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

This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/43272 since

Terms of use:
Open Access
Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.
Ischaemic preconditioning modulates the activity of Kupffer cells during \textit{in vivo} reperfusion injury of rat liver

Barbara Cavalieri\textsuperscript{1,1}, Maria-Giulia Perrelli\textsuperscript{1,1}, Manuela Aragno\textsuperscript{2}, Pierluigi Ramadori\textsuperscript{1}, Giuseppe Poli\textsuperscript{1} and Juan C. Cutrìn\textsuperscript{1,*}

\textsuperscript{1}Laboratory of Experimental Liver Pathology, Department of Clinical and Biological Sciences, University of Torino, Italy

\textsuperscript{2}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 \textit{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.\textsuperscript{1,3–5} 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. GdCl\textsubscript{3}, dichloromethylene diphosphonate), methyl-xanthine phosphodiesterase inhibitors (i.e. pentoxifylline) or by the use of calcium blocking agents (e.g. nisoldipine). However, the side-effects of these treatments cannot be entirely excluded.\textsuperscript{1,3–9}

The term IP was coined to describe a protective mechanism against ischaemia–reperfusion injury and consists of previous short periods of ischaemia followed by reperfusion.\textsuperscript{10} It was originally described in the heart, where it decreases infarct size after subsequent prolonged ischaemia and reperfusion.\textsuperscript{11}

In the liver, the exact mechanisms involved in the status of cytoprotection conferred by IP against ischaemia/reperfusion injury are not fully understood. However, experimental data suggest that modifications of the endothelin-1/nitric oxide ratio in favour of the latter, changes in energy metabolism, attenuation of \textit{in situ} TNF-\textalpha production and release, as well as a blocking effect on the ROS-generating systems, are actively implicated.\textsuperscript{12–17} Moreover, since the liver is composed...
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 ischemia/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 anestheasia 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 cytolysis

Irreversible hepatic damage was evaluated by determination of alanine aminotransferase and aspartate aminotransferase in blood-plasma obtained from the posterior cava 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 M 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. 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 Peterson 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

![Image of plasma transaminases levels](image_url)

Figure 1. Ischaemic preconditioning and gadolinium chloride treatment attenuate the post-ischaemic irreversible damage of the liver. Data are presented as means ± SE. p < 0.05; a versus sham; b versus Isch/Rep; ☆ versus Isch/Rep from 0.9% NaCl-treated rats.

Cytolysis was also significantly attenuated in animals previously treated with GdCl₃ (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 H₂O₂ 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. To exclude neutrophils as possible sources of 5-lipoxygenase-derived hydroxyperoxides, 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 H₂O₂ 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 GdCl₃ treatment (Table 1). Similarly, IP was able to protect against post-reperfusion generation of H₂O₂ 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 H₂O₂ production was observed (Table 1).

Similarly, GdCl₃ 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 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 H₂O₂ production was observed (Table 1).

Similarly, GdCl₃ 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 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 H₂O₂ production was observed (Table 1).

### Table 1. Hepatic concentration of hydrogen peroxide after reperfusion (µmoles)

<table>
<thead>
<tr>
<th>Groups</th>
<th>0.9% NaCl treated</th>
<th>GdCl₃ treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.20 ± 0.08</td>
<td>1.40 ± 0.06</td>
</tr>
</tbody>
</table>
| Isch/Rep                      | 3.20 ± 0.09*      | 2.20 ± 0.17*+*|<ref>
| Prec+Isch/Rep                 | 1.70 ± 0.17*+*    | 2.00 ± 0.17*+*|

Data are presented as means ± SE. 
*p < 0.05; *versus sham; †versus Isch/Rep; ‡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; ★ versus Isch/Rep from 0.9% NaCl-treated rats
Data are presented as means ± SE. Differences were considered significant at *p < 0.05; **versus sham.

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.15

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.35 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).36

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 pre-conditioning 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: H$_2$O$_2$ 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/monocyte lineage.$^{38–41}$

ACKNOWLEDGEMENTS

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

REFERENCES

29. Wanner GA, Müller PEM, Ertel W, Bauer M, Menger MD, Messmer K. Differential effect of anti-TNF-α antibody on...


