Original Article

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Pioglitazone attenuates myocardial ischemia-reperfusion injury *via* up-regulation of ERK and COX-2

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Summary **Our previous study demonstrated that the peroxisome proliferator-activated receptor (PPAR) γ agonist, pioglitazone (PIO), may be cardioprotective against ischemia-reperfusion injury; however, modulation of p42/p44 extracellular signal-regulated kinases (ERK1/2) and cyclooxygenase (COX)-2 by PIO in the myocardium with respect to ischemia-reperfusion (I/R) is only partially understood. We determined if PIO reduces I/R-induced apoptosis in cardiomyocytes, and whether or not this protective effect is due to modulation of ERK1/2 and COX-2. Sixty male Sprague-Dawley rats were randomized and assigned to 1 of 6 groups: I/R; I/R + PIO (5 mg•kg^{-1}•day^{-1}); I/R + PIO (10 mg•kg^{-1}•day^{-1}); I/R + PIO (10 mg•kg^{-1}•day^{-1}) + the ERK1/2 inhibitor, PD98059; I/R + PIO (10 mg•kg^{-1}•day^{-1}) + GW9662; and I/R + PD98059. Rats underwent 30 min of myocardial ischemia and 120 min of reperfusion, and then hearts were harvested for analysis. RT-PCR and Western blotting were performed to detect expression of ERK1/2 and COX-2. The number of TUNEL-positive cardiomyocytes and NEC in the PIO groups (5 and 10 mg•kg^{-1}•day^{-1}) was much lower than the I/R group. The cardioprotective effect of PIO was abrogated by PD98059 and GW9662. Phosphorylation of ERK1/2 and COX-2 was increased in the PIO-treated group compared with the I/R group. GW9662 reversed the expression of ERK1/2 and COX-2 phosphorylation induced by PIO. PD98059 reversed the expression of COX-2 induced by PIO. PIO was shown to be cardioprotective in an I/R injury model in rats *via* inhibition of cardiomyocyte apoptosis. PIO limited the infarct size in a PPAR-γ-dependent manner. These results show that PIO triggers the MAPK signaling pathway involving ERK1/2 using COX-2 as the downstream target.**

Keywords: Ischemia-reperfusion injury, apoptosis, peroxisome proliferator-activated receptor γ, pioglitazone, ERK1/2, COX-2

1. Introduction

Ischemia-reperfusion (I/R) injury is a major factor contributing to cardiac dysfunction and infarct size. In fact, I/R injury determines the prognosis after acute myocardial infarction. Cell death during I/R suggests two processes underlying the pathology of myocardial infarction (apoptosis and necrosis). Considerable evidence indicates that apoptosis is a significant contributor to myocardial cell death after I/R, particularly during the early stages (*1*). Studies suggest that targeting the reperfusion-induced apoptotic component of cell death can affect the apoptotic and necrotic components of cell death, the consequences of which are a reduction in infarct size and improved contractile function.

Our previous study demonstrated that the peroxisome proliferator-activated receptor (PPAR) γ agonist, pioglitazone (PIO), may protect the heart from I/R injury; the protective effect is likely to occur by inhibiting cardiomyocyte apoptosis (*1*). PPARs are nuclear hormone receptors that stimulate transcription of specific genes by binding to specific DNA

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sequences after activation by an appropriate ligand. Thiazolidinediones (TZDs: rosiglitazone, troglitazone, and PIO) are synthetic PPARγ ligands with well-recognized effects on glucose and lipid metabolism. It has recently become evident that the therapeutic effects of TZDs reach far beyond use as insulin sensitizers. Recently, several lines of evidence have suggested that TZDs protect the heart and other organs against tissue damage caused by I/R injury (*2*).

I/R has been shown to activate the pro-survival kinase signaling cascades, p42/p44 extracellular signal-regulated kinases (ERK1/2), which have been implicated in cell survival through recruitment of anti-apoptotic pathways of protection (*3*). The ERK1/2 signaling cascade is a mitogen-activated protein kinase (MAPK). The ERK1/2 signaling cascade is a family of serine-threonine kinases that are involved with regulation of the proliferation, differentiation, and survival of cells which are activated in response to the occupation of tyrosine kinase and G-protein-coupled receptors (*4*). The ERK1/2 cascade, when activated in the setting of I/R, can mediate cellular protection (*5,6*). Hausenloy *et al*. (*7*) demonstrated that ischemic pre-conditioning (IPC) protects the heart by phosphorylating the pro-survival kinases, ERK1/2, at reperfusion. The pharmacologic manipulation and up-regulation of pro-survival kinase cascades (the reperfusion injury salvage kinase (RISK) pathway) as an adjunct to reperfusion may therefore protect the myocardium from lethal reperfusion-induced cell death and provide a novel strategy to salvage viable myocardium and limit infarct size.

Data have shown that one of the downstream targets of ERK1/2 in IPC is cyclooxygenase (COX)-2 (*8*). COX-2 mediates the protective effects of ischemia-induced late pre-conditioning in rabbits and mice (*9*). Analyses of COX byproduct levels suggest that COX-2 mediates the late phase of cardioprotection *via* increased production of cytoprotective prostanoids (mainly prostaglandin (PG) I_2 and PGE_2) (*10,11*).

Ye *et al*. (*12*) reported that 3-day pre-treatment with pioglitazone (2.5 mg/kg/d) increased ERK1/2 phosphorylation in rat heart. Another TZD, rosiglitazone, augments ERK1/2 activation in myocardium of hypercholesterolemic rabbits subjected to I/R injury (*13*). Ye *et al*. (*14*) demonstrated that the infarct size-limiting effect of pioglitazone is COX-2-dependent. PIO, but not sitagliptin, increases cytosolic phospholipase A_2 and COX-2 activity in I/R models (*15*); however, modulation of COX-2 by the PPARγ agonist, PIO in the myocardium in relation to I/R has not been shown to be dependent on ERK.

We determined if PPAR-γ activation by PIO reduces I/R-induced apoptosis in cardiomyocytes and the area of necrosis in tissue, and if the underlying mechanism is related to modulation of the ERK1/2 and COX-2 pathways in the myocardium.

2. Materials and Methods

Experiments were carried out in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the Chinese PLA General Hospital (Beijing, China).

2.1. *Materials*

PIO was provided by Beijing Taiyang Pharmaceutical Industry Company, Ltd. (Beijing, China). Rabbit anti-ERK1/2 and anti-phosporylated ERK1/2 primary antibodies were purchased from Beijing Zhongshan Golden Bridge Biotechnology Company, Ltd. (Beijing, China). Rabbit anti-COX-2 primary antibody was purchased from Beijing Jingmei Biotech (Beijing, China). All other chemicals were reagent grade.

2.2. *Experimental preparation*

Sixty male Sprague-Dawley rats (190-240 g; Experimental Animal Center of the Academy of Military Medical Sciences, Beijing, China) were housed in a controlled environment at 25 ± 2°C with alternating 12 h light and dark cycles. All rats were acclimatized in our animal facility for ≥ 7 days before experiments. Stressful stimuli were avoided. Rats were randomized to 6 experimental groups of 10 rats each. Group 1 was the (I/R) group. Rats in group 1 were given 5% (v/v) dimethyl sulfoxide (DMSO, 10 $mL \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days before I/R. The second group was the I/R + PIO (5 $mg \cdot kg^{-1} \cdot day^{-1}$) group. Rats in group 2 were administered PIO (5 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days before I/R. The drug was initially dissolved in DMSO, then diluted in water, so that the final concentration of DMSO was < 5%, and adjusted to pH 7.4. After drug administration, rats had free access to standard rat food and water. The third group was the I/R + PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) group. Rats in group 3 were given PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days before I/R. Group 4 was the I/R + PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) + PD98059 group. Rats in group 4 were given PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days, then the ERK1/2 inhibitor, PD98059 (1 $mg \cdot kg^{-1}$, *iv*), 30 min before I/R. Group 5 was the I/R + PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) + GW9662 group. Rats in group 5 were given PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days, then the selective PPARγ antagonist, GW9662 (1 $mg \cdot kg^{-1}$, *ip*) 30 min before I/R. Group 6 was the I/R + PD98059 group. Rats in group 6 were given 5% (v/v) DMSO (10 $mL \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days, then the ERK1/2 inhibitor, PD98059 (1 $mg \cdot kg^{-1}$, *iv*) 30 min before I/R.

2.3. *Surgical procedures*

Rats were anesthetized with 10% urethane (10 $mL \cdot kg^{-1}$) before surgery. Anesthesia was maintained by supplementary injections of 10% urethane, as required.

The trachea was cannulated and the rats were ventilated with a ventilator (inspiratory oxygen concentration, 30%; 70 strokes•min^{-1}; tidal volume, 8-10 mL•kg^{-1}). A polyethylene catheter was inserted into the right carotid artery and connected to a pressure transducer to monitor blood pressure using an eight-channel polygraph recorder (Q1-160G; Nihon Kohden Corporation, Tokyo, Japan). Hemodynamic parameters were continuously monitored. The heart rate and mean blood pressure were noted at baseline (10 min after completion of surgery); at 10 min of ischemia; and at 10 min of reperfusion.

A snare occluder was placed around the left anterior descending artery (LAD). After completion of the surgical procedure, rats were allowed to stabilize for 30 min before LAD ligation. The coronary artery was occluded by tightening of the occluder. After 30 min of acute myocardial ischemia, the occluder was reopened to allow reperfusion for 120 min. The experiments were performed once.

After completion of the experiment, four hearts in each group were used to measure infarct size, and the other hearts were saved to determine apoptosis and expression of ERK1/2, pERK1/2, and COX-2. The number of hearts was 5, 5, 4, 6, 6, and 6 in groups 1-6, respectively.

2.4. *Quantification of injury to myocardial tissue*

At the end of the 120 min reperfusion period, the LAD was re-occluded and 1 mL of Evans blue dye (2% w/v) was injected into the right carotid artery. The Evans blue dye stained the tissue through which it could circulate, so that non-perfused vascular (occluded) tissue remained colorless. The rats were sacrificed by decapitation after aortic exsanguination. The heart was excised, and excess dye was washed off.

The heart was sectioned into slices 3-4 mm in thickness, and the wall of the right ventricle was removed. The ischemic area (area at risk (AAR)) was distinguished from the area not at risk by Evans blue dye staining. The infarcted portion of the myocardium (necrotic area (NEC)) was determined using the triphenyl tetrazolium chloride (TTC) method. The two portions (AAR and NEC) of the left ventricle were quantified by use of Image-Pro software.

2.5. *Preparation for histologic examination*

The hearts were quickly sectioned at the end of reperfusion. The I/R tissue was separated, fixed in 10% neutral formalin in phosphate-buffered saline (PBS; pH 7.4), and embedded in paraffin wax. The I/R tissue was then cut into 4 μm-thick sections for terminal dUTP deoxynucleotidyltransferase nick end-labeling (TUNEL) assay after deparaffinization and rehydration.

2.6. *Detection of in-situ cell death*

Apoptotic cells were identified using an *In Situ* Cell Death Detection Kit POD (Roche Applied Science, Mannheim, Germany). The kit permits immunohistochemical detection and quantification of apoptosis at the level of a single cell based on labeling of DNA strand breaks. Tissue sections were incubated with freshly prepared 3% hydrogen peroxide solution for 10 min at room temperature to block endogenous production of peroxidase, then with terminal deoxynucleotidyl transferase for 1 h at 37°C. Total and TUNEL-positive cardiomyocytes were counted in five random visual fields per tissue section using image analysis software. The results are expressed as the apoptosis index, as follows: apoptosis index = ((number of TUNEL-positive cardiomyocytes/total cardiomyocytes) ×100).

2.7. *Reverse transcription-polymersase chain reaction (RT-PCR) for COX-2*

Heart tissues were removed from the thoracic cavity, immediately frozen in liquid nitrogen, and stored at –70°C until RNA extraction. Total RNA was extracted from tissues with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 2 g of RNA was treated with ribonuclease-free deoxyribonuclease, and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Two microliters of synthesized cDNA were subjected to 30 cycles of PCR, which resulted in a single specific amplification product of expected size. The PCR conditions were as follows: 45 sec denaturation at 94°C; 45 sec annealing at 60°C (COX-2, β-actin); and 45 sec extension at 72°C. The PCR primers used were as follows: COX-2 sense, 5′-AGTATCAG AACCGCATTGCC-3′ and antisense, 5′-TAAGGTTTCAGGGAGAAGCG-3′; and β-actin sense, 5′-AAGTACCCCATTGAACACGG-3′ and antisense, 5′-ATCACAATGCCAGTGGTACG-3′. β-Actin was used as an internal control for the PCR. The cycle numbers were 30 (COX-2) and 26 (β-actin). The RT-PCRs were in the linear range of amplification for the target mRNA, as well as for the control. Each RT-PCR product was revealed on 1% agarose gels stained with ethidium bromide. The bands were documented, scanned, and quantified using Quantity One software (PDI, New York City, NY, USA) and normalized with the internal control (β-actin). Three repeated tests were performed for each set of measurements and the resulting data were averaged. The results are expressed as the fold change over the I/R group.

2.8. *Western blot analyses for ERK1/2, pERK1/2, and COX-2*

Rat heart tissues were washed twice in ice-cold PBS.

Cytosolic extracts were prepared by homogenizing the tissues in an extraction buffer with freshly added 1 mM Na_3VO_4, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 g/mL leupeptin, and 2 g/mL aprotinin. Protein concentrations were determined using the bicinchoninic acid assay. Approximately 15 μg of total protein was loaded. Proteins were separated on 10% or 12% sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Blots were blocked in blocking buffer containing PBS with 5% albumin. Membranes were incubated with primary antibody (rabbit anti-ERK1/2, rabbit anti-phosphorylated ERK1/2, and rabbit anti-COX-2). Blots were then incubated with secondary antibody conjugated with horseradish peroxidase (1:10,000) for 30 min at room temperature. Blots were developed with an enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK). Three repeated tests were performed for each set of measurements and the resulting data were averaged. The results are expressed as the fold change over the I/R group.

2.9. *Statistical analyses*

Data are the mean ± standard error (S.E.) of *n* observations, where *n* represents the number of rats in the group. For multiple group comparison, data were analyzed by one-way ANOVA, followed by the Student's *t*-test for comparison between two groups. A $p < 0.05$ was considered significant.

3. Results

Sixty rats were included in the protocol. Overall, there were nine rats in the I/R group (one rat was excluded because of a lack of ischemia). There were 9 rats in the PIO 5 mg•kg^{-1} group (1 died during ischemia). There were 8 rats in the PIO 10 mg•kg^{-1} group (2 rats died during reperfusion). None of the rats were excluded from the other groups.

There were no differences in body weight among the groups (Table 1). Heart rate and mean blood pressure are presented in Tables 2 and 3. Overall, there were no statistically significant differences among groups with respect to heart rate and mean blood pressure.

3.1. *PIO decreased the size of myocardial necrosis induced by I/R injury in rat hearts*

The mean values for the AAR, determined by Evans blue dye after reperfusion and expressed as percentages of LV (AAR/LV), showed no difference among the groups (Figure 1). NEC/left ventricle was significantly reduced in the PIO 5 mg•kg^{-1} (by 20%) and PIO 10 mg•kg^{-1} (by 23%) groups compared with the I/R group. NEC/left ventricle was significantly increased in the PIO 10 mg•kg^{-1} + PD98059 group (by 12% and 15%) compared with the PIO 5 mg•kg^{-1} and PIO 10 mg•kg^{-1} groups, respectively. NEC/left ventricle was significantly increased in the PIO 10 mg•kg^{-1} + GW9662 group (by 17% and 20%) compared with the PIO 5 mg•kg^{-1} and PIO 10 mg•kg^{-1} groups, respectively. There were no significant differences between the I/R

Table 1. Body weights

Items	I/R	PIO (5)	PIO (10)	PIO (10) + PD	PIO (10) + GW9662	PD	*p* value
n	9	9	8	10	10	10	
Body weights	228 ± 6	219 ± 7	231 ± 6	214 ± 8	223 ± 5	216 ± 6	0.4069

Values are the mean ± S.E.; *n*, no. of rats.

Table 2. Average heart rate during IS experiments

Items	I/R	PIO (5)	PIO (10)	PIO (10) + PD	PIO (10) + GW9662	PD	*p* value
n	9	9	8	10	10	10	
Baseline	207 ± 4	202 ± 6	209 ± 6	208 ± 5	210 ± 7	207 ± 4	0.9311
10-Min occlusion	212 ± 4	205 ± 5	213 ± 4	208 ± 7	209 ± 5	209 ± 5	0.9094
10-Min reperfusion	211 ± 6	203 ± 6	212 ± 4	206 ± 7	207 ± 3	206 ± 4	0.8482

Values (in beats/min) are the mean ± S.E. heart rate. At baseline, during coronary occlusion and reperfusion, there were no differences among the groups.

Table 3. Mean blood pressure during IS experiments

Items	I/R	PIO (5)	PIO (10)	PIO (10) + PD	PIO (10) + GW9662	PD	*p* value
n	9	9	8	10	10	10	
Baseline	108 ± 4	113 ± 3	109 ± 7	110 ± 5	105 ± 5	109 ± 4	0.9156
10-Min occlusion	106 ± 3	109 ± 5	108 ± 7	108 ± 3	102 ± 4	104 ± 5	0.8863
10-Min reperfusion	100 ± 6	105 ± 4	106 ± 4	105 ± 5	99 ± 3	100 ± 4	0.7735

Values (in mmHg) are the mean ± S.E. blood pressure (MBP). At baseline, during coronary occlusion and reperfusion, there were no differences among the groups.

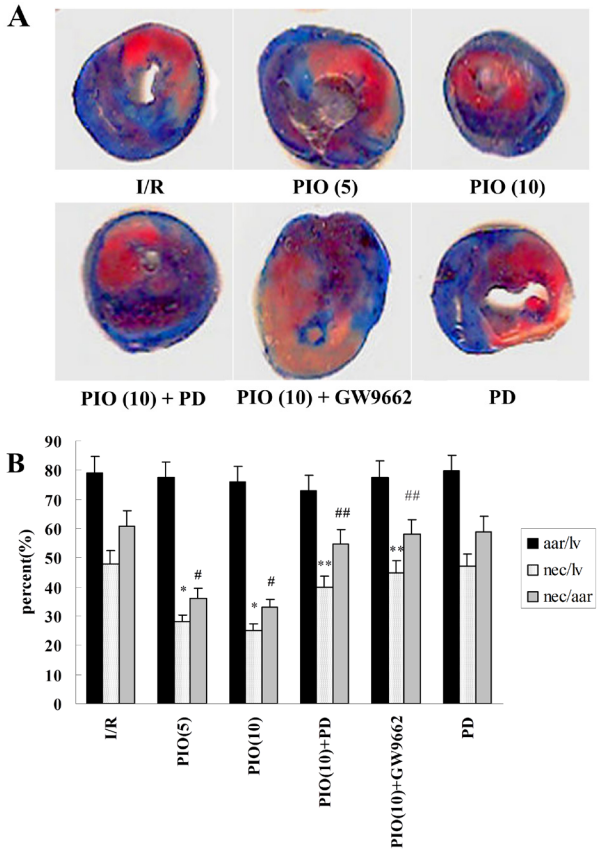

Figure 1. Effects of pioglitazone on ischemia–reperfusion injury. (A), Representative heart sections from I/R and PIO groups with the ischemic area consisting of both the red and pale regions. The ischemic area was determined by negative stain with Evans blue and the infarct area was detected by TTC staining as described in methods section . Note that the area of infarction is the pale zone, indicative of dead tissue. The area not at risk is stained blue. (**B)**, Effects of pioglitazone on MI size..Results are the mean ± S.E. #, * $p <$ 0.05 *vs*. I/R group, ##, ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups. aar: areas at risk; nec: area of necrosis.

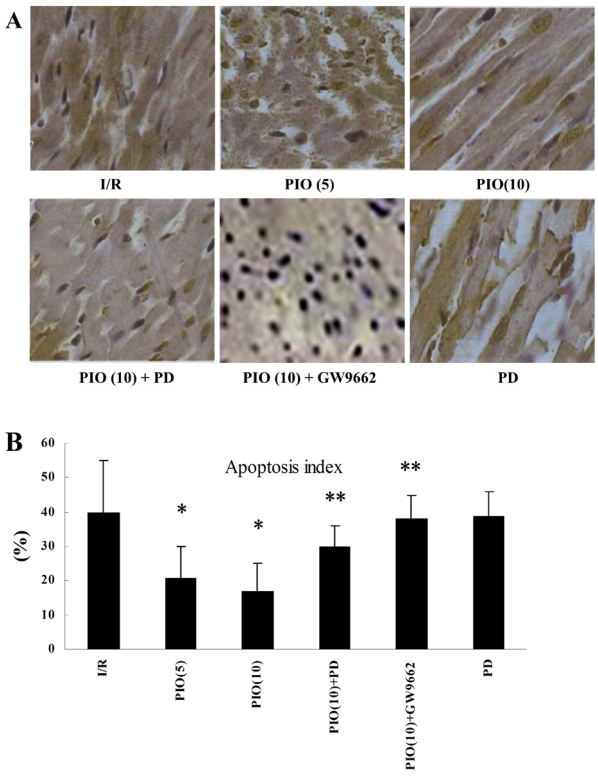

Figure 2. Effect of pioglitazone on cardiomyocyte apoptosis in the ischemia–reperfusion model of rat hearts analyzed by the TUNEL assay (×400). Representative TUNEL showed apoptotic cardiomyocytes from rats along with statistic analysis of apoptotic index. Apoptotic cells were determined using an *In Situ* Cell Death Detection Kit POD. PIO reduced the number of apoptotic cardiomyocytes compared with the ischemia-reperfusion group. Results are the mean ± S.E. * $p < 0.05$ *vs*. I/R group; ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups.

and PD98059 groups, indicating that PD98059 did not affect infarct size when given alone. NEC/AAR was noticeably reduced in the PIO 5 mg•kg^{-1} (by 24%) and 10 mg•kg^{-1} (by 28%) groups compared with the I/R group. NEC/AAR was significantly increased when PIO was used along with PD98059 and GW9662. There was no significant difference in NEC/left ventricle and NEC/AAR between the two PIO subgroups.

3.2. *PIO reduced the myocardial apoptosis index*

In the PIO 5 mg•kg^{-1} and PIO 10 mg•kg^{-1} groups, the number of TUNEL-positive cardiomyocytes was much lower than the I/R group (Figure 2). The apoptosis index was significantly increased when PIO was used along with PD98059 and GW9662. There were no significant differences between the I/R and PD98059 groups.

3.3. *PIO activated ERK1/2 signaling pathways*

Pre-treatment of rat hearts with PIO (5 and 10 mg•kg^{-1}) significantly enhanced the phosphorylation of ERK1/2 compared with the I/R group. The ERK1/2 levels were not significantly different among the groups. The PIO-mediated increase in phosphorylation of ERK1/2 (Figure 3) was reduced by PD98059 and GW9662.

3.4. *PIO up-regulated COX-2 expression in I/R myocardium*

COX-2 levels were significantly increased by pre-treatment with PIO (5 and 10 mg•kg^{-1}) at the mRNA and protein levels compared with the I/R group (Figures 4 and 5). The increased levels of COX-2 were significantly reduced by PD98059 and GW9662.

4. Discussion

We report here that pre-treatment with PIO for 7 days caused a substantial reduction in the apoptosis index and infarct size in rats undergoing regional myocardial I/R. Furthermore, these beneficial changes were accompanied by activation of the ERK1/2 pathway and increased expression of COX-2 at the mRNA and protein levels, which was blocked by GW9662, the

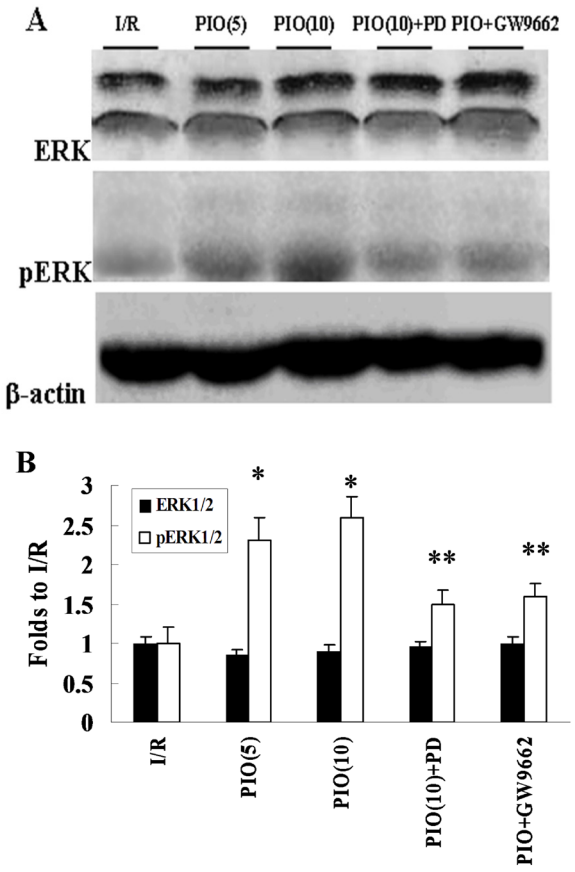

Figure 3. The WB analysis was performed on individual samples of each group. Representative immunoblot **(A)** and densitometric analysis **(B)** of ERK expression. Data are fold change compared with expression in the I/R group. Overall, there were significant differences among groups ($P < 0.05$). Pre-treatment with PIO (5 and 10 mg•kg^{-1}) significantly enhanced the phosphorylation of ERK compared with the I/R group. PIO-mediated increased phosphorylation of ERK1/2 was reduced by PD98059 and GW9662. * $p < 0.05$ *vs*. I/R; ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups.

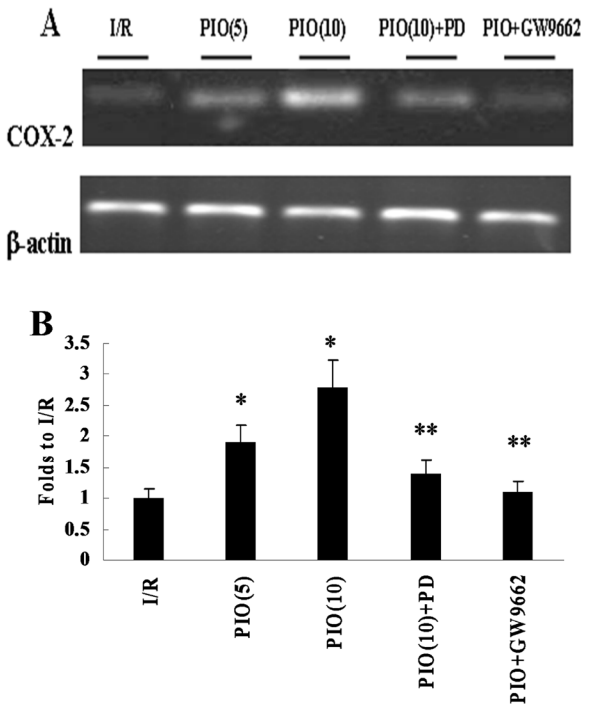

Figure 4. Effects of pre-treatment with pioglitazone on COX-2 mRNA expression. The RT-PCR analysis was performed on individual samples of each group. Relative densitometric units of COX-2/β-actin are shown in the histogram, with the density of the I/R bands set at 1.0. * $p < 0.05$ *vs*. I/R; ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups.

selective PPARγ antagonist. Pre-treatment with the ERK1/2 inhibitor, PD98059, partially blocked the effects exerted by PIO. Increased levels of COX-2 by PIO were significantly reduced by PD98059.

Myocardial ischemia-reperfusion is a problem associated with re-establishment of blood flow in coronary bypass surgery, thrombolysis, and angioplasty. Evidence indicates that apoptosis is a significant contributor to myocardial cell death after I/R. Studies have demonstrated that the pharmacologic inhibition of the apoptotic signaling cascade can attenuate the apoptotic and necrotic components of cell death (*16,17*). In addition to the apoptotic component of cell death contributing to the extension of infarct size during reperfusion, Zhao *et al*. (*18*) demonstrated that pharmacologic inhibition of the reperfusion-induced apoptotic component of cell death also resulted in improved contractile function of ischemic canine hearts. In healthy, diabetic, or obese animals, PPARγ agonists reduced the size of myocardial infarcts (*19*). These effects are associated with increased uptake of glucose

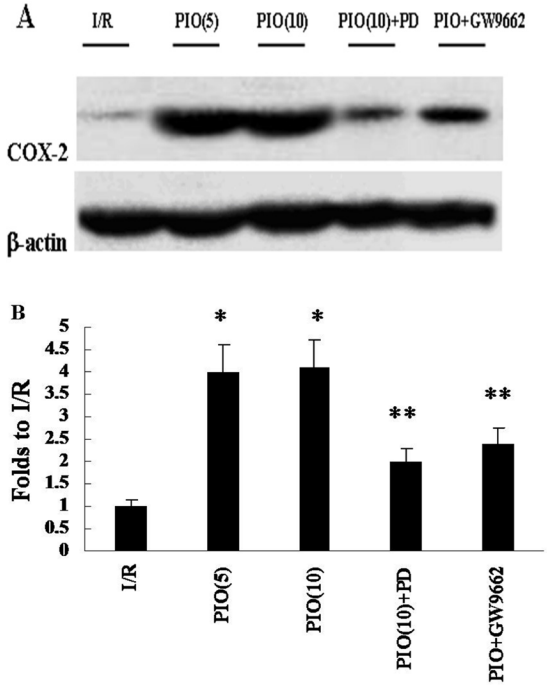

Figure 5. Effects of pre-treatment with pioglitazone on COX-2 protein expression. Each immunoblot is from a single experiment and is representative of all separate experiments. Relative densitometric units of COX-2/β-actin are shown in the histogram, with the density of the I/R bands set at 1.0. Densitometry results are the mean ± S.E. of individual experiments. * $p < 0.05$ *vs*. I/R; ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups.

and improved sensitivity to insulin. PPARγ agonists also reduce post-ischemic myocardial apoptosis (*13*). One study demonstrated the neuroprotective activity of PIO in global cerebral I/R injury and attributed the neuroprotective effects to a reduction in oxidative stress and DNA fragmentation (*20*). Based on the available evidence, the signaling molecules involved in the protection of PPAR-γ agonists include ERK (*12,13*), COX-2 (*14,15*), phosphatidylinositol-3 kinase (PI3K)/Akt, and microRNA-29. The PI3K pathway plays an important role in regulating numerous biological processes, including survival, proliferation, adhesion, migration, insulin activity, and cell activation. The protection of PIO against I/R injury is abolished by PI3K inhibitors (LY294002 or wortmannin); thus, the protection of PIO appears to involve PI3K-Akt (*21*). Nevertheless, there are conflicting reports regarding the effect of TZDs on Akt phosphorylation. In another study, PIO caused a minor, but insignificant increase in myocardial P-Akt expression (*14*). This suggests that the IS-limiting effect of PIO is probably independent of Akt phosphorylation. Down-regulation of miR-29 by an antisense inhibitor or by PIO protects H9c2 cells from simulated I/R injury, as indicated by increased cell survival and decreased caspase-3 activity (*22*); however, it has not been clarified that modulation of COX-2 by the PPARγ agonist, PIO, in the myocardium in relation to I/R is dependent on ERK.

In the present study, pre-treatment with the PPARγ agonist, PIO, markedly increased the level of phosphorylated ERK1/2 in rat hearts. PD98059 reversed the cardioprotection exerted by PIO. Increased levels of COX-2 by PIO were significantly reduced by PD98059. These results imply that PIO decreases apoptosis induced by I/R injury through the ERK1/2 signaling pathway using COX-2 as the downstream target.

The role of ERK1/2 activation in mediating TZD-induced cardioprotection is not clear. Administration of U0126 or PD98059, both of which are P42/44 MAPK inhibitors, before ischemia did not attenuate the infarct size-limiting effects of pioglitazone in Langendorff perfused rat hearts. In contrast, when administered just before reperfusion, both inhibitors blocked the protective effects of PIO, suggesting that ERK1/2 activation only affects reperfusion injury (*21*).

Activation of the ERK1/2 cascade would be expected to reduce cell death through several anti-apoptotic mechanisms (*23*). Based on the available evidence, it appears that BAD, BAX, p70S6K, and eNOS appear to be the downstream components responsible for mediating the protection associated with activation of the ERK1/2 cascade at the time of reperfusion. Data have shown that cardiac damage caused by oxidative stress after I/R was limited by ERK1/2-mediated induction of COX-2 (*8*). The present study confirmed that PIO increased the expression of COX-2 at the mRNA and protein levels. Increased expression of COX-2 was significantly reduced by PD98059, thus COX-2 may be downstream of ERK1/2. Recent studies have demonstrated that IPC up-regulates the expression and activity of COX-2 in the heart, and this increase in COX-2 activity mediates the protective effects of the late phase of IPC against myocardial stunning and infarction. Shibata *et al*. (*24*) reported that COX-2 mediated the cardioprotection from I/R injury due to adiponectin. Neilan *et al*. (*25*) reported that genetic disruption of COX-2 increases cardiac dysfunction after treatment with doxorubicin due to an increase in apoptosis of cardiac cells, suggesting that COX-2 and prostacyclin modulate the expression of genes encoding for proteins involved in apoptosis. Ye *et al*. (*26*) reported that the myocardial protective effect of PIO is iNOS-independent and may be only partially dependent on eNOS. Up-regulation of COX-2 by PIO is independent of NOS (*26*).

In the present study, the selective PPARγ antagonist, GW9662, abolished the protective effects of PIO, suggesting that the protective effects of PIO is likely PPARγ-dependent.

In general, pre-treatment with PIO in rats may protect the myocardium from I/R injury, as demonstrated by the reduction in the apoptosis index and size of the myocardial infarct. PIO might exert protection against I/R injury through the ERK1/2 pathway using COX-2 as the downstream target.

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References

1. Cao Z, Ye P, Long C, Chen K, Li X, Wang H. Effect of pioglitazone, a peroxisome proliferator-activated receptor gamma agonist, on ischemia-reperfusion injury in rats. Pharmacology. 2007; 79:184-192.
2. Abdelrahman M, Sivarajah A, Thiemermann C. Beneficial effects of PPAR-γ ligands in ischemia–reperfusion injury, inflammation and shock. Cardiovasc Res. 2005; 65:772-781.
3. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM. Serine/threonine protein kinases and apoptosis. Exp Cell Res. 2000; 256:34-41.
4. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiol Rev. 1999; 79:143-180.
5. Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B, Ohlstein EH. Inhibition of extracellular signal-regulated kinase enhances Ischemia/Reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. Circ Res. 2000; 86:692-

699.
6. Shimizu N, Yoshiyama M, Omura T, Hanatani A, Kim S, Takeuchi K, Iwao H, Yoshikawa J. Activation of mitogen-activated protein kinases and activator protein-1 in myocardial infarction in rats. Cardiovasc Res. 1998; 38:116-124.
7. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. Am J Physiol Heart Circ Physiol. 2005; 288: H971-H976.
8. Adderley SR, Fitzgerald DJ. Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. J Biol Chem. 1999; 274:5038-5046.
9. Shinmura K, Nagai M, Tamaki K, Tani M, Bolli R. COX-2-derived prostacyclin mediates opioid-induced late phase of preconditioning in isolated rat hearts. Am J Physiol Heart Circ Physiol. 2002; 283:H2534- H2543.
10. Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A, Bolli R. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. Proc Natl Acad Sci U S A. 2000; 97:10197-10202.
11. hinmura K, Xuan YT, Tang XL, Kodani E, Han H, Zhu Y, Bolli R. Inducible nitric oxide synthase modulates cyclooxygenase-2 activity in the heart of conscious rabbits during the late phase of ischemic preconditioning. Circ Res. 2002; 90:602-608.
12. Ye Y, Keyes KT, Zhang CF, Perez-Polo JR, Lin Y, Birnbaum Y. Additive effect of TAK-491, a new angiotensin receptor blocker, and pioglitazone, in reducing myocardial infarct size. Cardiovasc Drugs Ther. 2010; 24:107-120.
13. Liu HR, Tao L, Gao E, Lopez BL, Christopher TA, Willette RN, Ohlstein EH, Yue TL, Ma XL. Anti-apoptotic effects of rosiglitazone in hypercholesterolemic rabbits subjected to myocardial ischemia and reperfusion. Cardiovasc Res. 2004; 62:135-144.
14. Ye Y, Lin Y, Atar S, Huang MH, Perez-Polo JR, Uretsky BF, Birnbaum Y. Myocardial protection by pioglitazone, atorvastatin, and their combination: Mechanisms and possible interactions. Am J Physiol Heart Circ Physiol. 2006; 291:H1158-1169.
15. Ye Y, Keyes KT, Zhang C, Perez-Polo JR, Lin Y, Birnbaum Y. The myocardial infarct size-limiting effect of sitagliptin is PKA-dependent, whereas the protective effect of pioglitazone is partially dependent on PKA. Am J Physiol Heart Circ Physiol. 2010; 298:H1454-1465.
16. Yaoita H, Ogawa K, Maehara K, Maruyama Y. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. Circulation. 1998; 97:276-281.
17. Mocanu MM, Baxter GF, Yellon DM. Caspase inhibition and limitation of myocardial infarct size: Protection against lethal reperfusion injury. Br J Pharmacol. 2000; 130:197-200.
18. Zhao ZQ, Morris CD, Budde JM, Wang NP, Muraki S, Sun HY, Guyton RA. Inhibition of myocardial apoptosis reduces infarct size and improves regional contractile dysfunction during reperfusion. Cardiovasc Res. 2003; 59:132-142.
19. Lee TM, Chou TF. Troglitazone administration limits infarct size by reduced phosphorylation of canine myocardial connexin43 proteins. Am J Physiol Heart Circ Physiol. 2003; 285:H1650-H1659.
20. Kaundal RK, Iyer S, Kumar A, Sharma SS. Protective effects of pioglitazone against global cerebral ischemic-reperfusion injury in gerbils. J Pharmacol Sci. 2009; 109:361-367.
21. Wynne AM, Mocanu MM, Yellon DM. Pioglitazone mimics preconditioning in the isolated perfused rat heart: A role for the prosurvival kinases PI3K and P42/44MAPK. J Cardiovasc Pharmacol. 2005; 46:817-822.
22. Ye Y, Hu Z, Lin Y, Zhang C, Perez-Polo JR. Downregulation of microRNA-29 by antisense inhibitors and a PPAR-γ agonist protects against myocardial ischaemia-reperfusion injury. Cardiovasc Res. 2010; 87:535-544.
23. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: Targeting the reperfusion injury salvage kinase (RISK)-pathway. Cardiovasc Res. 2004; 61:448-460.
24. Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K. Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. Nat Med. 2005; 11:1096-1103.
25. Neilan TG, Doherty GA, Chen G, Deflandre C, McAllister H, Butler RK, McClelland SE, Kay E, Ballou LR, Fitzgerald DJ. Disruption of COX-2 modulates gene expression and the cardiac injury response to doxorubicin. Am J Physiol Heart Circ Physiol. 2006; 291:H532-H536.
26. Ye Y, Lin Y, Manickavasagam S, Perez-Polo JR, Tieu BC, Birnbaum Y. Pioglitazone protects the myocardium against ischemia-reperfusion injury in eNOS and iNOS knockout mice. Am J Physiol Heart Circ Physiol. 2008; 295:H2436-H2446.

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