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Activation of protein kinase C delta reduces hepatocellular damage in ischemic preconditioned rat liver

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ABSTRACT

Background: Liver ischemic preconditioning (IPC), pre-exposure of the liver to transient ischemia, has been applied as a useful surgical method to prevent liver ischemia and reperfusion (I/R) injury. Although activation of protein kinase C (PKC), especially novel PKCs, has been known as central signaling responsible for the liver protection of IPC, determination of the involved isozyme in strong protection afforded by IPC has not been elucidated.

Materials and methods: Rats were subjected to 90 min of partial liver ischemia followed by 3, 6, and 24 h of reperfusion. IPC was induced by 10 min of ischemia after 10 min of reperfusion before sustained ischemia. Rottlerin, a PKC-δ selective inhibitor; PKC-εV1-2 peptide, a selective PKC-ε inhibitor; and 3,7-dimethyl-1-[2-propargyl] xanthine, an adenosine A_2 receptor antagonist, were intravenously injected before IPC. N-acetyl-L-cysteine, a strong antioxidant, and Nω-nitro-L-arginine methyl ester, a nonselective nitric oxide synthase inhibitor, were injected intraperitoneally before IPC.

Results: IPC resulted in strong protection against liver I/R injury as evidenced by biochemical and histologic analyses. Inhibition of PKC-δ strongly attenuated the IPC-induced liver protection, whereas PKC-ε inhibition did not exert any effect on IPC-induced protection. Although inhibition of reactive oxygen species, adenosine, and nitric oxide attenuated the beneficial effects of IPC, inhibition of adenosine only attenuated PKC-δ and -ε translocation.

Conclusions: Our findings suggest that IPC protects against I/R-induced hepatic injury through activation of PKC-δ.

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1. Introduction

Ischemic preconditioning (IPC) refers to endogenous cellular protective mechanisms against ischemia and reperfusion (I/R) injury induced by pre-exposure to one or more brief ischemic stimuli followed by reperfusion [1]. IPC occurs in a biphasic pattern: an early phase, which wanes several hours (early IPC), that involves production of adenosine, reactive oxygen species (ROS), and/or nitric oxide (NO) and a late phase (late IPC), which reappears 24–48 h after reperfusion, that requires synthesis of multiple stress-response proteins, NO, and heme oxygenase 1 (HO-1) [2]. A large body of evidence has demonstrated that IPC effectively reduces liver injury in an animal model of I/R [3]. Although the benefit of IPC in the liver already has been suggested in a clinical pilot [4] and large randomized studies [5], facts regarding the exact molecular mechanisms remain unclear.

It is now generally accepted that the development of IPC largely depends on the activation of protein kinase C (PKC) signaling. PKC is a serine/threonine kinase that is activated by

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lipid cofactors derived from the breakdown of membrane lipids by phospholipase C. There are multiple isozymes of PKC, which is linked to distinctive function in different organs: classic PKCs (α, β, and γ), which are dependent on diacylglycerol (DAG) and calcium; novel PKCs (δ, ε, and η) which are calcium-independent, needing only DAG for their activation; and atypical PKC (ζ) which is neither dependent on calcium nor DAG [6]. Among these isozymes, IPC was found to cause translocation of PKC-α, -δ, and -ε, but PKC-ε alone was responsible for early IPC in rabbit cardiomyocytes [7] and isolated rat myocardium [8]. In the liver, however, activation of novel PKCs, especially both PKC-δ and -ε, was reported as a central signaling cascade in response to IPC stimuli [9]. In our recent reports, we demonstrated that activation of novel PKCs plays differential roles in both early and late phases of IPC, showing that translocation of PKC reduces oxidative stress and hepatocellular necrosis in the early phase of IPC whereas it reduces apoptosis in the late phase of IPC by inducing the synthesis of inducible nitric oxide synthase (iNOS), HO-1, and cyclooxygenase 2 [3]. Although two isozymes, PKC-δ and -ε, are members of the same subgroup, previous reports have demonstrated that they mediate contrastingly and induce opposing effects [10–12].

Therefore, in the present study, we aimed to evaluate which PKC isozyme confers protection afforded by liver IPC and its molecular mechanisms.

2. Materials and methods

2.1. Liver I/R and IPC procedures

All animal protocols were approved by the Animal Care Committee of Sungkyunkwan University and performed in accordance with the guidelines of the National Institutes of Health (publication number 86-23, revised 1985). Male Sprague–Dawley rats (Orient Bio, Inc, Gapyeong, South Korea) were fasted for 18 h but allowed free access to tap water. Under ketamine (60 mg/kg, intraperitoneally [IP]) and xylazine (8 mg/kg, IP) 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) for 90 min; reperfusion was initiated by removing the clamp. In the preconditioned group, preconditioning was induced by 10 min of ischemia followed by 10 min of reperfusion before 90 min of sustained ischemia, according to our previous report [3]. The sham group underwent the same protocol except for vascular occlusion. Because there were no significant differences between rats in the drug-treated and sham groups, the results of these groups were pooled and referred to as sham. Blood and liver tissues were obtained and stored at −75°C for biochemical analysis, except for the part in the left lobe, which was used for histologic analysis.

2.2. Drug treatment

Rottlerin (Sigma-Aldrich, St. Louis, MO), a selective PKC-δ inhibitor, was dissolved in dimethyl sulfoxide and injected intravenously at a dose of 0.3 mg/kg 10 min before IPC. PKC-εV1-2 peptide (Sigma-Aldrich), a selective PKC-ε inhibitor, was dissolved in 1:3 ethanol–0.9% saline and injected intravenously at a dose of 1 mg/kg for 10 min before IPC. 3,7-Dimethyl-1-[2-propargyl] xanthine (DMPX; Sigma-Aldrich), an adenosine A_2 receptor antagonist, was dissolved in distilled water and administered intravenously at a dose of 1 mg/kg 15 min before IPC. N-acetyl-L-cysteine (NAC; Sigma-Aldrich) dissolved in phosphate-buffered saline was IP injected twice at a dose of 300 mg/kg for 3 and 1 h before IPC. Nω-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich), a nonselective NOS inhibitor, was dissolved in phosphate-buffered saline and injected IP at a dose of 10 mg/kg for 5 min before IPC. The dose and timing of drug treatment were selected based on previous reports and our preliminary studies [3,13,14].

2.3. Histologic analysis

Fresh liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 5-μm thickness were then cut and stained with hematoxylin and eosin. The stained sections were evaluated at ×200 magnification with an Olympus CKX 41 microscope (Olympus Optical Co, Tokyo, Japan). Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining using a commercially available kit (In situ Apoptosis Kit; TaKaRa, Shiga, Japan) according to the manufacturer's instructions.

2.4. Serum aminotransferase and caspase 3 activities

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using a ChemiLab ALT and AST assay kit (IVDLab Co, Ltd, Uiwang, South Korea), respectively. Caspase 3 activity was measured using a Caspase 3 Colorimetric Assay Kit (Sigma-Aldrich) as described previously [15].

2.5. 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 [16] using 1,1,3,3-tetraethoxypropane as the standard. Total glutathione in liver homogenate was determined spectrophotometrically at a wavelength of 412 nm using yeast glutathione reductase, 5,5′-dithiobis(2-nitrobenzoic acid), and NADPH according to the method reported by Tietze [17]. 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.

2.6. Protein extraction of whole liver tissue

Isolated liver tissue was homogenized in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology Inc, Seongnam,

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