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## C-phycoyanin protects against ischemia-reperfusion injury of heart through involvement of p38 MAPK and ERK signaling

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**Khan, Mahmood, Saradhadevi Varadharaj, Latha P. Ganesan, Jagdish C. Shobha, Madireddi U. Naidu, Narasimham L. Parinandi, Susheela Tridandapani, Vijay Kumar Kutala, and Periannan Kuppusamy.** C-phycoyanin protects against ischemia-reperfusion injury of heart through involvement of p38 MAPK and ERK signaling. *Am J Physiol Heart Circ Physiol* 290: H2136–H2145, 2006. First published December 22, 2005; doi:10.1152/ajpheart.01072.2005.—We previously showed that C-phycoyanin (PC), an antioxidant biliprotein pigment of *Spirulina platensis* (a blue-green alga), effectively inhibited doxorubicin-induced oxidative stress and apoptosis in cardiomyocytes. Here we investigated the cardioprotective effect of PC against ischemia-reperfusion (I/R)-induced myocardial injury in an isolated perfused Langendorff heart model. Rat hearts were subjected to 30 min of global ischemia at 37°C followed by 45 min of reperfusion. Hearts were perfused with PC (10 μM) or *Spirulina* preparation (SP, 50 mg/l) for 15 min before the onset of ischemia and throughout reperfusion. After 45 min of reperfusion, untreated (control) hearts showed a significant decrease in recovery of coronary flow (44%), left ventricular developed pressure (21%), and rate-pressure product (24%), an increase in release of lactate dehydrogenase and creatine kinase in coronary effluent, significant myocardial infarction (44% of risk area), and TdT-mediated dUTP nick end label-positive apoptotic cells compared with the preischemic state. PC or SP significantly enhanced recovery of heart function and decreased infarct size, attenuated lactate dehydrogenase and creatine kinase release, and suppressed I/R-induced free radical generation. PC reversed I/R-induced activation of p38 MAPK, Bax, and caspase-3, suppression of Bcl-2, and increase in TdT-mediated dUTP nick end label-positive apoptotic cells. However, I/R also induced activation of ERK1/2, which was enhanced by PC treatment. Overall, these results for the first time showed that PC attenuated I/R-induced cardiac dysfunction through its antioxidant and antiapoptotic actions and modulation of p38 MAPK and ERK1/2.

oxidative stress; apoptosis; *Spirulina*; antioxidant; reactive oxygen species; free radicals

THE HEART IS SUBJECTED to episodes of ischemia, followed by reperfusion, in a number of situations, including angina, myocardial infarction, and cardiac surgery. These stresses can result in cell injury and death. The pathogenesis of myocardial ischemia-reperfusion (I/R) injury involves the interplay of multiple mechanisms. Numerous studies have indicated the role of oxidative damage mediated by activation of xanthine oxidase and subsequent formation of reactive oxygen species (ROS), including O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, ·OH, and peroxynitrite, lipid peroxidation of myocardial membranes, action of iron, de-

crease in GSH, altered GSH-to-GSSG ratio, depletion of high-energy phosphates including ATP, depressed energy metabolism, and altered calcium homeostasis in the pathogenesis of I/R injury (31). Several reports describe the efficacy of antioxidants and free radical scavengers superoxide dismutase (SOD), catalase, melatonin, and vitamin E in minimizing I/R injury (17). Overexpression of Mn-SOD, Cu-Zn-SOD, or glutathione peroxidase has been reported to protect the heart from I/R injury, further supporting the involvement of oxidative stress in I/R injury (12, 13, 66).

Studies have suggested that reperfusion of the ischemic myocardium results in cardiomyocyte apoptosis and necrosis in human and animal models of I/R injury (27, 45, 56, 59). Although necrosis represents the classic manifestation of hypoxia-induced cell damage, myocyte apoptosis appears to be an early event in cardiac I/R injury (69). I/R-induced apoptosis is mediated by different apoptotic signaling cascades that also involve the mitochondria-initiated pathway and are mediated by free radicals and oxidative stress, resulting in the release of cytochrome *c* from mitochondria, activation of caspase-9, and downregulation of the antiapoptotic protein Bcl-2 (28). Myocytes lacking the proapoptotic Bax gene reduced I/R injury through blockade of the necrotic and apoptotic pathways (32). Recently, several studies demonstrated the involvement of mitogen-activated protein kinases (MAPKs), which play a role in the induction of apoptosis in myocytes exposed to I/R in ischemic injury (43, 54, 67). Earlier studies demonstrated the activation of ERK1/2 after reperfusion, which is cardioprotective (23, 29, 41, 68). Phosphatidylinositol 3-kinase (PI3K)-Akt signaling is an important mediator of cell survival and promotes survival of cardiomyocytes in vitro and in vivo (2, 61, 71). In addition, it protects against acute I/R injury in the mouse heart (2, 71).

C-phycoyanin (PC), a biliprotein pigment and an important constituent of the blue-green alga *Spirulina platensis*, contains phycocyanobilin, an open-chain tetrapyrrole chromophore that is covalently attached to the apoprotein and plays a major role in some of its important biological properties (40). PC has been reported to possess significant antioxidant and radical-scavenging properties, offering protection against oxidative stress (4). Recently, we demonstrated that PC significantly inhibited doxorubicin-induced oxidative stress and apoptosis in rat cardiomyocytes in vitro (36). In view of these findings, in the

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present study, we investigated the cardioprotective role of PC and *S. platensis* preparation (SP) against I/R injury and the underlying signaling mechanism(s) therein. For the first time, our results revealed that PC significantly enhances the recovery of I/R-induced cardiac dysfunction and protects the heart from apoptosis and injury through its antioxidant action, modulation of p38 MAPK and ERK1/2, and antiapoptotic activity.

## MATERIALS AND METHODS

Fine dark blue-green spray-dried powder of *S. platensis* was obtained from New Ambadi Estates (36). SP was made with the desired amounts of powder in sterile distilled water and used for perfusion. Pure C-phycoerythrin (PC) was provided by Prof. P. Reddanna (University of Hyderabad, Hyderabad, India). SP and PC were prepared fresh each time they were used and filtered (1- $\mu$ m filter, Millipore) before use. Stock solutions of wortmannin, SB-203580, and U-0126 were prepared in DMSO and diluted in Krebs-Henseleit (KH) buffer (DMSO final concentration  $\leq 0.1\%$ ).

**Isolated heart preparation.** Sprague-Dawley rats (300–350 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) along with a heparin solution (500 IU/kg). A thoracotomy was performed, and the hearts were rapidly excised and immersed in ice-cold KH perfusion buffer. The aorta was cannulated, and the heart was perfused in a retrograde fashion, at a hydrostatic pressure of 80 mmHg, with modified KH buffer containing (in mM) 120 NaCl, 25 NaHCO<sub>3</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 CaCl<sub>2</sub>, and 11 glucose. The perfusion buffer was prepared and filtered (0.45- $\mu$ m Whatmann filter) before use and maintained at pH 7.4. The perfusate was equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub> at 37°C, and the hearts were maintained in a water-jacketed organ chamber at 37°C (72). All procedures were performed with approval of the Institutional Animal Care and Use Committee at Ohio State University (Columbus, OH) and conformed to the *Guide for the Care and Use of Laboratory Animals*. (NIH Publication No. 86-23).

**Measurement of heart hemodynamic parameters.** A fluid-filled balloon made of latex was inserted into the left ventricle (via the mitral valve) of the isolated heart and connected to a pressure transducer (ADI Instruments). The balloon was inflated with 0.4 ml of distilled water, sufficient to produce an end-diastolic pressure of 8–12 mmHg. Left ventricular pressure was measured using a pressure transducer connected via a hydraulic line to the ventricular balloon. The heart was continuously monitored with a computer-based data acquisition system (PC PowerLab with Chart 5 software, ADI Instruments). The following functional parameters were measured: left ventricular systolic pressure, left ventricular end-diastolic pressure, left ventricular developed pressure (LVDP), and heart rate (HR). Rate-pressure product (RPP) was calculated from LVDP  $\times$  HR. The coronary flow (CF) rate was measured using a flowmeter with an in-line probe (model T106, Transonic).

**I/R experimental protocol.** Isolated rat hearts were perfused for 15 min to stabilize the functions and then subjected to 30 min of ischemia followed by 45 min of reperfusion. The hearts were randomly divided into three groups of at least eight hearts in each group: the control group, which received no treatment, and the experimental groups to which PC (10  $\mu$ M) and SP (50 mg/l) were added to the perfusate 15 min before ischemia and the treatments were continued throughout reperfusion. Coronary effluent was collected for the determination of lactate dehydrogenase (LDH) and creatine kinase (CK) activities before ischemia and during reperfusion. Myocardial tissue was collected at the end of reperfusion, quickly frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analysis.

**Treatment with Akt, p38 MAPK, and ERK1/2 inhibitors.** Wortmannin (PI3K-Akt pathway inhibitor, 200 nM), SB-203580 (p38 MAPK-specific inhibitor, 10  $\mu$ M), and U-0126 (upstream inhibitor of ERK1/2, 2.5  $\mu$ M) were infused along with the perfusate for 10 min before the onset of ischemia. After 10 min of infusion of these

inhibitors, the hearts were subjected to the I/R protocol described above.

**LDH and CK assay.** Myocardial tissue damage was assessed by determination of the activities of LDH and CK in the coronary effluents collected before ischemia and at 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 min of reperfusion as indexes of release of LDH and CK from the damaged tissue. LDH and CK were determined in the coronary effluents using commercially available assay kits (Sigma Diagnostics and Catachem, respectively). The rate of change in absorbance (NADH) was determined by spectrophotometric measurement at 340 nm for 5 min at 25°C (model 50, Varian, Cary, NC). The enzyme activities were calculated using the molar extinction coefficient of NADH ( $\epsilon = 6.22$ ).

**Evaluation of myocardial infarct size.** Risk area and infarct size were measured by triphenyltetrazolium chloride (TTC) staining (64). TTC stains all living tissue brick red and leaves the infarct area unstained (white). To determine the infarct size, hearts were frozen, stored at  $-20^{\circ}\text{C}$  for 30 min, and then sliced perpendicularly along the long axis from apex to base in 2-mm sections. Sections were incubated for 20 min at 37°C in 1% TTC in PBS (pH 7.4). Once the color was established, the slices were fixed in 10% formalin for 20 min and digitally imaged using a Nikon microscope. The areas of infarct size (TTC-negative) and risk (TTC-positive) were determined using MetaMorph software (Molecular Devices, Downingtown, PA). Infarct size was expressed as percentage of risk area.

**Measurement of ROS by electron paramagnetic resonance spectroscopy.** ROS, mostly O<sub>2</sub> $\cdot$  and  $\cdot$ OH, react with the spin trap to form stable radical adducts, which can be measured by electron paramagnetic resonance (EPR) spectroscopy. The concentration of oxygen free radicals at reperfusion was measured by infusion of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (73) directly into the heart through a side arm using an infusion pump. DMPO (40 mM final concentration) was infused for 20 s before ischemia (control) and again on reperfusion. Effluent samples in 1.0-ml volumes were collected every 20 s for up to 120 s and then at 1- to 2-min intervals for up to 10 min of reperfusion and immediately frozen in liquid nitrogen for EPR measurements. Samples were measured using an ER-300 X-band (9.7-GHz) spectrometer. Spectral simulations were performed to identify specific radicals. The free radical signals were analyzed quantitatively by comparison of the intensity of the observed signal with that of a known concentration of a free radical standard in aqueous solution (52).

**TdT-mediated dUTP nick end labeling assessment of apoptosis.** The TdT-mediated dUTP nick end labeling (TUNEL) protocol is based on the preferential labeling of TdT at the 3'-OH ends of DNA (26). The TUNEL assay was performed using a bromodeoxyuridine (BrdU)-FITC kit (BioVision) according to the manufacturer's instructions. Briefly, 4- $\mu$ m-thick tissue sections were cut from the cryosamples and mounted on slides. The slides were washed with 1 ml of wash buffer provided in the kit and labeled with DNA labeling solution for 60 min at 37°C. After the slides were rinsed and incubated with anti-BrdU-FITC antibody for 30 min in the dark, they were counterstained with propidium iodide (PI) and finally examined by confocal fluorescent microscopy (Zeiss) within 3 h of staining. The brominated deoxyuridine triphosphate nucleotides are more readily incorporated into DNA strand breaks. TUNEL-positive cells are identified by a fluorescent anti-BrdU monoclonal antibody, which shows green staining for apoptotic cells over an orange-red PI counterstaining. The number of TUNEL-positive cells was analyzed using the Image J program.

**Western blot analysis of Bcl-2 and Bax.** Heart tissue samples were incubated with lysis buffer (25 mM sucrose, 20 mM HEPES, 1% Triton X-100, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 17  $\mu$ g/ml PMSF, 8  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, and 5  $\mu$ g/ml pepstatin, pH 7.4) homogenized, and centrifuged at 10,000 g for 10 min at 4°C. Western blot was used to analyze Bax and Bcl-2 protein expression in the supernatants. The

protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce Chemical). Protein (30  $\mu$ g) was resolved by 12% SDS-PAGE along with a See-blue Plus 2 protein ladder (Invitrogen). The blots were then transferred onto a polyvinylidene difluoride membrane, blocked with PBS + 0.1% Tween 20 containing 5% milk (pH 7.4) for 1 h, and incubated with the primary antibody (1:1,000 dilution) with PBS + 0.1% Tween 20 containing 5% milk overnight at 4°C. The membranes were washed three times at 15-min intervals and incubated with mouse IgG peroxidase conjugate at a 1:5,000 dilution for 1 h at room temperature. Protein bands were detected using enhanced chemiluminescence Western blot detection reagents (Amersham Biosciences) and Biomax-Kodak imaging film. The image was scanned, and the intensity of the bands was digitized using Un-Scan-it software (version 5.1, Silk Scientific, Orem, UT).  $\alpha$ -Actin was used as a loading control to correct the pixel values for Bcl-2 and Bax.

**Caspase-3 assay.** A caspase-3/CPP-32 colorimetric assay kit (Bio-Vision) was used to assay caspase-3 activity in heart tissue homogenate. Heart tissue homogenate was prepared using lysis buffer provided in the kit, and 150  $\mu$ g of lysate from each heart tissue were used for the assay, which is based on spectrophotometric detection of the chromophore *p*-nitroanilide after cleavage from the labeled substrate DEVD-*p*-nitroanilide. The activity of caspase-3 was expressed per microgram of protein.

**Detection of Akt, p38 MAPK, and ERK1/2 phosphorylation.** Heart tissues were homogenized in TN1 lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM  $\text{Na}_3\text{VO}_4$ , and 10  $\mu$ g/ml each aprotinin and leupeptin). Ten micrograms of protein from each sample were boiled in SDS sample buffer (60 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 1% 2-mercaptoethanol) for 5 min, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed first with the phosphospecific antibodies for Akt, ERK1/2, and p38 (1:1,000 dilution; Cell Signaling, Beverly, MA) and then with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The filters were developed by enhanced chemiluminescence and then reprobed with antibodies for total Akt, ERK1/2, and actin, respectively (Santa Cruz Biotechnology). The enhanced chemiluminescence signal was quantified using a scanner and a densitometry program (Scion Image). To quantify the phosphospecific signal in the activated samples, we subtracted background, normalized the signal to the amount of actin or total target protein in the lysate, and plotted the values as fold increase over preischemic samples, as previously described (25).

**Data analysis.** The statistical significance of the results of the assays was evaluated by ANOVA and Student's *t*-test. Values are means  $\pm$  SD.  $P < 0.05$  was considered significant.

## RESULTS

**PC enhances recovery of I/R-altered hemodynamic parameters.** The effects of treatment on CF, LVDP, and RPP during I/R in untreated control and PC (10  $\mu$ M)- or SP (50 mg/l)-treated hearts are shown in Fig. 1. The values are expressed as percentage of preischemic baseline values in each group. The preischemic baseline values were as follows: CF =  $16 \pm 5$  ml/min, LVDP =  $120 \pm 20$  mmHg, and HR =  $280 \pm 28$  beats/min. Administration of PC or SP had no effect on the preischemic cardiac function. The untreated control hearts subjected to 30 min of global ischemia followed by 45 min of reperfusion showed a significant decrease in the recovery of CF (44%), LVDP (21%), and RPP (24%). The percent recovery of LVDP and RPP after 45 min of reperfusion was significantly higher ( $P < 0.01$ ) in hearts infused with PC or SP than in control hearts.

To evaluate whether the cardioprotective actions of PC were mediated by the Akt, p38 MAPK, or ERK1/2 pathway, studies were performed using their inhibitors, namely, wortmannin (61), SB-203580 (42), or U-0126 (60), respectively. Stock solutions of wortmannin, SB-203580, and U-0126 were prepared in DMSO and diluted in Krebs buffer. The final concentration of DMSO, which was  $\leq 0.1\%$ , had no significant effect on heart function and recovery compared with the control (data not shown). LVDP and RPP were decreased in hearts perfused with wortmannin compared with untreated control hearts ( $P < 0.05$ ; Fig. 1). The wortmannin-induced decrease of LVDP and RPP was not affected by treatment with PC. Administration of the p38 MAPK-specific inhibitor SB-203580 resulted in an increase in LVDP and RPP compared with control. The increase in LVDP and RPP was much greater in hearts treated with PC + SB-203580 than SB-203580 alone ( $P < 0.01$ ). Treatment with the ERK1/2 upstream inhibitor U-0126 resulted in a decrease in CF, LVDP, and RPP compared with control that was not affected by cotreatment with PC.

**PC attenuates I/R-induced enzyme release by the heart.** LDH activity was measured before and after I/R in the coronary effluent in control and PC- and SP-treated hearts. LDH levels in the effluent from control hearts subjected to I/R significantly increased during reperfusion and attained a maximum at 10–15 min (0.026 U/ml) of reperfusion (Fig. 2). LDH activity significantly decreased in coronary effluent from hearts treated with PC (0.017 U/ml,  $P < 0.05$  vs. control) and SP (0.019 U/ml,  $P < 0.05$  vs. control). In coronary effluent of wortmannin-treated hearts, LDH activity increased, and this increase was significantly attenuated in hearts treated with wortmannin + PC (0.016 U/ml,  $P < 0.001$  vs. wortmannin). On the other hand, LDH activity decreased in hearts treated with SB-203580 (0.013 U/ml) and SB-203580 + PC (0.015 U/ml). There was no significant difference in LDH activity in coronary effluent between hearts treated with U-0126 and U-0126 + PC and untreated hearts.

Similar to LDH, CK activity in coronary effluent significantly increased after I/R and reached a maximum at 15 min (68 U/l) of reperfusion (Fig. 2). The hearts treated with PC or SP exhibited significant attenuation of the I/R-induced increase in CK activity in coronary effluent. CK activity in coronary effluent of hearts treated with PC and SP at 15 min of reperfusion was 29 U/l ( $P < 0.001$  vs. control) and 26 U/l ( $P < 0.001$  vs. control), respectively. CK activity was significantly higher in wortmannin-treated (81 U/l) than in control ( $P < 0.001$ ) hearts, whereas CK activity decreased in the PC + wortmannin-treated group (48 U/l,  $P < 0.001$  vs. wortmannin). CK activity significantly decreased in hearts treated with SB-203580 (22 U/l) and SB-203580 + PC (25.8 U/l). Similarly, CK activity significantly decreased in the coronary effluent of hearts treated with U-0126 (53 U/l) and PC + U-0126 (49 U/l).

**PC reduces I/R-induced infarct size.** TTC staining of control hearts subjected to 30 min of ischemia and 120 min of reperfusion showed  $44 \pm 7\%$  infarct of risk area. PC and SP treatments significantly reduced the infarct size (Fig. 3). Infarct sizes in PC- and SP-treated hearts were  $24.0 \pm 4.5$  and  $26.0 \pm 6.2\%$  of risk area, respectively. Compared with the I/R (control) group, the percentage of infarct size was reduced by SB-203580 and SB-203580 + PC. In hearts treated with wortmannin and U-0126, the infarct size was apparently unchanged as compared with control hearts.

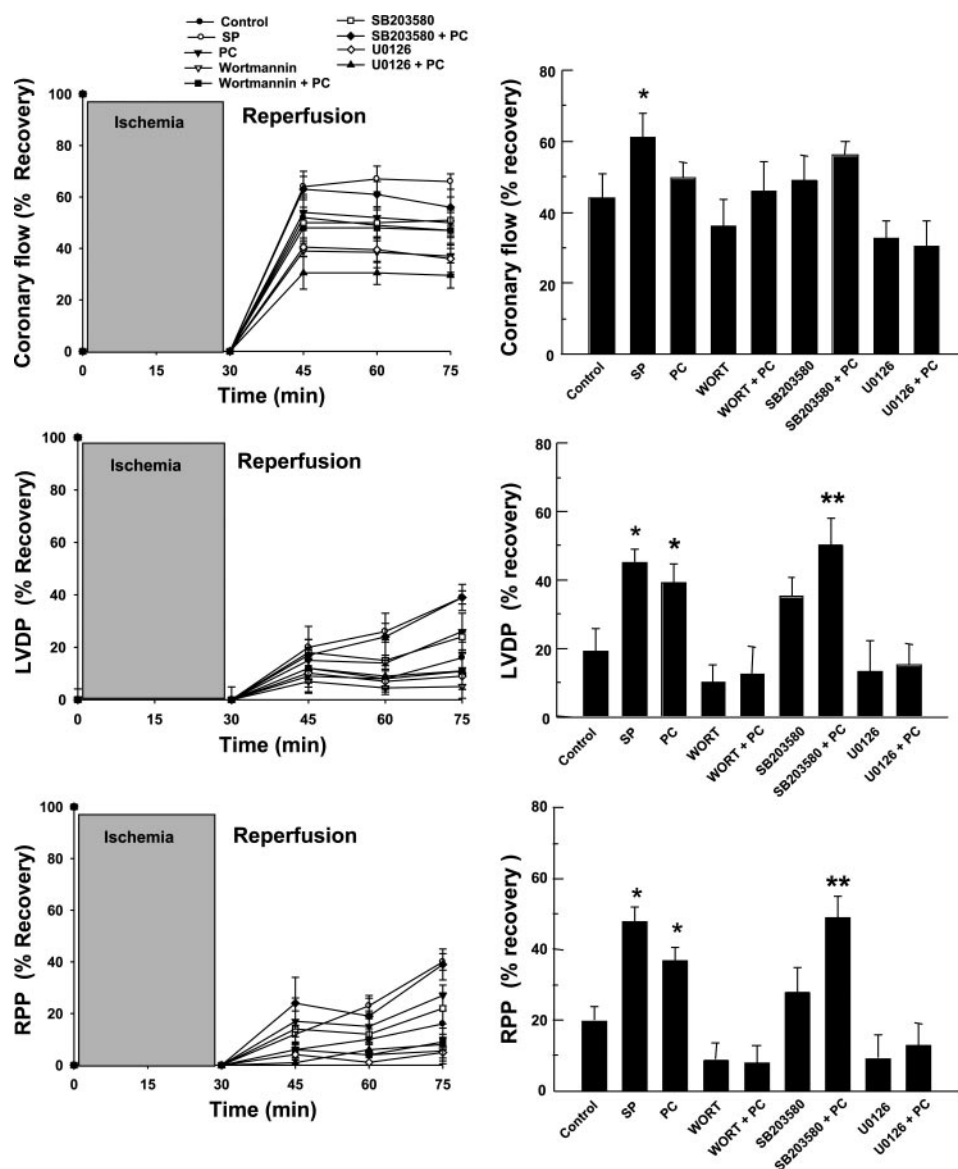


Fig. 1. C-phycocyanin (PC) ameliorates recovery of ischemia-reperfusion (I/R)-altered hemodynamic parameters and contractile functions. Isolated hearts were subjected to 30 min of global ischemia followed by 45 min of reperfusion. *Left*: time course of changes in coronary flow, left ventricular developed pressure (LVDP), and rate-pressure product (RPP). *Right*: recovery of coronary flow, LVDP, and RPP after 45 min of reperfusion. Hearts were treated with *Spirulina platensis* (SP, 50 mg/l) or PC (10  $\mu$ M) for 15 min before ischemia and maintained throughout I/R with and without wortmannin (Wort, 200 nM), SB-203580 (10  $\mu$ M), or U-0126 (2.5  $\mu$ M). Values are means  $\pm$  SD from 6 independent experiments. \* $P$  < 0.01 vs. control. \*\* $P$  < 0.05 vs. SB-203580. PC and SP significantly attenuated cardiac dysfunction induced by I/R.

PC attenuates generation of free radicals during reperfusion. Free radical concentration in effluent of DMPO-perfused hearts was determined using EPR spectroscopy. In untreated control and PC- and SP-treated hearts, oxygen free radical formation (measured as DMPO adduct) peaked at 1 min of reperfusion (Fig. 4). However, DMPO adduct formation was significantly attenuated in PC- and SP-treated hearts compared with control ( $P$  < 0.05).

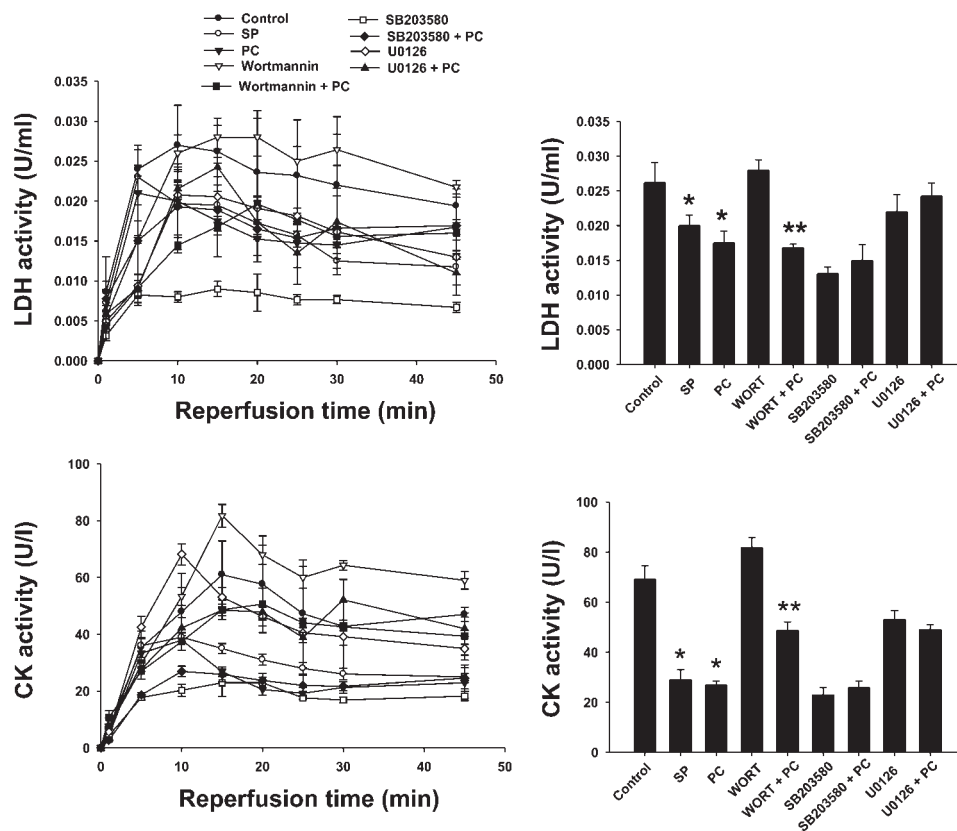
PC attenuates I/R-induced TUNEL-positive apoptotic cells. The nuclei of TUNEL-positive cells are stained green by the incorporation of fluorescein-conjugated deoxyuridine triphosphate nucleotides into the 3'-OH ends of cleaved DNA. PI, a dye that stains cell nuclei, allows quantification of the proportion of apoptotic cells. Stained sections were viewed through a rhodamine filter. TUNEL-negative nuclei appear red, and TUNEL-positive nuclei are bright yellow (Fig. 5A). More TUNEL-positive cells (23%) were seen in hearts subjected to 30 min of ischemia followed by 120 min of reperfusion than in control hearts (0.5%). The number of TUNEL-positive cells

was reduced in hearts treated with PC (4%), SB-203580 (1.8%), SB-203580 + PC (1.6%), U-0126 (8.1%), and U-0126 + PC (2.1%) (Fig. 5B).

PC modulates I/R-induced alterations of Bax and Bcl-2 protein expression. To investigate whether PC could modulate the expression of antiapoptotic Bcl-2 and proapoptotic Bax proteins, Western blot analysis was performed to determine the levels of Bcl-2 and Bax in heart tissue after I/R without and with PC treatment. Compared with preischemic hearts, I/R resulted in an increase in Bax and a decrease in Bcl-2 protein expression (Fig. 6). These changes were attenuated in PC-treated hearts. In addition, the increased Bax-to-Bcl-2 ratio in hearts subjected to I/R ( $P$  < 0.05) was attenuated by PC treatment.

PC attenuates I/R-induced enhancement of caspase-3 activity. Caspase-3 activity increased fourfold in hearts subjected to I/R compared with control hearts (Fig. 7). PC and SP treatments significantly inhibited the I/R-induced activation of caspase-3 activity. The I/R-induced activation

Fig. 2. PC attenuates I/R-induced lactate dehydrogenase (LDH) and creatine kinase (CK) release in coronary effluent from hearts subjected to 30 min of global ischemia followed by 45 min of reperfusion. LDH and CK data are shown as a function of reperfusion time (left) and at 15 min of reperfusion (right). See Fig. 1 legend for treatment protocol. Values are means  $\pm$  SD from 6 independent experiments. \* $P < 0.001$  vs. control. \*\* $P < 0.001$  vs. wortmannin. PC and SP significantly attenuated LDH and CK release.



of caspase-3 was inhibited by SB-203580 and SB-203580 + PC ( $P < 0.01$ ). In wortmannin-treated hearts, caspase-3 activity decreased. However, in hearts treated with wortmannin + PC, U-0126, and U-0126 + PC, caspase-3 activity remained higher and similar to that in control hearts subjected to I/R.

*PC modulates I/R-induced phosphorylation of p38 MAPK, ERK1/2, and Akt.* After 30 min of ischemia, Western blot was used to detect and quantify the extent of phosphorylation of p38 MAPK, ERK1/2, and Akt in tissue homogenates of hearts perfused for 5, 10, 20, and 30 min. I/R increased the extent of phosphorylation of Akt in hearts, with a maximal increase observed at 10 min of reperfusion (3.8-fold over basal level; Fig. 8). PC treatment showed no effect on the I/R-induced increase in Akt activation (Fig. 8B). I/R caused an increase (1.8-fold) in the phosphorylation of ERK1/2, which was further enhanced (4.2-fold,  $P < 0.01$ ) by PC treatment. A significant increase in the activation of p38 MAPK during reperfusion reached a maximum (7.8-fold) at 10 min of reperfusion, declined thereafter, and was markedly and significantly inhibited by PC treatment ( $P < 0.01$ ).

## DISCUSSION

The present study demonstrated that preischemic infusions of PC and SP were cardioprotective against I/R injury, leading to enhanced recovery of contractile function, attenuation of infarct size, decrease of apoptosis, and suppression of oxidative stress in postischemic reperfused heart. This protection is primarily due to the inhibition of apoptosis and necrosis, which was clearly revealed by our present finding that PC treatment decreased TUNEL-positive staining, Bax expression, caspase-3

activity, and infarct size. Several studies demonstrated that ROS produced in the reperfused myocardium cause oxidative stress-mediated injury that is protected by antioxidants (10, 11, 15, 16, 35, 38, 44, 48, 50, 61, 63). Our present results showed that PC significantly suppressed ROS generation in hearts subjected to I/R. Previous studies established that PC scavenges ROS, including superoxide, peroxy, hydroxyl (3, 51), and peroxynitrite (4), and significantly attenuates doxorubicin-induced free radical generation and apoptosis in cardiomyocytes (4, 36). Thus the antioxidant action can be attributed to its cardioprotection against I/R injury.

Apoptosis has been recognized as one of the important mechanisms of cell death during I/R in cultured cardiomyocytes and isolated hearts (5, 27, 34, 46, 55, 57, 62, 70). Our present results demonstrated that, in the heart, I/R was associated with upregulation of proapoptotic protein (Bax), downregulation of antiapoptotic protein (Bcl-2), and increase in caspase-3 activity, all of which were attenuated by PC treatment, suggesting the antioxidant and apoptosis-modulating properties of PC. This suggestion is further supported by experimental evidence that, in the heart subjected to I/R, ROS play a crucial role in apoptotic signaling (24, 44, 45, 49, 53), involving downregulation of the antioxidant gene Bcl-2 with increased DNA fragmentation (46).

MAPKs, namely, p38 MAPK, ERK1/2, and JNK, in different cell systems are activated by stress, including I/R (21, 42, 54, 58, 67). Previous studies demonstrated that ERK1/2 is activated in the first few minutes of reperfusion and offers cardioprotection against oxidative stress by blocking apoptosis (23, 29, 30, 41, 68). Our results demonstrated the involvement of MAPK signaling in PC-mediated attenuation of apoptosis

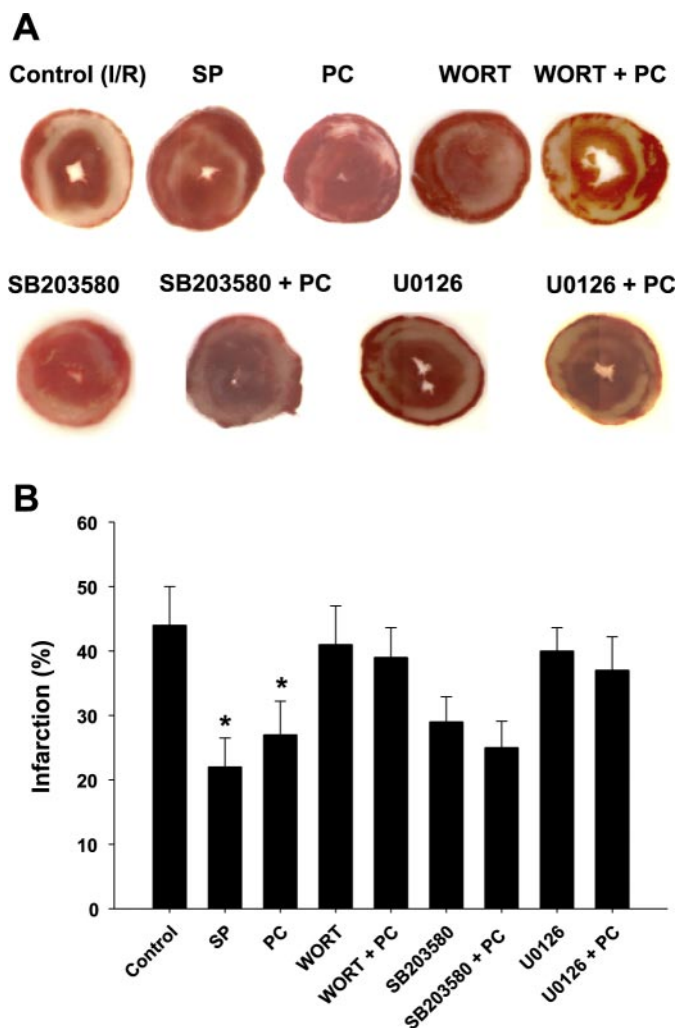


Fig. 3. PC reduces I/R-induced infarct size. Irreversible infarction was determined in triphenyltetrazolium chloride (1%)-stained ventricular sections. Treatment protocol is same as that described in Fig. 1 legend, except reperfusion time was 120 min. A: representative photomicrograph of tissue showing infarct (white) zones after triphenyltetrazolium chloride staining. B: infarct area as percentage of total area of sections measured using Metamorph software. Values are means  $\pm$  SD ( $n = 3$ ). \* $P < 0.01$  vs. control. PC and SP significantly attenuated I/R-induced infarct.

and caspase-3 activation in the heart after I/R, as evidenced by PC-mediated enhancement of I/R-induced activation of ERK1/2. Involvement of the ERK1/2 pathway (Fig. 9) in the cardioprotection provided by PC was further supported by recovery of heart function, reduction in infarct size, decrease in enzyme release, decline in apoptosis, and attenuation of caspase-3 activity induced by U-0126 (an ERK1/2 upstream inhibitor). p38 MAPK inhibition has been reported to be cardioprotective, possibly through suppression of apoptosis after a decrease in caspase-3 activity (43). Inhibition of p38 MAPK suppresses cardiomyocyte apoptosis and improves cardiac function after myocardial I/R (42). Oxidative stress has also been reported to play a role in p38 MAPK activation during I/R (14). Our present results clearly demonstrate that SB-203580, a p38 MAPK-specific inhibitor, not only improved cardiac function after reperfusion but, in addition, attenuated I/R-induced myocardial apoptosis and necrosis in hearts, further suggesting the activation of p38 MAPK in myocardial I/R injury. Studies have provided evidence that ischemia alone and I/R activate p38 MAPK in the heart and cultured cardiomyocytes and that administration of SB-203580 reduces myocardial apoptosis, causing recovery after reperfusion (41, 42, 68). PC treatment, as shown in the present study, significantly attenuated the I/R-induced activation of p38 MAPK and offered cardioprotection against I/R injury, possibly through modulation of p38 MAPK activity.

Our present study revealed a fourfold activation of Akt at 10 min of reperfusion. Recent studies showed the important role of the PI3K-Akt signaling pathway in survival of cardiomyocytes as well as protection against myocardial I/R injury in mice (2, 61, 71). The antiapoptotic effect of Akt is mediated by direct phosphorylation and inactivation of proapoptotic proteins, including caspase-9, an upstream activator of caspase-3 (18). In the present study, the PI3K-Akt pathway inhibitor wortmannin (200 nM) partially blocked the effects of Akt. Utilization of wortmannin to dissect out the role of Akt in apoptosis poses limitations, inasmuch as the doses of the inhibitor used in a particular study are critical. Experimental evidence showing failure to attain complete inhibition of activation of Akt and total blockade of activation of Akt and apoptosis during I/R by wortmannin at 100 nM and 1  $\mu$ M, respectively, supports this limitation (9, 61). Although the findings of the present study revealed that PC did not affect the Akt pathway significantly, the role of the Akt signaling path-

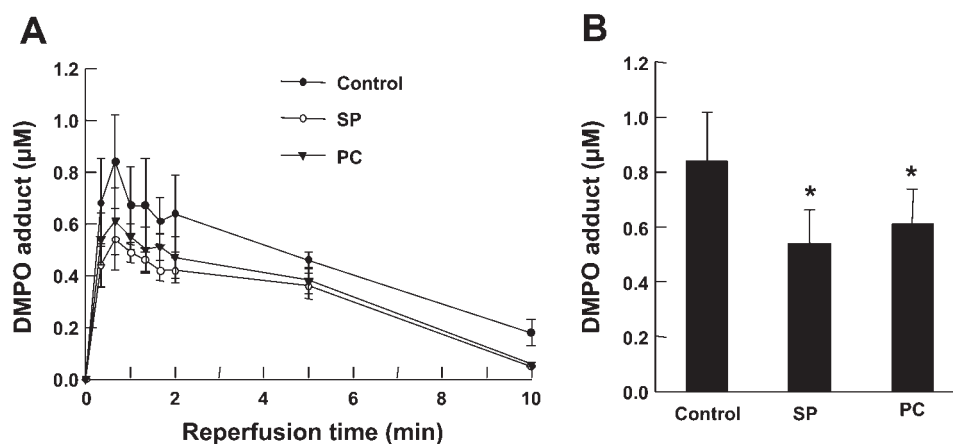


Fig. 4. PC attenuates generation of free radicals during reperfusion. A: time course of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) adduct. B: DMPO adduct at 1 min of reperfusion. Free radical generation was measured using the spin-trap DMPO with and without PC or SP in hearts subjected to 30 min of ischemia followed by 45 min of reperfusion. DMPO (40 mM final concentration) was infused through a sidearm during reperfusion, coronary effluent was collected at 0.5–10 min, and DMPO adduct formation was measured by electron paramagnetic resonance spectroscopy. Values are means  $\pm$  SD from 3 independent experiments. \* $P < 0.05$  vs. control. PC and SP significantly attenuated I/R-induced free radical formation.

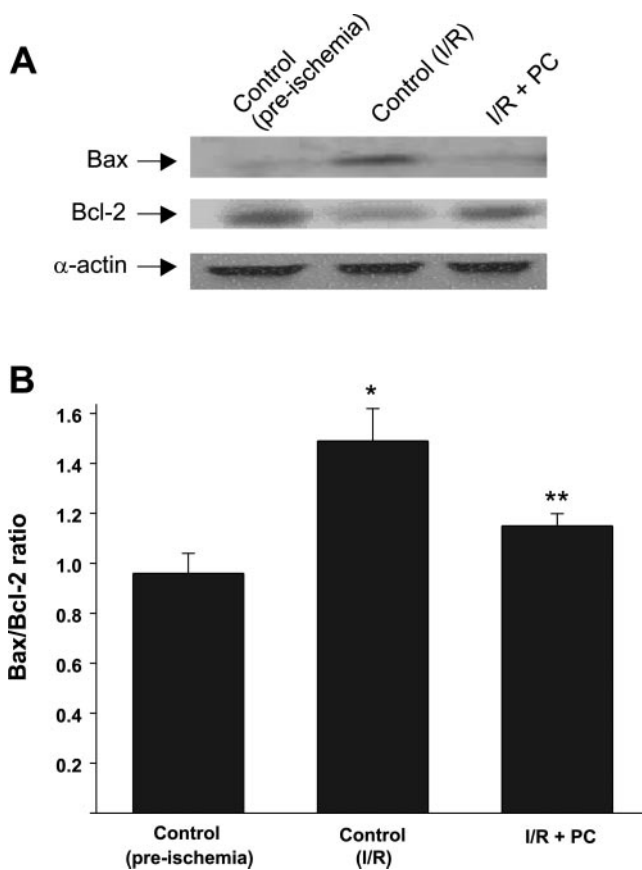
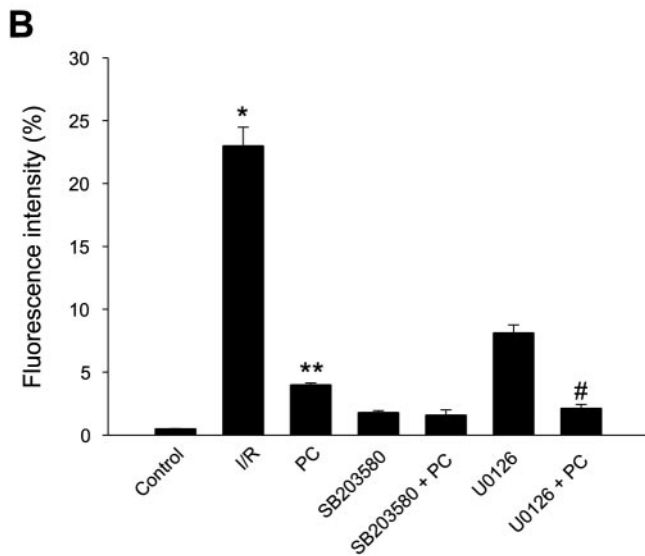
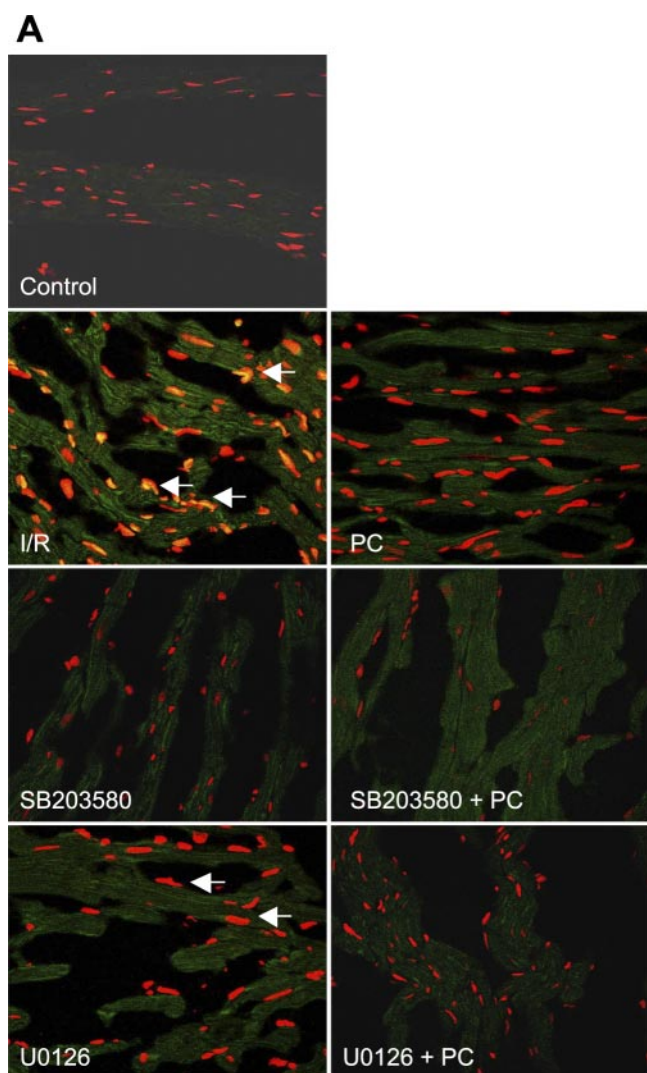


Fig. 6. PC modulates I/R-induced alterations of Bax and Bcl-2 protein expression. Hearts were treated without and with PC and subjected to 30 min of ischemia followed by 45 min of reperfusion. Bax and Bcl-2 protein expression in myocardial tissue was analyzed by Western blot. *A*: representative Western blots from 3 separate experiments. *B*: ratio of Bax to Bcl-2 protein expression. Values are means  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  vs. control (preischemia). \*\* $P < 0.05$  vs. control (I/R). I/R-induced increase in Bax-to-Bcl-2 ratio was attenuated by PC.

way in the protective action of PC against I/R myocardial injury is not ruled out. The kinetics of activation of Akt and the effective dose of PC and wortmannin to attain significant Akt activation in the myocardium after I/R need to be addressed thoroughly.

ERK and p38 MAPK are activated by several extracellular stimuli and through different signaling pathways (7, 8, 19). Activation of ERKs is important in protecting cardiomyocytes from oxidative stress-induced apoptosis (1). Despite activation of the prosurvival kinases, such as ERK1/2 and Akt, during I/R, activation of p38 MAPK may surpass that of the prosurvival kinases, thus mediating myocardial apoptosis and necrosis. Our present results also demonstrated that PC significantly

Fig. 5. PC attenuates I/R-induced TdT-mediated dUTP nick end label (TUNEL)-positive apoptotic cells. Hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion. Frozen cryosections were treated with antibromodeoxyuridine-FITC antibody and propidium iodide and monitored by confocal microscopy. *A*: representative photomicrographs. Counterstaining with propidium iodide results in TUNEL-positive cells with yellow-orange nuclei. *B*: percentage of TUNEL-positive nuclei. Values are means  $\pm$  SD from 3 independent experiments. \* $P < 0.001$  vs. (preischemic) control. \*\* $P < 0.001$  vs. I/R (control). # $P < 0.05$  vs. U-0126. I/R-induced apoptosis was attenuated by PC and SP.



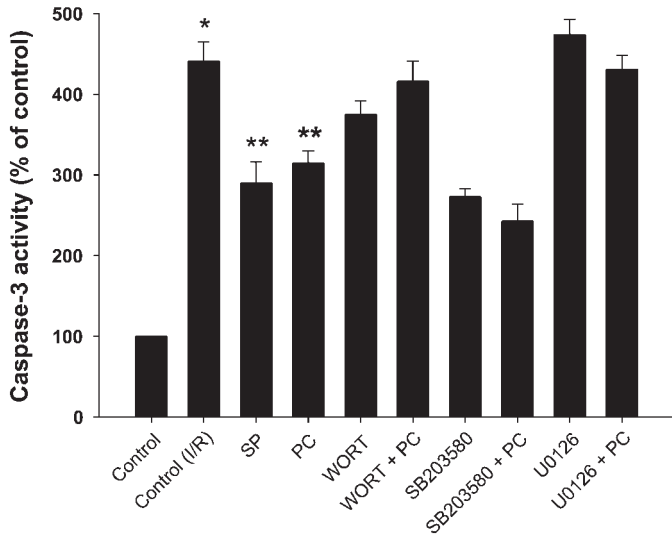


Fig. 7. PC attenuates I/R-induced enhancement of caspase-3 activity. Treatment protocol is described in Fig. 1 legend. Caspase-3 activity was measured in myocardial tissue by colorimetric assay. Data were normalized to total amount of protein. Values are means  $\pm$  SD ( $n = 3$ ). \* $P < 0.001$  vs. (preischemic) control. \*\* $P < 0.01$  vs. control (I/R). I/R-induced increase in caspase-3 activity was inhibited by PC and SP.

enhanced ERK activation and increased the abundance of ERK relative to p38 (4.3-fold vs. control), suggestive of subsequent cardiomyocyte survival. The exact mechanism of PC-mediated inhibition of p38 MAPK and enhancement of ERK activity is

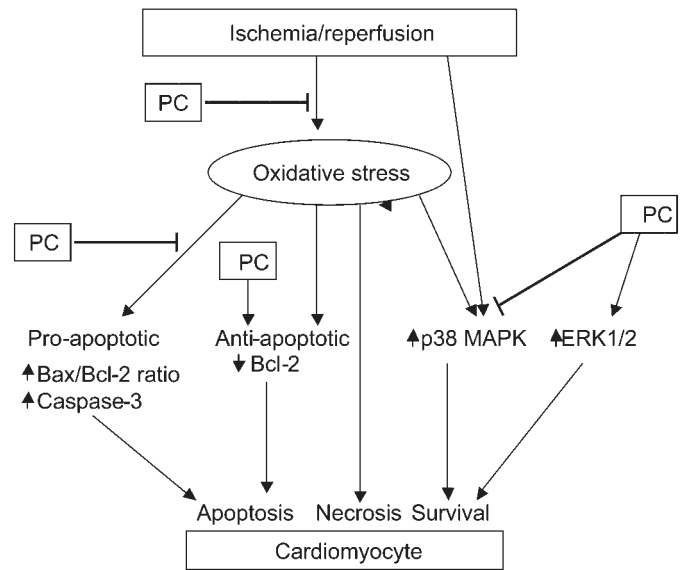


Fig. 9. Schematic description of mechanism of I/R-induced oxidative stress and apoptosis and protection by PC.

yet to be understood. However, we postulate that one possible mechanism is MAPK phosphatase-1 (MKP-1)-mediated regulation of p38 MAPK. Recent studies showed that dual-specificity MAPK phosphatases such as MKP-1 preferentially inactivate p38 MAPK by dephosphorylation (6, 22, 65). Therefore,

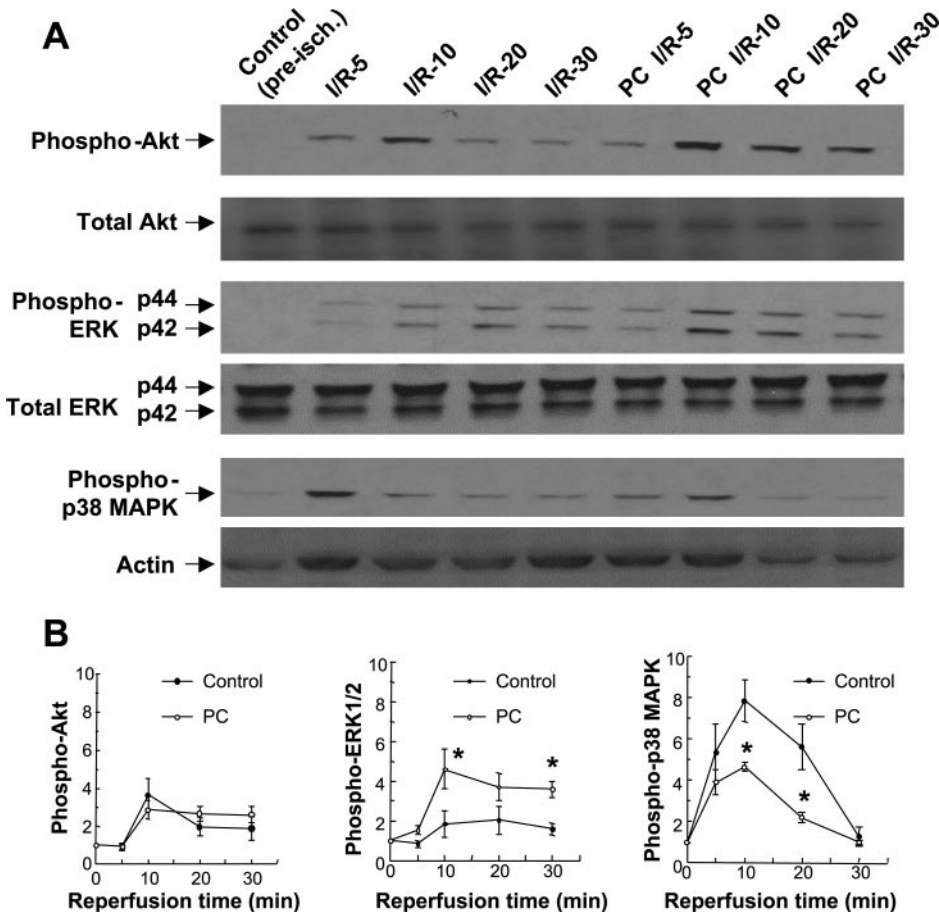


Fig. 8. PC modulates I/R-induced phosphorylation of p38 MAPK, ERK1/2, and Akt. Effect of PC on Akt, ERK1/2, and p38 MAPK phosphorylation was measured in hearts subjected to 30 min of ischemia and 0–30 min of reperfusion without and with 10  $\mu$ M PC. A: Western blot analysis of phosphorylated Akt, ERK1/2, and p38 MAPK. B: quantitative analysis of phosphorylated Akt, ERK1/2, and p38 MAPK. Values are means  $\pm$  SD from 3 independent experiments and expressed as fold increase above respective basal levels. \* $P < 0.01$  vs. control. PC treatment significantly attenuated I/R-induced activation of p38 MAPK and enhanced ERK1/2 activity.

we propose that PC may be activating MKP-1, thereby inhibiting the phosphorylation of p38 MAPK induced by I/R. Activation of the MEK-ERK pathway has been identified to play a role in cell proliferation, survival, and migration, a transformation that apparently depends on cell type and the extent and kinetics of ERK activation (47). Activation of the ERK1/2 pathway by a wide variety of phytochemicals (flavonoids and triterpenoids), leading to modulation of apoptosis, has been reported in different human cell systems (33, 39, 47). Recently, sphingosine-1-phosphate was shown to offer cytoprotection through activation and stimulation of phosphorylation of ERK (37). With these studies as the premise, it is highly possible that PC, in part, as observed in the present study, might have provided cardioprotection against I/R injury through its ability to activate ERK. As the present study has revealed, the antioxidant action of PC, the other possible mechanism by which PC attenuated the activation of p38 MAPK and enhanced ERK1/2 activity after myocardial I/R, might very well be through redox regulation of MAPKs (20). However, the modulation of MKP-1 and/or redox regulation of p38 MAPK and ERK1/2 by PC as a mechanism of PC protection against I/R myocardial injury warrant further investigation.

In conclusion, the results of the present study, for the first time, revealed that PC improved the recovery of cardiac function during I/R-induced myocardial injury by enhancing the activation of ERK1/2 and the expression of Bcl-2 and also by attenuating the activation of p38 MAPK and caspase-3 (Fig. 9). Our results also suggest that PC can act as an effective cardioprotectant against myocardial I/R injury.

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