(S)-1-(α-Naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (CKD712) Reduces Rat Myocardial Apoptosis against Ischemia and Reperfusion Injury by Activation of Phosphatidylinositol 3-Kinase/Akt Signaling and Anti-inflammatory Action in Vivo

Yong Chun Jin, Young Soo Lee, Young Min Kim, Han Geuk Seo, Jae Heun Lee, Hye Jung Kim, Hye Sook Yun-Choi, and Ki Churl Chang

Department of Pharmacology (Y.S.L., Y.M.K., H.G.S., J.H.L., H.J.K., K.C.C.) and Institute of Health Sciences (Y.C.J., K.C.C.), School of Medicine, Gyeongsang National University, Jinju, Korea; Natural Products Research Institute and College of Pharmacy (H.S.Y.-C.), Seoul National University, Seoul, Korea

Received December 29, 2008; accepted May 19, 2009

ABSTRACT
We examined our hypothesis that (S)-1-(α-naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (CKD712) inhibits apoptosis in myocardial ischemia and reperfusion (I/R) injury in vivo via activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and by reducing inflammation during I/R. To do this, we induced a 30-min period of ischemia by occlusion of the left anterior descending coronary artery of the rat followed by a 2-h (for phosphorylation of Akt), 6-h (for biochemical analysis), or 24-h (for functional analysis) period of reperfusion to determine the effect of CKD712 treatment. Pretreatment with CKD712 significantly improved myocardial function as evidenced by an increase in the dP/dt and a decrease in the infarct size, which were antagonized by a PI3K inhibitor, wortmannin (WT). Interestingly, CKD712 increased the phosphorylation of Akt and cAMP-response element-binding protein and increased the expression of the Bcl-2 gene, but it reduced the expression of the Bax gene. CKD712 decreased not only the expression but also the activity of the caspase-3 protein in the myocardium after reperfusion. Thus, all of the antiapoptotic effects of CKD712 were significantly inhibited by WT. Furthermore, the antiapoptotic effects of CKD712 and its inhibition by WT in myocardium after reperfusion were confirmed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining. Finally, CKD712 was found to reduce the serum levels of the high-mobility group box 1 protein, tumor necrosis factor-α, and the cardiac troponin I protein in addition to tissue levels of malondialdehyde and myeloperoxidase activity in I/R hearts. Taken together, both the activation of PI3K/Akt and its anti-inflammatory action prevent apoptosis in myocardial I/R injury by CKD712.

Early reperfusion during an episode of evolving myocardial infarction is essential for saving the myocardium and the patient’s life. Nevertheless, sublethal reperfusion injury is unavoidable in angioplasty, coronary bypass surgery, transplantation, and thrombolysis, which are all commonly used to re-establish the blood flow to minimize damage to the heart because of severe myocardial ischemia, and as such, limits myocardial salvage. In fact, myocardial reperfusion causes deleterious effects to endothelial cells and cardiomyocytes, leading to myocardial apoptosis (Stephanou, 2004). Despite multiple significant therapeutic advances, ischemia and reperfusion (I/R)-induced myocardial injury is still a major unsolved health problem. Protection of cardiomyocytes from I/R-induced cell death would in principle increase the viable myocardium, thus providing acute and chronic bene-

This work was supported by the Korea Research Foundation [Grant 03-2007-0487]; and the Korea Healthcare Technology R&D Project, Ministry of Health and Welfare, Republic of Korea [Grant A080506]. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.108.150342.

ABBREVIATIONS: I/R, ischemia and reperfusion; AAR, area at risk; CKD712, (S)-1-(α-naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; CREB, cAMP-response element-binding protein; DPPH, 1,1-diphenyl-2-picryl-hydrazil; cTnI, cardiac troponin I; HMGB1, high-mobility group box 1; IA, infarct area; dP/dt, maximum first derivatives of developed pressure; LV, left ventricle; LVEDP, left ventricular end-diastolic pressure; LVP, left ventricular systolic pressure; MDA, malondialdehyde; MPO, myeloperoxidase; NF-κB, nuclear factor κB; PI3K, phosphatidylinositol 3-kinase; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; WT, wortmannin; HR, heart rate.
fits after myocardial infarction. Therefore, cardiomyocyte apoptosis may be an excellent target for therapeutic modulation in the context of myocardial infarction and I/R injury. During I/R of the heart, reactive oxygen species (ROS), reactive nitrogen species, and inflammation are thought to play important roles in the pathogenesis of myocardial infarction (Burton et al., 1984; López-Neblińa and Toledo-Pereyra, 2006). In the intact organism, ischemic myocardial injury initiates an acute inflammatory response in which polymorphonuclear leukocytes are the major participants (Go et al., 1988). In fact, myocardial reperfusion induces a significant accumulation of neutrophils into the area of the previously ischemic myocardium, leading to an increased expression or up-regulation of numerous adhesion molecules and inflammation mediators in the endothelium and in the cardiomyocytes. Inflammatory conditions are characterized by the activation of the nuclear transcription factor-kappa B (NF-κB) and the expression of inflammatory mediators. Recently, we reported that CKD712, a synthetic tetrahydroisoquinoline alkaloid that is an investigational drug for the treatment of sepsis (Yun-Choi et al., 2003), inhibited lippopolysaccharide (LPS)-mediated NF-κB activity in RAW 264.7 cells (Tsöy et al., 2008). Moreover, recent observations suggest that the PI3K/Akt pathway inhibits the LPS-induced expression of inflammatory mediators by monocytes and endothelial cells and suppresses the activation of the innate immune response during endotoxemia (Hazeki et al., 2007). Thus, we hypothesized that CKD712 inhibits apoptosis by activation of the PI3K/Akt pathway and inhibits inflammation during I/R, which leads to a reduction of the infarct size and improves myocardial function after I/R injury in rats.

Materials and Methods

Experimental Animals. Male Sprague-Dawley rats between 7 and 8 weeks of age were used for this study. All of the animals were maintained in accordance with the *Guidelines for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication number 85-23, revised in 1996). The protocol was approved before the start of the animal study by the Care of Animal Research Committee of the Gyeongsang National University, Korea.

Materials. CKD712 was synthesized as a hydrobromide salt according to a method described previously (Pyo et al., 2008). Antibodies to Bax, Bcl-2, the cleaved caspase-3, and high-mobility group box 1 (HMGB1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Akt and p-Akt were purchased from Cell Signaling Technology Inc. (Danvers, MA). A caspase-3 activity assay kit was purchased from Millipore Bioscience Research Reagents (Temecula, CA). Unless otherwise indicated, all other reagents used in this study were purchased from Sigma-Aldrich Korea (Seoul, Korea).

Experiment Protocol. Reperfusion time was intentionally administered in different doses depending on the experimental objectives. For example, a 2-h reperfusion period was performed before the Western blot analysis of p-Akt and for the measurement of the tumor necrosis factor (TNF)-α and myeloperoxidase (MPO) activity, whereas a 6-h reperfusion period was performed for the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) analysis, the measurement of HMGB1, cardiac troponin I (cTnl), malondialdehyde (MDA), and for the expression of Bel-2 and phosphorylation of cAMP-response element-binding protein (p-CREB). In addition, a 24-h reperfusion period was performed for myocardial function and for the measurement of the infarct size. The rats were randomly distributed into four groups:

- group 1 (Sham), sham-operated (2 h after surgery, n = 8; 6 h after surgery, n = 24; 24 h after surgery, n = 8) rats in which no tightening of the coronary artery sutures was performed;
- group 2 (I/R), rats pretreated with a placebo (0.1% dimethyl sulfoxide 0.25 ml, intraperitoneal injection 1 h before ischemia) and subjected to 30 min of ischemia followed by a 2-h (n = 8), 6-h (n = 27), or 24-h (n = 10) period of reperfusion; and group 3 (I/R + CKD712), rats pretreated with CKD712 (intraperitoneal injection 1 h before ischemia) and subjected to 30 min of ischemia followed by a 2-h (10 mg/kg, n = 8), 6-h (5 mg/kg, n = 10; 10 mg/kg, n = 27), or 24-h (5 mg/kg, n = 8; 10 mg/kg, n = 10) period of reperfusion; and group 4 (I/R + CKD712 + WT), rats pretreated with both CKD712 (10 mg/kg, intraperitoneal injection 1 h before ischemia) and wortmannin (WT, 20 μg/kg, intravenous injection 15 min before reperfusion) and subjected to 30 min of ischemia followed by a 2-h (n = 4), 6-h (n = 20), or 24-h (n = 10) period of reperfusion.

Surgical Preparation. Animals underwent general anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg). Endotracheal intubation was performed with a 14-gauge angiocatheter that was coupled to mechanical ventilation. The chest was opened via a single cut along the left side of the sternum through the ribs followed by a pericardiotomy. A 4-0 Black silk on a curved taper needle was passed below the left descending vein and the coronary artery from the area immediately below the left atrial appendage to the right portion of the left ventricle, and the ends were pulled through a small vinyl tube to form a snare. On tightening of the snare, which was then fixed by clamping the tube with a small hemostat, the coronary artery branch was effectively occluded. Coronary artery occlusion was verified by epicardial cyanosis. After occlusion for 30 min, the snare was released, and the removal of the tube initiated reperfusion. Reperfusion was confirmed by epicardial hyperemia. The loosened suture was left in place and then re tied for the purpose of evaluating the ischemic area.

Hemodynamic Measurements. After 24 h of reperfusion, the rats were anesthetized with ketamine (75 mg/kg) and xylazine (7.5 mg/kg). The right common carotid artery was exposed and cannulated with a 2 F Millar Catheter (Millar Instruments, Inc. Houston, TX) placed into the ascending aorta to measure the systolic and diastolic blood pressure, the mean arterial pressure (MAP), and the heart rate (HR). The pressure transducer was then advanced into the left ventricle (LV) to measure the left ventricular systolic pressure (LVSP), the left ventricle end-diastolic pressure (LVEDP), and the first derivatives (positive and negative) of the LV pressure over time (dP/dt).

Determination of the Area at Risk and Infarct Size. After measurement of heart function, the left anterior descending coronary artery was occluded again, and 2 ml of 1% Evans blue dye was injected into the femoral vein (Zacharowski et al., 1999) to distinguish between perfused and nonperfused (area at risk [AAR]) sections of the heart. The Evans blue solution stains the perfused myocardium, and the occluded vascular bed remains uncolored. The atria, right ventricles, and major vessels were removed from the hearts, and the LV was separated according to staining from the remainder of the heart and weighed on the basis of wet weight to determine the infarct size as a percentage of the weight of the AAR, and the AAR was separated from the nonischemic area. AAR was expressed as a percentage of the LV. The AAR was cut into small pieces and incubated with 2,3,5-triphenyltetrazolium chloride for 20 min at 37°C to visualize the infarct area. The AAR of infarction was colored red because of the formation of a precipitate that resulted from the reaction of 2,3,5-triphenyltetrazolium chloride with dehydrogenate enzymes. The loss of these enzymes from the infarcted myocardium prevents the formation of the precipitate, and the infarcted area within the region at risk remains pale yellow.

Western Blot Analysis. The total protein was extracted from the “at risk zones” of the heart. In brief, 2 h after reperfusion (for the p-Akt and t-Akt) or 6 h after reperfusion (for the Bcl-2, Bax, and cleaved caspase-3), myocardial tissue was placed in a lysis buffer.
containing protease inhibitors, homogenized, and then centrifuged. For Western blot analysis of HMGBl, the blood was collected by heart puncture from the I/R, the I/–+CKD712, or the I/R+/+CKD712 + WT animal groups after 6-h reperfusion. The resulting serum was then filtered and concentrated by use of a Centricon YM-100 and YM-10 (Millipore Corporation, Billerica, MA), respectively. The proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and incubated with antibodies to Bcl-2, Bax, cleaved caspase-3, p-Akt, t-Akt, and HMGBl at 4°C overnight. The proteins were detected with horseradish peroxidase conjugated to a secondary antibody (1:5000 dilution in Tris-buffered saline with Tween 20 containing 5% skim milk powder, at room temperature for 1 h) and were visualized by use of an enhanced chemiluminescence method. Blots were quantified by laser-scanning densitometry.

**Measurement of Caspase-3 Activity.** The activity of caspase-3 was evaluated by using a colorimetric activity assay kit (Millipore Bioscience Research Reagents) according to the manufacturer’s instructions. Myocardial tissue specimens were obtained from the ischemic area. The samples were homogenized in ice-cold buffer provided by this kit and centrifuged. After determining the protein concentration, the supernatant was incubated with the caspase-3 substrate (Ac-DEVD-pNA) on a 96-well plate. The activity of caspase-3 was determined by use of a spectrophotometer (Infinite F200; Tecan Group Ltd., Männedorf, Switzerland) at 405 nm, and the results were expressed as the -fold increase over the mean value of sham.

**TUNEL Staining.** The hearts were isolated from the animals of each group (after a 6-h reperfusion period) for the TUNEL assay. After the blood and the fixation solution were washed out, the heart was sliced transversely into 2.5-mm-thick sections. Paraaffin-embedded, 9-μm-thick myocardial sections were used as described previously (Scarabelli et al., 1999). Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed by using a TUNEL kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Fluorescence staining was viewed with a fluorescent microscope (Fluoview 500; Olympus, Tokyo, Japan). Three sections from each myocardial sample were randomly selected, and 10 microscopic fields per section were evaluated. In each field, the nuclei were counted, and the percentage of TUNEL-positive nuclei was calculated. The heart tissue samples for TUNEL analyses were obtained from the margins of the noninfarcted ischemic areas.

**Free Radical Scavenging Activity.** The free radical scavenging activity of CKD712 was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method described by Brand-Williams et al. (1995). In brief, a 0.2 mM solution of DPPH in ethanol was prepared, and (DPPH) using the method described by Brand-Williams et al. (1995). The content of MDA was measured by use of commercial kits (Cayman Chemical, Ann Arbor, MI) with a spectrophotometer (Infinite F200; Tecan Group Ltd.).

**Measurement of Cardiac Troponin I.** Blood serum samples after 6-h reperfusion were collected to measure the levels of the myocardial specific enzyme, cTnI. An enzyme-linked immunoabsorbent assay kit for cTnI determination was purchased from Life Diagnostics Inc. (West Chester, PA), and its activity was measured according to the manufacturer’s instructions. In brief, the extracted serum was reacted with the assay reagents in cTnI kits and analyzed spectrophotometrically (Infinite F200; Tecan Group Ltd.). The levels of cTnI were expressed as nanograms per milliliter of serum.

**Statistical Analysis.** All of these data were expressed as the mean ± S.D. The differences between groups were assessed by a one-way ANOVA followed by a Scheffé test. The values of *P* < 0.05 were considered to be statistically significant.

**Results**

**Hemodynamics.** To test whether CKD712 improves myocardial function by activation of PI3K activity, we utilized the pharmacological PI3K inhibitor, WT, in myocardial I/R-induced rats. Twenty-four hours after reperfusion, the LVEDP, the +dP/dt, the HR, and the MAP were investigated. As shown in Table 1, a marked elevation of LVEDP was observed in the I/R group (10 ± 1 mm Hg), which was significantly reduced by treatment with 10 mg/kg CKD712 (6 ± 2 mm Hg, *P* < 0.05), but not with 5 mg/kg CKD712. However, WT significantly inhibited these effects of CKD712 on LVEDP (from 6 ± 2 mm Hg to 9 ± 1 mm Hg, *P* < 0.05). The positive and negative maximal values of the first derivatives of the left ventricular pressure (+dP/dt) were significantly improved by the addition of 10 mg/kg CKD712 compared with the I/R control group (3557 ± 448 versus 3207 ± 375 mm Hg/s, *P* < 0.05; –2826 ± 490 versus –2557 ± 387 mm Hg/s, *P* < 0.05, respectively). These parameters were also inhibited by WT (3557 ± 448 mm Hg/s to 3275 ± 176 mm Hg/s, *P* < 0.05; –2826 ± 490 mm Hg/s to –2618 ± 177 mm Hg/s, *P* < 0.05, respectively), indicating that CKD712 improved the LV systolic and the diastolic function after reperfusion in ischemic myocardium through the PI3K pathway. The other parameters such as MAP and HR were not significantly different between the CKD712 and the sham group.
However, the MAP was decreased significantly in the I/R group ($P < 0.05$). In addition, the LVSP also decreased significantly ($P < 0.05$) in the I/R rats, but this value increased significantly ($P < 0.05$) after administration of 10 mg/kg CKD712. CKD712 may have influenced $+dP/dt$ and $-dP/dt$ by decreasing preload and afterload of the heart. To exclude this possibility, we evaluated the hemodynamic effect of CKD712 (10 mg/kg) on sham animal rats, in which no tightening of the coronary artery sutures was performed. Although CKD712 showed the tendency to decrease preload and afterload during the infusion period (30 min), it was not statistically significant compared with vehicle infusion (Table 2).

**Area at Risk and Infarct Size.** Because CKD712 improved myocardial function in I/R rats, we measured the infarct size as described under Materials and Methods. As represented by the percentage of the total area at risk, the AAR/LV, IA/LV, and IA/AAR are shown in Fig. 1. The ischemic area induced by the left anterior descending snare (AAR/LV, %) did not differ among the experimental groups. However, the administration of 10 mg/kg, but not 5 mg/kg, CKD712 produced a statistically significant reduction in the IALV and IA/AAR values compared with the I/R control group ($16 \pm 6$ versus $27 \pm 4$, $P < 0.05$; $35 \pm 9$ versus $58 \pm 3$, $P < 0.05$, respectively). The IALV values tended to be higher in the CKD712+WT group than in the CKD712 group ($20 \pm 4$ versus $16 \pm 6$), although the difference was not statistically significant, but the IA/AAR value was significantly higher in the CKD712+WT group than in the CKD712 group ($46 \pm 5$ versus $35 \pm 9$; $P < 0.05$).

**CKD712 Activates Akt through the PI3K Pathway.** Numerous experimental observations have indicated that the Akt (protein kinase B) protein, a serine-threonine kinase, is activated by PI3K and plays a critical role in facilitating cell survival by inhibiting the activation of caspases, which are known to function as effectors of the apoptotic signaling cascade program (Datta et al., 1999). Because we had evidence from these results that the effect of CKD712 was antagonized by administration of WT, we directly measured the phosphorylation status of Akt (p-Akt) by CKD712 in the I/R-injured myocardium. As shown in Fig. 2B, treatment with CKD712 resulted in a 2.5-fold (relative to sham, $P < 0.05$) increase in the p-Akt level. Furthermore, CKD712 increased the expression of p-Akt in intact animal heart (Fig. 2A). Cotreatment of CKD712 with WT significantly reduced the level of p-Akt induced by CKD712 (1.2-fold increase relative to sham versus a 2.5-fold increase relative to sham, $P < 0.05$). Thus, there was no difference in total Akt among the groups studied. These results demonstrated that in vivo treatment with CKD712 activated Akt through the PI3K pathway.

**Antia apoptotic Effect of CKD712.** To confirm that CKD712 inhibits apoptosis in the postischemic myocardium by reperfusion, the expression of apoptosis-related proteins in the I/R-injured myocardium was assessed by Western blot

### Table 1
Summary of hemodynamic measurements in myocardial ischemia and reperfusion

<table>
<thead>
<tr>
<th></th>
<th>LVSP/LVEDP</th>
<th>$\pm dP/dt$</th>
<th>HR</th>
<th>MAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm Hg</td>
<td>min mm Hg/s</td>
<td>bpm</td>
<td>mm Hg</td>
</tr>
<tr>
<td>Sham (n = 6)</td>
<td>99 $\pm$ 95 $\pm$ 2</td>
<td>3896 $\pm$ 466$^*/$2074 $\pm$ 553</td>
<td>301 $\pm$ 29</td>
<td>81 $\pm$ 5</td>
</tr>
<tr>
<td>I/R (n = 10)</td>
<td>83 $\pm$ 6$^<em>/$10 $\pm$ 1$^</em>$</td>
<td>3207 $\pm$ 375$^<em>/$2557 $\pm$ 387$^</em>$</td>
<td>334 $\pm$ 31</td>
<td>67 $\pm$ 5$^*$</td>
</tr>
<tr>
<td>CKD5 (n = 8)</td>
<td>90 $\pm$ 9$^*/$4</td>
<td>3223 $\pm$ 422$^*/$2568 $\pm$ 433</td>
<td>315 $\pm$ 23</td>
<td>69 $\pm$ 6</td>
</tr>
<tr>
<td>CKD10 (n = 10)</td>
<td>92 $\pm$ 5$^<em>/$6 $\pm$ 2$^</em>$</td>
<td>3557 $\pm$ 448$^<em>/$2826 $\pm$ 490$^</em>$</td>
<td>327 $\pm$ 22</td>
<td>71 $\pm$ 7</td>
</tr>
<tr>
<td>CKD10 + WT (n = 10)</td>
<td>83 $\pm$ 4$^<em>/$9 $\pm$ 1$^</em>$</td>
<td>3275 $\pm$ 176$^<em>/$218 $\pm$ 177$^</em>$</td>
<td>268 $\pm$ 27</td>
<td>72 $\pm$ 7</td>
</tr>
</tbody>
</table>

CKD5, CKD712 (5 mg/kg); CKD10, CKD712 (10 mg/kg).

$^*$ $P < 0.05$ vs. sham.

$^*$ $P < 0.05$ vs. I/R.

$^*$ $P < 0.05$ vs. CKD712.

### Table 2
The time course of hemodynamic changes of CKD712 (10 mg/kg) and vehicle after intraperitoneal infusion

<table>
<thead>
<tr>
<th>Time</th>
<th>LVSP/LVEDP</th>
<th>HR</th>
<th>$\pm dP/dt$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10 mg/kg, n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>103 $\pm$ 6/5.0 $\pm$ 0.6</td>
<td>260 $\pm$ 50</td>
<td>4018 $\pm$ 323$^*/$2813 $\pm$ 265</td>
</tr>
<tr>
<td>0.5 h</td>
<td>98 $\pm$ 4/5.4 $\pm$ 0.9</td>
<td>374 $\pm$ 16</td>
<td>4229 $\pm$ 256$^*/$3042 $\pm$ 245</td>
</tr>
<tr>
<td>1.0 h</td>
<td>104 $\pm$ 9/6.5 $\pm$ 1.3</td>
<td>375 $\pm$ 60</td>
<td>4373 $\pm$ 410$^*/$2934 $\pm$ 296</td>
</tr>
<tr>
<td>1.5 h</td>
<td>106 $\pm$ 12/6.5 $\pm$ 10</td>
<td>382 $\pm$ 19</td>
<td>4411 $\pm$ 258$^*/$3067 $\pm$ 368</td>
</tr>
<tr>
<td>24 h</td>
<td>100 $\pm$ 4/6.5 $\pm$ 0.4</td>
<td>257 $\pm$ 43</td>
<td>3987 $\pm$ 245$^*/$2913 $\pm$ 247</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>LVSP/LVEDP</th>
<th>HR</th>
<th>$\pm dP/dt$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.1% DMSO 0.25 ml, n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 h</td>
<td>105 $\pm$ 6/5.3 $\pm$ 0.4</td>
<td>239 $\pm$ 34</td>
<td>3380 $\pm$ 362$^*/$3412 $\pm$ 421</td>
</tr>
<tr>
<td>1.0 h</td>
<td>98 $\pm$ 6/4.8 $\pm$ 0.2</td>
<td>346 $\pm$ 19</td>
<td>4234 $\pm$ 264$^*/$2348 $\pm$ 320</td>
</tr>
<tr>
<td>1.5 h</td>
<td>95 $\pm$ 6/6.4 $\pm$ 0.6</td>
<td>356 $\pm$ 41</td>
<td>4031 $\pm$ 258$^*/$2377 $\pm$ 317</td>
</tr>
<tr>
<td>24 h</td>
<td>98 $\pm$ 5/6.6 $\pm$ 0.6</td>
<td>356 $\pm$ 62</td>
<td>4118 $\pm$ 159$^*/$2660 $\pm$ 557</td>
</tr>
</tbody>
</table>

DMSO, dimethyl sulfoxide.
analysis. Figure 3A shows that I/R injury obviously reduced the Bcl-2 expression levels by 0.7 - 0.1-fold and increased the Bax expression levels by 5.1 - 0.3-fold in comparison with the sham group. In contrast to the I/R control, CKD712 increased the Bcl-2 expression levels by 0.9 - 0.1-fold but significantly attenuated the Bax expression levels by 3.4 - 0.2-fold (P < 0.05, respectively). In addition, the cleaved caspase-3 expression was also significantly reduced in the CKD712 group compared with the I/R control group (1.5 ± 0.2 versus 2.2 ± 0.1, P < 0.05). Furthermore, all of these CKD712 effects were again reversed by the addition of WT (0.6 ± 0.1 versus 0.9 ± 0.1 for Bcl-2, P < 0.05; 5.0 ± 0.1 versus 3.4 ± 0.2 for Bax, P < 0.05; and 2.1 ± 0.1 versus 1.5 ± 0.2, for active caspase-3, P < 0.05; respectively; Fig. 3A). As shown in Fig. 3B, treatment with CKD712 significantly reduced the caspase-3 activity compared with the I/R controls.

**Fig. 2.** The effect of CKD712 on the expression levels of p-Akt. CKD712 was administered intraperitoneally to rats. After 6 h, hearts were collected under anesthesia from intact animals (A) or from sham, I/R, I/R tissues with CKD712, and I/R+CKD712+WT animals (B). Western blot analysis was performed by the use of intact cardiac tissues (A) or the ischemic zone of the cardiac tissues (B) as described under Materials and Methods. The results are expressed as the mean ± S.D. CKD10, CKD712 (10 mg/kg). †, P < 0.05 versus sham; *, P < 0.05 versus I/R; ‡, P < 0.05 versus CKD10.

**Fig. 3.** The effect of CKD712 on apoptosis-related gene expression. The expression levels of Bcl-2, Bax, and cleaved caspase-3 (A) and caspase-3 activity (B) were analyzed in sham, I/R, I/R tissues with CKD712, and I/R+CKD712+WT-treated myocardial tissues. A, Western blot analysis was performed with use of the ischemic zone of the cardiac tissues as described under Materials and Methods. B, caspase-3 activity from the ischemic zone of cardiac tissue was measured in rats treated for 6 h with reperfusion. The results are expressed as the mean ± S.D. CKD10, CKD712 (10 mg/kg). †, P < 0.05 versus sham; *, P < 0.05 versus I/R; ‡, P < 0.05 versus CKD10.
(1.2 ± 0.3 versus 2.3 ± 0.5, P < 0.05), which were again reversed by the addition of WT (2.0 ± 0.2 versus 1.2 ± 0.3, P < 0.05). Figure 4A shows that CKD712 significantly enhanced the p-CREB in intact heart. In the I/R heart, CKD712 also augmented the p-CREB (1.2 ± 0.2 versus 0.6 ± 0.1, P < 0.05), but these effects were blocked by WT (1.2 ± 0.2 versus 0.7 ± 0.1, P < 0.05). As shown in Fig. 4B, I/R injury caused apoptosis in the rat heart that was confirmed further by the TUNEL staining assay. TUNEL-positive cells were found to increase significantly from 4 ± 2 to 35 ± 8% after I/R injury (P < 0.01). However, treatment with CKD712 significantly reduced the number of TUNEL-positive cells in the I/R heart (14 ± 6 versus 35 ± 8%, P < 0.01), which again was able to be reversed by treatment with WT (27 ± 7 versus 14 ± 6%, P < 0.05). These results, coupled with the gene expression data reported above, strongly suggest that CKD712 has significant antiapoptotic effects that require the activity of PI3K. However, CKD712 by itself did not affect caspase-3 activity in intact myocardium (data not shown).

**Antioxidant Action of CKD712.** The antioxidant effect is considered to be one of the important mechanisms for the inhibition of oxidative stress (I/R injury)-induced apoptosis. Thus, we directly measured the antioxidant effect of CKD712 with use of a cell-free system. Compared with the two known radical scavengers, BHA and BHT, the radical scavenging effect of CKD712 was found to be most potent. The scavenging effects on the DPPH radical decreased in the order of CKD712 > BHA > BHT (Fig. 5).

**The Effect of CKD712 Inhibition on Lipid Peroxidation and Phagocyte Infiltration.** Oxidative stress and

---

**Fig. 4.** The effect of CKD712 on the expression levels of p-CREB and TUNEL staining. CKD712 was administered intraperitoneally to rats, and 6 h later, hearts were collected under anesthesia from intact animals (A) or from sham, I/R, I/R tissues with CKD712, and I/R + CKD712 + WT animals (B). Western blot analysis was performed on intact rat heart (A) and the ischemic zone of cardiac tissues (B). C, a representative photomicrograph of a TUNEL-stained rat cardiac myocyte with the corresponding percentage of TUNEL-positive nuclei. The results are expressed as the mean ± S.D. CKD10, CKD712 (10 mg/kg). †, P < 0.05 versus sham; *, P < 0.05 versus I/R; ‡, P < 0.05 versus CKD10.
phagocyte infiltration have been proposed as key mediators capable of triggering cardiac damage after reperfusion (Zhao, 2004). Therefore, we measured the MDA levels and MPO activity as markers of lipid peroxidation and infiltration of ROS-generating inflammatory cells (i.e., neutrophils and macrophages), respectively. The results from Fig. 6A indicate that the MDA levels in the tissue from the ischemic area were increased significantly compared with the nonischemic tissue (sham group, \( P < 0.01 \)). In addition, treatment with CKD712 (10 mg/kg) resulted in a significant reduction in the myocardial MDA levels compared with the I/R control group (30 ± 4 versus 65 ± 5 nM/mg protein, respectively; \( P < 0.05 \)). However, treatment with WT significantly (\( P < 0.05 \)) inhibited the effect of CKD712 (Fig. 6A). The MPO activity in the tissues from the ischemic area increased significantly compared with the nonischemic tissue (sham group, \( P < 0.01 \)) from the I/R and the CKD712 groups, indicating that extravasation of neutrophils into the ischemic tissues is evident (Fig. 6B). The MPO activity in the CKD712 (10 mg/kg) group was significantly lower than that observed for the I/R group (40 ± 5 U/g weight versus 62 ± 7 U/g tissue, \( P < 0.05 \)). Treatment with WT reversed the effect of CKD712 (40 ± 5 versus 55 ± 4 U/g weight, \( P < 0.05 \)). Finally, we measured the levels of TNF-α, HMGB1, and cTnI to know whether or not CKD712 displays an anti-inflammatory effect. As shown in Fig. 6C, CKD712 (10 mg/kg) significantly reduced the levels of circulating TNF-α (10.2 ± 0.7 versus 16.4 ± 1.5 ng/ml plasma, \( P < 0.05 \)). Figure 7 shows that CKD712 (10 mg/kg) significantly reduced the levels of circulating HMGB1 (0.55 ± 0.2-fold compared with I/R, \( P < 0.05 \)) and cTnI, a marker of cardiac tissue injury (10.3 ± 1.5 versus 19.5 ± 2.5 ng/l serum, \( P < 0.05 \)). However, treatment of WT significantly reversed the effect of CKD712 in both HMGB1 and cTnI levels (15.4 ± 1.3 versus 10.3 ± 1.5 ng/l serum, \( P < 0.05 \)).

**Discussion**

The present study clearly demonstrated that CKD712 significantly reduced the effects of myocardial infarction and preserved left ventricular function, as reflected by a significant increase in the indices of contractility (\( +dP/dt \)) and relaxation (\( -dP/dt \)) in addition to a decrease in preload (LVEDP). Furthermore, CKD712 attenuated MDA and MPO activity in the ischemic myocardium. The mechanism under-
lying this beneficial effect on I/R injury was closely related to the antiapoptotic effect of CKD712 via activation of the PI3K signal pathway and of the anti-inflammatory response. This conclusion was based on results indicating that CKD712 increased the phosphorylation of Akt, increased the expression of Bcl-2 and the subsequent phosphorylation of CREB coupled with a decrease in Bax and cleaved caspase-3, reduced the infarct size, and improved cardiac function. However, most importantly, all of these effects of CKD712 were antagonized to a significant degree by WT. In fact, Akt has been shown to reduce the possibility of undergoing myocardial apoptosis, thereby preventing myocardial injury after I/R injury (Matsui et al., 2001). We demonstrated that CKD712 significantly increased the p-Akt levels in resting cardiac tissues, which was significantly augmented in I/R cardiac tissues compared with the placebo treatment. Therefore, we investigated the signal pathways that lead to anti-apoptosis by CKD712. First, we addressed whether or not CKD712 can regulate genes associated with apoptosis, such as Bcl-2 and Bax, because of the activation of PI3K during I/R injury. The role of the Bcl-2 family in the development of apoptotic cell death in different species has been widely investigated (Hockenbery et al., 1993; Misao et al., 1996; Kirschenbaum and de Moissac, 1997). Furthermore, the balance in the expression levels between the antiapoptotic Bcl-2 and the proapoptotic Bax proteins plays a major role in regulating myocardial apoptotic cell death (Misao et al., 1996; Kirschenbaum and de Moissac, 1997). As expected, treatment with CKD712 increased the expression of the antiapoptotic protein Bcl-2 but reduced the abundance of the proapoptotic protein, Bax. We speculated that this might be possible because of the pharmacological action of CKD712 on the heart. Previously, we reported that CKD712 and its congeners, such as higenamine (Chang et al., 1994) and YS49 (Lee et al., 1994), increased cAMP levels in the heart muscle by activating cardiac β-adrenergic receptors that induce positive inotropic action in vitro and in vivo (Yun-Choi et al., 2003). Thus, we investigated the expression of the p-CREB protein because the transcription factor CREB has been identified as a positive regulator of Bcl-2 expression (Wilson et al., 1996; Pugazhenthi et al., 1999). Interestingly, the promoter region of Bcl-2 contains a cAMP-response element site. We also found that CKD712 increased the phosphorylation of CREB both in resting and I/R heart. However, the increased levels of the p-CREB and the Bcl-2 protein were significantly reversed by treatment with the PI3K inhibitor, wortmannin, indicating that the antiapoptotic action of CKD712 appears to act through the activation of the PI3K/Akt-dependent pathway. This was consistent with previous results indicating that Akt inhibited the activation of the proapoptotic protein, Bax (Le Good et al., 1998). However, we did not address other possible pathways for CKD712 activity, such as PKA activation or via the protective action against I/R injury, because our hypothesis was to test whether or not the antiapoptotic effect of CKD712 on I/R myocardial tissue was associated with PI3K activation. Next, we focused our attention on the anti-inflammatory or antioxidant action of CKD712 that may also reduce myocardial damage during I/R. Previously, CKD712 and its congeners reduced pro-inflammatory cytokines in LPS-treated macrophages (Kang et al., 1999; Tsoyi et al., 2008) and induced heme oxygenase (HO)-1 in many cells, including I/R hearts (Lee et al., 2006). We demonstrated that CKD712 showed powerful antioxidant activity in a cell-free system. This effect may limit free radical damage by scavenging ROS generated during the I/R process. It is well known that lipid peroxidation provides a direct measurement of free radical stress in cells or tissues. To assess the level of myocardial ROS, we measured MDA, a surrogate marker of oxidative stress. These results clearly showed that MDA was increased in the hearts of an I/R rat yet were significantly decreased by the administration of CKD712, which was significantly reversed by the presence of WT. Because the inflammatory response is known to play a crucial role in myocardial I/R injury, numerous anti-inflammatory drugs can alleviate reperfusion injury. Indeed, myocardial reperfusion induces an intense accumulation of neutrophils into the area of previously ischemic myocardium, leading to the expression or up-regulation of numerous adhesion molecules and inflammation mediators, not only in the endothelium but also in the cardiomyocytes. We found that CKD712 significantly reduced the activity of MPO in the tissues and of TNF-α in the serum in the I/R rats. We also found that CKD712 significantly reduced the release of HMGB1 in the I/R myocardium in the current study, which
was also reversed by the presence of WT. Evidence increasingly suggests that HMGB1 plays a major role in the early event of I/R injury by binding the receptor for advanced glycation end products, which results in the activation of proinflammatory pathways and enhanced myocardial injury (Andrassy et al., 2008). However, compelling evidence has been reported indicating that HMGB1 may be beneficial in chronically failing rat hearts upon infarction or in I/R rat hearts (Takahashi et al., 2008). Thus, the role and function of HMGB1 in the I/R rat heart are controversial. Because cardiac troponin I, 100% tissue-specific for the heart, is an excellent serum marker for detecting myocardial injury, HMGB1 can be leaked passively from injured tissues in cases involving the I/R myocardium. If this is the case, CKD712 reduces the passive leakage of HMGB1 by reducing myocardial damage. Currently, the exact mechanism of action of CKD712 on HMGB1 release remains to be elucidated. However, it is evident that CKD712 reduces the release of HMGB1, although this reduction may be caused by a reduction of inflammation or myocardial injury. We believe that the anti-inflammatory action of CKD712 probably inhibited HMGB1 release by reducing the levels of proinflammatory cytokines such as TNF-α. In this context, it is noteworthy that CKD712 significantly reduced the serum TNF-α levels in I/R rats. We believe that significant decrease of the serum TNF-α, HMGB1, and cTnI levels in I/R rats may be attributed to inhibition of NF-κB activation because of its strong antioxidant action of CKD712. Previously, CKD712 has been shown to significantly inhibit the production of proinflammatory cytokines in LPS-treated mice in vivo (Yun-Choi et al., 2003) and macrophages in vitro (Tsoyi et al., 2008) by inhibition of NF-κB activation. As expected, these anti-inflammatory effects of CKD712 were significantly reversed by the presence of WT, suggesting that CKD712 acts through the PI3K/Akt signaling pathway.

In summary, we determined that CKD712 can limit myocardial I/R injury by inhibition of apoptosis via the activation of the survival signal through the phosphorylation of Akt. We concluded that both anti-inflammatory action and the activation of the PI3K/Akt signaling pathway are important cellular mechanisms that have been devised for the protective effect of CKD712 against myocardial I/R injury. Thus, CKD712 may be beneficial in myocardial infarction or in situations at risk for oxidant injury.

References

Address correspondence to: Dr. Ki Churl Chang, Department of Pharmacology, School of Medicine Gyeongsang National University, Jinju, 660-751, Korea. E-mail. kcchang@gnu.kr