Ischaemic preconditioning modulates the activity of Kupffer cells during in vivo reperfusion injury of rat liver

Barbara Cavalieri, Maria-Giulia Perrelli, Manuela Aragno, Pierluigi Ramadori, Giuseppe Poli and Juan C. Cutrìn

Laboratory of Experimental Liver Pathology, Department of Clinical and Biological Sciences, University of Torino, Italy
Department of Experimental Medicine and Oncology, University of Torino, Italy

This work was performed to elucidate further the main cellular events underlying the protective effect of ischaemic preconditioning in an in vivo rat liver model of 90 min ischaemia followed by 30 min reperfusion. A significant attenuation of the various aspects of post-ischaemic injury, namely necrosis and the levels of hydrogen peroxide and 5- and 15-hydroperoxyeicosatetraenoic acids, was afforded by the prior application of a short cycle of ischaemia/reperfusion (10 + 10 min) or when rats were previously treated with gadolinium chloride. However, when preconditioning was applied on Kupffer cell-depleted livers, no additional level of ischaemic tolerance was obtained. In terms of cellular pathology, this result could be suggestive of Kupffer cells as the target of the preconditioning phenomenon during the warm ischaemia/reperfusion injury. Accordingly, modulation of Kupffer cell activity was associated with a well-preserved hepatocyte integrity, together with low levels of pro-oxidant generation during reperfusion. As activated Kupffer cells can generate and release potentially toxic substances, their modulation by ischaemic preconditioning could help to provide new surgical and/or pharmacological strategies to protect the liver against reperfusion damage. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS — liver ischaemic preconditioning; Kupffer cell; oxidative stress

ABBREVIATIONS — HPETE, 5- and 15-hydroperoxyeicosatetraenoic acids; IP, ischaemic preconditioning; ROS, reactive oxygen species

INTRODUCTION

Substantial evidence indicates that Kupffer cells play a central role in the pathogenesis of liver parenchymal cell damage during the early post-reperfusion phase, mainly through releasing ROS and other toxic mediators (i.e. cytokines) able to modulate hepatocyte metabolism and function. In light of this and in order to improve liver function after reperfusion, various attempts have been made to block Kupffer cell activity, either through administration of specific toxins (e.g. GdCl₃, dichloromethylene diphosphonate), methyl-

Received 22 September 2002
Revised 29 October 2002
Accepted 31 October 2002

Copyright © 2003 John Wiley & Sons, Ltd.
of a heterogeneous cell population, it is likely that during preconditioning complex cross-talk could take place between parenchymal and non-parenchymal cells. Accordingly, we investigated the effects of IP on Kupffer cell activity in a rat liver model of ischaemia/reperfusion injury.

MATERIALS AND METHODS

Animal model

Male Sprague-Dawley rats (224 ± 5 g body weight) were used; they had free access to standard chow diet and water. After anesthesia with 10% ketamine chloride solution, they were positioned under a heat lamp to maintain constant body temperature. After a midline laparotomy, selective inflow occlusion of the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes was performed. Reperfusion was initiated by clamp removal. Samples from the post-ischaemic lobes were taken at the end of the reperfusion phase, and biochemical and morphological studies were carried out. All animals received humane care according to the criteria outlined in Guide for the Care and Use of Laboratory Animals.18

Depletion of Kupffer cells

To inhibit Kupffer cell activity, a randomized set of rats were treated with gadolinium chloride (GdCl₃; 10 mg kg⁻¹ in sterile 0.9% NaCl solution, i.v.) 18 h before the ischaemia and reperfusion cycle.³

Experimental design

The rats were subdivided into six groups, each group being of five animals.

Group 1. Sham saline: animals treated with sterile 0.9% NaCl solution were subjected to anaesthesia and laparotomy alone.

Group 2. Sham GdCl₃: as in group 1, but rats were treated with GdCl₃ instead of sterile 0.9% NaCl solution.

Group 3. Standard Ischaemia/Reperfusion (Isch/Rep): rats were subjected to 90 min of ischaemia followed by 30 min of reperfusion.

Group 4. IP + Isch/Rep: before ischaemia/reperfusion, rats were subjected to 10 min ischaemia followed by 10 min reperfusion.¹⁷

Group 5. GdCl₃ + Isch/Rep: before ischaemia/reperfusion, rats were treated with GdCl₃.

Group 6. GdCl₃ + IP + Isch/Rep: before IP procedure, as in group 4, rats were treated with GdCl₃.

Biochemical assessment of liver cytolyis

Irreversible hepatic damage was evaluated by determination of alanine aminotransferase and aspartate aminotransferase in blood-plasma obtained from the posterior caval vein after 30 min of reperfusion, using a commercial kit from Sigma Chemical Co. (St. Louis MO, USA).

H₂O₂ analysis

Specimens from sham-operated and reperfused livers were homogenized (10%, w/v) in a buffer containing 0.033 mol l⁻¹ Na₂HPO₄ and 0.9% KCl, pH 7.4 at 4°C. The homogenates were centrifuged at 40,000 g for 45 min and the supernatant was used for H₂O₂ analysis. The H₂O₂-mediated oxidation of Fe²⁺ to Fe³⁺ ion, under acidic conditions and in the presence of xylenol orange dye, was quantified as described elsewhere.¹⁹ For the working reagent, 1 ml solution A containing ammonium ferrous sulphate (25 mm) and sulphuric acid (2.5 mm) was mixed with 100 ml of solution B containing sorbitol (100 mm) and xylenol orange (125 µmol l⁻¹ in water. A 0.1-ml volume of supernatant was added to 1 ml of working reagent, mixed and incubated at room temperature for 15 min. The absorbance was read at 560 nm. The values were read against a standard curve prepared with 1 to 50 µmol l⁻¹ concentrations of H₂O₂. The absolute values of H₂O₂ were expressed in µmol l⁻¹.

Determination of 5- and 15-HPETE

The concentration of 5- and 15-HPETE in the lipid fraction from cytosol samples extracted by the method of Folch et al.²⁰ was determined by the method of Tordijman et al.,²¹ slightly modified. Briefly, determination was by high performance liquid chromatography analyses with a Symmetry C₁₈ column. The mobile phase used was a mixture of 0.05 mol NaH₂PO₄, pH 5/methanol/acetonitrile (30:35:35, v:v). The HPETE levels in the samples were calculated by comparison with standard solutions of 5- and 15-HPETE (Sigma, St Louis, MO, USA). The results were expressed as µM.

Number of neutrophils

Fragments from the left lateral lobe of about 1.0 × 0.5 × 0.3 cm were placed overnight in a solution of 4% formaldehyde in 50 mm phosphate buffer solution,
pH 7.4. Polymorphonuclear leukocytes (PMN) were identified on 5-μm paraffin sections by means of the naphthol AS-D chloroacetate technique for esterase, following the method described by Moloney et al.22 with slight modifications; the substrate was dissolved in dimethyl sulphoxide/Triton X-100 (9:1, v/v) and then 0.1% Fast Garnet dGBC in 0.1 M phosphate buffer solution, pH 8.5 was added. Red stained PMN were counted in 20 non-consecutive, randomly chosen ×500 histological fields. Results were expressed as the number of neutrophils per 40 × histological power field (hpf).

Protein determination
The protein content of the liver homogenates was determined by the spectrophotometric method described by Peterson23 using bovine serum albumin as standard.

Statistical analysis
Data are presented as means ± SE; the statistical significance of differences was analysed using the one-way analysis of variance (ANOVA) corrected by the Tukey–Kramer Multiple Comparisons test and with the Student t test where p values ≤ 0.05 were considered to be statistically significant.

RESULTS
In our experimental model, long-term ischaemia followed by reperfusion induced hepatic necrosis in both non-preconditioned and preconditioned animals. However, a marked reduction of irreversible cell injury was detectable when IP had previously been applied (Figure 1).

Among non-parenchymal liver cells, Kupffer cells are thought to constitute the major source of toxic mediators, namely ROS, released immediately after
reperfusion.\textsuperscript{1,3,6} Cytolysis was also significantly attenuated in animals previously treated with GdCl\textsubscript{3} (Figure 1). However, the degree of ischaemic tolerance was not improved if preconditioning was previously applied to Kupffer cell-depleted rats (Figure 1).

In this study, we examined the changes in concentrations of H\textsubscript{2}O\textsubscript{2} and 5- and 15-HPETE during the course of the ischaemia/reperfusion injury, as by-products of the activity of the Kupffer cell’s NADPH oxidase and 5-lipoxygenase.\textsuperscript{4,25,26} To exclude neutrophils as possible sources of 5-lipoxygenase-derived hydroperoxides,\textsuperscript{27} we evaluated the number of naphthol-AS-D-chloroacetate-positive infiltrating leukocytes.

After ischaemia and reperfusion, a 2.6-fold increase in the concentrations of H\textsubscript{2}O\textsubscript{2} was seen in the group of rats treated with 0.9\% NaCl. This level of oxidative stress was reduced by 31\% however, when the animals were previously submitted to GdCl\textsubscript{3} treatment (Table 1). Similarly, IP was able to protect against post-reperfusion generation of H\textsubscript{2}O\textsubscript{2} in the 0.9\% NaCl-treated group (47\% decrease). However, when the IP procedure was applied to the Kupffer cell-depleted livers, no additional attenuation over the level of H\textsubscript{2}O\textsubscript{2} production was observed (Table 1).

Similarly, GdCl\textsubscript{3} treatment and IP were able to attenuate the increased production of 5-lipoxygenase-derived lipid hydroperoxides following ischaemia/reperfusion (Figure 2). However, in the liver

### Table 1. Hepatic concentration of hydrogen peroxide after reperfusion (\textmu{}moles)

<table>
<thead>
<tr>
<th>Groups</th>
<th>0.9% NaCl treated</th>
<th>GdCl\textsubscript{3} treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.20 ± 0.08</td>
<td>1.40 ± 0.06</td>
</tr>
<tr>
<td>Isch/Rep</td>
<td>3.20 ± 0.09\ast</td>
<td>2.20 ± 0.17\ast\dagger</td>
</tr>
<tr>
<td>Prec+Isch/Rep</td>
<td>1.70 ± 0.17\ast\dagger</td>
<td>2.00 ± 0.17\ast\dagger</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE.
p < 0.05; \ast versus sham; \dagger versus Isch/Rep; \dagger versus Isch/Rep from 0.9\% NaCl-treated rats.

Figure 2. Ischaemic preconditioning and gadolinium chloride treatment attenuate the post-ischaemic generation of 5- and 15-hydroperoxyeicosatetraenoic acids. Data are presented as means ± SE. p < 0.05; a versus sham; b versus Isch/Rep; \dagger versus Isch/Rep from 0.9\% NaCl-treated rats
Depleted of Kupffer cells, IP did not accentuate the inhibitory effect over 5- and 15-HPETE production, so differences brought about by preconditioning of the liver cannot be discerned. Moreover, the fact that there was no change in the number of neutrophils seen in the reperfused livers allowed these cells to be ruled out as possible sources of 5- and 15-HPETE (Table 2).

**DISCUSSION**

A prominent feature of ischaemia and reperfusion leading to liver damage is the activation of Kupffer cells. Activated Kupffer cells are involved in the development of liver cell injury through production of ROS and inflammatory mediators, which directly contribute to the sinusoidal dysfunction and hepatocyte damage and death during the early phase of reperfusion, and indirectly by promoting neutrophil recruitment and initiation of inflammatory responses. Modulation of Kupffer cell activity attenuated the early post-reperfusion damage. Accordingly, depletion of Kupffer cells with GdCl₃ was associated with a well-preserved hepatocyte integrity, together with low levels of pro-oxidant generation during reperfusion.

Products of lipid peroxidation, other than 4-hydroxy-2, 3-trans-nonenal and malondialdehyde, namely 5-lipoxygenase-derived lipid hydroperoxides, are also known to be toxic to cells. As the enzyme 5-lipoxygenase is present only in the non-parenchymal cells of the liver, namely Kupffer cells, measurement of its biological by-products at the end of the early phase of reperfusion studied here, could provide a functional index of Kupffer cell activity.

Are Kupffer cells targets of IP? A hallmark of this study is that application of IP to the livers allows us to reproduce practically identical findings to those obtained through GdCl₃ treatment, i.e. cytolysis and tissue oxidative stress were attenuated. However, when Kupffer cell-depleted livers were preconditioned, no additional level of tolerance against post-ischaemic damage was obtained. Moreover, the levels of H₂O₂ and 5- and 15-HPETE were almost identical to those obtained by either GdCl₃ treatment or IP alone. In our opinion, this fact constitutes evidence that a main mechanism of action of preconditioning against *in vivo* reperfusion injury could be the modulation of Kupffer cells activity. Accordingly, recent experiments from an isolated perfused rat liver model have shown that IP protects sinusoidal endothelial cells and suppresses Kupffer cell activation after cold storage and reperfusion.

Could there be any mechanism(s) of cell damage not susceptible to modulation by IP? This question emerges because of the residual cytolysis and tissue oxidative stress found at the end of the reperfusion phase in both preconditioned and GdCl₃-treated livers. A probable explanation for these results could be that neither IP nor GdCl₃ treatment are sufficient to attenuate the irreversible cell damage and the pro-oxidant levels generated during the sustained ischaemic phase.

The present study does not address the mechanisms by which IP offers tolerance against sustained ischaemia/reperfusion injury, but in terms of cellular pathology, it indicates Kupffer cells as a target of IP during this *in vivo* model of warm ischaemia/reperfusion injury to the liver. We would like to think that some mediator(s) formed during the brief cycle of ischaemia/reperfusion (10 min + 10 min) are able to modulate Kupffer cell activity. In agreement with this, adenosine has been implicated as an important mediator of cardiac and liver IP, and it has been reported that adenosine is able to prevent activation of Kupffer cells and to suppress TNF-α release from them.

During ischaemia, ATP is rapidly degraded to adenosine. Adenosine modulates several tissue functions through receptor-mediated mechanisms. Adenosine receptors differ in their biological and pharmacological responses to adenosine agonists and antagonists. Adenosine A₁ receptors stimulate inhibitory G-proteins that block adenylate cyclase and decrease cAMP. By contrast, activation of A₂ receptors stimulates adenylate cyclase and increases cAMP. Accordingly, in recent experiments carried out with a cold storage model of liver injury, it was reported that adenosine mediates IP to sinusoidal endothelial cells through A₂ receptors and that adenosine A₂ receptor agonists, as well as cAMP, protect preconditioned sinusoidal endothelial cells against cold storage/reperfusion injury to the same extent as brief cycles of ischaemia–reperfusion (preconditioning phenomenon).

Because adenosine suppresses endotoxin-stimulated tumour necrosis factor-α formation by Kupffer cells via an adenosine A₂ receptor pathway, it seems...
reasonable to believe that adenosine released by preconditioning interferes in some step(s) of the mechanism involved in the activation of the Kupffer cells’ NADPH oxidase and 5-lipoxygenase enzymes, i.e. blocking the post-reperfusion release of toxic mediators: H₂O₂ and 5- and 15-HPETE. Accordingly, it has been documented that intracellular cAMP-elevating agents have been shown to be associated with the downregulation of the oxidative burst and the inhibition of the 5-lipoxygenase translocation and activation in both neutrophils and cells of the macrophage/macrophage lineage.

ACKNOWLEDGEMENTS

This work was supported by grants from Italian Ministry of the University (PRIN 1999, 2000, 2001), Centro Nazionale delle Ricerche (Progetto Finalizzato Biostrategia), Turin University.

REFERENCES


29. Wanner GA, Müller PEM, Ertel W, Bauer M, Menger MD, Messmer K. Differential effect of anti-TNF-α antibody on...
proinflammatory cytokine release by Kupffer cells following liver ischemia and reperfusion. 


32. Arai M, Thurman RG, Lemasters J. Involvement of Kupffer cells and sinusoidal endothelial cells in ischemic preconditioning to rat livers stored for transplantation. 


34. Cohen MV, Baines CP, Downey JM. Ischemic preconditioning: from adenosine receptor to K<sub>ATP</sub> channel. 

35. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. 

36. Arai M, Thurman RG, Lemasters JJ. Contribution of Adenosine A<sub>2</sub> receptors and cyclic adenosine monophosphate to protective ischemic preconditioning of sinusoidal endothelial cells against storage/reperfusion injury in rats livers. 


38. Ottonello L, Morone MP, Dallegri F. cAMP-elevating agents down regulate the oxidative burst induced by granulocytemacrophage colony stimulating factor (GM-CSF) in adherent neutrophils. 

39. Berger CEM, Horrocks BR, Datta HK. CAMP-dependent inhibition is dominant in regulating superoxide production in the bone-resorbing osteoclasts. 

40. Bigby TD. The Yin and the Yan of 5-lipoxygenase pathway activation. 