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• BASIC RESEARCH •

Protein kinase C-dependent activation of P44/42 mitogen-activated protein kinase and heat shock protein 70 in signal transduction during hepatocyte ischemic preconditioning

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Abstract

AIM: To investigate the significance of protein kinase C (PKC), P44/42 mitogen-activated protein kinase (MAPKs) and heat shock protein (HSP)70 signal transduction during hepatocyte ischemic preconditioning.

METHODS: In this study we used an *in vitro* ischemic preconditioning (IP) model for hepatocytes and an *in vivo* model for rat liver to investigate the significance of protein kinase C (PKC), P44/42 mitogen-activated protein kinase (P44/42 MAPKs) and heat shock protein 70 (HSP70) signal transduction in IP. Through a normal liver cell hypoxic preconditioning (HP) model in which cultured normal liver cells were subjected to 3 cycles of 5 min of incubation under hypoxic conditions followed by 5 min of reoxygenation and subsequently exposed to hypoxia and reoxygenation for 6 h and 9 h respectively. PKC inhibitor, activator and MEK inhibitor were utilized to analyze the phosphorylation of PKC, the expression of P44/42 MAPKs and HSP70. Viability and cellular ultrastructure were also observed. By using rat liver as an *in vivo* model of liver preconditioning (3 cycles of 10-min occlusion and 10-min reperfusion), *in vivo* phosphorylation of PKC and P44/42MAPKs, HSP70 expression were further analyzed. AST/ALT concentration, cellular structure and ultrastruture were also observed. All the data were statistically analyzed.

RESULTS: Similar results were obtained in both *in vivo* and *in vitro* IP models. Compared with the control without IP (or HP), the phosphorylation of PKC and P44/42 MAPKs and the expression of HSP70 were obviously increased in IP (or HP) treated model in which cytoprotection could be found. The effects of preconditioning were mimicked by stimulating PKC with 4β phorobol-12-myristate13-acetate (PMA). Conversely, inhibiting PKC with chelerythrine abolished the protection given by preconditioning. PD98059, inhibitor of MEK (the upstream kinase of P44/42MAPKs), also reverted the cytoprotection exerted by preconditioning.

CONCLUSION: The results demonstrate that preconditioning induces a rapid activation of P44/42MAPKs and PKC activation plays a pivotal role in the activation of P44/42 MAPKs pathway that participates in the preservation of liver cells. HSP expression is regulated by signals in PKC dependent P44/42 MAPKs pathway.

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INTRODUCTION

The term ischemic preconditioning (IP) was first coined by Murry *et al*. to describe a phenomenon where brief periods of sublethal ischemia protected the heart against infarction caused by a subsequently more prolonged period of coronary artery occlusion[1]. Preconditioning occurs in 2 phases: an early phase, also known as acute preconditioning, in which protection lasts up to 1-2 h following preconditioning, and a second phase, known as the second window of protection, in which protection reappears 24-72 h following preconditioning. Although a variety of mediators and effectors have been proposed to be essential for conferring preconditioning, including the adenosine receptor[2], protein kanase C[3], and the ATP-sensitive K^+channel[4]. The importance of PKC to ischemic preconditioning has been shown in a variety of studies in whole heart and isolated ventricular cardiocytes[5]. Whereas it is widely accepted that PKC plays a pivotal role in ischemic preconditioning, the relevant downstream signaling molecules remain a topic of intense investigation and controversy.

Over the last few years, a number of studies in both whole hearts and isolated cardiomyocytes have described the activation of members of the MAPK family of signaling proteins during ischemica and ischemic reperfusion[6,7]. All of the MAPKs are proline-directed, serine/threonine-protein kinases are activated by dual phosphorylation on tyrosine and threonine residues by upstream kinases. The family consists of 3 members, extracellular signal-regulated kinases 1 and 2 (ERK1/2; P44/42MAPKs), c-jun NH2-terminal kinases 1 and 2 (JNK1/2), P38MAPKs and ERK5/BMK1 (big mitogen-activated protein kinase, BMK1) which was found recently. ERK1/2 is predominantly activated by growth factors, and JNKs and P38MAPKs are generally activated by stresses such as ultraviolet light, inflammatory cytokines, heat shock, and ischemic reperfusion. Recent evidence implicates PKC is in the activation of 2 members of this kinase family, *i.e,* the P44 and P42MAPKs[8]. But whether PKC-dependent activation of P44/42 MAPKs in signal transduction pathways during hepatocyte ischemic preconditioning contributes to cytoprotective effect is largely unknown. Thus, in the present investigation we tested it by using an *in vitro* IP model for hepatocytes and an *in vivo* model for rat liver. Similar results were obtained both *in vivo* and *in vitro* IP models. The phosphorylation of PKC and P44/42 MAPKs was obviously increased in IP treated. The effects of

preconditioning were mimicked by stimulating PKC with 4β phorobol-12-myristate13-acetate (PMA). Conversely, inhibiting PKC with chelerythrine abolished the protection given by preconditioning. PD98059, an inhibitor of MEK (the upstream kinase of P44/42MAPKs), also reverted the cytoprotection exerted by preconditioning. This suggests that preconditioning induces a rapid activation of P44/42MAPKs and PKC activation plays a pivotal role in the activation of P44/42 MAPKs pathway that participates in the preservation of liver cells.

Athough it has been reported that IP conveys protective signals to hepatocytes, few studies have been published on intracellular protective mechanism. It remains to be elucidated whether PKC-dependent P44/42MAPKs pathways are involved in the regulation of protective proteins, such as heat shock protein (HSP70). Some substantial literature describes the induction of HSP70 by ischemia[9], the potential role of HSP70 in ischemic preconditioning[10], and an inverse correlation between expression of HSP70 induced by ischemic or thermal preconditioning and infart size in animal models[11]. In addition, enhanced expression of HSP70 conveys a cytoprotective effect in cultured cells, including cardiac myocytes subjected to simulated ischemia[12,13]. Specifically, overexpression of HSP70 in transgenic mice improves myocardial function[14,15], preserves metabolic functional recovery, and reduces infart size after ischemic preconditioning. Several recent studies suggest that PKC, a ubiquitous intracellular mediator, may play a role in mediating the protective effects of ischemic preconditioning while the activators such as PMA mimic the protective effect via phosphorylation of unknown effector protein. PKC plays a crucial role in the signal transduction for the activation of many cellular functions. Many transcription factors are known to be activated by various PKC subtypes[16]. These include heat shock protein transcription factors (HSF). The synthesis of HSPs is mediated by the activation of heat shock gene transcription which is mediated by the binding of HSF to the heat shock element in the promoter region of HS genes. The gene knockout model of HSF1 *in vitro* demonstrated the essentisl requirement of this regulatory pathway in cellular protection[17]. But whether P44/42 MAPKs signal pathways during hepatocyte ischemic preconditioning mediate the synthesis of protective protein (HSP70) remains elusive and represents an unresolved problem. In this study, we observed that HSP70 expression was increased in IP treated models, while it was inhibited by PKC inhibitor chelerythrine, the cytoprotective effect was reduced, but the activator of PKC (PMA) could induce the activation of PKC, HSP70 expression and cytoprotection apparently. Thus the data presented here suggested that PKC could regulate HSP70 expression directly or indirectly. While MEK inhibitor PD-98059 abolished the activation of P44 and P42 MAPKs. The synthesis of HSP70 was reduced and the protective effect of preconditioning was blocked. We propose that activation of P44 and P42 MAPKs correlates with the regulation of HSP70 expression.

MATERIALS AND METHODS

In vivo model

Nine to twelve-week male Sprague-Dawley rats weighing 220-230 g were obtained from the Animal Center of the First Military Medical University. Chelerythrine chloride (CHE) was purchased from Calbiochem Co. Phorobol 12-myristate 13-acetate (PMA) was obtained from Gibco/BRL Co. PD98059 was purchased from Sigma. All other chemicals in this study were of analytical reagent quality.

Grouping and experimental protocol Male Sprague-Dawley rats were fasted with free access to water 18 h before experiment. Animals were randomly divided into one of the 6 subgroups (6 rats in each group) and subjected to the following experimental protocals. According to the method of Kobayashi *et al*.[18], the model of rat local ischemic reperfusion was established. (1) Group C (control): The abdomen was opened by a midline incision and the liver hilus was exposed, but not occluded. (2) Group IR (ischemic reperfusion): All vessels (hepatic artery, portal vein, and bile duct) to the left and median liver lobes were occluded for 40 min with a vascular clamp. Thereafter the clamp was removed and blood flow was reperfused for 3 h. Since blood vessels to the remaining parts of the liver were not occluded with this method, portal stasis could be avoided, which was of special relevance for circulatory stability in rats. The abdominal walls were closed during reperfusion. (3) Group IP (ischemic precondition): To induce ischemic preconditioning, mice underwent a sequence of three 10-min liver hilus occlusions separated by 10-min of reperfusion prior to the 40-min occlusion and 3 h reperfusion. (4) Group PMA (IR +PMA): PMA (4 μg/kg[19]) total volume 5 mL was slowly injected through dorsal veins of penis for 10 min, beginning 10 min before the start of ischemic rerperfusion. (5) Group CHE (IP + chelerythrine chloride): Total volume of 5 mL chelerythrine chloride (5 mg/kg[20]) was slowly injected through dorsal veins of penis for 10 min, 10 min before the start of ischemic preconditioning. (6) Group PD (IP + PD98059): Total volume of 5 mL PD98059 (5 mg/kg[20]) was slowly injected through dorsal veins of penis over 10 min, 10 min before the start of ischemic preconditioning. The dose of PD98059 was shown to effectively block the activation of p44/p42MAPKs[21].

Measurement of serum ALT and AST Three hours after the last reperfusion, the abdoman of each group was re-opened. Blood samples of infrahepatic vena cava were obtained and centrifuged to get serum in order to detect the concentration of ALT and AST.

PKC activity assay The rats were euthanized 3 h after the last reperfusion. 0.3 cm×0.3 cm×0.3 cm tissue samples from left liver lobe were rapidly removed. PKC activity assay kit was used. The tissue in 5 mL of cold extraction buffer was homogenized using a cold homogenizer then the lysate was centrifuged for 5 min at 4 ℃, 14 000 *g* in a microcentrifuge and the supernatant was saved. The supernatant was passed over an 1-mL column of DEAE cellulose that was pre-equilibrated in extraction buffer and the column was washed with 5 mL of extraction buffer. The PKC-containing fraction was eluted using 5 mL of extraction buffer containing 200 mmol/L NaCl. Then enzyme sample, PKC coactivation buffer, PKC activation buffer, PKC biotinylated peptide substrate, and [γ-32P]ATP were mixed gently and incubated at 30 ℃ for 5 min. All samples were spotted on SAM2 membrane, and the washing and rinsing steps were followed. The SAM2 membrane was dried and placed into individual scintillation vials added with scintillation fluid and analysed using a phosphorimaging system.

Western blotting analysis of P44/42 MAPKs The rats were euthanized 3 h after the last reperfusion (a time point at which marked activation of P44/42 MAPKs begins[21]). 0.3 cm×0.3 cm×0.3 cm tissue samples from left liver lobe were rapidly removed. P44/42 MAPKs test kit (New England Biolabs) was utilized.

Western blotting analysis of HSP70 The rats were euthanized 3 h after the last reperfusion and tissue samples were obtained as described above. When extracted from hepatocytes, protein samples were separated on 100 g/L SDS-polyacrylamide gel and transferred to nitrocellulose membranes. After membrances were transferred, they were blocked for 1 h with 50 mL/L nonfat milk in Tris-buffered saline. Membranes were incubated with murine monoclonal antibody to HSP70 overnight at 4 ℃, washed 3 times for 5 min in TBST before addition of goat anti-mouse-HRP conjugated secondary antibody for 1 h at room temperature. Membrances were washed 3 times for 5 min with TBS, and peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting

detection system.

Changes of cellular structure and ultrastructur The rats were euthanized 3 h after the last reperfusion. 0.3 cm×0.3 cm×0.3 cm tissue samples from left liver lobe were also removed in order to observe the cellular structure and ultrastructure.

In vitro model

Liver cell line L02 was obtained from Shanghai Institute of Cell, (Academy of Medical Sciences of China). The necessary reagents and test kits were similar to *in vivo* setting.

Grouping and experimental protocol Cell viability, estimated at the beginning of the experiments, ranged between 90% to 95%. Hepatocytes were suspended in Krebs-Henseleit-Hepes (KHH)[22] buffer containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $KH2PO_4$, 1.3 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, and 20 mmol/L Hepes at pH7.4. Hypoxic preconditioning was obtained by 3 cycles of 5 min of incubation at 37 ℃ in KHH buffer equilibrated with 900 mL/L N_2, 80 mL/L CO_2, 20 mL/L H_2 followed up by 5 min of reoxygenation obtained by fluxing the incubation flasks with 950 mL/L O_2, 50 mL/L CO_2 gas mixture. After reoxyenation, the cells were transferred in deoxygenated KHH buffer and further incubated at 37 ℃ in sealed bottles in a 900 mL/L N_2, 80 mL/L CO_2, 20 mL/L H_2 atmosphere for 6 h followed up by reoxygenation for 9 h. Nonpreconditioned cells were incubated in KHH buffer until the beginning of hypoxic treatment. Cells were randomly divided into one of the 6 subgroups and subjected to the following experimental protocals. (1) Group C (control): Untreated hepatocytes were incubated in 950 mL/L O_2, 50 mL/L CO_2 atmosphere. (2) Group HR (Hypoxia reoxygenation,HR): Untreated hepatocytes were incubated in 900 mL/L N_2, 80 mL/L CO_2, 20 mL/L H_2 atmosphere for 6 h followed up by reoxygenation for 9 h. (3) Group IP (hypoxic precondition, HP): preconditioned cells were incubated at 37 ℃ in sealed bottles in a 900 mL/L N_2, 80 mL/L CO_2, 20 mL/L H_2 atmosphere for 6 h followed up by reoxygenation for 9 h. (4) Group PMA (HR + PMA): Hepatocytes suspended in KHH medium were preincubated for 10 min at 37 ℃ with 150 nmol/L PMA[23] and then subjected to hypoxic incubation for 6 h in sealed flasks in a 900 mL/L N_2, 80 mL/L CO_2, 20 mL/L H_2 atmosphere followed up by reoxygenation for 9 h. (5) Group CHE (HP + chelerythrine chloride): Hepatocytes suspended in KHH medium were pretreated with 50 mmol/L[24] chelerythrine for 10 min before preconditioning. (6) Group PD (HP + PD98059): Hepatocytes were pretreated with 50 mmol/L[21] PD98059 for 10 min before preconditioning.

Determination of cell viability Treated with the methods above, the cells of each group were incubated in KHH buffer containing 20 uL (4,5-dimethylthioazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) for 4 h at 37 ℃ in room air containing 50 mL/L CO_2. During this incubation tetrazolium component of the dye was reduced, in metabolically active cells, to a formazan dye. Thereafter the reaction was terminated by addition of 100 μL solubilization solution (ethanol) and the absorbance of the lysate was recorded at 570 nm using an ELISA reader.

PKC activity assay The protocol was the same as the above.

Western blotting analysis of P44/42 MAPKs The protocol was the same as the above.

Western blotting analysis of HSP70 The protocol was the same as the above.

Changes of cellular ultrastructure Having been treated with the methods above, the cellular ultrastructure of each group was observed.

Statistical analysis

Data were presented as mean±SD. Statistical comparisons were made by analysis of variance. When significant differences were observed, SNK-*q* test was used for multiple comparions. Statistical significance was inferred at $P<0.05$.

RESULTS

Cytoprotective effects on hepatocytes induced by PC

The serum concentration of ALT and AST was increased and the cell viability was decreased significantly in group IR (HR) ($P<0.01$) compared with the control group. In contrast, compared with group IR (HR), the serum concentration of ALT and AST was reduced and the cell viability was increased markedly in group IP (HP) ($P<0.01$) (Tables 1, 3). In group C, cells had no apparent degeneration and necrosis where Kupffer cells did not proliferate markedly and the structure of portal area was not changed. Organellae were intact and mitochondria were lined up in order. In group IR, cells of lobules of liver were swollen and had hydropic degeneration where Kupffer cells proliferated significantly and had active phagocytosis. The spotty necrosis could be easily found. Portal area was enlarged and infiltrated with mononuclear cells. Hepatocytes were swollen and endothelial cells had malformation with mitochondria ballooned. Matrix was reduced, densely and floccularly degenerated. Bar was decreased and disarranged (Figures 3A, 6A). In group IP heptocytes were slightly swollen and had no degeneration with no proliferation of Kupffer cells. Portal area was normal. Organellae were not swollen apparently with some neutrophils and lymphocytes infiltrated. Mitochondria were well distributed (Figures 3B, 6B). This indicated that PC could elicit the cytoprotective effects on hepatocytes subjected to a subsequent lethal ischemia reperfusion or hypoxia reoxygenation.

Table 1 Changes in serum concentration of ALT and AST (n=6, mean±SD)

Group	AST(U/L)	ALT(U/L)
C	99.3±13.2	61.90±12.1
IR	820.9±111.3[d]	762.80±130.5[d]
IP	407.7±73.7[b]	281.00±35.6[b]
IR+PMA	553.2±58.67[b]	354.37±53[b]
IP+PD	732.9±91.1[f]	466.20±82.8[f]
IP+CHE	678.6±136.5[f]	645.61±90.4[f]

[b]$P<0.01$ versus group IR, [d]$P<0.01$ versus group C, [f]$P<0.01$ versus group IP.

Table 2 Changes in levels of phosphorylation activity of PKC in the liver (n=6, mean±SD)

Group	Phosphorylation activity of PKC (fmol/mg/min)
C	36.28±3.8
IR	42.78±2.22
IP	112.61±4.86[b]
IP+CHE	34.07±2.77[d]
IR+PMA	165.47±27.25[b]

[b]$P<0.01$ versus group IR, [d]$P<0.01$ versus group IP.

Effect of ischemic PC or hypoxic PC on PKC activity

Several studies performed in the heart implicated the activation of PKC as one of the key events in the development of ischemic PC[25,26]. Similar results were obtained both *in vivo* and *in vitro* IP models. Compared with group IR (HR), the phosphorylation of PKC was obviously increased in group IP (HP) (Tables 2, 4) and the cytoprotective effect developed during ischemic PC (Tables 1, 3; Figures 3B, 6B). PMA, a well known activator of PKC, decreased cell killing by ischemia reperfusion or hypoxia reoxygenation (Tables 1, 3; Figures 3C, 6C). Conversely, PKC inhibitor chelerythine reverted the effect of preconditioning on hepatocytes. Chelerythrine also abolished the protection against the damage caused by ischemia reperfusion or hypoxia

reoxygenation (Tables 1, 3; Figures 3D, 6D), suggesting that PKC might play a pivotal role in ischemic PC or hypoxic PC.

Table 3 Protection of hypoxic preconditioning against cytotoxicity caused by hypoxic hepatocyte incubation (n=6, mean±SD)

Group	Hepatocyte viability (%)
C	95±10.8
HR	35.57±3.99[b]
HP	81.49±12.1[d]
HR+PMA	75.29±11.9[d]
HP+PD	51.03±9.09[f]
HP+CHE	47.21±5.42[f]

[b]P<0.01 *vs* group C, [d]P<0.01 *vs* group HR, [f]P<0.01 *vs* group HP.

Table 4 Changes in PKC phosphorylation activity during hypoxia in control and preconditioned hepatocytes (n=6, mean±SD)

Group	Phosphorylation activityof PKC (fmol/mg/min)
C	32.67±5.11
HR	42.63±4.73
HP	109.42±16.09[b]
HR+PMA	152.47±19.59[b]
HP+CHE	65.28±5.36[d]

[b]P<0.01 *vs* group HR, [d]P<0.01 *vs* group IP.

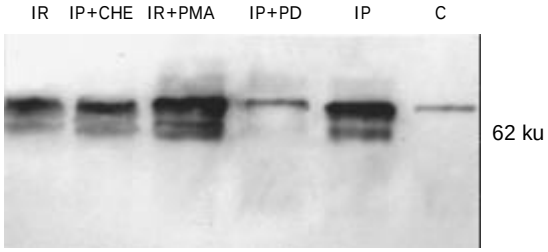


Figure 1 Expression of P44 and P42 MAPKs in rat liver.

Effect of ischemic PC or hypoxic PC on MAPK activity

Both *in vivo* and *in vitro* IP models, our results showed that hepatocyte lysates exhibited the sharp signal at 60 ku as detected by Western immunoblotting. Compared with group IR (HR), the expression of P44 and P42 MAPKs was markedly increased in group IP (HP) (Figures 1, 4), and the cytoprotective effect developed during ischemic PC (Tables 1, 3; Figures 3B, 6B). This result implied that the activation of P44 and P42 MAPKs was associated with the cytoprotection. The effect was abolished by the MEK inhibiter PD-98059. Compared with group IP (HP), the serum concentration of ALT and AST was increased and the cell viability was decreased significantly in group IP+PD (P<0.01). We observed that hepatocytes were swollen with mitochondria ballooned and Kupffer cells proliferated. The spotty necrosis was found. Portal area was slightly enlarged and a few mononuclear cells infiltrated (Figures 3E, 6E). The results indicateed that P44 and P42 MAPKs as a major signal transduction molecule played an important role in cytoprotection during hepatocyte ischemic PC or hypoxic PC. To determine whether activation of P44/42 MAPKs during ischemic PC or hypoxic PC was dependent on PKC activation, we measured P44/42 MAPKs activity undergoing PC after pretreatment with chelerythrine (group IP or HP + CHE). Previous studies demonstrated that chelerythrine completely blocked the translocation of PKC and development of late PC in conscious rabbit model (39 rabbits). In the present study, we found that chelerythrine completely blocked the activation of P44/42 MAPKs and cytoprotection, and the changes in serum concentration of ALT and AST, cell viability, cellular structure and ultrastructure were similar to those in group IR (HR)(Tables 1, 3; Figures 3D, 6D). PMA, an activator of PKC resulted in increased P44/42 MAPKs activity and mimiced the cytoprotection. The changes in serum concentration of ALT and AST, cell viability, cellular structure and ultrastructure were similar to those in group IP (HP) (Tables 1, 3; Figures 3C, 6C). These data demonstrated that the activation of P44 and P42 MAPKs during ischemic PC or hypoxic PC occurred via a PKC-dependent pathway.

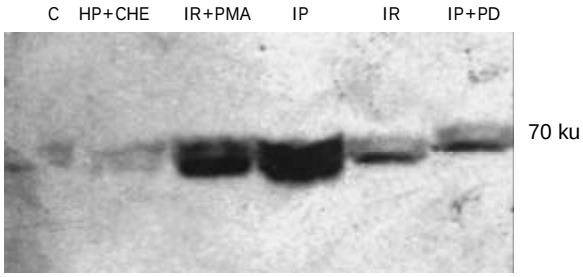


Figure 2 Expression of HSP70 in rat liver.

Effect of ischemic PC or hypoxic PC on HSP70 expression

The expression of HSP70 exhibited a sharp signal at 70 ku as detected by Western immunoblotting. Compared with group IR (HR), the expression of HSP70 was apparently increased in group IP (HP) (Figures 2, 5). In addition, IP conveyed a cytoprotective effect on hepatocytes suffering ischemia reperfusion or hypoxia reoxygenation (Tables1, 3; Figures 3B, 6B). While PKC inhibitor chelerythrine and MEK inhibitor PD-98059 inhibited the protein expression, and the cytoprotective effect was reduced (Tables1, 3; Figures 2, 5, 3D, 6D, 3E, 6E), but the activator of PKC (PMA) could induce HSP70 expression and cytoprotection apparently (Tables 1, 3; Figures 2, 5, 3C, 6C). Thus the data presented here suggested that expression of HSP70 had a cytoprotective effect on hepatocytes and PKC could regulate HSP70 expression directly or indirectly.

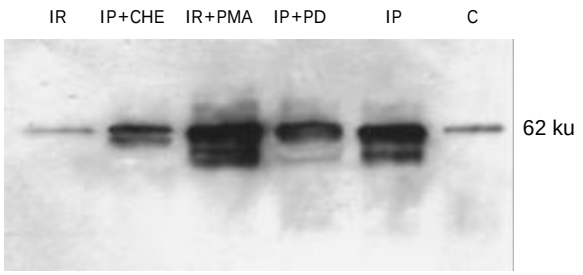


Figure 4 Expression of P44 and P42 MAPKs in isolated human hepatocytes.

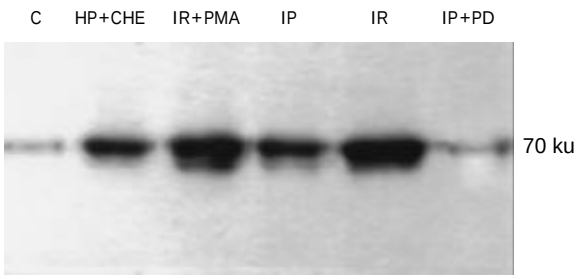
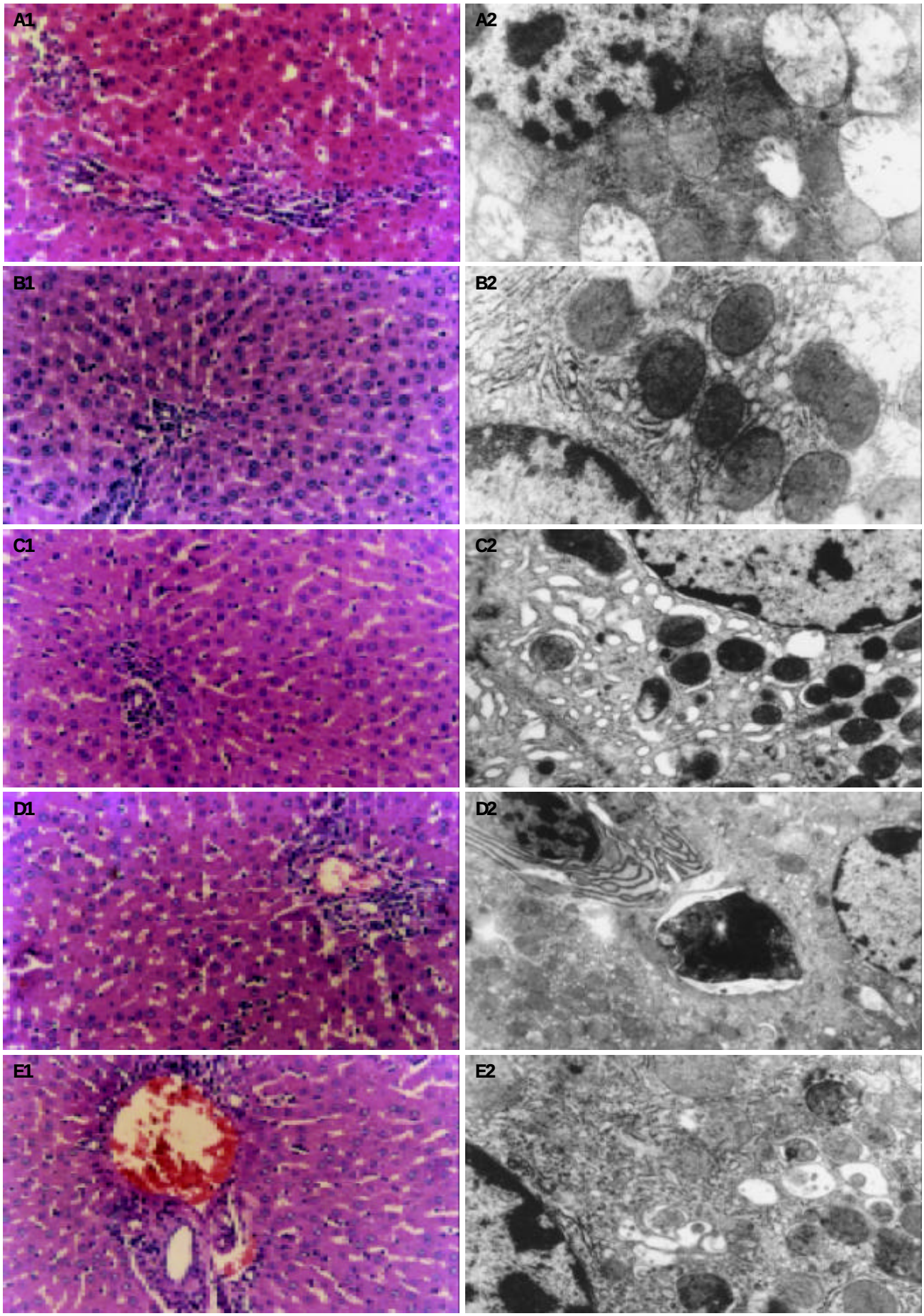


Figure 5 Expression of HSP70 in isolated human hepatocytes.



Figures 3 Changes of cellular structure and ultrastructure after treatment with IR, IP, IR+PMA, IP+CHE, and IP+PD98059. A1-2: Effect of ischemia and reperfusion on hepatocytes, B1-2: Cytoprotective effects of ischemia preconditioning, C1-2: Effects of ischemia preconditioning after stimulation of PKC with PMA, D1-2: Proteetive effect of ischemia preconditioning abolished by inhibition of PKC with chelerythrine, E1-2: Cytoprotective effect of ischemia preconditioning reverted by PD98059

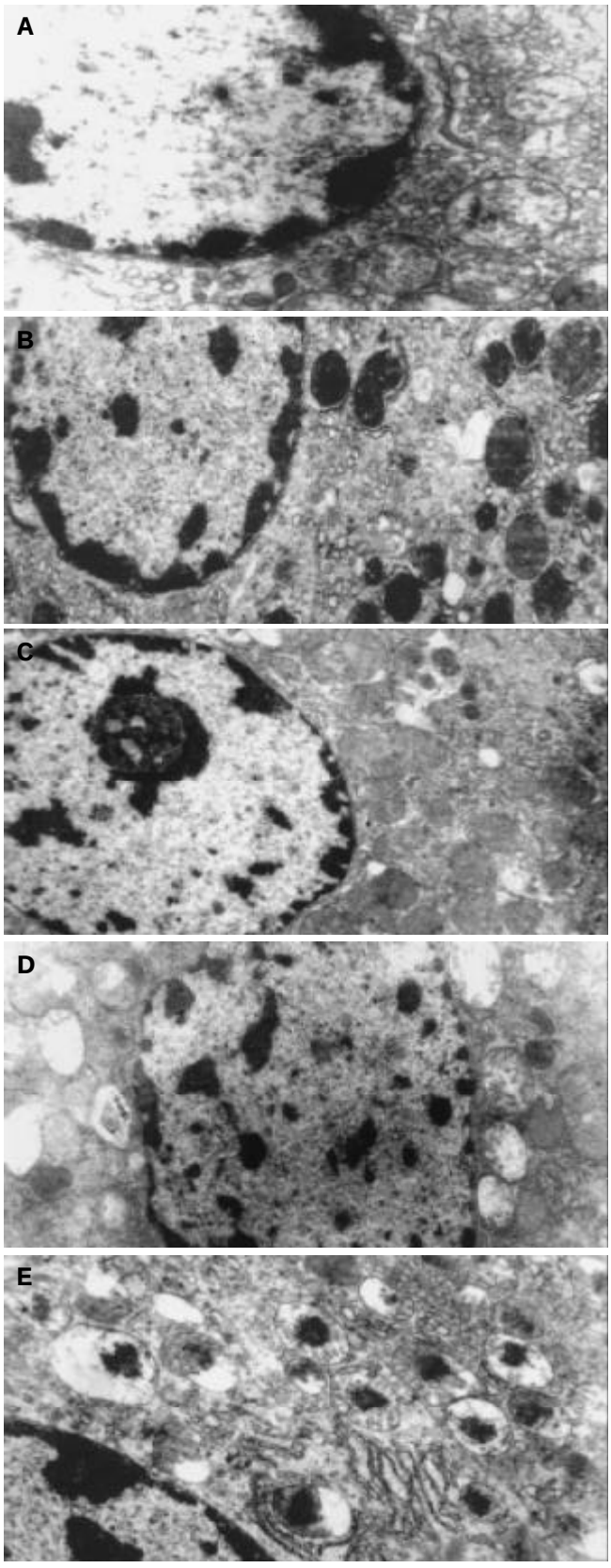


Figure 6 Changes of cellular ultrastructure after treatment with IR, IP, IR+PMA, IP+CHE, and IP+PD98059. A: Effect of hypoxia reoxygenation on hepatocytes, B: Cytoprotective effects of hypoxic preconditioning, C: Preconditioning effects mimicked by stimulation of PKC with PMA, D: Preconditioning protection abolished by inhibition of PKC with chelerythrine, E: Cytoprotection of preconditioning reverted by PD98059.

DISCUSSION

Ischemic preconditioning refers to the resistance to ischemic injury acquired by tissues following one or more brief periods of ischemia followed by reperfusion. Ischemic preconditioning was first described in myocardium, but has been shown in several other organs, including the brain, skeletal muscles, and small intestine[27-30]. Recent studies have shown that the same phenomenon could also be observed in the liver[31]. Particularly, a 10-min interruption of liver blood supply in anesthetized rats followed by 10 min of reperfusion reduced the release of transaminases during a subsequent 90-min period of ischemia and 90 min of reperfusion[32]. A similar effect has also been observed in steatotic liver after heat shock preconditioning[33]. Furthermore, ischemic preconditioning before cold prevervation of rat liver grafts increased the survival rate of rats receiving transplanted livers[34]. The use of isolated hepatocyte suspensions has confirmed that the hepatoprotective action of liver preconditioning observed *in vivo* could be reproduced *in vitro*. In accordance with the *in vivo* experiment, hepatocyte preconditioning developed after a transient hypoxia lasting more than 5 min and not exceeding 10 min and reduced the cytotoxicity during a subsequently prolonged hypoxic incubation by about 40%. But the signal pathway that mediated the development of hepatocyte ischemic preconditioning or hypoxic preconditioning was also largely unknown[35,36]. PKC-dependent activation of P44/42 MAPKs and HSP70 in signal transduction pathways during hepatocyte ischemic preconditioning or hypoxic preconditioning has not been reported.

There were several major findings in this study. First, ischemic PC or hypoxic PC had cytoprotective effects on hepatocytes subjected to a subsequent lethal ischemia reperfusion or hypoxia reoxygenation. Second, PKC appeared to play a pivotal role during hepatocyte ischemic PC. Third, the ischemic PC or hypoxic PC inducing activation of P44/42 MAPKs was completely abolished by the PKC inhibitor chelerythrine, indicating that the activation of P44/42 MAPKs is downstream of, and dependent on, PKC activation, and that P44/42 MAPKs may play a role in PKC mediated ischemic PC or hypoxic PC. Finally, protective proteins, such as HSP70, were induced by PC and regulated by signals in PKC dependent P44/42 MAPKs pathway.

The protective effect of ischemic preconditioning in myocardium involved the interstitial accumulation of endogenous mediators among which adenosine played a major role[25]. Large quantities of adenosine were released within seconds from the beginning of myocardial ischemia. By interacting mainly with adenosine A1 receptors, adenosine mediated mycardiocyte protection through the activation of a signaling pathway involving Gi-proteins, phospholypase C, diacylglyccerol and PKC[25]. Indeed, inhibiting PKC with polymyxin B, calphostin C, or chelerythrine could abolish the protective effect of myocaardialk ischemic preconditioning, whereas the stimulation of PKC by PMA or by diacylglyccerol analogues significantly reduced the infart size[26]. The role of PKC was further supported by membrane translocation of the deta and epsilon PKC isoforms in isolated rat hearts exposed to preconditioning[37]. We have observed that the cytoprotection exereted by preconditioning in the *in vivo* and *in vitro* setting was abolished by inhibiting PKC with chelerythrine. Moreover, PKC stimulation by PMA could reduce cell killing by ischemia reperfusion or hypoxia reoxygenation, mimicking the effect of preconditioning. This indicates that PKC activation might be involved in the signaling pathway responsible for the development of preconditioning in hepatocytes. Such a conclusion was consistent with a number of reports indicating that PKC stimulation was critical for the development of preconditioning in isolated rat, rabbit, and human myocardiocytes[38,39].

Previous studies have addressed the effect of ischemia on MAPK in *in vitro* models of global ischemia (isolated rat heart) and have yielded conflicting results. Maulik *et al*.[40] showed

that 4 cycles of 5-min ischemia/10-min reperfusion caused a significant increase in total MAPK phosphorylation activity and in the activity of MAPK-activated protein kinase 2. Knight and Buxton[41] reported that a single episode of ischemia ≤10 min followed by 15 min of reperfusion had no effect on total MAPK phosphorylation activity, a 15-min period of ischemia in itself had no effect but was associated with increased MAPK activity after 5-min rerperfusion. The reason for these discrepancies is unclear. Maulik *et al*.[40] Knight and Buxton[41] determined total MAPK activity using a phosphorylation assay. To a certain extent, P38 MAPKs activity, or P44/42 MAPKs activity was not individually assessed. Saurin *et al*[42]. reported that sustained P38 activation occurred during lethally simulated ischemia in cultured rat neonatal cardiocytes. This activation could be attenuated by cardioprotective treatments such as preconditioning and over expression of active PKC-δ[42]. Ping *et al*.[43] demonstrated that P44/42 MAPKs were activated during preconditioning stimuli in both isolated rabbit cardiomyocytes and rabbit hearts. In the present study we examined P44/42 MAPKs and found that P44/42 MAPKs were activated during hepatocyte preconditioning stimuli in both *in vivo* and *in vitro* models. Furthermore, activition of P44/42 MAPKs was PKC dependent. Previous studies suggested that PKC activated MAPKs in neonatal cardiac cells[44] and isolated hearts[45]. Virtuslly no information is available whether PKC activates MAPKs during hepatocyte ischemic preconditioning *in vivo*. If so, whether, in the setting of ischemic PC, mobilization of PKC occurs in parallel to MAPK activation or is a distal event. The results both *in vivo* and *in vitro* models showed that P44/42 MAPKs expression was markedly increased and the cytoprotective effect developed during ischemic PC, implying that the activation of P44 and P42 MAPKs was associated with the cytoprotection. The effect was abolished by the MEK inhibiter PD-98059, indicating that P44/42 MAPKs as a major signal transduction molecule played an important role in cytoprotection during hepatocyte ischemic preconditioning or hypoxic preconditioning. The PC-induced activation of P44 and P42 MAPKs was completely abolished by the PKC inhibitor chelerythrine, and PMA, an activator of PKC resulted in increased P44/42 MAPKs activity and mimicked the cytoprotection. These data demomstrate three important points: **(1)** PKC plays an obligatory role in the stimulation of P44/42 MAPKs during ischemic PC; (2) PKC activation precedes MAPK activation in the cascade that leads to PC ; (3) P44/42 MAPKs may play an important role in PKC-mediated ischemic PC. Because PKC activation was required for PC to develop[46,47], P44/P42 MAPKs might be downstream phosphorylation targets of PKC and the PKC-induced signaling pathways that mediate ischemic PC or hypoxic PC.

Ping *et al*.[43] indicated that PKC-dependent activation of P44/P42 MAPKs during hepatocyte preconditioning in both isolated rabbit cardiomyocytes and rabbit hearts produced a marked reduction in infarct size and the serum level of LDH, which had cardioprotective effects. Their results were coincident with what we presented. In preconditioned isolated rat hepatocytes, Carini *et al*.[35] observed that interfering with P44 and P42 MAPKs activition using MEK inhibition PD98509 did not affect cytoprotection, whereas SB203580, a specific inhibition of P38 MAPKs completely abolished the effects of preconditioning. So they proposed that P44 and P42 MAPKs in signal transduction pathway was not responsible for the development of liver ischemic preconditioning. Their results were diff was from ours. The possible reasons might be as follows. (1) The experimental environment different *in vitro* Ip model for hepatocyte and *in vivo* model for rat liver. *In vitro* experiment imitated the ischemia *in vivo* merely by depriving of the oxygen and blood serum, but it could not be mimicked completely. Ischemic preconditioning *in vivo* could be affected by body temperature, homeostasis, anesthesia and some other factors. But in our experiment, both in the *in vivo* and *in vitro* setting, we got the same result. (2) By passing compensatory activation, cross talking existed in intracelluar signal transduction pathways[48], while P44 and P42 MAPKs signal transduction pathway was inhibited, which continued to convey the signals and induced the expression of cytoprotective proteins, such as P38MAPKs signal transduction pathway[49]. (3) Different methods were used in ischemic preconditioning. Fryer *et al*.[50] reported that mutiple-cycle-induced IPC could activate more pathways than a single-cycle IPC stimulus and that this difference could be attributable to the recruitment of another PKC-independent signal transduction pathway. The major signal pathway eliciting the cytoprotection would change while using different IP method. Our results were in agreement with those results of Ping' s group[43] obtained in the rabbit heart also via repetitive IPC, but Carini *et al*.[35] drew the different conclusion using a single IPC. (4) Dose and timing dependence. The activation of intracellular signal molecule was time dependent, and the reverse feedback regulation occurred at a time point. P44/42 MAPKs activity increased as early as 6 h following precondition, and peaked at 48 h. Preincubation with PD98059 (a selective MEK inhibitor) was associated with a dose-dependent inhibition which was statistically significant for concentrations higher than 10 μmol/L, and maximal at 100 μmol/L, and near-complete inhibition of activation of P44 and P42 MAPKs was observed with 50 μmol/L PD98059[21]. Referring to the time point of activation of P44/42 MAPKs and the dosage of PD98059, eventually we got the similar results[43]. Carini *et al*.[35] got various results by using different dosages of PD98059 (20 μmol/L) to treat hepatocytes and detect the effect of inhibition at different time points (after exposure to 90 min of hypoxia).

HSP is one of the most highly conserved proteins in existence, and has been found in every organism[51]. These proteins are known to protect cells from the toxic effects of heat and other stresses and were synthesized quickly and intensely in response to stressors[52]. Exactly how HSPs protect cells is unclear, however, everal explanations have been offered. These include the renaturation of damaged proteins or facilitation of the folding and targeting of newly synthesized proteins to organellae[52]. HSPs may also maintain newly synthesized proteins in a translocational configuration (linear or unfolded). Induction of heat shock protein has been shown to subsequently protect cells in signal transduction pathways in liver ischemic preconditioning[53], but few studies have been published on intracellular protective mechanism. Whether PKC dependent P44/42 MAPKs pathways are involved in the regulation of protective proteins, such as HSP70 is largely unknown. Some substantial literature described the induction of HSP70 by ischemia[21], the potential role of HSP70 in ischemic preconditioning[24], and an inverse correlation between expression of HSP70 induced by ischemic or thermal preconditioning and infart size in animal models[25,26]. In addition, enhanced expression of HSP70 conveyed a cytoprotective effect in cultured cells, including cardiac myocytes subjected to simulated ischemia[29,30]. Specifically, overexpression of HSP70 in transgenic mice could improve myocardial function[32,33], preserve metabolic functional recovery, and reduce infart size after ischemic preconditioning. Several recent studies suggested that PKC might play a role in mediating the protective effects of ischemic preconditioning while the activators such as PMA mimicked the protective effect via phosphorylation of unknown effector protein. PKC played a crucial role in the signal transduction for the activation of many cellular functions. Many transcription factors have been known to be activated by various PKC subtypes[36]. These include heat shock protein transcription factors (HSF). The synthesis of HSPs is mediated by the activation of heat shock gene transcription which is mediated by

the binding of HSF to the heat shock element in the promoter region of HS genes. The gene knockout model of HSF1 *in vitro* demonstrated the essentisl requirement of this regulatory pathway in cellular protection[37]. But whether P44/42 MAPKs signal pathways during hepatocyte ischemic preconditioning mediates the synthesis of HSP70 remains elusive and represents an unsolved problem. In this study, expression of HSP70 exhibited a sharp signal at 70 ku as detected by Western immunoblotting in both *in vivo* and *in vitro* models. HSP70 expression was increased in IP (HP) treated models which provided cytoprotective effect on hepatocytes suffering from ischemia reperfusion or hypoxia reoxygenation. While PKC inhibitor chelerythrine and MEK inhibitor PD-98059 inhibited the protein expression. The cytoprotective effect was reduced, but the activator of PKC (PMA) could induce HSP70 expression and cytoprotection apparently. Thus, HSP70 was recognized as molecular chaperones and could protect cells under the hazardous conditions such as ischemia reperfusion or hypoxia reoxygenation. It was induced by PC and regulated by signals in PKC dependent P44/42 MAPKs pathway.

In summary, PKC-dependent activation of P44/42 MAPKs and HSP70 in signal transduction pathways during hepatocyte ischemic preconditioning is an important part of endogenous protective mechanisms. PKC is upstream of P44/42 MAPKs, and PKC regulates the activation of P44 and P42 MAPKs positively. HSP expression is regulated by signals in P44/42 MAPKs pathway, but this passway is just one of the most important signal transduction pathways during liver ischemic preconditioning. PKC–dependent activation of MAPKs family such as p38MAPK, JNK and ERK5 can regulate HSP70 expression and deserve further study.

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