Ischemic preconditioning in hepatic ischemic–reperfusion injury

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Received 2 February 2016; received in revised form 18 February 2016; accepted 1 March 2016
Available online 5 July 2016

KEYWORDS
ischemia–reperfusion injury; liver; preconditioning

Abstract Background/Introduction: Ischemic preconditioning is a method in which brief periods of ischemia render tissues resistant to injury resulting from prolonged ischemia and reperfusion, so-called ischemia–reperfusion injury. Purpose: To elucidate the possible protective role of ischemic preconditioning in rat livers with ischemia–reperfusion injury. Methods: Rats were first allocated to either a sham control or an ischemic preconditioning group. On the following day, the rats from each group were administered either 30 minutes or 45 minutes hepatic ischemia. Next, rat livers were harvested for measuring proliferating cell nuclear antigen, heme oxygenase-1, inducible nitric oxide synthase, and heat shock protein 70 mRNA levels. Results: Both proliferating cell nuclear antigen and heme oxygenase-1 expression increased significantly after 45 minutes hepatic ischemia compared with those after 30 minutes hepatic ischemia, but they decreased significantly with ischemic preconditioning. However, ischemic preconditioning did not affect inducible nitric oxide synthase or heat shock protein 70 expression. Conclusion: From the preliminary findings, further elucidation is warranted.

1. Introduction

During ischemic preconditioning, brief periods of tissue ischemia are used to render tissue resistant to injury resulting from prolonged ischemia and reperfusion, so-called ischemia–reperfusion injury. Hepatic ischemia–reperfusion injury plays a major role in hepatic resection and shock, particularly during liver transplantation.1–4 Hepatic vascular control is commonly used to prevent massive hemorrhage during hepatectomy. Hepatic vascular occlusion provides a relatively bloodless operative field, enabling unhurried, meticulous, and accurate intrahepatic dissection and hemostasis.5–7

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Hemeoxygenases (HOs) catalyze the formation of carbon monoxide (CO), biliverdin, and Fe²⁺ ions during heme degradation. HO-1 is the inducible isof orm of HO and is induced for cytoprotection under various stress-related conditions including hyperemia, hypoxia, and ischemia—reperfusion.⁸,⁹

Nitric oxide (NO) plays a crucial role in maintaining the hepatic vascular tone.¹⁰ NO synthase (NOS) metabolically produces NO from L-arginine, oxygen, and nicotinamide adenine dinucleotide phosphate. In the liver, inducible NOS (iNOS) is a key molecule catalyzing NO synthesis.¹¹,¹²

Proliferating cell nuclear antigen (PCNA), also called cyclin, is an auxiliary protein of DNA polymerase-δ;¹³ its expression levels are directly correlated with cell proliferation and DNA synthesis rates.¹⁴ PCNA is synthesized in the late G1 and S phases and remains present in the nuclei throughout the cell cycle; thus, the PCNA levels are correlated with the cell proliferative state.¹⁵

2. Methods

This study elucidated the pathophysiology of ischemia—reperfusion injury in a rat liver model and evaluated the possible protective role of ischemic preconditioning in rat livers with ischemia—reperfusion injury.

2.1. Samples and experimental design

We used male Sprague–Dawley rats, weighing 250–300 g, housed in stainless-steel cages under controlled temperature, humidity, and 12-hour light—dark cycles. They were allowed free access to food and water before and after surgery. All the rats received humane care, and the study protocol complied with the guidelines and animal research laws of our institution. The study protocol was reviewed and approved by the Chang Gung Memorial Hospital Animal Care and Use Committee, Kaohsiung, Taiwan. An intramuscular dose of anesthesia containing 30 mg/kg of Zoletil 50 (VIRBAC, France) and 10 mg/kg of Xylazine (Bayer Vital GmbH, Germany) was administered before surgery. After surgery, 2200 IU/100 g of penicillin (Y. F. Chemical Ltd., Taiwan) and 3 mg/kg of ketorolac tromethamine (Standard Chem & Pharm Co. Ltd., Taiwan) were administered intramuscularly for infection prophylaxis and pain relief, respectively. The rats were euthanized by the experiments with surgical removal of the heart under anesthesia.

All the rats were divided into six groups (n = 6 in each group): Group 1 underwent sham operation (laparotomy and closure only); Group 2 underwent laparotomy and total hepatic ischemia, performed using a smallatraumatic clamp to occlude the hepatoduodenal ligament for 10 minutes (preconditioning); Group 3 underwent laparotomy and closure, and after 24 hours (Day 2) again underwent laparotomy followed by total hepatic ischemia for 30 minutes; Group 4 underwent the 10-minute preconditioning performed in the Group 2 rats, and after 24 hours (Day 2) again underwent laparotomy followed by total hepatic ischemia for 30 minutes; Group 5 underwent laparotomy and closure, and after 24 hours (Day 2) again underwent laparotomy followed by total hepatic ischemia for 45 minutes; and Group 6 underwent the 10-minute preconditioning performed in the Group 2 rats, and after 24 hours (Day 2) again underwent laparotomy followed by total hepatic ischemia for 45 minutes.

Tissue samples were harvested as follows: Groups 1 and 2, 24 hours later (Day 2); and Groups 3–6, 15 minutes (reperfusion) after removal of the temporary occlusion on Day 2. All harvested hepatic and intestinal tissue samples were stored in liquid nitrogen until analysis.

2.2. RNA extraction and reverse-transcription polymerase chain reaction for measuring PCNA, HO-1, iNOS, and heat shock protein 70 mRNA levels

Total tissue RNA was extracted using guanidine isothiocyanate, according to the method described by Chomczynski and Sacchi.¹⁶ First-strand cDNA was synthesized using oligo-dT primers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA).

Hot start Taq polymerase was used for cDNA polymerase chain reaction (PCR) performed at 88°C in a Thermal Cycler (Perkin–Elmer, Norwalk, CT, USA). PCNA-specific primers were added to each tube at a final concentration of 0.2 μmol/L. The 5’–3’ sequences of the primer pairs and predicted sizes of the amplified PCR fragments have been provided previously.¹⁷,¹⁸ After a 5-minute initial melting step at 97°C, 35–40 PCR cycles were performed as follows: denaturation at 94°C for 1 minute, followed by annealing at 50°C–60°C (52°C for lipopolysaccharide binding protein (LBP), 50°C for tumor necrosis factor-α, and 60°C for CD14) for 1 minute and then extension at 72°C for 2 minutes. The final cycle was followed by a 10-minute soak at 72°C.

We used the constitutively expressed β-actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as internal controls to confirm the validity of the target gene expression observed under different experimental conditions for standardizing the PCR products. The cycle numbers of each primer pair were selected in a linear range to prevent the plateau effect. The ratios of PCNA/G3PDH, HO-1/β-actin, INSO/β-actin, and heat shock protein (HSP) 70/β-actin signals were calculated for each sample. Each experiment included a negative control (a sample RNA, not subjected to reverse transcription).

2.3. Statistical analysis

All data were statistically analyzed and the results are presented as means ± standard deviations. The data were compared using the Mann–Whitney U test. Differences with p < 0.05 were considered statistically significant.

3. Results

PCNA expression did not differ significantly between Group 3 (30-minute hepatic ischemia only) and Group 4 (30-minute hepatic ischemia with preconditioning; p = 0.9813). It significantly increased in Group 5 (45-minute hepatic ischemia only) compared with that in Group 3 (p = 0.0007), whereas it decreased significantly in Group 6 (45-minute ischemia with preconditioning) compared with that in Group 5 (p = 0.0065; Figure 1).
Similarly, HO-1 expression did not differ significantly between Group 3 and Group 4 ($p > 0.1797$). It significantly increased in Group 5 compared with that in Group 3 ($p = 0.0024$), whereas it decreased significantly in Group 6 compared with that in Group 5 ($p = 0.049$; Figure 2).

The difference in iNOS expression of Groups 3 and 5 was nonsignificant ($p = 0.2131$). Preconditioning did not cause any significant difference in inOS expression in either the 30- or 45-minute ischemia groups (Group 3 vs. Group 4, $p = 0.2997$; Group 5 vs. Group 6, $p = 0.0673$; Figure 3).

Similarly, HSP70 expression did not differ significantly between Groups 3 and 4 ($p = 0.6376$), and preconditioning was also ineffective in both the 30- and 45-minute ischemia groups (Group 3 vs. Group 4, $p = 0.5041$; Group 5 vs. Group 6, $p = 0.9124$; Figure 4).

### 4. Discussion

Ischemic preconditioning before a brief period of vascular occlusion can render an organ resistant to the damaging effects of ischemia–reperfusion.1–4,18 This protective effect was originally described for the heart19 and subsequently for the brain, intestine, skeletal muscle, and liver.18–22

Pang et al23 reported that only a brief period of hepatic ischemia is required for preconditioning. Xue et al18 hypothesized that the short reperfusion phase of ischemic

**Figure 1** Proliferating cell nuclear antigen (PCNA) expression significantly increased after 45-minute hepatic ischemia alone (45) compared with that after 30-minute hepatic ischemia alone (30). PCNA expression after 45-minute ischemia with preconditioning (45P) was significantly lower than that after 45-minute ischemia alone (45). * $p = 0.9813$, ** $p = 0.0065$, *** $p = 0.0007$.

**Figure 2** Hemeoxygenase (HO)-1 expression significantly increased after 45-minute hepatic ischemia alone (45) compared with that after 30-minute hepatic ischemia alone (30). HO-1 expression after 45-minute ischemia with preconditioning (45P) was significantly lower than that after 45-minute ischemia alone (45). * $p = 0.1797$, ** $p = 0.049$, *** $p = 0.0024$.

**Figure 3** The difference in inducible nitric oxide synthase expression between 30- and 45-minute ischemia alone (30 and 45, respectively) was nonsignificant. Preconditioning induced no significant difference in either the 30-minute ischemia groups (30 vs 30P) or the 45-minute ischemia groups (45 vs 45P). * $p = 0.2997$, ** $p = 0.0673$, *** $p = 0.2131$.

**Figure 4** The difference in heat shock protein 70 expression after 30- and 45-minute ischemia alone (30 and 45, respectively) was nonsignificant. Preconditioning induced no significant difference in either the 30-minute ischemia groups (30 vs 30P) or the 45-minute ischemia groups (45 vs 45P). * $p = 0.5041$, ** $p = 0.9124$, *** $p = 0.6376$. 

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preconditioning generates reduced reactive oxygen species (ROS) sufficient for activating redox-sensitive intracellular signaling pathways. Furthermore, the downstream consequences of such ROS-induced signaling pathways are cytotoxic to liver cells through the abrogation of cell death pathways, simulation of antioxidant and other cytoprotective mechanisms, and initiation of the cell cycle. However, the actual protective mechanisms of ischemic preconditioning remain unclear.

HO is the rate-limiting enzyme in heme degradation, resulting in the formation of CO, biliverdin, and Fe^{2+} ions.24 Thus far, three distinct HO isoforms—HO-1, HO-2, and HO-3—have been identified. HO-1 is constitutively expressed in the normal gastric, intestinal, and colonic mucosa.25,26 It is also expressed by all liver and circulating nucleated cells and can be induced under several stress-related conditions such as ischemia—reperfusion. HO-1 induction is considered cytotoxic.8,9 HO-1 inducers may reduce the instances of hepatic ischemia-reperfusion injury, whereas HO-1 inhibitors may exacerbate them.27,28 The protective mechanism of HO-1 is attributable to its byproducts, CO and biliverdin, which are vasodilators that improve liver microcirculation and reduce hepatocellular apoptosis and necrosis.29

NO is synthesized from L-arginine by the NOS family of enzymes. Two NOS isoforms were previously identified: constitutive NOS and iNOS.30 Constitutive NOS comprises endothelial NOS (eNOS) and neuronal NOS. NO plays a crucial role in the homeostasis of hepatic circulation under physiological conditions.31,32 Furthermore, a large amount of NO is produced in response to various stress-related conditions including hepatic ischemia—reperfusion.33 Under a stable condition, only the physiological amount of NO produced by eNOS maintains hepatic vascular tone.34 Nevertheless, the large amount of NO produced by iNOS induces vasodilatation.35 Therefore, the induction and activation of iNOS constitute a crucial biological mechanism for coping with stress-related conditions.32

The heat shock response is induced by various stimuli including thermal, chemical, and physical stresses as well as short-term ischemia.36 Organisms typically respond to such detrimental insults by inducing a group of protective proteins called HSPs. HSPs are classified into multimember families on the basis of the molecular weights of protein they encode (HSP90, HSP70, HSP60, and the small HSP family). HSP70 is one of the most extensively studied mammalian HSPs.36,37 HSPs, particularly HSP70, play a major role in thermodilatation as well as in protecting against oxygen radical toxicity and ischemia-reperfusion injury.38

PCNA levels are directly correlated with cell proliferation and DNA synthesis rates and with the cell proliferative state.14,39 Prellich et al.40 studied post-hepatectomy regeneration in mice and reported that regeneration with PCNA-positive cell augmentation increased after 24 hours. Tanoue et al.41 demonstrated that a 50% reduced liver promoted hepatic remnant regeneration, which was accentuated by N-acetylcysteine, according to the total hepatocyte number and PCNA levels.

In previous studies, the effects of ischemic preconditioning have been generally evaluated immediately or a few hours after the maneuver is performed. To the best of our knowledge, our study is the first to evaluate the long-term effects of ischemic preconditioning (on the 2nd day, 24 hours later) and demonstrate that both PCNA and HO-1 expressions significantly increased after the longer hepatic ischemia (45 minutes), which decreased with ischemic preconditioning. Because longer ischemic insults probably induce more reactive production of PCNA and HO-1, PCNA and HO-1 expressions may have significantly increased after 45-minute ischemia compared with that after 30-minute ischemia. Nevertheless, why ischemic preconditioning significantly reduced PCNA and HO-1 expressions in the 45-minute ischemia groups remains unknown. Whether the long-term effects of ischemic preconditioning stabilize the liver—resulting in reduced PCNA and HO-1 expressions after 45-minute ischemia—warrants further elucidation.

Hypoxia-inducible factor (HIF)-1 and HIF-2 are hetrodimeric basic helix—loop—helix transcription factors from the Per—ARNT—Sim family.42 Their basic helix—loop—helix domains mediate dimerization and bind to contact the DNA of target genes and activate transcription.43 HIFs are universal cellular oxygen-sensitive transcription factors that activate several hypoxia-responsive genes, some of which are responsible for protective cellular functions.44 During organ donation, allografts are exposed to hypoxia and ischemia for significant periods. Exploiting this pathway during donor management and organ preservation may prevent or reduce allograft injury and improve organ transplantation outcomes.45 The role of HIFs warrants exploration in a future study.

Acknowledgments

This study was supported by Grant No. NSC102-2314-B-182A-073 from the National Science Council of the Republic of China.

References


