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Differential consequences of protein kinase C activation during early and late hepatic ischemic preconditioning

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Abstract Activation of protein kinase C (PKC) has been implicated in the protection of ischemic preconditioning (IPC), but the exact role of PKC in early and late hepatic IPC is still unclear. The present study was conducted in order to investigate the differential role of PKC during early and late hepatic IPC. Rats were subjected to 90 min of partial hepatic ischemia followed by 3 (early IPC) and 24 h (late IPC) of reperfusion. IPC was induced by 10 min of ischemia following 10 min of reperfusion prior to sustained ischemia, and chelerythrine, a PKC inhibitor, was injected 10 min before IPC (5 mg/kg, i.v.). Chelerythrine abrogated the protection of early IPC, as indicated by increased serum aminotransferase activities and decreased hepatic glutathione content. While the IPC-treated group showed a few apoptotic cell deaths during both phases, chelerythrine attenuated these changes only at late IPC and limited IPC-induced inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1) overexpression. Membrane translocation of PKC-δ and -ε during IPC was blocked by chelerythrine. Our results suggest that PKC might play a differential role in early and late IPC; activation of PKC-δ and -ε prevents necrosis in early IPC through preservation of redox state and prevents apoptosis in late IPC with iNOS and HO-1 induction. Therefore, PKC represents a promising target for hepatocyte tolerance to ischemic injury, and understanding the differential role of PKC in early and late IPC is important for clinical application of IPC.

Introduction

Hepatic ischemia and reperfusion (I/R) injury is one of the commonly encountered problems in a variety of clinical settings, such as liver transplantation, trauma, and elective liver resection [1]. Among the various attempts to determine an ideal treatment for prevention of hepatic I/R injury, ischemic preconditioning (IPC), an endogenous mechanism of protection against a sustained ischemic insult after an initial but brief ischemic stimulus, has received increasing interest, since it reduces release of transaminases [2], increases rat survival after liver transplantation [3], and even improves human hepatic functions after major liver surgery in patients subjected to 30 min of ischemia [4]. IPC occurs in a biphasic pattern: an early phase, which wanes for several hours (early IPC), and involves direct modulation of cell function as a result of accumulation of adenosine and/or nitric oxide and activation of protein kinase C (PKC), and a late phase (late IPC), which reappears 24–48 h after reperfusion, and requires synthesis of multiple stress-response proteins, including heat shock proteins (HSP), nitric oxide synthase (NOS), and heme oxygenase-1 (HO-1) [5–7]. Indeed, recent advances in understanding of molecular mechanisms of protection provided by IPC have enabled application of this phenomenon to a variety of surgical and pharmacological interventions in the clinic [8]. However, there is still a need to better understand this issue, because the data so far have been obtained primarily from in vitro study, which does not recapitulate the systemic and hemodynamic stress components of I/R injury in vivo.

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Mode of cell death in the process of reperfusion injury has been extensively investigated during the last decades. Indeed, hepatic I/R has resulted in necrotic cell death within minutes of reperfusion [9] while others have shown the occurrence of apoptosis as early as 2 h of reperfusion [10]. Despite the concurrent induction of these two distinctive forms of cell death, we have recently reported that necrosis might be the main form of cell death in the reperfused rat liver during the early phase, which then shifts to apoptosis during the late period of reperfusion [11]. Development of early and late IPC has been shown to protect cells from both necrotic and apoptotic cell death. IPC-induced tolerance to reactive oxygen species (ROS) is thought to be responsible for this prevention, since ROS provokes cell death either by directly attacking various cellular molecules or by indirectly promoting synthesis of various protease [12]. Specifically, IPC decreased necrotic cell death in the early phase of reperfusion and this effect lasted up to 48 h [13]. IPC prior to partial warm hepatic I/R in mice also reduced apoptotic cell death through a caspase-dependent pathway during early IPC [10]. However, in the heart, IPC was found to attenuate apoptotic cell death, not only during early IPC but also during late IPC [14]. In this regard, clarification of the effect of IPC on the main mode of cell death that occurs in a sequential manner after hepatic I/R is of clinical importance.

PKC, a family of serine/threonine kinases, has been implicated as an important mediator in IPC; isozyme-selective translocation of PKC has been previously documented, even though multiple PKC isozymes were identified. During early and late hepatic IPC, PKC activation and subsequent overexpression of mitogen-activated protein kinase (MAPK) have been shown to prevent necrotic cell death [15]. Using isolated hepatocytes, Carini et al. [16] reported that activation of PKC-δ and -ε isozymes coupled with p38 MAPK activation results in reduction of acidosis and Na$^+$ overload during early IPC. In cardiomyocytes, translocation of PKC-ε induced by IPC was responsible for prevention of apoptotic cell death during late IPC [17].

This study aimed to evaluate the role of PKC and its downstream signaling as a possible cellular mechanism in prevention of cell death promoted by hepatic I/R during early and late IPC.

Materials and methods

Hepatic I/R and IPC procedures

All animal protocols were approved by the Animal Care Committee of Sungkyunkwan University and were performed in accordance with the guidelines of the National Institutes of Health. Male Sprague–Dawley rats (body weight 270–300 g; Orient bio, Gapyeong, Korea) were fasted for 18 h, but allowed free access to tap water. Under ketamine (60 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.) anesthesia, a midline laparotomy was performed. Using an operating microscope, the liver hilum was exposed, and portal structures to the left and median lobes were occluded with a microvascular clamp (Biomedical Research Instruments, Rockville, MD, USA) for a period of 90 min; reperfusion was initiated by removal of the clamp. In the preconditioned group, preconditioning was induced by 10 min of ischemia followed by 10 min of reperfusion prior to 90 min of sustained ischemia, according to our preliminary studies. Liver tissues and blood samples were taken at 3 and 24 h. Liver tissues were stored at −75°C for later analysis, except for the part in the left lobe, which was used for histological analysis.

Drug treatment

Chelerythrine (Sigma-Aldrich, St. Louis, MO, USA), dissolved in distilled water, was administered by intravenous injection via the penile vein, 10 min prior to IPC at a dose of 5 mg/kg of body weight; an equal volume of saline was injected for the controls. Briefly, after placing the rat in a supine position, the glans penis was extruded by sliding the prepuce downwards and pressing at the base of the penis. The penile vein was visible and the 23-gauge needle was used to achieve intravenous injection. The dose and injection time of chelerythrine treatment were based on previous reports [18]. Rats were randomly divided into 14 groups: (1) vehicle-treated sham, 3 h ($n = 4$); (2) chelerythrine-treated sham, 3 h ($n = 4$); (3) vehicle-treated sham, 24 h ($n = 4$); (4) chelerythrine-treated sham, 24 h ($n = 4$); (5) rottlerin (ROT)-treated sham, 10 min of ischemia ($n = 4$); (6) PKC-εV1-2 (εV1-2)-treated sham, 10 min of ischemia ($n = 4$); (7) vehicle-treated ischemic (I/R), 3 h ($n = 9$); (8) vehicle-treated ischemic preconditioned I/R (IPC + I/R), 3 h ($n = 10$); (9) chelerythrine-treated ischemic preconditioned I/R (CHE + IPC + I/R), 3 h ($n = 10$); (10) I/R, 24 h ($n = 9$); (11) IPC + I/R, 24 h ($n = 10$); (12) CHE + IPC + I/R, 24 h ($n = 10$); (13) ROT-treated ischemic ($n = 6$); and (14) PKC-εV1-2-treated ischemic ($n = 6$). Because there were no differences in any of the parameters between chelerythrine-, vehicle-, ROT-, and PKC-εV1-2-treated rats in the sham groups, the results of groups (1) and (2) were pooled and were referred to as sham, 3 h ($n = 8$), groups (3) and (4) were pooled as sham, 24 h ($n = 8$), and groups (5) and (6) as sham, ischemic ($n = 8$).

Histological analysis

Formalin-fixed samples were embedded in paraffin and cut into 5-μm sections. Tissues were stained with hematoxylin

and eosin (H&E), and slides were assessed for inflammation and tissue damage using Olympus microscopy (Olympus Optical, Tokyo, Japan) at ×400 magnification.

Serum aminotransferase activities

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using the standard spectrophotometric procedure using ChemiLab ALT, and AST assay kits (IVDLab, Uiwang, Korea), respectively.

Hepatic lipid peroxidation and glutathione content

The steady-state level of malondialdehyde (MDA), the end-product of lipid peroxidation, was analyzed in liver homogenates by spectrophotometric measurement of the level of thiobarbituric acid-reactive substances at a wavelength of 535 nm according to the method described by Buege and Aust [19] using 1,1,3,3,-tetraethoxypropane as the standard. Total glutathione in the liver homogenate was determined spectrophotometrically at a wavelength of 412 nm using yeast glutathione reductase, 5,5′-dithio-bis(2-nitrobenzoic acid), and NADPH according to the method reported by Tietze [20]. The oxidized glutathione (GSSG) level was measured using the same method in the presence of 2-vinylpyridine, and the reduced glutathione (GSH) level was determined from the difference between the total glutathione and GSSG levels.

Protein extraction of whole liver tissue

Isolated liver tissue was homogenized in PRO-PREP^{TM} Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea) in a microcentrifuge tube. After standing in a cold ice-bath for a period of 30 min for cell lysis, the whole homogenate was centrifuged at $13,000g$ for 5 min. The supernatant was collected, and the protein concentrations of the whole homogenates were determined using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA).

Isolation of liver mitochondrial and cytosolic fraction

Liver mitochondrial fraction was prepared according to previous reports [21]. Briefly, the tissues were homogenized on ice using a Teflon pestle homogenizer (Thomas Scientific, Swedesboro, NJ, USA) in medium containing 250 mM sucrose, 10 mM Tris–HCl, and 1 mM EDTA, pH 7.2 at 4°C. The homogenate was centrifuged at $600g$ for 10 min and the supernatant was centrifuged for 5 min at $15,000g$ to obtain the mitochondrial pellet. The mitochondrial pellet was then washed with a medium containing no added EDTA, and centrifuged for 5 min at $15,000g$, resulting in a final pellet containing approximately 50 mg protein/ml. The supernatant was centrifuged at $100,000g$ for 30 min. The resulting supernatant was used as the particulate-free cytosolic fraction. The BCA Protein Assay kit (Pierce Biotechnology) was used for determination of protein concentration of the mitochondrial and cytosolic fractions.

Immunoblots

Protein samples were loaded on 10–15% polyacrylamide gels and were then separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using the Semi-Dry Trans-Blot Cell (Biorad Laboratories, Hercules, CA, USA). After transfer, the membranes were washed with 0.1% Tween-20 in 1× Tris-buffered saline (TBS/T) and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBS/T. Blots were then incubated overnight at 4°C with primary antibodies. After being washed three times for 5 min each in TBS/T, the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature and detected using and ECL detection system (iNtRON Biotechnology), according to the manufacturer's instructions. ImageQuant^{TM} TL software (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA) was used for densitometric evaluation of visualized immunoreactive bands. Primary antibodies against PKC-α, -βI, -βII, -δ, -ε, and -ζ (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), total p38, extracellular signal-related kinase (ERK), and c-Jun N-terminal kinase (JNK) (1:1,000 dilution; Cell Signaling Technology, Beverly, MA, USA), p-p38, p-ERK, and p-JNK (1:1,000 dilution, Cell Signaling Tecnology), cytochrome c (1:1,000 dilution; BD Pharmingen^{TM}, San Jose, CA, USA), HO-1 (1:1,000 dilution; StressGen Biotechnologies, Victoria, BC, Canada), inducible NOS (iNOS) (1:500 dilution; Transduction Laboratory, Lexington, KY, USA), and cyclooxygenase-2 (COX-2) (1:500 dilution; Cayman Chemical, Ann Arbor, MI, USA) were used and the signals were standardized to that of β-actin (1:2,000 dilution; Sigma-Aldrich).

Caspase-3 activity

Caspase-3 activity was measured using an in vitro fluorogenic peptide substrate, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (DEVD-AFC; BioMol, Plymouth Meeting, PA, USA), according to the procedure reported by Morin et al. [22]. Liver tissue (1 g) was homogenized in 6 ml of a buffer containing 25 mM Tris, 5 mM MgCl$_2$, 1 mM EGTA, and 50 μl of a protease

inhibitor cocktail (Sigma-Aldrich). The homogenate was centrifuged at 600g for 10 min. The supernatant was then centrifuged again for 15 min at 40,000g, and the resulting supernatant was collected for determination of caspase-3 activity. Dithiothreitol (10 mM) was added to the samples immediately before freezing. Caspase-3 activity was assayed in a total volume of 100 μl. Then, 30 μg of the cytosolic protein were incubated in a buffer containing 30 mM HEPES, 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100, and 10 mM dithiothreitol. The samples were incubated at room temperature for 15 min. The reaction was started by addition of 200 μM DEVD-AFC, and the samples were incubated at 37°C. Change in fluorescence (excitation at 400 nm and emission at 490 nm) was monitored after 120 min of incubation.

Detection of apoptotic cells

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining using a commercially available kit (In situ Apoptosis Detection Kit; TaKaRa, Shiga, Japan), according to the manufacturer's instructions. Under microscopy, the number of TUNEL-positive cells in ×400 histological fields was counted per liver section.

Statistical analysis

The overall significance of the results was examined using two-way analysis of variance (ANOVA). The difference between the groups was considered statistically significant at $P < 0.05$ with the appropriate Bonferroni correction made for multiple comparisons. Results are presented as the mean ± standard error of mean values (SEM).

Results

Effects of IPC and chelerythrine on hepatocellular damage

In sham-operated animals, serum ALT and AST activities remained constant at basal level throughout the experimental period. In rats undergoing I/R, ALT activity showed a dramatic increase at 3 h after reperfusion, and then a gradual decrease, but did not recover completely to the normal basal level, even after 24 h of reperfusion. These increases were significantly attenuated by IPC at both 3 and 24 h of reperfusion. Pretreatment with chelerythrine abolished the beneficial effect of IPC at 3 h of reperfusion, but did not exert any effect on the ALT level at 24 h of reperfusion. Similarly, serum AST activity in rats undergoing I/R showed a dramatic increase at 3 h of reperfusion

and this increase was gradually decreased by 24 h of reperfusion. Whereas IPC significantly attenuated I/R-induced increase in AST activity at both 3 and 24 h of reperfusion, chelerythrine treatment resulted in significant reversal of this decrease at 3 h of reperfusion (Fig. 1a). As shown in Fig. 1b, liver sections isolated from sham-operated animals showed normal lobular architecture and cell structure. However, liver obtained from rats undergoing I/R showed multiple and extensive areas of hepatocyte necrosis randomly distributed throughout the parenchyma. In contrast, in the liver of animals subjected to IPC before the primary I/R, only mild centrizonal necrosis and Kupffer cell hyperplasia were observed in scattered areas; these protective effects of IPC were reversed by chelerythrine pretreatment.

Effects of IPC and chelerythrine on oxidative stress

Hepatic GSH content in sham-operated animals was 7.2 ± 0.6 and 7.2 ± 0.7 μmol/g liver at 3 and 24 h after reperfusion, respectively. Reperfusion causes a significant decrease in hepatic GSH content at 3 h of reperfusion and tends to be gradually restored by 24 h of reperfusion. The most significant decrease, which occurred at 3 h after reperfusion, was attenuated by IPC, while there was no difference in hepatic GSH content between the I/R group and IPC-treated I/R group at 24 h after reperfusion. The effect of IPC on GSH content was abolished by chelerythrine. The level of MDA showed a marked increase at 3 h after reperfusion and this elevation persisted until 24 h of reperfusion. The elevated MDA level was significantly attenuated by IPC at 3 h of reperfusion and chelerythrine attenuated this beneficial effect. At 24 h of reperfusion, neither IPC nor chelerythrine exerted any effect on the level of MDA (Table 1).

Effects of IPC and chelerythrine on cytosolic cytochrome c expression, caspase-3 activity, and apoptotic cell death

The level of cytochrome c protein expression was barely detectable in liver cytosol fractions obtained from sham-operated animals. However, cytochrome c content showed a dramatic increase at 3 h after reperfusion and remained elevated even after 24 h of reperfusion. Although IPC prevented an increase in cytosolic cytochrome c content at all time points measured, pretreatment with chelerythrine abolished the effect of IPC at only 24 h of reperfusion (Fig. 2a). Results shown in Fig. 2b indicate that caspase-3 activity in sham-operated animals was quite low; caspase-3 activity in liver isolated after 3 h of reperfusion was considerably higher than that of sham-operated animals, and
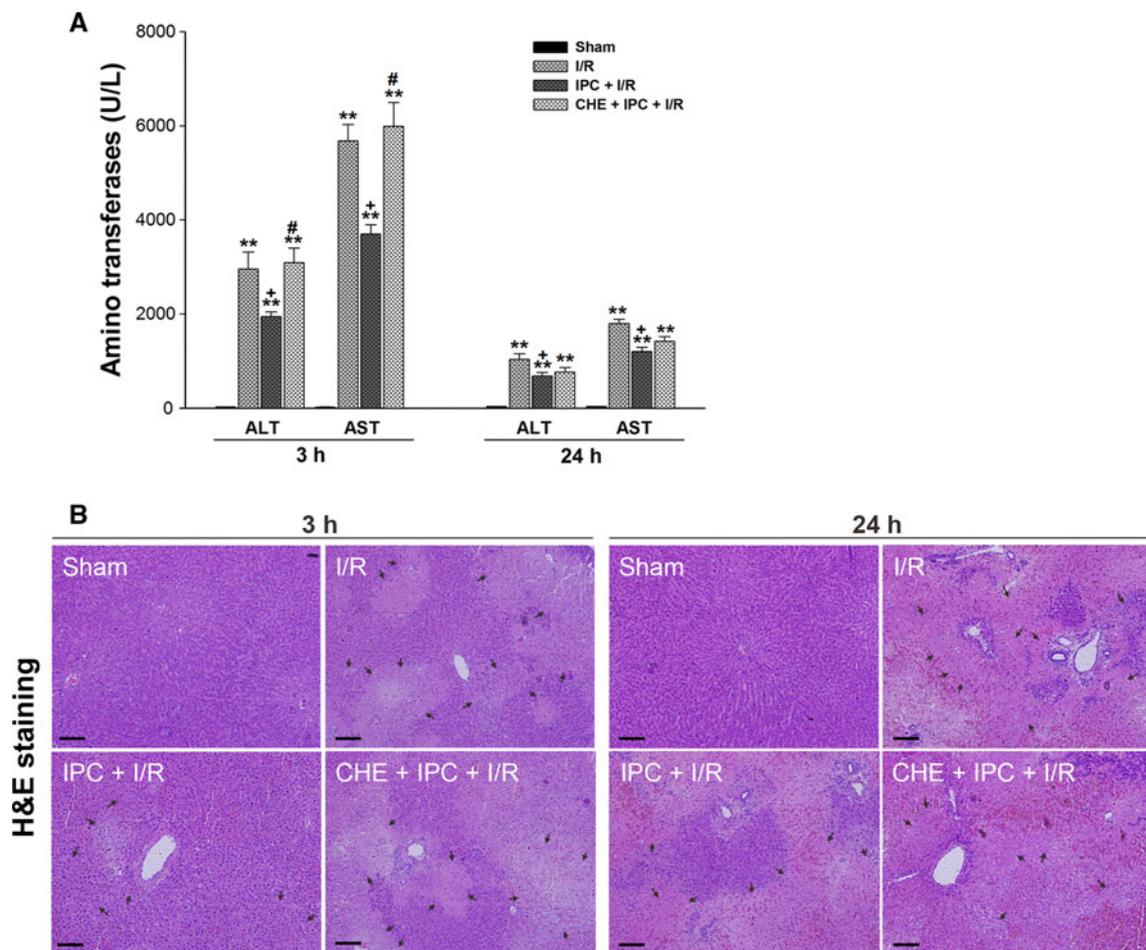
Fig. 1 Effects of IPC and chelerythrine on serum aminotransferase activities (**a**) and histological features of liver sections with H&E staining (**b**) at 3 and 24 h after reperfusion. Typical images were chosen from each experimental group ($\times 200$, *scale bar* 200 µm) and *arrows* indicate the necrotic area. Results are presented as mean ± SEM of *sham*, 3 h ($n = 8$); *I/R*, 3 h ($n = 9$); *IPC + I/R*, 3 h ($n = 10$); *CHE + IPC + I/R*, 3 h ($n = 10$); *sham*, 24 h ($n = 8$); *I/R*, 24 h ($n = 9$); *IPC + I/R*, 24 h ($n = 10$); *CHE + IPC + I/R*, 24 h ($n = 10$). **Significant differences ($P < 0.01$) versus the sham group; [+]significant differences ($P < 0.05$) versus the I/R group. [#]significant differences ($P < 0.05$) versus the IPC + I/R group

Table 1 Effects of IPC and chelerythrine on *GSH* and *MDA* levels following hepatic I/R

Group	GSH (µmol/g liver)		MDA (nmol/mg protein)	
	3 h	24 h	3 h	24 h
Sham	7.2 ± 0.6	7.2 ± 0.7	0.3 ± 0.0	0.3 ± 0.0
I/R	2.8 ± 0.5**	4.9 ± 0.5	0.6 ± 0.0**	0.7 ± 0.1**
IPC + I/R	5.0 ± 0.5*,[+]	5.6 ± 0.7	0.4 ± 0.0*,[+]	0.6 ± 0.1**
CHE + IPC + I/R	2.4 ± 0.4**,[##]	4.1 ± 0.3*	0.7 ± 0.1**,[##]	0.6 ± 0.1**

The levels of GSH and MDA were determined at 3 h and 24 h after reperfusion. Results are presented as mean ± SEM of sham, 3 h ($n = 8$); I/R, 3 h ($n = 9$); IPC + I/R, 3 h ($n = 10$); CHE + IPC + I/R, 3 h ($n = 10$); sham, 24 h ($n = 8$); I/R, 24 h ($n = 9$); IPC + I/R, 24 h ($n = 10$); CHE + IPC + I/R, 24 h ($n = 10$)

GSH glutathione, *MDA* malondialdehyde, *I/R* ischemia and reperfusion, *IPC* ischemic preconditioning, *CHE* chelerythrine

*,**Significantly different ($P < 0.05$, $P < 0.01$) from sham

[+] Significantly different ($P < 0.05$) from I/R

[##] Significantly different from IPC + I/R
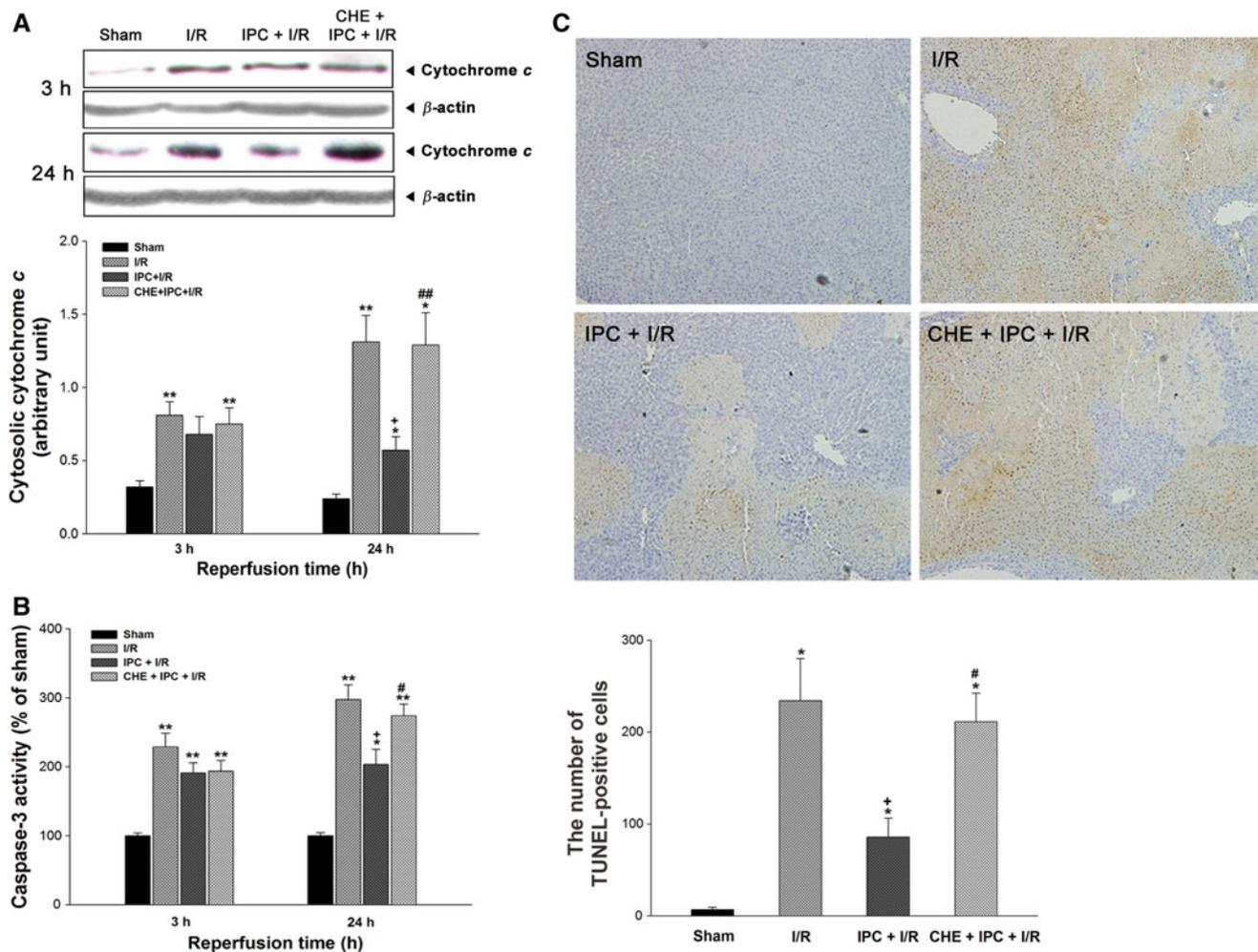
Fig. 2 Effects of IPC and chelerythrine on cytosolic cytochrome c protein expression (a), caspase-3 activity (b), and TUNEL-stained histology of rat liver (c). Cytosolic cytochrome c protein expression in the liver was measured by immunoblots at 3 and 24 h after reperfusion. Caspase-3 activity in the liver was measured at 3 and 24 h after reperfusion and the level of the sham group was arbitrarily set at 100.0%. TUNEL-stained histology of rat liver was measured at 24 h after reperfusion (×400). Results are presented as mean ± SEM

of sham, 3 h ($n = 8$); I/R, 3 h ($n = 9$); IPC + I/R, 3 h ($n = 10$); CHE + IPC + I/R, 3 h ($n = 10$); sham, 24 h ($n = 8$); I/R, 24 h ($n = 9$); IPC + I/R, 24 h ($n = 10$); CHE + IPC + I/R, 24 h ($n = 10$). *,**Significant differences ($P < 0.05$, $P < 0.01$) versus the sham group; +significant differences ($P < 0.05$) versus the I/R group; #,##significant differences ($P < 0.05$, $P < 0.01$) versus the IPC + I/R group

further increased by 24 h of reperfusion. While IPC did not prevent an increase in caspase-3 activity, which occurred at 3 h of reperfusion, the increase in caspase-3 activity at 24 h of reperfusion was significantly prevented by IPC. This effect of IPC was reversed with chelerythrine pretreatment. These biological parameters for apoptotic cell death paralleled with the morphological changes observed with TUNEL staining. A large number of TUNEL-positive hepatocytes were observed in liver tissues obtained 24 h after reperfusion. However, in livers treated with IPC, significantly fewer TUNEL-positive hepatocytes were observed and this was reversed by chelerythrine pretreatment (Fig. 2c).

Effects of IPC and chelerythrine on HO-1, iNOS, and COX-2 protein expression

As shown in Fig. 3a, the level of HO-1 protein expression was unchanged among any of the experimental groups at 3 h of reperfusion. However, at 24 h of reperfusion, the level of HO-1 protein expression showed a marked increase in liver subjected to I/R and IPC potentiated this increase. Chelerythrine pretreatment prior to IPC resulted in significant attenuation of potentiated HO-1 expression by IPC. The level of iNOS protein expression in liver isolated after 3 h of reperfusion was considerably higher than that of sham-operated animals, and decreased by 24 h of
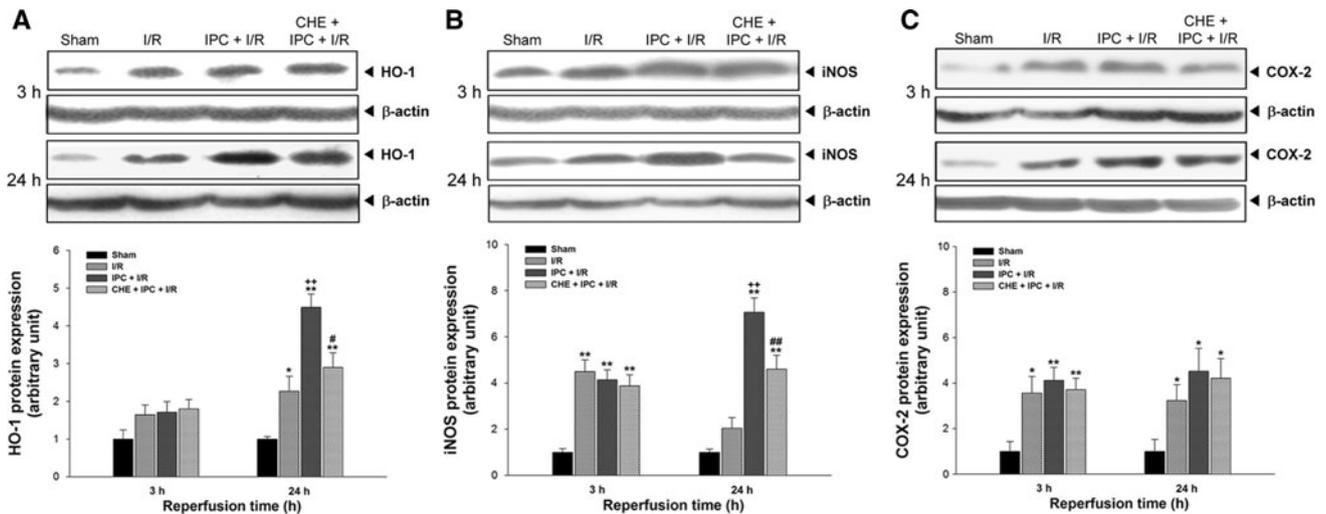
Fig. 3 Effects of IPC and chelerythrine on levels of HO-1 (**a**), iNOS (**b**), and COX-2 (**c**) protein expression. HO-1, iNOS, and COX-2 protein expression in the liver was measured by immunoblots at 3 and 24 h after reperfusion. Blot shown is representative of three experiments with similar results. *,**Significant differences ($P < 0.05$, $P < 0.01$) versus the sham group; $^{++}$significant differences ($P < 0.01$) versus the I/R group; $^{#,##}$significant differences ($P < 0.05$, $P < 0.01$) versus the IPC + I/R group

reperfusion. At 3 h after reperfusion, IPC did not affect the level of iNOS protein; however, IPC dramatically induced iNOS protein expression at 24 h after reperfusion and IPC-induced increase in iNOS protein expression was attenuated by chelerythrine pretreatment (Fig. 3b). Although COX-2 protein expression showed a dramatic increased in ischemic animals, neither IPC nor chelerythrine pretreatment contributed to the I/R-induced increase in COX-2 protein expression (Fig. 3c).

Effects of chelerythrine on PKC isozyme protein expression

Figure 4 shows isoform-specific translocation of PKC-δ and -ε by the IPC procedure. At 10 min of ischemia, the levels of PKC-δ and -ε in the particulate fraction increased, whereas those in the soluble fraction showed a marked decrease. The other PKC isoforms (PKC-α, -βI, -βII, and -ζ) were not altered by IPC treatment either in particulate or in soluble fractions. Chelerythrine pretreatment resulted in abolishment of isoform-specific translocation of PKC-δ and -ε. Further experiments were performed to confirm specific translocation of PKC-δ and -ε during IPC. Treatment with respective inhibitors of PKC-δ and -ε, rottlerin, and PKC-εV1-2 resulted in occurrence of significantly reversed translocation during IPC.

Effects of IPC and chelerythrine on MAPK protein expression

At 3 h after reperfusion, the total levels of p38 MAPK, ERK, and JNK did not differ significantly among

experimental groups. Reperfusion caused a dramatic increase in phosphorylated p38 MAPK, ERK, and JNK; IPC further increased the level of phosphorylated p38 MAPK and this was prevented by chelerythrine pretreatment. The levels of phosphorylated ERK and JNK were not significantly changed by either IPC or chelerythrine pretreatment (Fig. 5).

Discussion

IPC has been shown to prevent both necrotic and apoptotic cell death in several organs, including heart, brain, and liver [23, 24]. In liver, necrotic cell death caused by total hepatic I/R was significantly abolished during early IPC and this effect lasted even at 24 h after reperfusion [13, 25]. Although IPC has also been shown to prevent hepatocyte apoptosis through inhibition of caspase-3 dependent pathways during early IPC [10], studies in other organs, including heart, brain, and lung, have emphasized the role of late IPC in prevention of apoptotic cell death, since apoptosis often occurred in a delayed manner [14]. The signal cascades responsible for IPC, which consequently lead to limited cell death, have largely been unveiled in recent decades. However, data obtained so far have been primarily dependent on the heart and little information is available on differential cellular mechanisms during early and late hepatic IPC [26].

PKC plays an essential role in development of IPC: selective translocation of PKC-δ, but not PKC-ε, was reported in preconditioned Zucker rat liver and chelerythrine treatment abrogated protection of IPC, even at the late
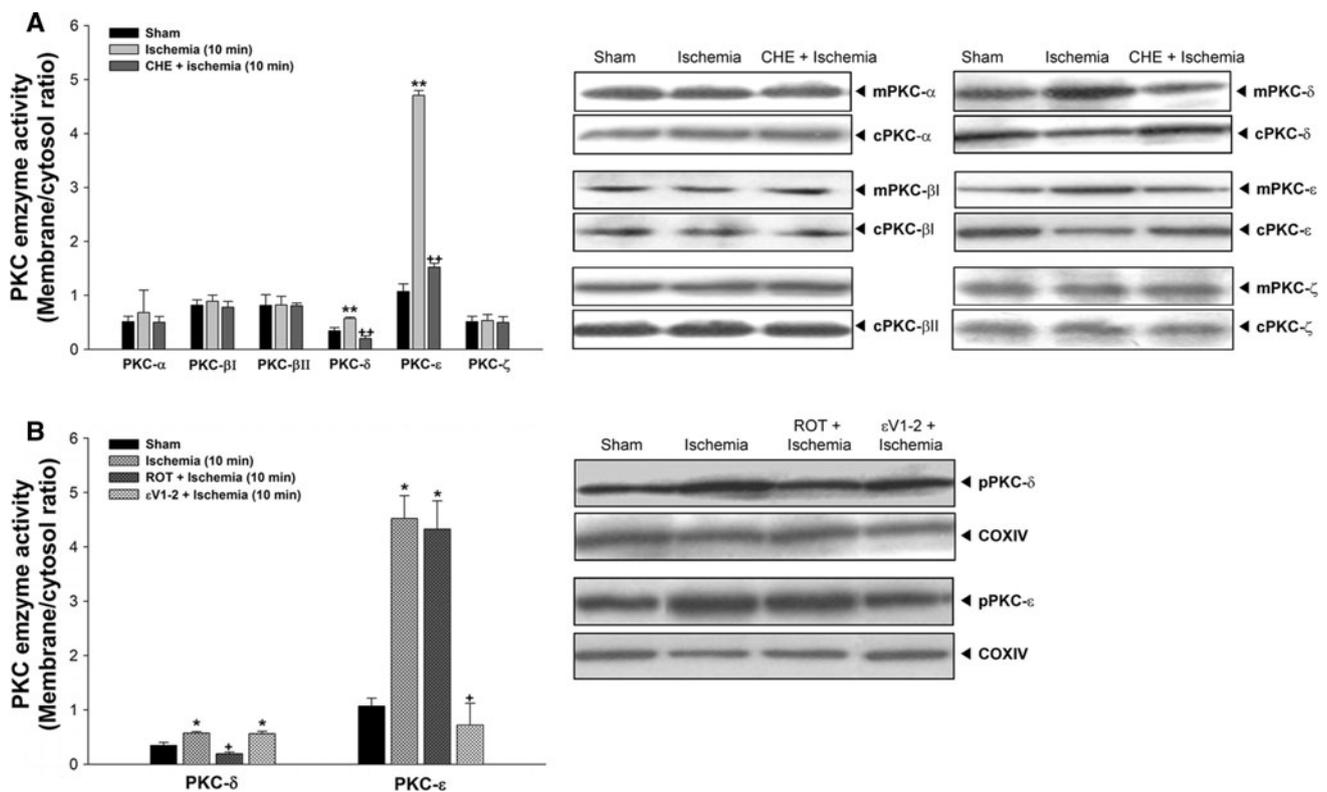
Fig. 4 Effects of chelerythrine on membrane translocation of PKC isoforms during IPC. **a** Membrane translocation of PKC-α, -βI, -βII, -δ, -ε, and -ζ isoforms in rat liver were measured by immunoblots immediately after 10 min of ischemia. **b** Effects of rottlerin and PKC-εV1-2 on membrane translocation of PKC-δ and -ε isoforms were measured by immunoblots immediately after 10 min of ischemia. Blot shown is representative of three experiments with similar results. *,**Significant differences ($P < 0.05$, $P < 0.01$) versus the sham group; +,++significant differences ($P < 0.05$, $P < 0.01$) versus the ischemia group
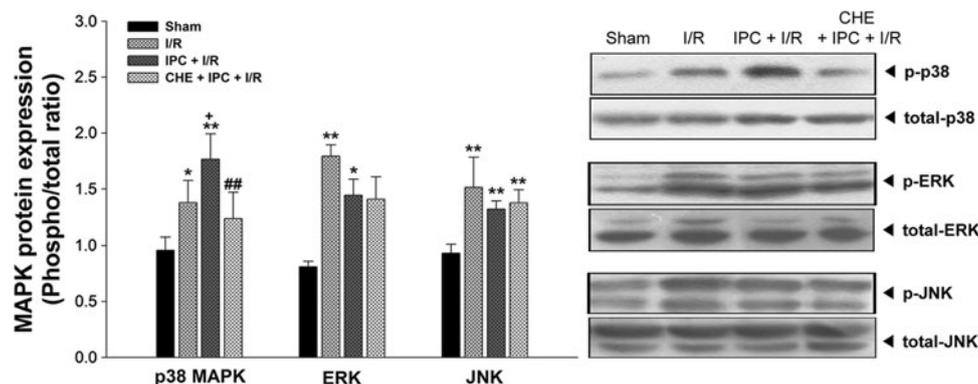
Fig. 5 Effects of IPC and chelerythrine on MAPK activation. p38, ERK, and JNK activation in the liver were measured by immunoblots at 3 h after reperfusion. Blot shown is representative of three experiments with similar results. *,**Significant differences ($P < 0.05$, $P < 0.01$) versus the sham group; +significant differences ($P < 0.05$) versus the I/R group; ##significant differences ($P < 0.01$) versus the IPC + I/R group

phase [7]. Activation of PKC-ε blocked cultured ventricular myocyte apoptosis, presumably by opening of ATP-dependent potassium channels [27], and similar results were also reported in chick cardiomyocytes as well as human myocardium [17, 28]; however, increased PKC activity induced apoptosis in the salivary epithelium [29].

In the present study, the levels of serum ALT and AST, marker of necrotic hepatocytes, showed marked increases at 3 h of reperfusion, and declined by 24 h of reperfusion. IPC induced significant reductions of both serum ALT and AST activities during early and late phases. Of particular interest, chelerythrine, a PKC inhibitor, induced almost

complete reversal of the beneficial effects of IPC at 3 h of reperfusion, but did not alter the decreases in serum ALT and AST levels at 24 h of reperfusion. Meanwhile, the representative biochemical parameters of apoptosis, cytochrome c, and caspase-3 activity were dramatically increased at 3 h after reperfusion, and remained elevated even after 24 h of reperfusion. However, these increases were abolished by IPC. Notably, chelerythrine pretreatment resulted in the abolishment of anti-apoptotic effects of IPC only at the late phase of reperfusion. These observations were further supported by H&E staining and TUNEL-positive cells. Our results suggest that PKC has a central role in prevention of hepatocyte necrosis during early IPC and hepatocyte apoptosis during late IPC.

During the brief period of ischemia, levels of PKC-δ and -ε in the particulate fraction showed a significant increase, whereas those in the soluble fraction showed a marked decrease. Upon pretreatment with chelerythrine, translocation of PKC-δ and -ε was completely abolished. Our results suggest that specific translocation of PKC-δ and -ε is responsible for protection of hepatic IPC.

Resistance toward I/R injury that develops during early IPC is likely to result from complex signal cascades. Transient hypoxia generates multiple ligands, including adenosine, bradykinin, and opioids, which are all capable of triggering protection through interaction with specific receptors [5]. Once activated, these receptors lead to activation of downstream signals and PKC appears to be the first element of the kinase cascade [30]. Current evidence largely shows that this cascade contains activation of at least one or more MAPKs, since PKC acts on this enzyme family [31]. Recently, several studies have examined the potential role of MAPKs, including p38 MAPK, ERK, and JNK in hepatic IPC. In isolated hepatocytes, selective enhancement of PKC-δ and PKC-ε is coupled with stimulation of p38 MAPK [32]. However, others have shown decreased p38 MAPK and JNK activities following IPC stimuli [7]. In our results, p38 MAPK was markedly stimulated during early IPC, while the levels of both ERK and JNK were not changed. Activation of p38 MAPK was completely blocked by chelerythrine pretreatment. We also measured the levels of these MAPKs during late IPC; expression of these enzymes was barely detectable and did not show any differences among the experimental groups (data not shown). Our results suggest that activation of p38 MAPK is followed by increased PKC-δ and -ε membrane translocation during early IPC.

In the early phase, IPC-triggered signal transduction appears to directly preserve several cell functions, including intracellular energy state [33], pH [34], and redox system [35]. Indeed, Peralta et al. [36] showed increased hepatic GSH content and reduced Kupffer cell activation after IPC stimuli. In addition, postischemic intravenous infusion of GSH mimicked IPC-mediated protection; however, combination of IPC with postischemic GSH infusion did not exert additional effects, suggesting that IPC confers protection by modulation of toxic effects of ROS. Hypoxic preconditioning-induced resistance towards Kupffer cell-derived H_2O_2 was associated with activation of p38 MAPK, preserving the GSH/GSSG redox system, though the upstream signal for this has not been investigated in detail [37]. In our results, the decreased GSH level and increased MDA level indicating increased oxidative stress, which might induce defense enzymes through perturbation of intracellular redox state, were significantly attenuated by IPC at 3 h after reperfusion. In addition, PKC inhibition resulted in abolishment of these changes, suggesting that PKC might play an important role in preservation of intracellular redox state, especially during early IPC.

Late IPC requires simultaneous activation of multiple stress-response genes and synthesis of new proteins, not simply activation of preexisting proteins [38]. Several proteins have been proposed as possible mediators, including NOS, COX-2, antioxidant enzymes, and HSPs [6, 39]. Delayed protection against both myocardial stunning and myocardial infarction was found to be completely abrogated when preconditioned animals were given selective iNOS inhibitor, implicating iNOS as the specific NOS isoform involved in mediation of late IPC [40]. Previous reports have shown that PKC can directly stimulate expression of iNOS in rat colon [41]. In our study, expression of iNOS protein was very high at 3 h of reperfusion, and gradually decreased by 24 h of reperfusion. IPC pretreatment did not affect iNOS protein expression at 3 h of reperfusion; iNOS protein expression at 24 h of reperfusion was significantly increased by IPC pretreatment and chelerythrine attenuated it. Thus, our data suggest that a strong induction of iNOS by PKC signaling during late IPC results in development of delayed protection against hepatic I/R injury.

HO-1, an endogenous, cytoprotective enzyme that is upregulated under conditions of oxidant stress, has been shown to contribute to development of late IPC; HO-1 overexpression by either pharmacological preconditioning or IL-13 gene transfer in hepatic I/R mimicked IPC and has been shown to prevent apoptotic cell death [42]. Specifically, a recent report by Amersi et al. [43] showed that carbon monoxide-mediated hepatoprotection is specifically blocked by inhibition of p38 MAPK. In our results, HO-1 protein expression showed a significant increase at 24 h after reperfusion in the I/R group, and this increase was augmented by IPC. The increase in HO-1 expression was dramatically attenuated by chelerythrine pretreatment, demonstrating involvement of PKC signaling on IPC-induced HO-1 overexpression. In addition to HO-1, HSPs

are also possible mediators of late IPC. The induction of HSPs, particularly HSP27 and HSP70, has been observed in preconditioned liver and they were shown to reduce apoptotic cell death [44, 45]. Given the plethora of substrates and the effectiveness of PKC in modulating diverse cellular responses, it cannot be excluded that direct phosphorylation of HSPs by PKC. Indeed, previous evidence showed that PKC-δ and -ε directly phosphorylate HSP27 and increase its activities [46, 47]. However, the direct link between PKC-δ, -ε and HSPs requires further investigation.

The full explanation of the role of PKC in early and late hepatic IPC has yet to be elucidated, and on the basis of clinical application, we cannot exclude the fact that the experimental model used in the present study, 70% of hepatic ischemia, had combined effects of local preconditioning (i.e., preconditioning of the left lobe) and remote IPC (i.e., of the right hepatic lobe), and is also irrelevant with the Pringle manoeuvre, which clamps the hepatoduodenal ligament during liver resection. However, judging from previous reports, this model has sufficient potential to be reproduced in human cases [4, 8, 10]. Our study evaluated the differential role of PKC and its downstream signaling responsible for early and late protection of hepatic IPC. Isozyme-selective translocation of PKC, PKC-δ, and -ε is thought to be involved in both phases; preventing necrotic cell death during early IPC through preservation of redox state and limiting apoptotic cell death in late IPC with iNOS and HO-1 induction.

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