Effect of ischemic preconditioning on P-selectin expression in hepatocytes of rats with cirrhotic ischemia-reperfusion injury

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INTRODUCTION

Ischemic preconditioning (IPC) refers to a phenomenon in which a tissue is rendered resistant to the deleterious effects of prolonged ischemia and reperfusion (I/R) by prior exposure to a short period of vascular occlusion. This phenomenon was first demonstrated in the heart a decade ago[1] and has been the subject of intensive investigation ever since. Although it is clear that activation of adenosine receptors and protein kinase C (PKC) is critical to the development of the beneficial action of IPC, the downstream effectors in the signaling cascade initiated by IPC are uncertain. Akimisi et al[2] and Kubes et al[3] have demonstrated that IPC prevents intestinal and skeletal muscle I/R injury by inhibiting posts ischemic leukocyte-endothelial cell interaction. However, identification of the end effectors of the ant adhesive effects of IPC remains unclear. A likely candidate effector molecule that may be targeted by signaling cascade initiated by IPC is P-selectin, because post-ischemic leukocyte rolling (and thus subsequent stationary adhesion and emigration) is critically dependent on the expression of P-selectin on venular endothelium[4]. IPC has been commonly studied in the heart, but few studies have been performed on cirrhotic liver IPC. This study was aimed to determine the effects and mechanisms of IPC on the I/R injury rats with liver cirrhosis and the effect of IPC on the expression of P-selectin.

MATERIALS AND METHODS

Reproduction of rat cirrhotic liver model

Sprague-Dawley (SD) Male rats initially weighing 200±20 g were used.

Subcutaneous injection of 60 % Ccl_{4}(0.3 mg/kg) was made once every 4 days for 8 weeks and 5% ethanol was allowed for 60 days[5].

Operative procedure

At first, ligamentous attachments around the liver were dissected. The common bile duct was then cannulated and bile output was measured. Ischemia was induced in the median and left lateral hepatic lobes by clamping the corresponding hepatic arterial and portal vein, while the blood flowing to the other lobe was left intact. When the assigned period of warm ischemia was completed, the clamp was removed and the pedicles to the non-ischemic lobe were ligated[6].

Grouping of animals

Forty male SD rats with liver cirrhosis were divided into 5...
groups randomly, eight rats in each group. Animals in sham Operation group (SO group) were subjected to anesthesia and laparotomy. Animals in ischemia/reperfusion group (I/R group) were subjected to 30 min of left and middle lobe hepatic ischemia, followed by 120 min of reperfusion. Animals in ischemic preconditioning group (IPC group) were same as I/R group, but subjected to 10 min of ischemia and 5 min of reperfusion prior to I/R. Animals in L-arginine preconditioning group (APC group) were same as IPC group, but treated with a continuous intravenous infusion of L-Arginine (10 mg/kg, portal vein) for 5 min before preconditioning. Animals in L-NAME preconditioning group (NPC group) were same as IPC group, but treated with a continuous intravenous infusion of L-NAME (10 mg/kg, portal vein) for 5 min before preconditioning.

The animals were killed after blood samples were collected from the inferior vena cava after 120 min of reperfusion. Liver samples were excised from the anterior edge of the median lobe before ischemia, after the induction of ischemia and 120 min after reperfusion respectively. The specimens were immersed in liquid nitrogen immediately after sampling to prevent metabolism. The liver samples were homogenized by a high-speed homogenizer using 4% perchloric acid at 4°C. After centrifugation, the supernatant was stored at 4°C for analysis.

**Energy metabolism**

ATP and its metabolites, ADP and AMP in the liver tissue were measured by high-performance liquid chromatography (HPLC). Energy charge (EC) was equal to (ATP+1/2ADP)/(ATP+ADP+AMP) [7].

**Measurement of serum cytosolic enzymes**

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured at 4°C using commercially available kits (Horizon, American) by an auto-biochemistry analyzer.

**Measurement of bile output**

Bile output from the ischemic liver was measured through a choleodochotomy tube placed in the common bile duct.

**P-selectin expression in liver tissues**

Immunohistochemical staining for P-selectin protein was performed using SP technique [8]. The immunostaining of P-selectin was visually classified into four groups: no staining present in any tumor cells (+), slight staining in most of the hepatocytes (++), most of the hepatocytes with moderate staining (+++), and strong staining in most of the hepatocytes (+++). Two senior pathologists who did not know the clinicopathological data did the classification.

**Histological examination**

Liver samples were excised from the anterior edge of the median lobe 120 min after reperfusion. Small portions (0.5 cm×0.5 cm) were fixed immediately in 4% buffered para formaldehyde (pH 7.2) and embedded in paraffin. These portions were cut into 4 μm thick sections and stained with hematoxylin and eosin (H & E). Leukocyte count in ischemic hepatic lobe could be calculated randomly under microscopy (×400).

**Statistical analysis**

The results were expressed as ±s. The one-way NOVA and H test were used for statistical significance of differences between groups. Correlation analysis between two factors was made by Spearman method. P<0.05 was considered significant.

**RESULTS**

**Change of ATP, ADP, AMP and EC levels in liver after ischemia and reperfusion**

At 30 min of hepatic inflow occlusion, the ATP and EC levels in liver tissues were significantly decreased in I/R, IPC, APC and NPC groups (P<0.05). At 120 min after reperfusion, the ATP and EC levels in IPC and APC groups were significantly higher than those in I/R group (P=0.000, P=0.001). There was no significant difference between NPC and I/R groups (P>0.05) (Table 1).

**Change of ALT, AST and LDH in serum**

Significant increases of ALT, AST and LDH levels in serum were observed in the group subjected to ischemia and reperfusion (I/R group) in comparison with the control group (SO group). When ischemia was preceded by 10 min of ischemia and 5 min of reperfusion (IPC), the increases of AST, ALT and LDH in serum were prevented (P=0.000). Administration of L-Arginine (APC group) resulted in the same effects on ALT, AST and LDH as above (P=0.001). However, infusion of L-NAME (NPC group) inhibited the beneficial effects of preconditioning (Table 2).

**Results of bile output and leukocyte count in ischemic hepatic lobe**

The livers produced more bile in IPC group than in I/R group during 120 min after reperfusion (0.101±0.027 versus 0.066±0.027 ml/g liver, P=0.002). There was a significant difference between APC and I/R, NPC and SO groups (P=0.001, P=0.000) respectively. However, there was no significant

### Table 1 ATP, ADP, AMP and EC levels in liver after ischemia and reperfusion (U/L)

| Groups | n | After Ischemia | After Reperfusion |
|--------|---|---------------|------------------|
|        |   | ATP | ADP | AMP | EC  | ATP | ADP | AMP | EC  |
| SO     | 8 | 5.4±1.3 | 3.1±0.8 | 1.0±0.2 | 0.7±0.0 | 5.5±0.8 | 3.2±1.0 | 1.0±0.1 | 0.7±0.0 |
| I/R    | 8 | 0.5±0.2  | 2.3±0.6 | 3.5±1.0 | 0.3±0.0 | 1.5±0.6 | 2.3±1.2 | 2.6±1.3 | 0.4±0.1 |
| IPC    | 8 | 0.5±0.1  | 2.1±0.5 | 3.6±1.5 | 0.3±0.1 | 4.1±1.6 | 2.3±0.8 | 1.9±0.9 | 0.6±0.1 |
| APC    | 8 | 0.5±0.1  | 2.2±0.5 | 3.4±0.7 | 0.3±0.0 | 4.0±1.6 | 2.5±1.1 | 2.2±1.2 | 0.6±0.1 |
| NPC    | 8 | 0.5±0.2  | 2.0±0.7 | 3.3±0.6 | 0.3±0.0 | 2.3±1.6 | 2.2±0.9 | 3.2±1.1 | 0.4±0.1 |

*P* <0.05, *P* <0.01, vs SO group; *P* <0.01, vs I/R group.
The degree of P-selectin expression was positively correlated with the counts of leukocytes in liver tissues. The degree of P-selectin expression positively correlated with the counts of leukocyte infiltration in liver. This is accomplished through a brief preceding episode of vascular occlusion which renders these tissues resistant to the deleterious effects of prolonged ischemia and reperfusion. The protective effects of IPC have been well documented in the previous studies involving different tissues and organs. These included cardiac muscle[1,9], skeletal muscle[10], small intestines[10] and more recently the liver[11]. Although the mechanism of IPC is still unclear up to now, several potential mediators (nitrogen monoxide, adenosine, oxide radical, bradykinin and so on) have been found to play different roles in different organs[11-14]. Adenosine and protein kinase C (PKC) were critical to the beneficial actions of IPC in the heart[13]. IPC-induced adenosine A1-receptor stimulation during the period of preconditioning ischemia increased phospholipase C (PLC) activity, an event that is coupled by pertussis toxin-sensitive G proteins[12,13]. Activation of PLC induced the formation of diacylglycerol, which in turn promotes the translocation and activation of PKC. Activation of PKC stimulated the activation of ATP-sensitive potassium (KATp) channels, and the beneficial actions of IPC in the heart were induced[13], while adenosine stimulated NO production in IPC to protect against the injury associated with I/R in liver[16]. In the case of the cirrhotic liver, our work revealed that the ATP and EC levels in IPC group were higher than those in I/R group. There was significantly more bile produced by the livers in IPC group too. However, the increase of AST, ALT and LDH release was attenuated, when IPC was performed before ischemia. This fact shows the protective effect of IPC on preventing ischemia-reperfusion damage of cirrhotic liver. In addition, we found that L-arginine administration in hepatic ischemia reperfusion attenuated the injury in a manner similar to that of IPC. Accordingly, inhibition of NO synthesis abolished the beneficial effects of IPC. Thus, our data suggest that NO is one of the potential mediators of the protective effects of IPC.

Results of bile output and leukocyte count in ischemic hepatic lobe

| Groups | Cases | Bile output (ml/g liver) | Leukocyte count (piece/HP) |
|--------|-------|-------------------------|---------------------------|
| SO     | 8     | 0.15±0.02              | 181.38±69.23             |
| I/R    | 8     | 0.07±0.03               | 442.3±64.10              |
| IPC    | 8     | 0.10±0.03               | 353.0±64.11              |
| APC    | 8     | 0.10±0.02               | 347.7±61.53              |
| NPC    | 8     | 0.07±0.04               | 407.8±60.40              |

**ab** <0.01, vs SO group; **a** <0.05, vs I/R group.

Correlation between leukocytes infiltration and P-selectin expression in liver tissues

Leukocytes infiltration was significantly correlated with P-selectin expression in liver tissues. The degree of P-selectin expression was positively correlated with the counts of leukocyte infiltration in liver (r=0.602, P=0.000).

**DISCUSSION**

IPC is a unique phenomenon which attenuates organ injury caused by I/R. This is accomplished through a brief preceding
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