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 P44/42 MAPKs, HSP70 expression were further analyzed. AST/ALT concentration, cellular structure and ultrastructure 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 phorbol-12--myristate 13-acetate (PMA). Conversely, inhibiting PKC with chelerythrine abolished the protection given by preconditioning. PD98059, inhibitor of MEK (the upstream kinase of P44/42 MAPKs), also reverted the cytoprotection exerted by preconditioning.

CONCLUSION: The results demonstrate that preconditioning induces a rapid activation of P44/42 MAPKs 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.

INTRODUCTION

The term ischemic preconditioning (IP) was first coined by Murty 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 kinase 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 ischemia 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; P44/42 MAPKs), c-jun NH2-terminal kinases 1 and 2 (JNK1/2), P38 MAPKs which was found recently. ERK1/2 is predominantly activated by growth factors, and JNKs and P38 MAPKs are generally activated by stresses such as ultraviolet light, inflammatory cytokines, heat shock, and ischemic reperfusion. Recent evidence implicates PKC in the activation of 2 members of this kinase family, i.e., the P44 and P42 MAPKs[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 phorbol-12-myristate-13-acetate (PMA). Conversely, inhibiting PKC with chelerythrine abolished the protection given by preconditioning. PD98059, an inhibitor of MEK (the upstream kinase of P44/42 MAPKs), also reverted the cytoprotection exerted by preconditioning. This suggests that preconditioning induces a rapid activation of P44/42 MAPKs and PKC activation plays a pivotal role in the activation of P44/42 MAPKs pathway that participates in the preservation of liver cells.

Although 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 P44/42 MAPKs 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, the potential role of HSP70 in ischemic preconditioning, and an inverse correlation between expression of HSP70 induced by ischemic or thermal preconditioning and infant size in animal models. In addition, enhanced expression of HSP70 conveys a cytoprotective effect in cultured cells, including cardiac myocytes subjected to simulated ischemia. Specifically, overexpression of HSP70 in transgenic mice improves myocardial function, preserves metabolic functional recovery, and reduces infarct 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. 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 essential requirement of this regulatory pathway in cellular protection. 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. Phorbol 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 protocols. According to the method of Kobayashi et al., 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 preconditioning): 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) total volume 5 mL was slowly injected through dorsal veins of penis for 10 min, beginning 10 min before the start of ischemic reperfusion. (5) Group CHE (IP + chelerythrine chloride): Total volume of 5 mL chelerythrine chloride (5 mg/kg) was slowly injected through dorsal veins of penis for 10 min, 10 min before the start of ischemic preconditioning. (6) Group PD (PD + PD98059): Total volume of 5 mL PD98059 (5 mg/kg) 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/p42 MAPKs.

Measurement of serum ALT and AST

Three hours after the last reperfusion, the abdomen of each group was re-opened. Blood samples of infranephatic 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 °C, 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 °C 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 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 membranes 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 °C, washed 3 times for 5 min in TBST before addition of goat anti-mouse-HRP conjugated secondary antibody for 1 h at room temperature. Membranes were washed 3 times for 5 min with PBS, and peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting...
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. Organella 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 hepatocytes were slightly swollen and had no degeneration with no proliferation of Kupffer cells. Portal area was normal. Organella 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  | 61.9±12.1  |
| IR      | 820.9±111.3 | 762.8±130.5 |
| IP      | 407.7±73.7  | 281.0±35.6 |
| IR+PMA  | 553.2±58.67  | 354.3±53 |
| IP+PD   | 732.9±91.1  | 466.2±82.8 |
| IP+CHE  | 678.6±136.5  | 645.6±50.4 |

\(P<0.01\) versus group IR, \(P<0.01\) versus group C, \(P<0.01\) 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       | 36.29±3.8 |
| IR      | 42.78±2.22 |
| IP      | 112.6±14.86 |
| IP+CHE  | 165.47±27.25 |

\(P<0.01\) versus group IR, \(P<0.01\) 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. 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 chelerythrine reverted the effect of preconditioning on hepatocytes. Chelerythrine also abolished the protection against the damage caused by ischemia reperfusion or hypoxia.
reperfusion (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               |
| HP                   | 81.42±12.1               |
| HR+PMA               | 75.29±11.9               |
| HP+PD                | 51.03±9.09               |
| HP+CHE               | 47.21±5.42               |

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

| Group            | Phosphorylation activity of PKC (fmol/ mg/ min) |
|------------------|-----------------------------------------------|
| C                | 32.67±5.11                                    |
| HR               | 42.63±4.73                                    |
| HP               | 109.42±16.09                                  |
| HR+PMA           | 152.47±19.59                                  |
| HP+CHE           | 65.28±5.36                                    |

**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 (Tables 1, 3; Figures 3B, 6B). While PKC inhibitor chelerythrine and MEK inhibitor PD-98059 inhibited the protein expression, and the cytoprotective effect was reduced (Tables 1, 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.
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: Protective effect of ischemia preconditioning abolished by inhibition of PKC with chelerythrine. E1-2: Cytoprotective effect of ischemia 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. Recent studies have shown that the same phenomenon could also be observed in the liver. 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. A similar effect has also been observed in steatotic liver after heat shock preconditioning. Furthermore, ischemic preconditioning before cold preservation of rat liver grafts increased the survival rate of rats receiving transplanted livers. 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. 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. Large quantities of adenosine were released within seconds from the beginning of myocardial ischemia. By interacting mainly with adenosine A1 receptors, adenosine mediated myocardocyte protection through the activation of a signaling pathway involving Gi-proteins, phospholipase C, diacylglycerol and PKC. Indeed, inhibiting PKC with polymyxin B, calphostin C, or chelerythrine could abolish the protective effect of myocardial ischemic preconditioning, whereas the stimulation of PKC by PMA or by diacylglycerol analogues significantly reduced the infarct size. The role of PKC was further supported by membrane translocation of the delta and epsilon PKC isoforms in isolated rat hearts exposed to preconditioning. We have observed that the cytoprotection exerted 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 myocardocytes.

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. 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 reperfusion. 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[43]. Ping et al.[44,45] 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 hepatic preconditioning stimuli in both in vivo and in vitro models. Furthermore, activation of P44/42 MAPKs was PKC dependent. Previous studies suggested that PKC activated MAPKs in neonatal cardiace[34] and isolated hearts[45]. Virtually no information is available whether PKC activates MAPKs during hepatic 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 inhibitor PD-98059, indicating that P44/42 MAPKs as a major signal transduction molecule played an important role in cytoprotection during hepatic 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 demonstrate 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[56-57], P44/42 MAPKs might be downstream phosphorylation targets of PKC and the PKC-induced signaling pathways that mediate ischemic PC or hypoxic PC.

Ping et al.[31] indicated that PKC-dependent activation of P44/42 MAPKs during 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 activation using MEK inhibition PD98059 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 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 intracellular signal transduction pathways[38], 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[29]. (3) Different methods were used in ischemic preconditioning. Fryer et al.[50] reported that muple-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 molecular 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[25]. 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[31]. 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, several explanations have been offered. These include the renaturation of damaged proteins or facilitation of the folding and targeting of newly synthesized proteins to organelae[51]. 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[32,33], the potential role of HSP70 in ischemic preconditioning[24], and an inverse correlation between expression of HSP70 induced by ischemic or thermal preconditioning and infarct size in animal model[28,29]. In addition, enhanced expression of HSP70 conveyed a cytoprotective effect in cultured cells, including cardiac myocytes subjected to simulated ischemia[30,31]. Specifically, overexpression of HSP70 in transgenic mice could improve myocardial function[22,33], preserve metabolic functional recovery, and reduce infarct 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[34]. 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 essential requirement of this regulatory pathway in cellular protection.

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 kDa 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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