the plates four times with PBS, TACS-Sapphire colorimetric substrate (25 μl/well; Trevigen Inc.) was added and allowed to stand for 10 min for color development. Finally, the reaction was stopped by adding 25 μl of 0.2 N HCl and optical densities were read at 450 nm by Victor II (PerkinElmer Oy, Turku, Finland). The average value of control wells containing only NAD^+ was set as 0% PARP-1 activity, while the average value of control wells containing NAD^+ and PARP-1 (but no inhibitor) was set as 100% PARP-1 activity. The values obtained from the various concentrations of inhibitors were converted to a percentage of PARP-1 activity and plotted. KR-33889 and its several mebolites such as KR-34285 (2-[carboxy(4-methoxyphenyl) methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide),

KR-34292 (2-[methoxycarbonyl(4-hydroxyphenyl)methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide), KR-34314 (2-[carboxy(4-hydroxyphenyl)methylsulfanyl]-1*H*-benzimidazole-4-carboxylic acid amide), and TM-0261 (2-mercapto-1*H*-benzimidazole-4-carboxylic acid amide), were dissolved in 100% dimethylsulfoxide (DMSO) and diluted with distilled water for *in vitro* study resulting in a final concentration of 5% DMSO. All solutions were freshly prepared immediately before the experiments. 6-[5*H*]-phenanthridione and 5-aminoisoquinolinone were used as references.

Cell Culture. H9c2 cells were purchased from the American Tissue Culture Collection (Manasas, VA, USA). Cells were subcultured weekly in culture flasks containing 10 ml Dulbecco's modified Eagle's medium (DMEM; Cambrex Bio Science, Walkersville, MD, USA) with 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD, USA) and antibiotics (25 U/ml penicillin and 25 U/ml streptomycin). Cell was cultured (37°C, 5% CO_2) in 24 well plates (for 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) activity) or in 6 well plates (for western blotting for measuring of PARP activity) until they reached confluence. Cells were used at the following passage numbers: H9c2 cells (P_{3-15}).

Cytoprotective Efficacy in H9c2 Cells. To elucidate the effects of KR-33889 on the

cell insult caused by hydrogen peroxide, cells were preincubated with the compounds for 10 min and then exposed to hydrogen peroxide (2 mM) for 3 h after which time cell insult was assessed. Insult rates in H9c2 cells were determined indirectly by measuring the mitochondrial-dependent reduction of MTT to formazan (i.e. mitochondrial respiration). The amount of formazan formed was quantified by measuring the absorbance of the solution at 540 nm. In addition, the release of LDH in the culture medium was determined as an indicator of cell necrosis by spectrophotometric analysis at 340 nm as described (Jung et al, 2006).

Determination of Radical Scavenging Activity. Free radical scavenging activity was estimated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction assay. A 100 μl of 0.15 mM DPPH solution in methyl alcohol was added to the indicated concentration of each compound in methyl alcohol (total 200 μl). Absorbance at 520 nm was determined after 30 min and scavenging activity was calculated as a percentage of radical reduction.

Western Blot Analysis for PARP Activation. To investigate whether KR-33889 affects hydrogen peroxide-induced PARP activation, we examined the effects of KR-33889 (1, 3 and 10 μM) on hydrogen peroxide-induced activation of PARP. As described above, cells were preincubated with the compounds for 10 min and then exposed to hydrogen peroxide (2 mM) for 10 min. After cell lysates were prepared, equal amounts of protein (30 μg) were

loaded in 10% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham, Piscataway, NJ, USA). The membranes were blocked in 5% dry milk for 1 h, and then proved with PARP monoclonal antibody overnight. Following washing and proving with horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody for 30 min, immunoreactive bands were detected by LumiGLO kit (Beverly, MA, USA). In separate experiments, poly (ADP-ribose) (PAR), the enzymatic end-product of PARP activation, was proved with PAR monoclonal antibody (10H, Alexis, San Diego, CA, USA). All antibodies were purchased from Cell Signaling (Beverly, MA, USA) except for PAR antibody and were used at a 1:1000 dilution. The results of Western blots were quantified by means of Scion Image (ver. 4.02 beta; Scion Corporation. Frederick, MD).

Rat Models of Ischemic Heart. This study conformed to the *Guidelines for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health. Male Sprague-Dawley rats (weighing 380–420 g; Orient, Seoul, Korea) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.; Hanlim Pharm., Seoul, Korea), intubated and connected to a rodent ventilator (SAR 830/P ventilator, CWE, Ardmore, PA, USA) for artificial ventilation with ambient air (stroke volume, 10 ml/kg; 60 strokes/min). Left anterior descending coronary artery occlusion was employed as described previously (Lee et al., 2004;

2005a). All rats were subjected to 30 min of coronary artery occlusion and 3 h of reperfusion. In all groups, arterial blood pressure was continuously monitored via an Isotec pressure transducer (Hugo Sachs Electronic, Hugstetten-March, Germany) connected to a physiograph (WR 3300 Linearcorder, Graphtec, Tokyo, Japan). Electrocardiogram and heart rate were measured by Lead II using an electrocardiogram/rate coupler (Type 576; Hugo Sachs Electronic), both being analyzed by the computer program (PONEMAH physiology platform - model P3 Plus, Gould Inc., Cleveland, OH, USA). KR-33889 was intravenously twice administered by bolus first injection at 5 min prior to ischemia and then at reperfusion. In addition, KR-34285 which is a major metabolite of KR-33889 was administered at same condition. In separate experiments to assess the effects of timing of treatment, KR-33889 (3 mg/kg) was intravenously given by bolus injection at 5 min before ischemia or simultaneously with reperfusion as well as both. Finally, to evaluate the oral activity of KR-33889, animals orally administered by gavage (using a 18-gauge stainless steel feeding needle) with vehicle (0.5% carboxymethylcellulose) or KR-33889 (1, 3 and 10 mg/kg) at 1 h before occlusion. At the end of the reperfusion period, the anatomic area at risk and the nonischemic area were differentiated as previously described (Lee et al., 2004; 2005a). After 3 h of reperfusion, the coronary artery was reoccluded and 2 ml of an 1% Evans blue was injected via tail vein. The heart was immediately removed, and the left ventricle was dissected free from other structures and sliced transversely into 2-mm thick sections. The image of the

sections was captured by Hi-Scope (KH-2200 MD2, HiROX Co., Japan) to determine the area at risk. The sections were then incubated in 1% triphenyltetrazolium chloride for 15 min at 37 ºC and then fixed for 20-24 h in a 10% formalin solution to determine the infarct size, which corresponds to the area not stained by triphenyltetrazolium chloride. The image of the sections was captured again, and analyzed with image analyzing program (Image Pro Plus®, Media Cybernetics, Silver Spring, MD, USA).

PAR Immunohistochemistry. Poly (ADP-ribose) (PAR), an enzymatic end-product of PARP activation, was detected in order to assess the activation of PARP in left ventricle from rat subjected to 30 min ischemia and 3 h reperfusion (Szabo et al., 2002). Heart biopsy specimens were isolated from sham-operated, vehicle and KR-33889 (3 mg/kg, i.v.) treated rats, and fixed in formalin and embedded in paraffin. After section of the probes, slides were deparaffinized and rinsed in water. The antigen was retrieved by incubation in boiling 0.01 M citrate buffer (pH 6.0). Slides were rinsed with PBS, and then endogenous peroxidase activity was quenched with 3% (vol/vol) hydrogen peroxide for 6 min. Non-specific binding sites were blocked using blocking solution (PK 6102, Vector Lab Inc., Burlingame, CA, USA) for 25 min. PAR activation was proved with PAR monoclonal antibody (10H, Alexis, San Diego, CA, USA) for 1 h and it was used 1: 1000 dilutions. After slides washed with PBS, a secondary antibody, biotinylated anti-mouse IgG (Vector Lab Inc.) was used for 30 min. After

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PBS washes, slides were incubated with VECTASTAIN Elite ABC (peroxidase) standard kit (Vector Lab Inc.) for 30 min and then developed using DAB substrate kit (SK-4100, Vector Lab Inc.). Slides were counterstained with Mayer's hematoxylin. Immunohistochemical images were obtained in the peri-infarction (border) zone and represent $n \geq 4$ sections per group.

Pharmacokinetic Study in Rats. Measurement of the plasma concentration of KR-33889 and its metabolite KR-34285 were performed in male Sprague-Dawley rats following intravenous or oral administration at 10 mg/kg. The plasma was collected at 0.03, 0.08, 0.17, 0.33, 0.5, 1, 2, 4, 6, 8, 10, and 24 h after dosing, and the plasma levels of KR-33889 and KR-34285 were determined by LC/MS/MS analysis (Q TRAP mass spectrometer, Applied Biosystem, USA). The separation was performed on a C18 Hypersil Gold column (3 μm, 2.1 × 100 mm; Thermo Electron Corporation, Bellefonte, PA, USA) using a mixture of acetonitrile-10 mM ammonium formate (80:20, v/v) at a flow rate of 0.25 ml/ml. The column temperature was 40°C. The pharmacokinetic parameters were calculated by a non-compartmental method with WinNolin professional Version 4.1 (Pharsight, Inc., Moutain, CA, USA).

Statistical Analysis. All values are expressed as means ± S.D. except the data from rat

models of ischemic heart (means ± S.E.M.). Data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons (Sigma Stat, Jandel Co., San Rafael, CA, USA). In all comparisons, the difference was considered to be statistically significant at $P < 0.05$.

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Results

PARP-1 Inhibitory Activity of KR-33889. To determine PARP-1 inhibitory activity of the compounds, human recombinant PARP-1 was used. As shown in Fig. 2, KR-33889 potently inhibited the enzyme activity with an IC_{50} value of 0.52 ± 0.10 μM. Additionally, KR-34285, a major metabolite of KR-33889, exhibited inhibitory effects (IC_{50} value: 3.70 ± 1.33 μM), while other metabolites such as KR-34292, KR-34314, and TM-0261 caused no PARP-1 inhibitory activity (IC_{50} value > 10 μM). As reference, the IC_{50} values of 6-[5H]-phenanthridione and 5-aminoisoquinolinone for PARP-1 inhibitory activity were 0.96 ± 0.18, and 3.55 ± 0.54 μM, respectively.

Cytoprotective Action of KR-33889 in H9c2 Myocardial Cells. As shown in Fig. 3A, exposure of H9c2 cell to hydrogen peroxide caused a substantial reduction in mitochondrial respiration. Pretreatment of these cells with KR-33889 significantly and concentration-dependently attenuated the impairment in mitochondrial respiration caused by hydrogen peroxide at a concentration higher than 0.1 μM. To confirm the results of cell viability, LDH release as an indicator of cell necrosis was measured in same condition. As shown in Fig. 3B, hydrogen peroxide-induced LDH release activity was markedly decreased at concentrations higher than 0.3 μM. The pretreatment with KR-34285 also caused a

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concentration-dependent attenuation of the impairment in mitochondrial respiration and LDH release activity caused by hydrogen peroxide (Fig. 4). To determine whether KR-33889 or KR-34285 has properties to reduce reactive oxygen species-induced cytotoxicity directly, radical scavenging activity was evaluated using DPPH free radical in the same concentration for cell viability test. KR-33889 and KR-34285 did not exhibit DPPH radical scavenging activity, even at a concentration of 1 mM (data not shown).

Inhibitory Effects of KR-33889 on Intracellular PARP Activation. The activation of intracellular PARP and PAR formation, the enzymatic end-product of PARP activation, in H9c2 cells by exposing hydrogen peroxide can be detected by Western blot. As shown in Fig. 5, hydrogen peroxide caused the rapid proteolytic cleavage of PARP after 10 min as revealed by the appearance of the p85 proteolytic fragment in H9c2 cells. The pretreatment with 3 or 10 μM of KR-33889 significantly decreased intracellular PARP activation and PAR formation caused by exposing hydrogen peroxide.

Cardioprotective Action of KR-33889 in Rat Model of Ischemic Heart. The effects of KR-33889 on myocardial infarct size expressed as a percentage of the area at risk and hemodynamic effects of KR-33889 were shown in Fig. 6 and Supplemental Table 1. In rats subjected to 30 min occlusion followed by 3 h reperfusion, twice administration of KR-33889

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at pre-ischemia and reperfusion resulted in significant reductions of the myocardial infarct size (35.9 ± 2.3, 33.7 ± 1.7, and 30.9 ± 2.2% at 0.3, 1.0 and 3.0 mg/kg, respectively, $P<0.05$) when compared to vehicle-treated group (51.7 ± 1.6%). In all experimental group, the area at risk was similar (approximately 35 - 40%), indicating that the rats in each respective group had same potential for ischemic damage as a result of coronary artery occlusion. KR-34285 also significantly decreased the myocardial infarct size in a dose-dependent manner (39.7 ± 1.5 and 34.8 ± 2.02% at 0.1 and 1.0 mg/kg, respectively, $P<0.05$) compared to vehicle-treated group (51.7 ± 1.6%) (Fig. 7 and Supplemental Table 2). However, other metabolites of KR-33889, such as KR-34292, KR-34314 and TM-0261, showed a minimal effect against myocardial infarction (data not shown). To compare the effects of KR-33889 on myocardial infarct size when KR-33889 was administrated at 5 min before ischemia (Pre-Isch), or with reperfusion (With-Rep), or both in anesthetized rats, we performed additional experiments (Fig. 8 and Supplemental Table 3). In rats, pre-ischemic, with reperfusion as well as both administration of KR-33889 (3 mg/kg, i.v.) significantly reduced the myocardial infarct size (33.6 ± 2.2, 41.4 ± 1.5 and 30.9 ± 2.2%, respectively, $P<0.05$) compared with their respective vehicle-treated group (49.0 ± 1.6, 49.2 ± 0.9 and 51.7 ± 1.6%, respectively) in similar condition of the area at risk. In study to evaluate experiments for oral activity of KR-33889, all doses of KR-33889 significantly reduced the myocardial infarct size (41.9 ± 2.2, 40.7 ± 1.6 and 38.7 ± 1.3% at 1, 3, and 10 mg/kg, p.o., respectively, $P<0.05$) compared to vehicle-treated

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group (49.0 ± 0.8%) (Fig. 9 and Supplemental Table 4). In all studies with rats, KR-33889 and KR-34285 caused no significant changes in the mean blood pressure and heart rate compared with the vehicle-treated group, although the mean arterial pressure of all groups decreased slightly throughout the experiment (Supplemental Table 1-4).

Poly (ADP-Ribose) Polymer Formation and Effect of KR-33889. While there is no detectable poly (ADP-ribose) polymer in sham-operated normal hearts, massive immunostaining of poly (ADP-ribose) polymer was observed in the peri-infarction (border) zone of left ventricle from rat subjected to 30 min ischemia and 3 h reperfusion (Fig. 10). The formation of poly (ADP-ribose) polymer in identical zone was strongly inhibited by administration of KR-33889 at a dose of 3 mg/kg.

Pharmacokinetic Study. The plasma concentration of KR-33889 and KR-34285 in rats were determined at 0.03, 0.08, 0.17, 0.33, 0.5, 1, 2, 4, 6, 8, 10, and 24 h after intravenous or oral administration at 10 mg/kg (Table 1). The serum concentration of KR-33889 could not be measured after intravenous or oral administration of 10 mg/kg KR-33889 because of its low metabolic stability. Although the serum concentration of its metabolite KR-34285 instead of KR-33889 was measured, maximum plasma concentration (C_{max}) of KR-34285 after intravenous or oral administration of 10 mg/kg KR-33889 were 112.0 ± 20.0 or 10.1 ± 5.2

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μg/ml, respectively. The bioavailability of KR-33889 (66.3%; measured as KR-34285) was approximately 6-times larger than that of KR-34285 (11.0%).

Discussion

This study demonstrates that KR-33889 and its metabolite KR-34285 have potent protective effects on hydrogen peroxide-induced cell insult and on ischemia/reperfusion-induced myocardial injury by both i.v. injection and oral administration in rats.

Recently, we have identified that KR-33889 is one of the most potent candidates through the rational discovery strategies, such as structure-based drug design and conventional structure-activity relationship. KR-33889 is a 2-benzylsufanyl-substituted benzimidazole-4-carboxamide derivative substituted with carboxylic ester group at the benzylic carbon, which is important group for *in vivo* cardioprotective effect and oral bioavailability. A molecular docking study indicated that the amide moiety of KR-33889 invariably interacts with the backbone atoms of Gly863 and Ser904, while the benzimidazole aromatic portion interacts, presumably through π-π interaction, with Try907, characteristics of this class of inhibitors (Ishida et al., 2006). As we expected, a novel PARP-1 inhibitor KR-33889 showed more potent inhibitory activity (0.52 μM) of human recombinant PARP-1 than that of 6-[5H]-phenanthridione and 5-aminoisoquinolinone (0.96 and 3.55 μM, respectively).

Consistent with the potent PARP-1 inhibitory activity, KR-33889 exerted cytoprotective activities against hydrogen peroxide-induced cell insult as judged by a mitochondrial-dependent reduction of MTT, an indicator for cell viability (Wayman et al.,

2001), and LDH release, an indicator for necrotic cell death (Filipovic et al., 1999) (Fig. 3). These results were confirmed by investigating intracellular PARP activation and PAR formation in rat myoblastic H9c2 cells (Fig.5). These results reflect that activation of PARP contributes to the cell death caused by oxygen-derived free radicals in rat cardio-myoblasts (Gilad et al., 1997; Bowes et al., 1998) and the inhibition by PARP-1 inhibitors like KR-33889 may attenuates the cell death associated with oxidant stress in rat cardiac myoblasts (Bowes et al., 1999). In contrast, in *in vitro* DPPH assay, KR-33889 showed no radical scavenging activity even at a concentration of 10 μM. These results indicate that KR-33889 in the condition for cell viability test does not modulate the reactive oxygen species-mediated pathway, but instead that the cytoprotective properties is likely due to the result of its specific PARP inhibitory activity.

The importance of the PARP pathway is well documented in various experimental models of myocardial ischemia-reperfusion injury (Zingarelli et al., 1997; Pieper et al., 2000; Yang et al., 2000). Consistent with the cardioprotective properties of other known PARP-1 inhibitors, twice administration of KR-33889 (0.3-3 mg/kg) by i.v. bolus first injection at 5 min prior to ischemia and then at reperfusion resulted in significant reductions of the myocardial infarct size in a dose-dependent manner (Fig. 6). However, the time between the onset of ischemia and the initiation of therapy may consider a critical factor in treating acute ischemic injury. Particularly, like overactivation of PARP, pharmacologically targeting a

downstream event in ischemic injury could theoretically need long time for initiation of therapy (Nakajima et al., 2005). Thus, we assessed the effects of timing of treatment in separate experiments. In our study of therapeutic time window, administration of 3 mg/kg KR-33889 before ischemia (33.6%) and with reperfusion (41.4%) also significantly reduced the myocardial infarct size compared with their respective vehicle-treated group (49.0 and 49.2%, respectively) although the magnitude of the reduction in infarct size was smaller than those of both treatments (30.9%; Fig. 8). These results suggest that treatment of KR-33889 in early ischemia or at reperfusion phase could improve ischemia-reperfusion injury and time window of therapeutic treatment with KR-33889 may be well tolerated.

Having established the cardioprotection of KR-33889, we next determined the cardioprotective potency of metabolites of this agent against *in vitro* model for cell death and the rat model of ischemic heart. According to our pharmacokinetic studies, KR-33889 has been known to undergo a rapid metabolism in rats and results in the formation of several metabolites, such as KR-34285, KR-34292, KR-34314 and TM-0261 (unpublished data). In the present study, KR-34285 exhibited potent PARP-1 inhibitory activity in converting a biotinylated NAD-based colorimetric assay (IC_{50} value: 3.70 μM), whereas other metabolites such as KR-34292, KR-34314 and TM-0261, caused no PARP-1 inhibitory activity (IC_{50} values > 10 μM). In study *in vitro* model for cell death, KR-34285 also exhibited a protective effect against hydrogen peroxide-induced cell injury (Fig. 4). Furthermore, KR-34285 showed

a significant cardioprotection after transient cardiac ischemia in rat model of ischemic heart (Fig. 7), while other metabolites, such as KR-34292, KR-34314 and TM-0261, showed a minimal effect against myocardial infarction. The potency of KR-34285 against myocardial infarction is similar to that of KR-33889, although PARP inhibitory activity of KR-34285 in *in vitro* colorimetric assay (IC_{50} value: 3.70 μM) is about 7 times weaker than that of KR-33889 (IC_{50} value: 0.52 μM). This potency differences observed between *in vitro* assay and *in vivo* assay might be related to low metabolic stability of KR-33889. In pharmacokinetic studies with male Sprague-Dawley rats, the serum concentration of KR-33889 could not be measured after intravenous administration of 10 mg/kg KR-33889 because of its low metabolic stability (Table 1). Accordingly, the serum concentration of its metabolites instead of KR-33889 was measured. The maximum serum concentration of KR-34285 (313.4 μM) was higher than those of other metabolites, KR-34292, KR-34314, and TM-0261 (35.0, 39.0, and 46.6 μM, respectively). Taken together, these results suggest that the cardioprotective effect of KR-33889 in *in vivo* study may be due to those of KR-34285 which is a unique metabolite with potent PARP inhibitory activity.

The next objective of our study was to investigate whether KR-33889 is an orally active against ischemic heart disease. Indeed, an orally active PARP inhibitor could offer significant advantages in terms of safety and convenience with respect to intravenous injection over current clinical therapies. The oral administration of KR-33889 (1-10 mg/kg) at

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1 h before occlusion significantly reduced the myocardial infarct size in a dose-dependent manner (Fig. 9). The serum concentration of KR-34285 reached a maximum of 28.3 μM (10.1 μg/ml) at 1 h after oral administration of KR-33889 (10 mg/kg), which is 7.6 times higher than its IC_{50} value of KR-34285 for the PARP inhibition (Table 1). Thus, these results imply that a concentration of KR-34285 sufficient to inhibit the PARP can be attained in serum 1 h after oral administration of 10 mg/kg KR-33889. The ability of KR-33889 to inhibit PARP in the cardiac myocytes was confirmed by immunohistochemical detection of poly (ADP-ribose) activation (Fig. 10). This result indicates that the cardioprotective properties of KR-33889 *in vivo* assay may be due to the result of its specific PARP inhibitory activity. Cardioprotective mechanisms may be largely influenced by the presence of major risk factors (Ferdinandy et al., 2007). In the present study, however, the efficacy of KR-33889 has not been studied in infarction model in the presence of diabetes or hyperlipidemia. To our best knowledge, this is a first report to show that oral administration of potent PARP inhibitor was effective in ischemic myocardial infarction model.

In conclusion, the present study demonstrates that KR-33889, a novel PARP inhibitor, exhibited significant cardioprotective properties both in *in vitro* and *in vivo*, presumably via inhibition of intracellular PARP activation. Furthermore, the oral administration of KR-33889 preserved the cardioprotective properties in rat model of ischemic heart, and its oral activity may be due to that of an active metabolite, KR-34285. Accordingly, KR-33889 is expected to

be an attractive therapeutic candidate with clinical benefit for cardiovascular disorder including myocardial infarction.

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Footnotes

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The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

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Legends for Figures

Fig. 1. Chemical structures of KR-33889 (A) and its metabolite KR-34285 (B).

Fig. 2. PARP-1 inhibitory activity of KR-33889 and its metabolite KR-34285. IC_{50} values (mean ± S.D.) were calculated from the concentration dependence of the inhibition curves by using computer-assisted nonlinear regression analyses.

Fig. 3. Cardioprotective effects of KR-33889 against hydrogen peroxide-induced oxidative insult in H9c2 cells. Exposure of 2 mM H_2O_2 for 3h produced severe cell damage as evaluated by MTT assay (A) and LDH release assay (B). Damage was significantly decreased by addition of 0.3 to 10 μM KR-33889 10 min before H_2O_2 exposure to the culture medium. Each point represents the means ± S.D. of at least three experiments. *, $p < 0.05$, significantly different from its respective control group.

Fig. 4. Cardioprotective effects of KR-34285 against hydrogen peroxide-induced oxidative insult in H9c2 cells. Exposure of 2 mM H_2O_2 for 3h produced severe cell damage as evaluated by MTT assay (A) and LDH release assay (B). Damage was significantly decreased by addition of 3 to 1000 μM KR-34285 10 min before H_2O_2 exposure to the culture medium. Each point represents the means ± S.D. of at least three experiments. *, $p < 0.05$, significantly different from its respective control group.

Fig. 5. Effects of KR-33889 on hydrogen peroxide-induced intracellular PARP activation and PAR formation in H9c2 cells. Exposure of 2 mM H_2O_2 for 10 min to H9c2 cells caused marked PARP activation and PAR formation, which were inhibited by treatment with KR-33889 (3 and 10 μM). β-tubulin is shown as loading control. Representative immunoblots from three experiments and densitometric evaluation are demonstrated. *, $p < 0.05$, significantly different from normal cell (-). $^{\#}$, $p < 0.05$, significantly different from control cell exposed only H_2O_2.

Fig. 6. Effects of KR-33889 on myocardial infarct size in rats subjected to 30 min occlusion of left anterior descending coronary artery followed by 3 h reperfusion. Drugs were given twice by i.v. bolus at first 5 min before occlusion and then at reperfusion. The area at risk (A) was expressed as a percentage of the left ventricle (LV). The infarct size (B) was expressed as a percentage of the area at risk. Values are mean percentage ± SEM. Vehicle (n = 8), 0.03 mg/kg (n = 8), 0.1 mg/kg (n = 8), 0.3 mg/kg (n = 10), 1 mg/kg (n = 14), and 3 mg/kg (n = 9) of KR-33889. *, $p < 0.05$, significantly different from the vehicle-treated group.

Fig. 7. Effects of KR-34285 on myocardial infarct size in rats subjected to 30 min occlusion of left anterior descending coronary artery followed by 3 h reperfusion. Drugs were given twice by i.v. bolus at first 5 min before occlusion and then at reperfusion. The

area at risk (A) was expressed as a percentage of the left ventricle (LV). The infarct size (B) was expressed as a percentage of the area at risk. Values are mean percentage ± SEM. Vehicle (n = 8), 0.1 mg/kg (n = 8) and 1 mg/kg (n = 10) of KR-34285. *, $p < 0.05$, significantly different from the vehicle-treated group.

Fig. 8. Cardioprotective effect of KR-33889 (3 mg/kg, i.v.) at different treatment timing in rats subjected to 30 min occlusion of left anterior descending coronary artery followed by 3 h reperfusion. KR-33889 were given by i.v. bolus at 5 min before occlusion (Pre-Isch: Vehicle, n = 8; KR-33889, n = 8), at reperfusion (With-Rep: Vehicle, n = 7; KR-33889, n = 8) or both (Both: Vehicle, n = 8; KR-33889, n = 9). The area at risk (A) was expressed as a percentage of the left ventricle (LV). The infarct size (B) was expressed as a percentage of the area at risk. Values are mean percentage ± SEM. *, $p < 0.05$, significantly different from its respective vehicle-treated group. $^{\#}$, $p < 0.05$, significantly different from the Pre-Isch group.

Fig. 9. Cardioprotective effect of KR-33889 (p.o.) on myocardial infarct size in rats subjected to 30 min occlusion of left anterior descending coronary artery followed by 3 h reperfusion. Drugs were orally administered at 1 h before occlusion. The area at risk (A) was expressed as a percentage of the left ventricle (LV). The infarct size (B) was expressed as a percentage of the area at risk. Values are mean percentage ± SEM.

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Vehicle (n = 8), 1 mg/kg (n = 8), 3 mg/kg (n = 9) and 10 mg/kg (n = 6) of KR-33889. *, $p < 0.05$, significantly different from the vehicle-treated group.

Fig. 10. Representative photographs of poly(ADP-ribose) polymer immunostaining in reperfused rat hearts. Light purple-stained cells were result from counterstaining with Mayer's hematoxylin for nucleus detection. Brown-stained cells show poly(ADP-ribose) polymer-positive cells. While there is no detectable polymer in sham-operated normal hearts, poly (ADP-ribose) immunoreactivity was markedly increased in left ventricle from rat subjected to 30-min ischemia and 3-h reperfusion (Vehicle). KR-33889 administration (3 mg/kg, i.v.) inhibited the formation of poly (ADP-ribose) polymer in left ventricle from rat subjected to ischemia/reperfusion. Higher magnification (×40) shows that PARP activation was mainly located in the nuclei of myocytes in the peri-infarction (border) zone. Immunohistochemical pictures represent $n \geq 4$ sections per group.

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TABLE 1

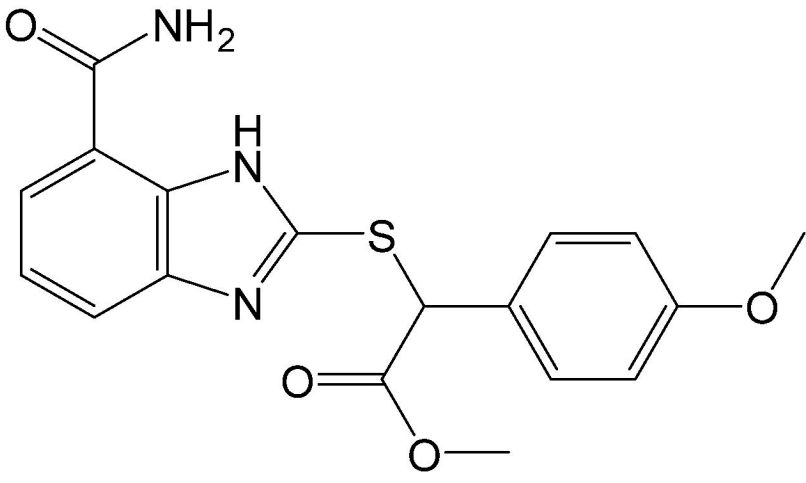
Pharmacokinetic parameters of KR-33889 and its metabolite KR-34285 intravenously or orally administered in male Sprague-Dawley rats *in vivo*.

Parameters	Intravenous administration			Oral administration		
	KR-33889 (10 mg/kg)		KR-34285 (10 mg/kg)	KR-33889 (10 mg/kg)		KR-34285 (10 mg/kg)
	Detected as KR-33889	Detected as KR-34285		Detected as KR-33889	Detected as KR-34285	
C_{max} (μg/mL)	NA	112.0±20.0	NA	ND	10.1±5.2	1.3±0.4
t_{max} (h)	NA	0.03±0.0	NA	NA	0.9±0.1	2.3±1.5
$t_{1/2}$ (h)	3.9±1.8	1.6±0.3	3.6±1.6	NA	17.4±8.2	5.2±0.24
$AUC_{0-\infty}$ (μg·h/mL)	0.2±0.1	66.5±8.4	100.2±21.8	NA	44.1±9.0	11.0±3.0
CL (L/h/kg)	NA	NA	0.10±0.02	NA	NA	0.9±0.2
*V*ss (L/kg)	NA	NA	0.24±0.11	NA	NA	NA
F (%)	NA	NA	NA	NA	66.3	11.0

Values are expressed as mean ± S.D. (n=3). C_{max}, maximum plasma concentration; t_{max}, the time of maximum concentration; $t_{1/2}$, terminal half-life; $AUC_{0-\infty}$, area under the time-concentration curve extrapolated to infinity; CL, clearance; *V*ss, volume of distribution at steady state; *F*, bioavailability; NA, not applicable; ND, not detectable.

Fig. 1.

A



B

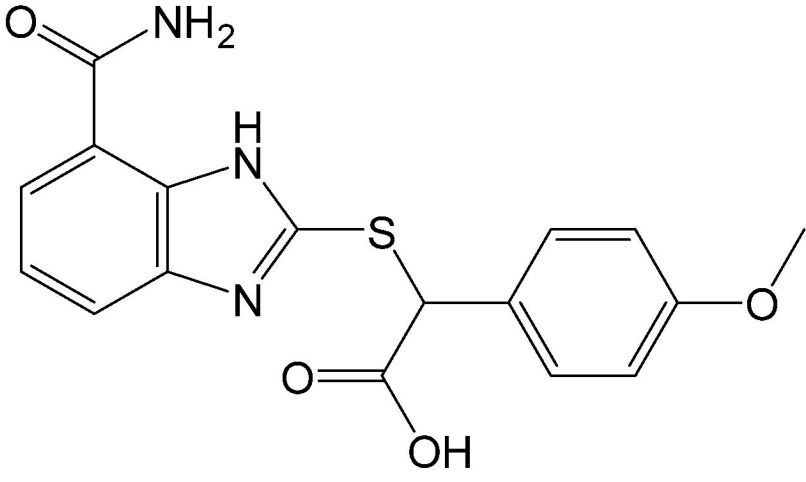


Fig. 2.

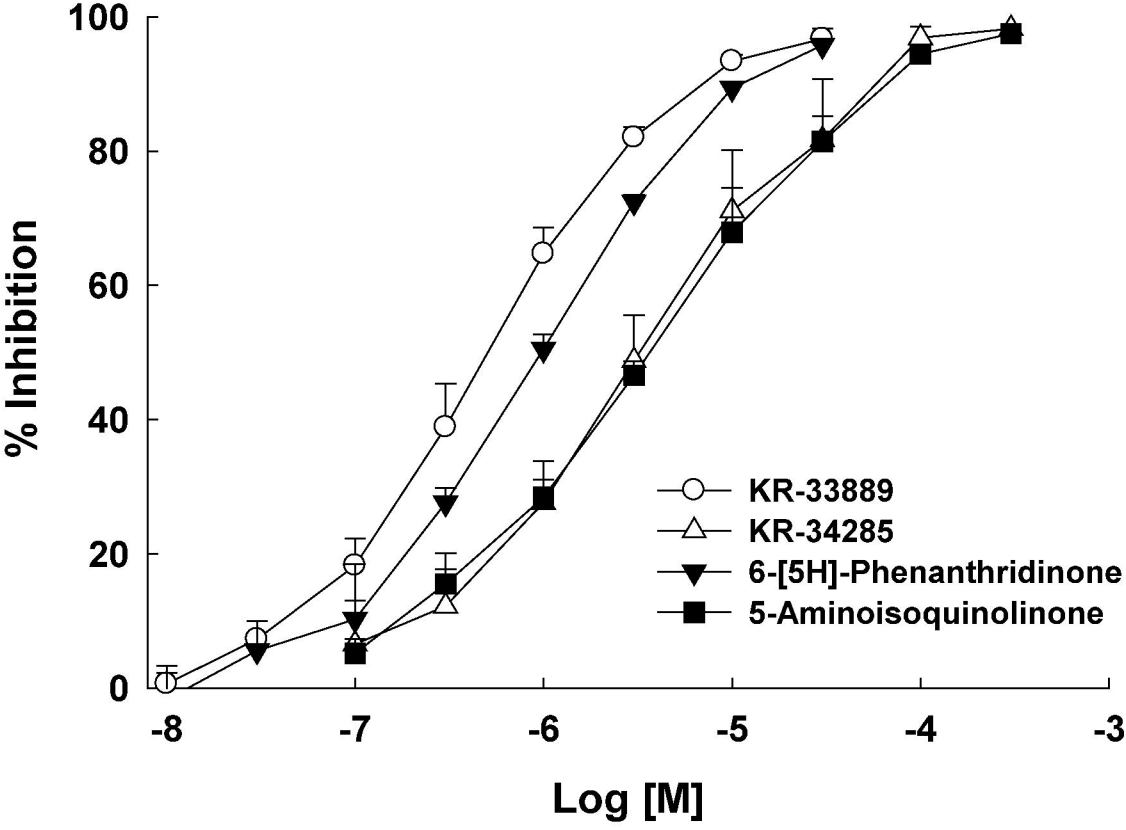

100
80
60
40
20
0
% Inhibition
-8
-7
-6
-5
-4
-3
Log [M]
KR-33889
KR-34285
6-[5H]-Phenanthridinone
5-Aminoisoquinolinone

Fig. 3.

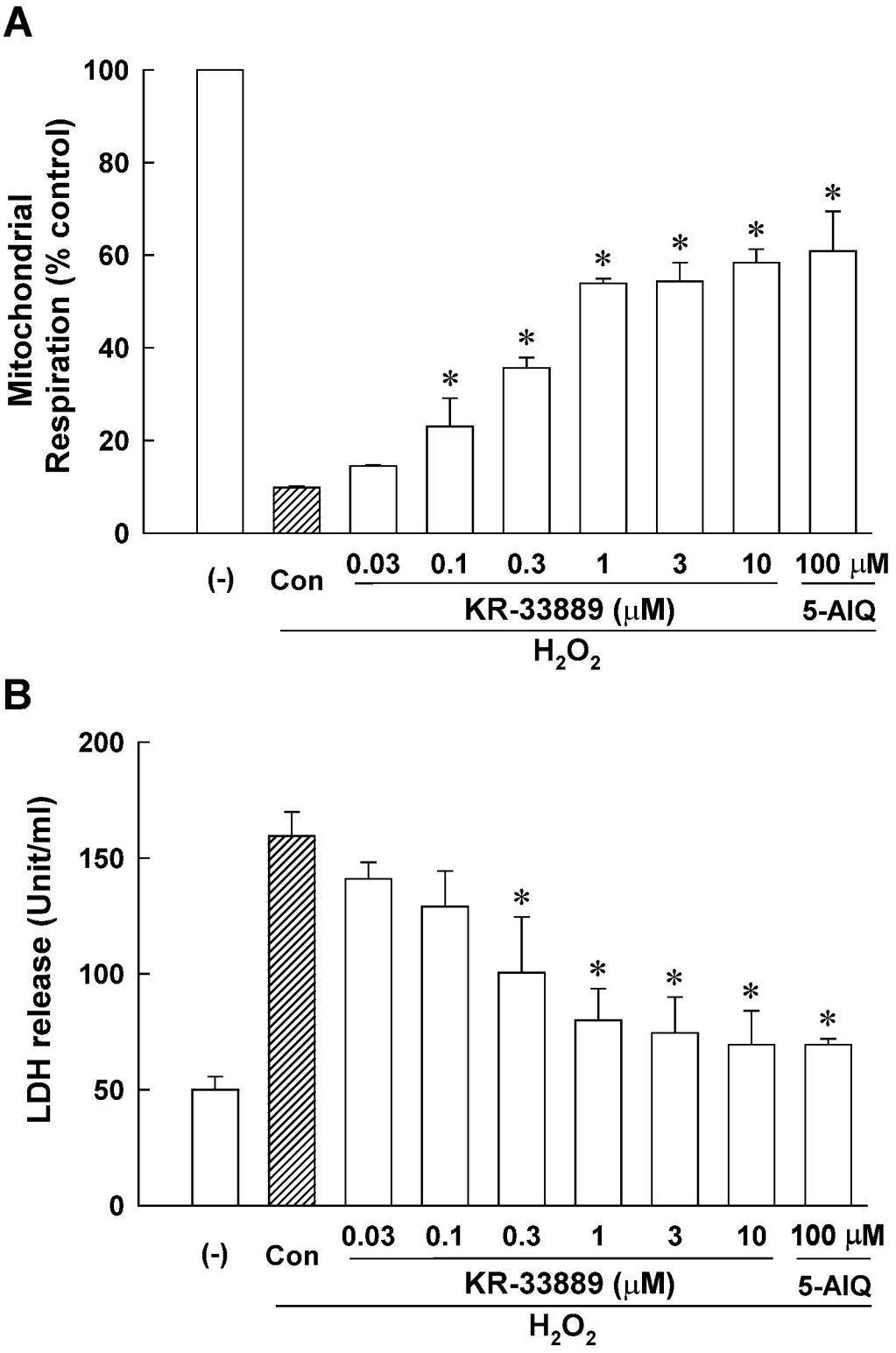

A
Mitochondrial Respiration (% control)
100
80
60
40
20
0
(-)
Con
0.03
0.1
0.3
1
3
10
100 μM
KR-33889 (μM)
5-AIQ
H2O2
B
LDH release (Unit/ml)
200
150
100
50
0
(-)
Con
0.03
0.1
0.3
1
3
10
100 μM
KR-33889 (μM)
5-AIQ
H2O2

Fig. 4.

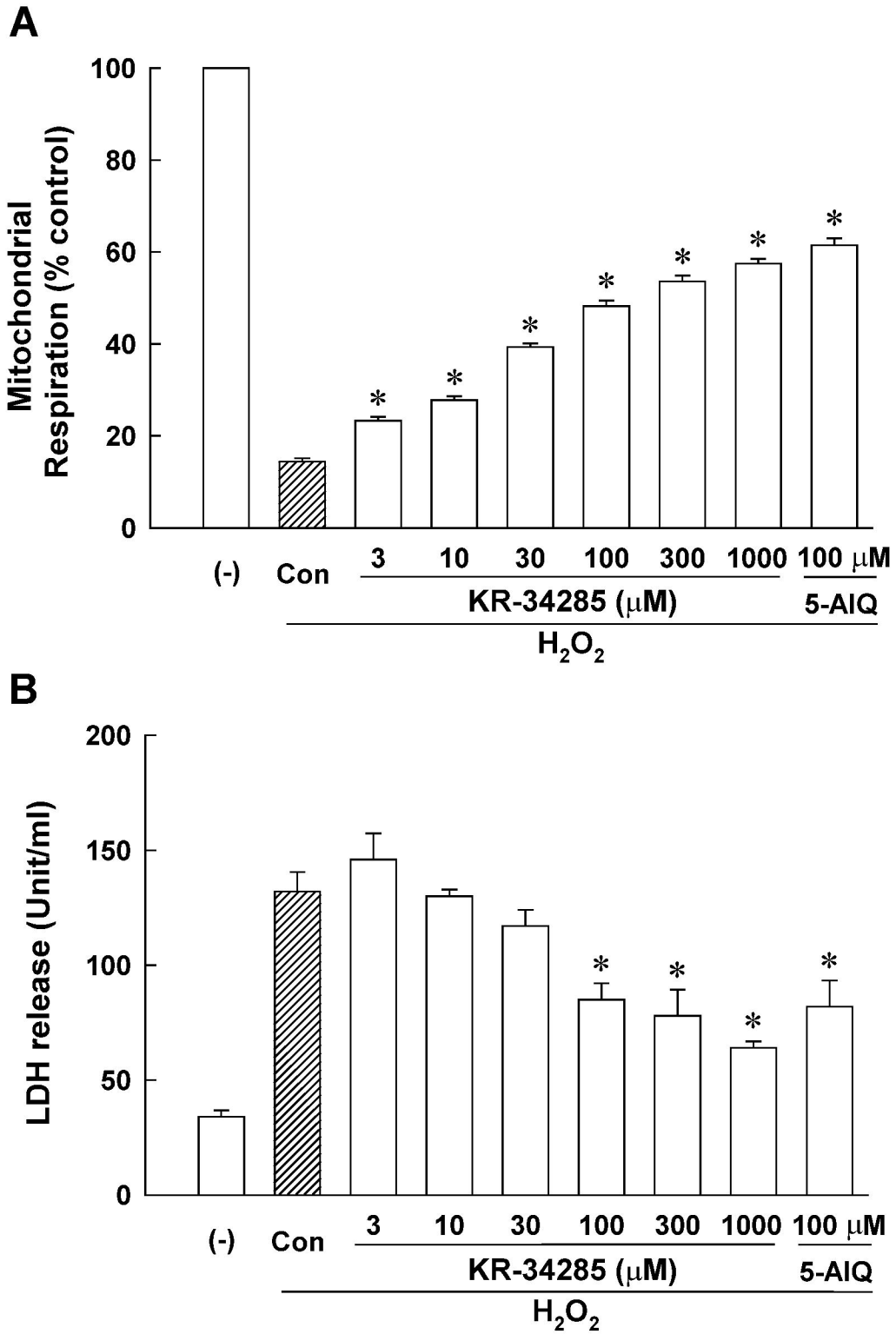
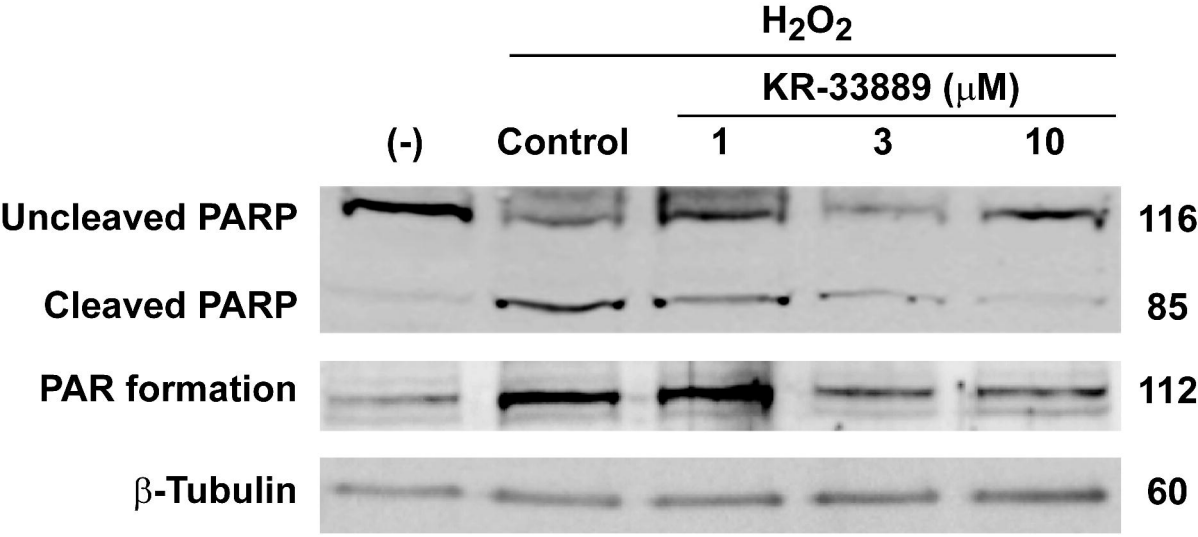

A
Mitochondrial Respiration (% control)
100
80
60
40
20
0
(-)
Con
3
10
30
100
300
1000
100 μM
KR-34285 (μM)
5-AIQ
H2O2
B
LDH release (Unit/ml)
200
150
100
50
0
(-)
Con
3
10
30
100
300
1000
100 μM
KR-34285 (μM)
5-AIQ
H2O2

Fig. 5.


H2O2
KR-33889 (μM)
(-)
Control
1
3
10
Uncleaved PARP
Cleaved PARP
PAR formation
β-Tubulin
116
85
112
60

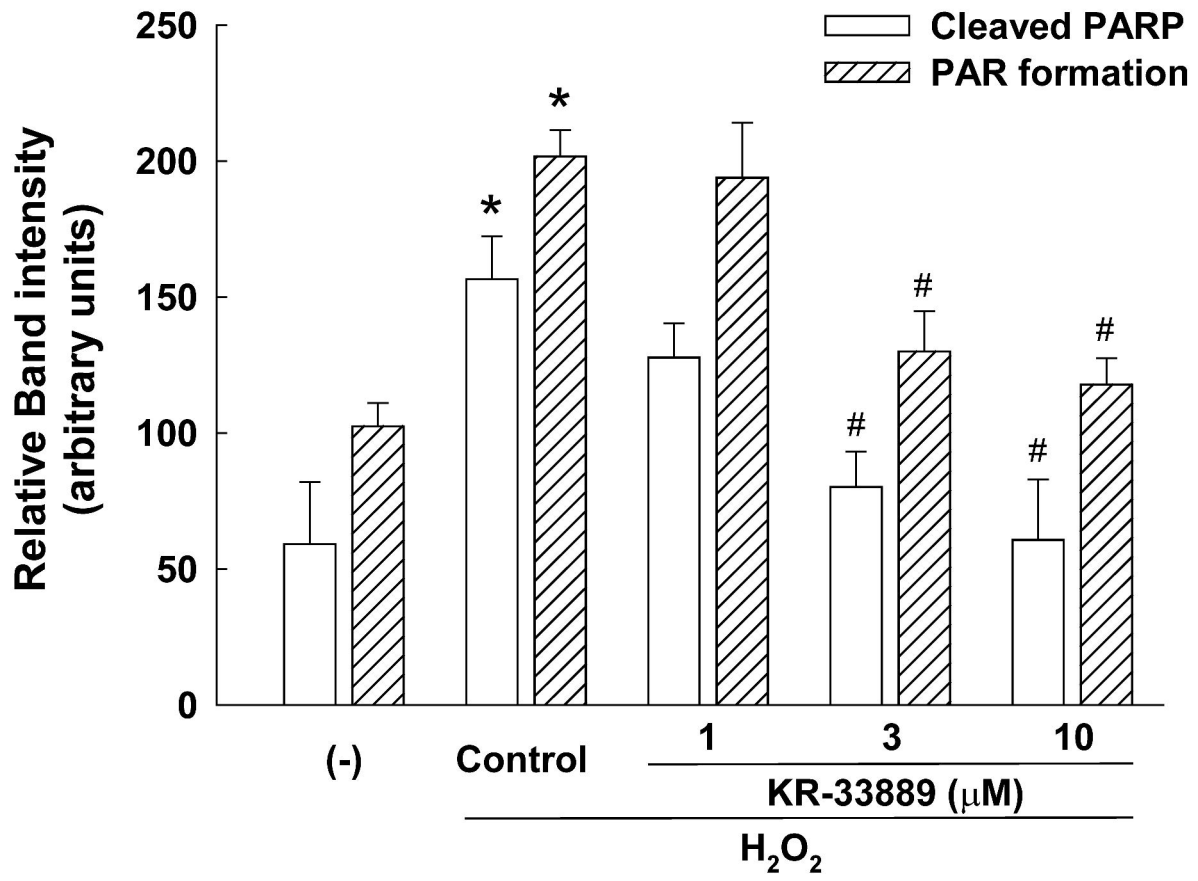

Cleaved PARP
PAR formation
Relative Band intensity (arbitrary units)
250
200
150
100
50
0
*
*
*
#
#
#
(-)
Control
1
3
10
KR-33889 (μM)
H2O2

Fig. 6.

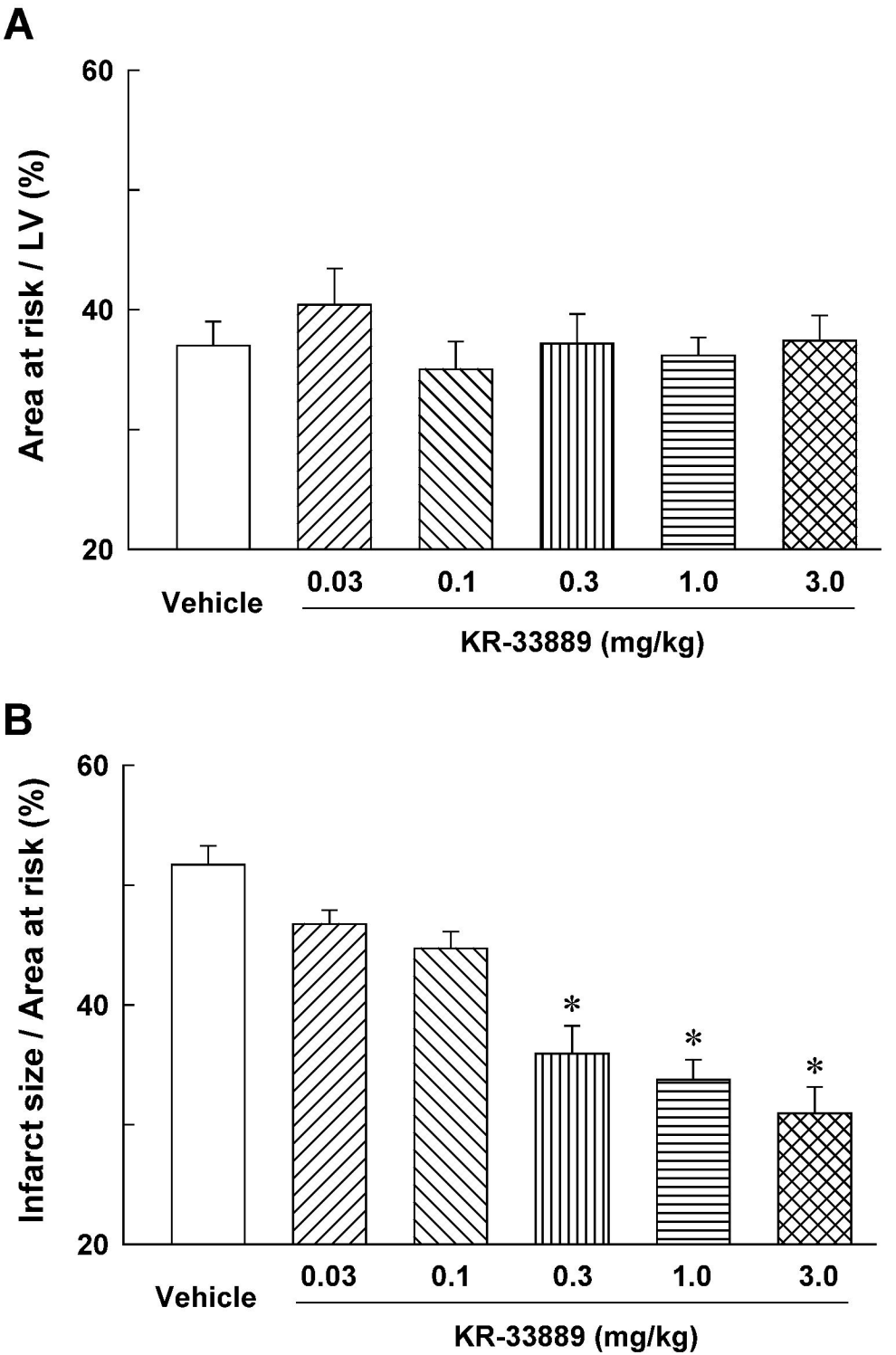

A
60
40
20
Area at risk / LV (%)
Vehicle
0.03
0.1
0.3
1.0
3.0
KR-33889 (mg/kg)
B
60
40
20
Infarct size / Area at risk (%)
*
*
*
Vehicle
0.03
0.1
0.3
1.0
3.0
KR-33889 (mg/kg)

Fig. 7.

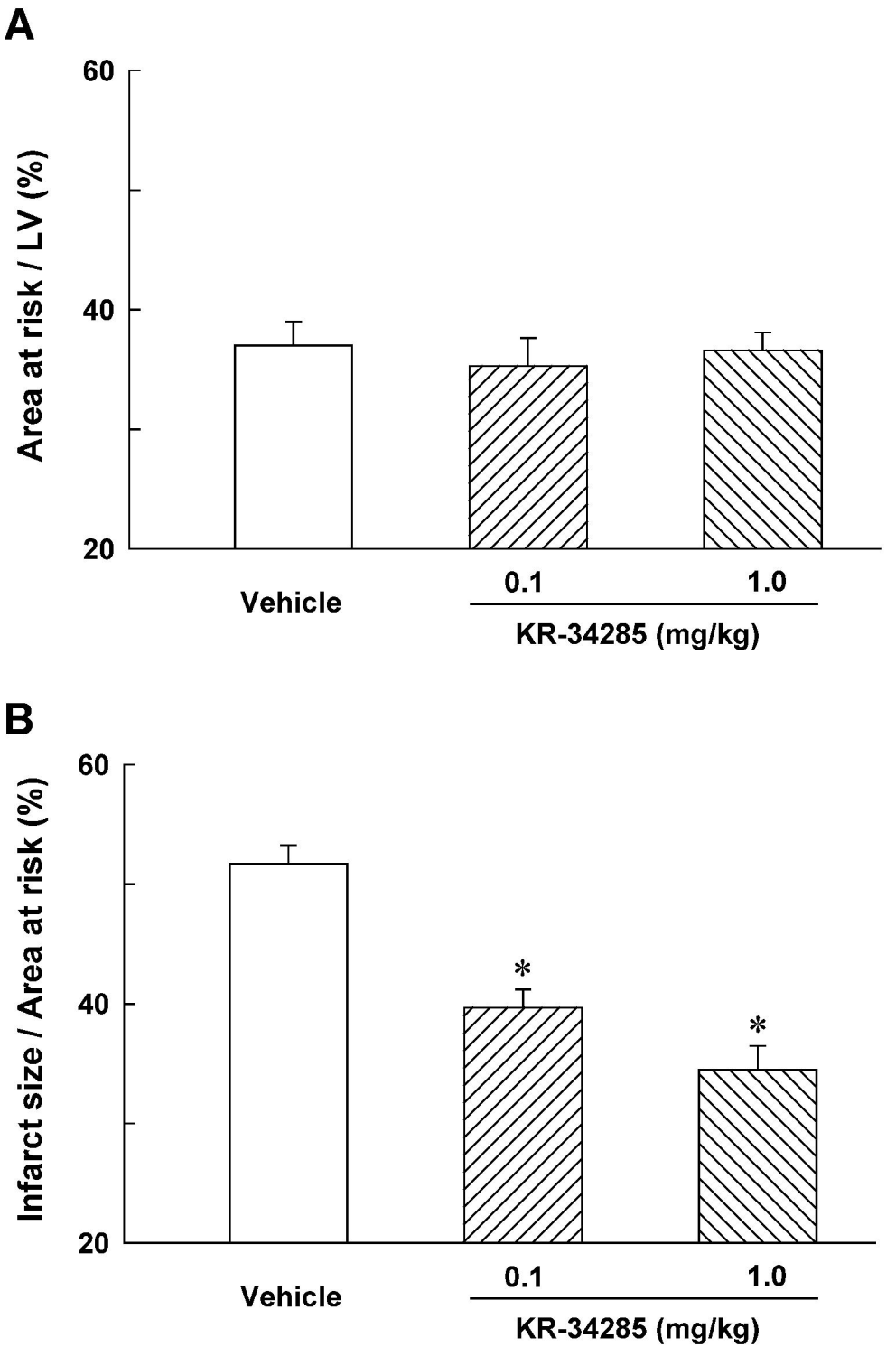

A
60
40
20
Area at risk / LV (%)
Vehicle
0.1
1.0
KR-34285 (mg/kg)
B
60
40
20
Infarct size / Area at risk (%)
*
*
Vehicle
0.1
1.0
KR-34285 (mg/kg)

Fig. 8.

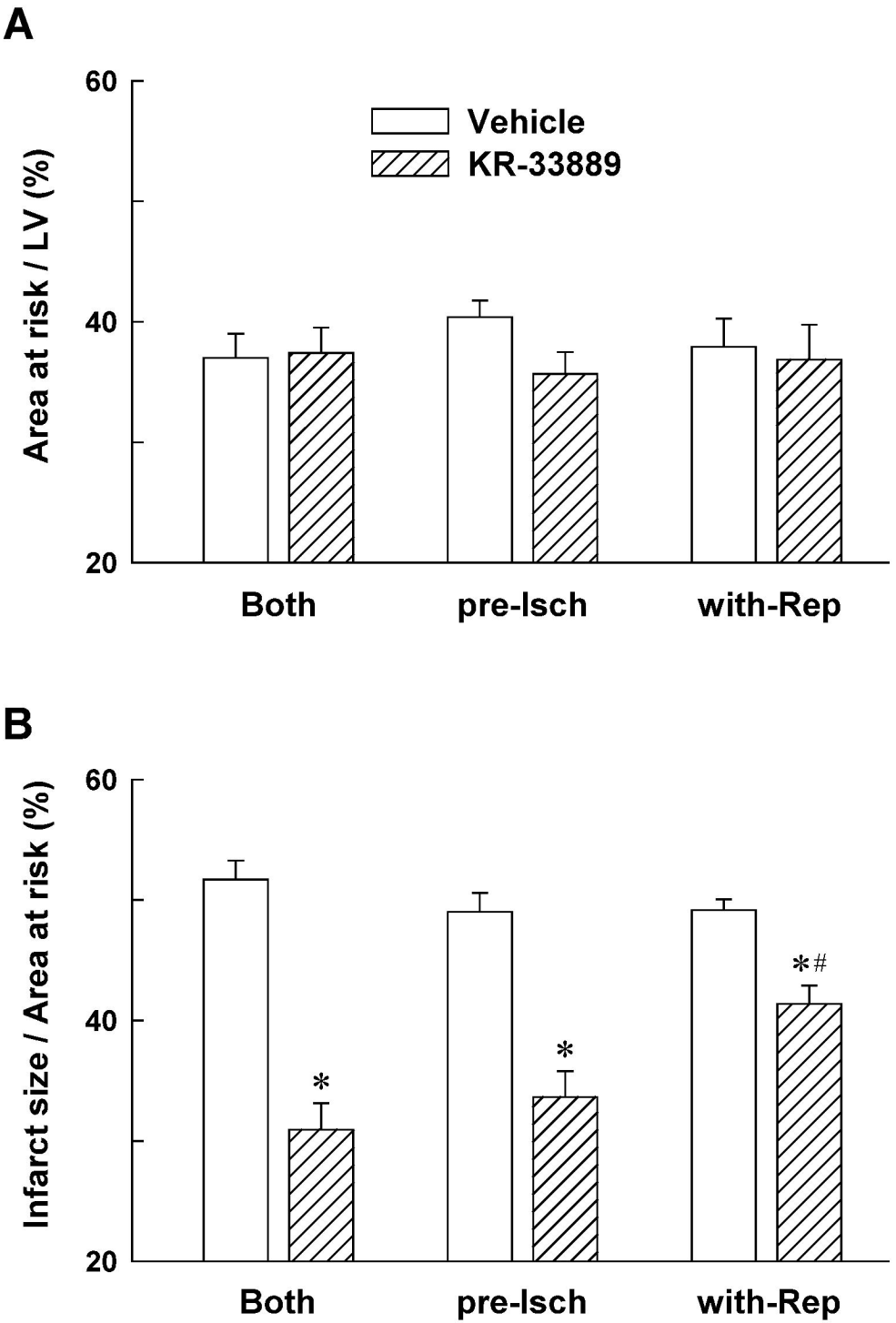
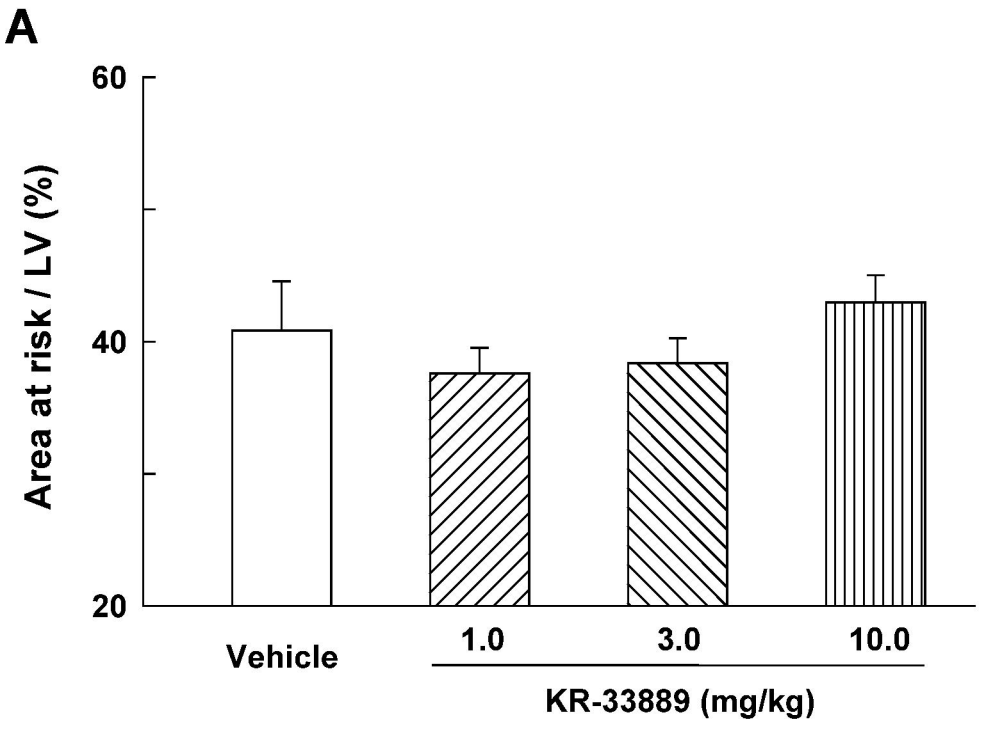

A
60
40
20
Area at risk / LV (%)
Vehicle
KR-33889
Both
pre-Isch
with-Rep
B
60
40
20
Infarct size / Area at risk (%)
*
*
*#
Both
pre-Isch
with-Rep

Fig. 9.


A
60
40
20
Area at risk / LV (%)
Vehicle
1.0
3.0
10.0
KR-33889 (mg/kg)

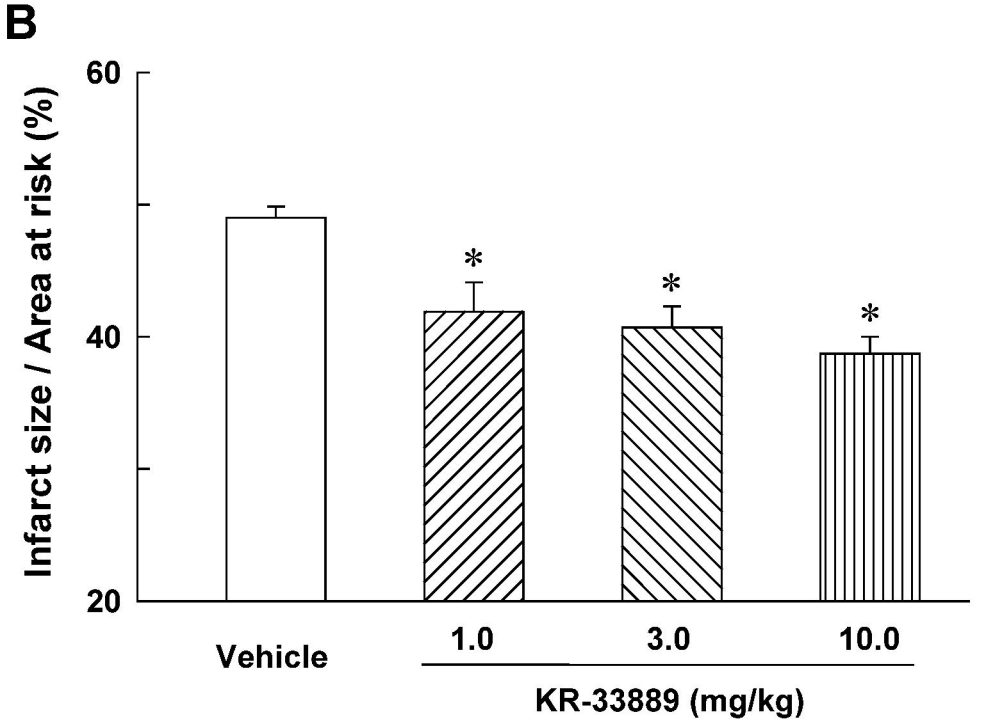

B
60
40
20
Infarct size / Area at risk (%)
*
*
*
Vehicle
1.0
3.0
10.0
KR-33889 (mg/kg)

Fig. 10.