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Ischemic Preconditioning Ameliorates Hepatic Injury from Cold Ischemia/Reperfusion

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Abstract – We investigated whether ischemic preconditioning (IPC) protects liver against cold ischemic injury using isolated perfused rat liver. Rat livers were preconditioned by 5 minutes of ischemia and 5 minutes of reperfusion and preserved for 30 hours at 4°C in University of Wisconsin solution. Livers were then reperfused for 120 minutes. Oxygen uptake and bile flow in ischemic livers markedly decreased during reperfusion. These decreases were prevented by IPC. Portal pressure was elevated in cold ischemic and reperfused livers and this elevation was prevented by IPC. Lactate dehydrogenase and purine nucleoside phosphorylase activities markedly increased during reperfusion. These increases were prevented by IPC. The ratio of reduced glutathione to glutathione disulfide was lower in ischemic livers. This decrease was prevented by IPC. Our findings suggest that IPC protects the liver against the deleterious effect of cold ischemia/reperfusion, and this protection is associated with the reduced oxidative stress.

Keywords □ Ischemic preconditioning, cold ischemia/reperfusion, uw solution, hepatic function, oxidative stress

INTRODUCTION

Primary nonfunction and dysfunction occur in 5 to 30% of liver transplantation cases, resulting in either a requirement for retransplantation or the death of the recipient (Ploeg *et al.*, 1993). Because liver transplantation is the therapy of choice in an increasing number of liver diseases (Pichlmayr *et al.*, 1987) and the organ pool is limited, there is an urgent need to understand the underlying mechanisms responsible for graft failure.

Cold ischemia occurs during storage of the organ. Multiple mechanisms, including the formation of oxygen free radicals (Marzi *et al.*, 1989), the activation and migration of leukocytes (Takei *et al.*, 1991), the injury of endothelial cells (Caldwell-Kenkel *et al.*, 1989), and the disturbance of hepatic microcirculation (Thurman *et al.*, 1988) have been identified as accounting for cold ischemia/reperfusion (I/R) injury in the liver.

Ischemic preconditioning (IPC), defined as brief periods of I/R before sustained ischemia, has been reported to confer a state of protection in several organs, resulting in increased tolerance towards organ hypoxia. IPC has shown to attenuate the tissue injury observed after reperfusion of the heart, brain and skeletal muscle (Murry *et al.*, 1986; Glazier *et al.*, 1994; Pang *et al.*, 1995). Quite recently, this procedure has been extended to experimental models of hepatic vessel interruption and liver resections (Clavien *et al.*, 2003). Furthermore, few studies concerning the effectiveness of IPC associated with liver transplantation have been reported. IPC improved graft survival after cold ischemic storage and orthotopic rat liver transplantation (Yin *et al.*, 1998). Contrary evidence existed that IPC makes livers more vulnerable to storage-reperfusion injury (Adam *et al.*, 1998).

The aim of the present study was to examine whether IPC could decrease cold ischemic injury in rat liver.

MATERIALS AND METHODS

Chemicals

University of Wisconsin solution (UW, Viaspan®, Bristol-Myers Squibb, Co., NY, USA) was donated by the Jeil Pharmaceutical Company of Korea. Sodium taurocholate acid, xanthine oxidase, inosine, glutathione reductase, reduced β-nicotinamide adenine dinucleotide phosphate and 5,5'-dithiobis(2-nitrobenzoic acid) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals used in this

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study were of reagent grades and were locally and commercially available.

Animals

Male Sprague-Dawley rats weighing 260-300 g were obtained from Jeil animal breeding company of Korea and were acclimatized to laboratory conditions at Sungkyunkwan University for at least one week. Rats were kept in a temperature and humidity controlled room (25 ± 1°C and 55 ± 5%, respectively) with a 12 h light-dark cycle and allowed to drink tap water *ad libitum*.

Hepatectomy and perfusion

Rats were fasted for 18 h before the experiment. They were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) and systemically heparinized (400 U/kg) via the penile vein. A midline incision was made to the abdomen. After IPC, the portal vein and common bile duct were cannulated with PE-190 and PE-10, respectively. The liver was flushed with Krebs-Henseleit bicarbonate buffer (KHBB) containing (in mmol/l) NaCl, 118; KCl, 4.6; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; EDTA, 0.1; glucose, 5; sodium taurocholate, 0.03 (pH 7.4, 37°C). The flow rate was ~4 ml/min/g liver essentially and the perfusate was saturated with 95% O_2-5% CO_2 gas mixture. After flushing had begun, the inferior vena cava was ligated above the right renal vein and cut distally. During flushing, the liver was dissected free from the rat and moved to the perfusion apparatus. The liver was flushed with 10 ml of UW (4°C) and then stored in UW for 30 h at 4°C. Livers were then reperfused at a rate of ~4 ml/min/g liver for 120 min essentially as previously described (Lee and Clemens, 1992). Samples of perfusate were taken 5, 30, 60 and 120 min after reperfusion. The liver was weighed at the end of reperfusion.

Ischemic preconditioning

Prior to hepatectomy, IPC was performed by 5 min of ischemia followed by 5 min of reperfusion. The left branches of the portal vein and hepatic artery were clamped to induce complete ischemia of the median and left hepatic lobes. The right lobes remained perfused in order to prevent intestinal congestion. Control livers were prepared in a similar manner except that a clip was not placed on the median and left lobes.

Experimental protocol

Control group: The livers were removed from the animals as described above, connected to the perfusion apparatus and perfused with oxygenated KHBB for 120 min at 37°C. *Cold I/R group*: The livers were treated as for cold ischemia and reperfused for 120 min. *IPC+cold I/R group*: After IPC, the livers were treated as for cold I/R.

Oxygen uptake, bile flow and portal pressure

Oxygen uptake in the effluent perfusate was monitored continuously with a Teflon-shielded, Clark-type oxygen electrode (Yellow Springers Instrument Co., OH, USA). Oxygen uptake of the whole livers was calculated from influent minus effluent oxygen concentration differences at constant flow rates and was normalized for the wet weight of the liver (Lee and Clemens, 1992). Bile flow was calculated as 1 ml/1 g from the weight of collected bile. A plastic tube (diameter, 2 mm) was connected to a cannula inserted into the portal vein and portal pressure was measured by increase in the height of a water column during 2 min of reperfusion (Charrueau *et al.*, 2002).

Analytical procedures

Lactate dehydrogenase (LDH) activity, a marker of hepatocyte injury, was determined by spectrophotometric procedures using ChemiLab LDH assay kit (IVDLab Co., Korea). Purine nucleoside phosphorylase (PNP) activity, a marker of endothelial cell injury, was measured according to the method of Hoffee *et al.* (1978). Total glutathione and glutathione disulfide (GSSG) were determined by the method of Brehe and Burch (1976). Reduced glutathione (GSH) was estimated by deducting GSSG from the total glutathione concentration.

Statistics

All data are expressed as means ± SEM. Overall significance was tested by two-way ANOVA. Differences between groups at specific time points were considered significant at $p < 0.05$ with appropriate Bonferronic correction made for multiple comparisons.

RESULTS

Oxygen uptake and bile flow

Oxygen uptake averaged 100 μmol/h/g liver in isolated perfused liver. After cold ischemia, oxygen uptake started to decrease and markedly decreased after 60 min of reperfusion. IPC prevented the decrease in oxygen uptake (Fig. 1). As shown in Fig. 2, bile flow in the ischemic livers was signifi-

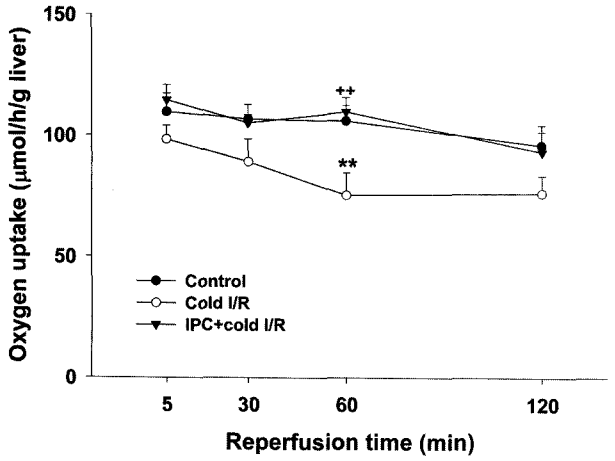

Fig. 1. Effect of IPC on oxygen uptake during hepatic cold I/R. Values are means ± SEM for 8-10 rats per group.
** = Significantly different ($p < 0.01$) from control group.
++ = Significantly different ($p < 0.01$) from cold I/R group.

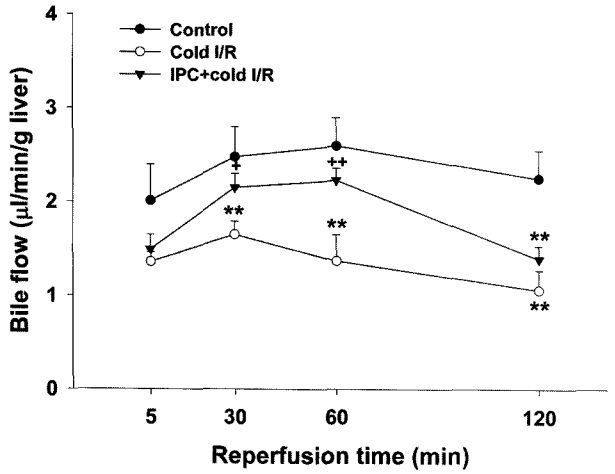

Fig. 2. Effect of IPC on bile flow during hepatic cold I/R. Values are means ± SEM for 8-10 rats per group.
** = Significantly different ($p < 0.01$) from control group.
+,++ = Significantly different ($p < 0.05$, $p < 0.01$) from cold I/R group.

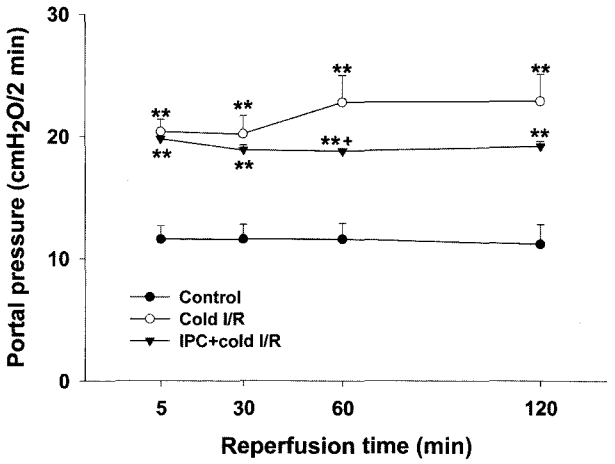

Fig. 3. Effect of IPC on portal pressure during hepatic cold I/R. Values are means ± SEM for 8-10 rats per group.
** = Significantly different ($p < 0.01$) from control group.
+ = Significantly different ($p < 0.05$) from cold I/R group.

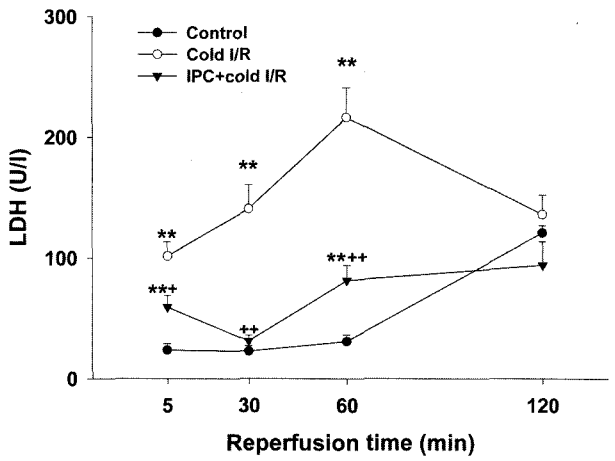

Fig. 4. Effect of IPC on the release of LDH during hepatic cold I/R. Values are means ± SEM for 8-10 rats per group.
** = Significantly different ($p < 0.01$) from control group.
+,++ = Significantly different ($p < 0.05$, $p < 0.01$) from cold I/R group.

cantly lower than that of the control livers during reperfusion. This decrease was prevented by IPC at 30 min and 60 min of reperfusion.

Portal pressure

Portal pressure in the control livers remained constant at approximately 12 $cmH_2O/2$ min throughout the period of reperfusion. After cold ischemia, however, portal pressure markedly increased to approximately 20 $cmH_2O/2$ min and there was an additional increase during reperfusion. The increase in portal pressure was prevented by IPC at 60 min of reperfusion (Fig. 3).

Lactate dehydrogenase and purine nucleoside phosphorylase

The release of LDH was negligible (23.6 ± 5.4 to 30.8 ± 5.4 U/l) until 60 min of reperfusion in the control. After cold ischemia, the release of LDH started to increase and peaked at 60 min of reperfusion. This increase was prevented by IPC (Fig. 4). The release of PNP in the ischemic livers significantly increased over the whole reperfusion period. The increase in the release of PNP was prevented by IPC (Fig. 5).

Glutathione efflux

As shown in Fig. 6, in the control livers, GSH efflux in the

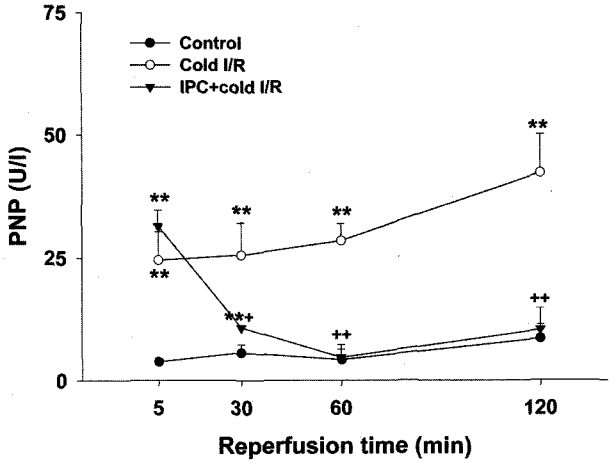

Fig. 5. Effect of IPC on the release of PNP during hepatic cold I/R. Values are means ± SEM for 8-10 rats per group. ** = Significantly different ($p < 0.01$) from control group. +,++ = Significantly different ($p < 0.05$, $p < 0.01$) from cold I/R group.

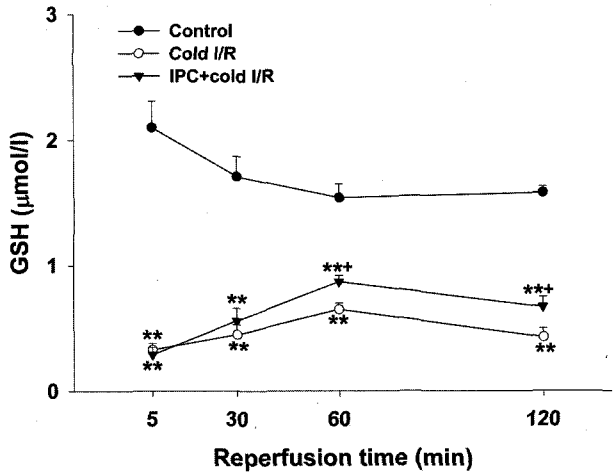

Fig. 6. Effect of IPC on GSH efflux during hepatic cold I/R. Values are means ± SEM for 8-10 rats per group. ** = Significantly different ($p < 0.01$) from control group. + = Significantly different ($p < 0.05$) from cold I/R group.

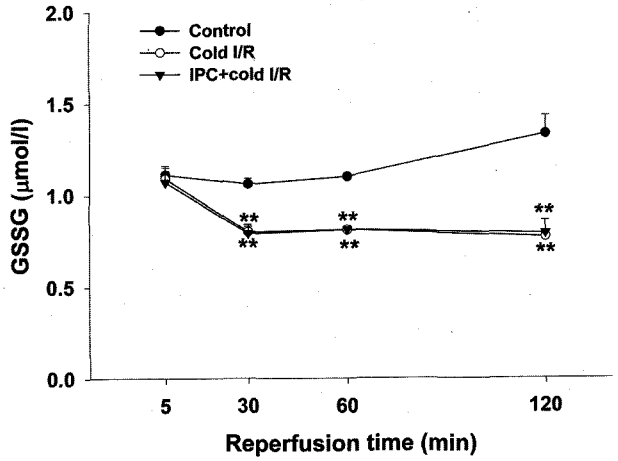

Fig. 7. Effect of IPC on GSSG efflux during hepatic cold I/R. Values are means ± SEM for 8-10 rats per group. ** = Significantly different ($p < 0.01$) from control group.

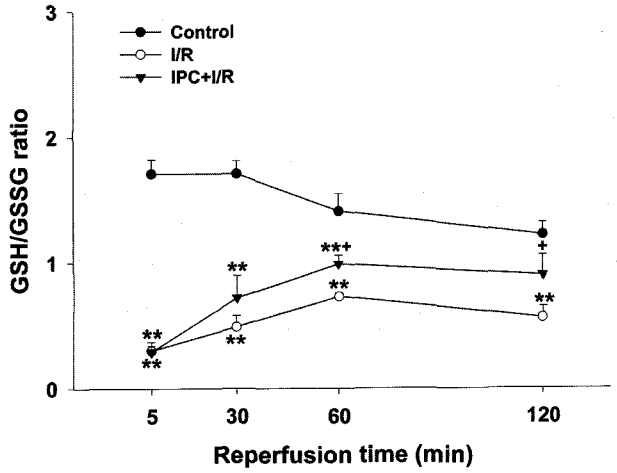

Fig. 8. Effect of IPC on GSH/GSSG ratio during hepatic cold I/R. Values are means ± SEM for 8-10 rats per group. ** = Signi-ficantly different ($p < 0.01$) from control group. + = Significantly different ($p < 0.05$) from I/R group.

perfusate remained constant at 2.10 ± 0.21 to 1.58 ± 0.05 μmol/l throughout the experiment. GSH efflux after cold I/R was significantly lower than that of control. The decrease in GSH efflux was prevented by IPC at both 60 min and 120 min of reperfusion. GSSG efflux in the ischemic livers significantly decreased during reperfusion. IPC did not affect the decrease in GSSG efflux (Fig. 7). Similar to GSH efflux, GSH/GSSG ratio in the ischemic livers markedly decreased, and this decrease was prevented at 60 min and 120 min of reperfusion by IPC (Fig. 8).

DISCUSSION

The present study demonstrates the beneficial effect of a transient episode of hepatic ischemia before sustained cold storage. In our cold I/R model, IPC improved oxygen uptake and bile flow, diminished endothelial cell and hepatocyte damages and reduced oxidative stress.

Although the protective effect of IPC on heart muscle has been known for many years, the role of IPC before hepatic cold storage is uncertain at this time. Studies evaluating IPC before hepatic cold storage have demonstrated inconsistent results. In the rat model, IPC was shown to negatively affect hepatic cold

preservation (Adam *et al.*, 1998). Rat livers treated with a short episode of warm ischemia before cold preservation demonstrated increased release of liver enzymes and enhanced vascular resistance. This study is contrasted by the study that reveals a beneficial role of IPC in the cold-preserved rat liver (Yin *et al.*, 1998). Given the conflicting data, we undertook a study to closely evaluate hepatic IPC. Our data indicate a beneficial or protective role of IPC before cold ischemia.

Hepatic metabolic activity is considered as one of the most important liver functions. In isolated perfused liver, oxygen uptake has been evaluated as the index of hepatic metabolic function (Lee *et al.*, 2000; Ricciardi *et al.*, 2001). In the present study, IPC prevented the decrease in the oxygen uptake after cold ischemia. Bile secretion has been observed to be suppressed during ischemia and was restored on reperfusion after a short period of ischemia. These changes in bile secretion have been ascribed to the depression of cellular ATP level. A correlation between the bile flow and the cellular level of ATP was reported by Slater and Delaney (1970) more than 30 years ago. Kamiike *et al.* (1985) have shown that extent of hepatic injury can be assessed simply by monitoring the bile flow, which is believed to reflect the cellular level of ATP. Our data showed that IPC inhibited the decrease of the bile flow in cold ischemic livers. Thus, the present results of oxygen uptake and bile flow show that IPC improves hepatic function after cold ischemia.

High portal pressure is an important parameter, as it is associated with pathological situations (Shibayama, 1988), and it is of clinical relevance as an indicator of the severity of end-stage hepatic diseases before transplantation (Paulsen and Klintmalm 1992), as well as in patients suffering from acute liver rejection (Hadengue *et al.*, 1993). Portal pressure is an indicator of hepatic microcirculation, and as high portal pressure is usually associated with vasoconstriction, one would expect a reduced vascular space after cold storage. In the present study, IPC improved the increase in the portal pressure after cold storage.

The greater metabolic activity in damaged hepatocytes or the shedding of cytosolic components in blebs, formed during I/R (Lemasters and Thurman, 1991), may account for the increased perfusate level of LDH. Our results showed that the release of LDH in perfusate increased during reperfusion. The hepatoprotective effects against cold I/R injury were clearly shown in the rats treated with IPC. Although the use of PNP as an indicator of endothelial injury is controversial (Brass and Mody, 1995), the enzyme is believed to originate primarily from damaged endothelial cells (Rao *et al.*, 1990). The data on PNP release presented here suggest that the beneficial effect of IPC may be related to maintaining the integrity of endothelial cells damaged during cold I/R.

Accumulating evidences indicate that oxygen-derived free radicals play a major role in producing the microvascular and parenchymal cell damage associated with reperfusion of the ischemic tissue (Drugas *et al.*, 1991). Glutathione (GSH) plays an important role as a free radical scavenger. Previous studies have shown that GSH is depleted from cells exposed to warm ischemia, and GSH-depleted cells are more sensitive to a loss of viability upon reoxygenation (Vreugdenhil *et al.*, 1990). In cold ischemic livers, the concentration of hepatic GSH significantly decreased after reperfusion. Because of the decreased GSH efflux, we expected that cold ischemic liver might increase the GSSG efflux. In the present study, however, GSSG efflux decreased in the cold ischemic liver. There is evidence that the secretion of GSSG out of various cell types is a carrier-mediated transport that requires ATP (Nicotera *et al.*, 1985). As hepatic ATP levels are significantly reduced during ischemia and are not fully recovered upon reperfusion (Marubayashi *et al.*, 1986), the GSSG secretion of the liver might be impaired due to the lack of ATP. These decreases in GSH and GSSG efflux were prevented by IPC treatment.

Our findings suggest that IPC protects the liver from sustained cold ischemia, and the protective effect is possibly mediated by reduced oxidative stress.

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