Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Hypoxia-reoxygenation-induced chemokine transcription is not prevented by preconditioning or intermittent hypoxia, in mice hepatocytes

Marina Laurens,[1] Virginie Defamie,[1] Gitana Scozzari,[1] Annie Schmid-Alliana,[2] Jean Gugenheim[1] and Dominique Crenesse[3]

1 Laboratoire de Recherches Chirurgicales, Faculté de Médecine, Université de Nice-Sophia Antipolis, Nice, France
2 INSERM U638 Faculté de Médecine, Université de Nice-Sophia Antipolis, Nice, France
3 Laboratoire de Physiologie, Faculté de Médecine, Université de Nice-Sophia Antipolis, Nice, France

Keywords
chemokines, hypoxia-reoxygenation, intermittent hypoxia, preconditioning, mice.

Correspondence
Dominique Crenesse, Laboratoire de Physiologie, Faculté de Médecine, Université de Nice-Sophia Antipolis, 06107 Nice cedex 2, France. Tel.: 00 334 93 37 77 19; fax: 00 334 93 37 77 70; e-mail: crenesse@unice.fr

Received: 13 January 2004
Revised: 25 May 2004
Accepted: 14 October 2004

doi:10.1111/j.1432-2277.2004.00064.x

Summary

Prolonged ischemia used in liver surgery and/or transplantation causes cellular damage resulting in apoptosis and necrosis. Ischemia–reperfusion (I/R) led Kupffer cells to pro-inflammatory cytokines secretion [tumor necrosis factor (TNF)-α, interleukin-1] which involve chemokines secretion by hepatocytes. These chemokines have neutrophil chemotactic properties and neutrophils are involved in the development of I/R-induced necrosis. The aim of this study was to specify the consequence of partial oxygen pressure variation on hepatocyte chemokines synthesis and to verify if intermittent hypoxia and/or preconditioning could decrease it. It was performed on primary cultured mice hepatocytes and Kupffer cells, subjected to continuous, intermittent hypoxia or preconditioning phases, mimicking surgical processes. The chemokine secretion was evaluated by RNase protection assay and enzyme-linked immunosorbent assay method. Only monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) mRNA formation were observed, especially after 1-h hypoxia followed by 10-h (for MCP-1) or 24-h reoxygenation (for MIP-2). In conclusion, TNF-α and coculture with Kupffer cells increased hepatocyte chemokines mRNA transcription, whereas surgical split up protocols (intermittent hypoxia and preconditioning) had no significant effect.

Introduction

In liver surgery and/or transplantation, prolonged ischemia is necessary to avoid excessive blood loss. Partial oxygen pressure (PO_2) variation is one of the most important damaging factors playing a part in ischemia–reperfusion (I/R) injuries. Its decrease during vascular clamp, and its abrupt increase during organ reperfusion, cause functional and structural damage to liver cells, resulting in apoptosis [1] and necrosis [2] which may occur in parallel. Both processes contribute to cell death and liver dysfunction, which represent an important cause of morbidity and mortality [1]. It is well known that the neutrophil plays an important role in the development of I/R-induced necrosis [3].

Hepatic injury following I/R consists of two periods: (i) the initial phase (1–6-h postreperfusion) is associated with oxygen-free radical generation [1], Kupffer cell activation, and initial release of neutrophils [3]. Several studies, especially in *in vivo* models have shown that I/R led to pro-inflammatory cytokine secretion [tumor necrosis factor (TNF)-α, interleukin-1 (IL-1)] by Kupffer cells [4,5]. In response to this cytokine secretion, hepatocytes release chemokines, which have neutrophil chemotactic properties (CXC chemokines) [6,7]. Chemokines are a family of leukocyte chemoattractant proteins which are

classified into groups dependent on the number and position of cysteine residues, which form internal disulfide bounds [8]; (ii) the later phase (6–24-h postreperfusion) is associated with more intense neutrophil influx in the liver from sinusoid capillaries. After their transendothelial migration, neutrophils adhere to parenchymal cells and release proteases (cathepsin G and elastase) which cause parenchymal necrosis [3,9–12].

The chemokines and their receptors participate intimately in many pathological conditions among them inflammatory diseases. They contribute to the inflammatory process that follows acute hepatic damage [13]. One characteristic of these proteins is their specificity according to the injury. For example, in the liver, monocyte chemoattractant protein-1 (MCP-1) is expressed at high levels and has protective function in acetaminophen-induced injury [14], whereas during I/R, this increase induced by reactive oxygen species (ROS) release contributes to inflammatory lesions [15,16]. In chronic alcoholic liver disease and cirrhosis, MCP-1 is required for infiltration of monocytes and subsequent inflammation, fibrosis, and expression levels correlate with AST levels and severity of injury [17,18].

The liver poorly tolerates prolonged ischemic periods without irreversible damage. Protective strategies have been developed such as intermittent inflow occlusion (intermittent Pringle maneuver) [19] and ischemic preconditioning [20,21]. Various protocols of intermittent ischemia have been used but Belghiti *et al.* [22] have shown that intermittent clamping using multiple cycles of 15 min of ischemia followed by 5 min of reperfusion was associated with decreased injury compared with similar periods of continuous ischemia. Liver preconditioning has been applied recently [23–26]. It consists of a short period of ischemia followed by a short period of reperfusion before the beginning of prolonged ischemia. This process protects against lethal ischemic stress [24,26–28]. We have shown recently [29] that intermittent ischemia in an *in vivo* I/R model or intermittent hypoxia, in cultured hepatocytes, significantly reduced apoptosis through reduction of $JNK_1/SAPK_1$ activation and downregulation of caspase 3 activity.

The first purpose of this study was to specify if, under oxygen partial pressure variations, hepatocytes alone could release chemokines, and to check the activating role of TNF-α, added or secreted by Kupffer cells. As we have previously shown the protective effect of these processes against apoptosis, the second aim of the study was to verify if intermittent hypoxia and/or preconditioning were able to decrease this chemokine secretion and, consequently, could reduce necrosis. These experiments were performed on primary cultured mice hepatocytes and/or Kupffer cells, isolated from other liver cells and blood flow, and subjected to continuous, intermittent hypoxia or preconditioning phases, mimicking surgical processes.

Materials and methods

Hepatocytes and Kupffer cells' isolation and culture

Hepatocytes

Male CD1 mice weighing 25–30 g (Harlan, Gannat, France) were used. All animal experiments were conducted in compliance with French rules on animal handling. All chemicals were obtained from Sigma (St Quentin Fallavier, France), unless otherwise stated. Hepatocytes were obtained according to the methods previously described for rats [30,31] and modified in our laboratory. Briefly, fed mice were killed by cervical dislocation. Immediately after this, livers were perfused through the portal vein with ethyleneglycoltetraacetic acid–HEPES solution (3 ml/min) for 5 min, followed by HEPES solution for 2 min and 2.3% liberase RH (Blendzyme 3; Roche Diagnostics, Meylan, France) for 5 min. Dissociated hepatocytes were collected in Leibovitz culture medium (L15; Eurobio, Les Ullis, France), supplemented with 5% newborn calf serum and centrifuged (40 *g*) at 20 °C for 2 min. Supernatant was removed and the pellet was collected in William's culture medium (Eurobio) supplemented with 5% fetal calf serum (FCS) and insulin (0.1 IU/ml). Cell viability was estimated to be superior to 90% by tryptan blue exclusion. Cells were then adjusted to a density of 0.5×10^6 cells/ml onto collagen-coated 60-mm plates and cultured at 37 °C in the presence of 95% air, and 5% CO_2. The culture medium, containing dexamethazone (1 μM), was renewed 4 h later. After overnight culture, hepatocytes were maintained subsequently for 24 h, in the same medium minus FCS and dexamethazone before experiment because both factors influence chemokine expression [6].

Selective magnetic cell sorting of Kupffer cells

After liberase perfusion, the liver was dissociated in L15 and the solution was centrifuged at 40 *g* for 2 min to remove hepatocytes (see above). Supernatant was centrifuged for 10 min at 600 *g* to pellet the other hepatic cells. The pellet was resuspended in phosphate-buffered saline containing 2 mM ethylenediaminetetraacetic acid, and 0.5% bovine serum albumin (PEB buffer) before addition of rat anti-mouse/human CD11b antibody conjugated with MicroBeads (1/10; Miltenyi Biotech, Paris, France). The suspension was incubated for 30 min at 4 °C. Cells were washed to eliminate excess of MicroBeads by adding PEB buffer and centrifuging at 600 *g* for 10 min. The supernatant was removed, the pellet was resuspended in PEB buffer and cell suspension was placed in a magnetic column for the separation (MACS separation columns; Miltenyi Biotech). After three washes in PEB buffer, the column was removed from the magnetic field, and the fraction containing the CD11b positive cells was eluted by PEB buffer and final flushing out. The cells were centrifuged at 600 *g* for

10 min, the pellet was then resuspended in William's E medium supplemented with 10% FCS, and 0.1 U/ml insulin. Kupffer cell purity was determined by light microscopic examination and cytometric flux measures (Faxscan; Becton Dickinson, Le Pont de Claix, France). Then they were adjusted to a density of 0.5×10^5 cells/ml and cocultured with hepatocytes (0.5×10^6 cells/ml) onto collagen-coated 60-mm plates as seen above.

Hypoxic stress conditions

Culture plates were maintained at 37 °C in a hermetic bag (Bioblock, Illkirch, France) in which PO_2 was adjusted to 50 ± 10 mmHg by an N_2 supply and maintained constant. During reoxygenation phase, plates were maintained at 37 °C under ambient air ($PO_2 = 150 \pm 20$ mmHg). The culture medium used, Leibovitz medium (L15; Eurobio), was nitrogen saturated. L15 medium, without $NaHCO_3$, used the buffering capacity of free-base amino acids for cell culture in free gas exchange with the atmosphere. A sample of each medium was left under the same conditions and immediately analyzed (Blood gas analyzer Corning 2504; Corning Cergy Pontoise, France), at the end of each experiment, to control PO_2, PCO_2 and pH values. This latter was stable at 7.20 ± 0.02.

Continuous hypoxia
Five groups were studied: group control in which cells were not subjected to stress. Group 1, in which cells were maintained in a hypoxic atmosphere for 1 h at 37 °C; group 2 corresponded to conditions of group 1, followed by 6 h in ambient air at 37 °C; and groups 3 and 4 corresponded to conditions of group 1, followed by 10, and 24 h, respectively, in ambient air at 37 °C.

When used, TNF-α (R&D Systems, Abington, UK) was added to culture medium (1.17×10^{-9} M) at the beginning of the stress. Although this concentration was higher than normal blood level, it was currently used in *in vitro* experiments [32,33].

Intermittent hypoxia
Cells were subjected to hypoxia for 15 min and reoxygenated for 5 min in ambient air, successively four times, followed by 6, 10, and 24 h in ambient air [29,34].

Preconditioning
Cells were subjected to hypoxia for 10 min, and reoxygenated for 15 min in ambient air before the beginning of 1-h hypoxia, followed by 6, 10, and 24 h in ambient air [28]. Studied experimental conditions were summarized in Table 1.

RNase protection assay

Total RNA was prepared as described by the Chomczynski protocol [35]. All protocols followed the instructions of the RiboQuant multiprobe RNase protection assay system (Pharmingen, Le Pont de Claix, France). With the use of an *in vitro* transcription kit and a template set [containing mouse Ltn, RANTES, eotaxine, macrophage inflammatory protein-1β (MIP-1β), MIP-1α, MIP-2, IP-10, MCP-1, and tricyclic antidepressant-3 (TCA-3)], a radiolabeled probe set was synthesized using [α-^{32}P]UTP (ICN, Irvine, CA, USA). These probes were hybridized with total RNA isolated from cultured cells. After digestion of nonhybridized RNA with RNase (for 45 min at 30 °C), the protected probes were separated on a denaturing polyacrylamide gel. The gel was dried (for 30 min at 80 °C) and exposed to X-ray film (Kodak X-OMat; Fisher Scientific, Pittsburg, PA, USA) overnight at −80 °C. The developed X-ray films were scanned and quantified with NIH-image software.

MCP-1 and MIP-2 secretion assay

At the end of stress time, cell supernatants were assayed for immunoreactive MCP-1 and MIP-2-specific enzyme-linked immunosorbent assay (ELISA) (R&D Systems). All reagents were prepared according to the manufacturer's instruction

Table 1. Experimental conditions.

Stress conditions		1-h Hypoxia 1	1-h Hypoxia + 6-h reoxygenation 2	1-h Hypoxia + 10-h reoxygenation 3	1-h Hypoxia + 24-h reoxygenation 4
+TNF-α	A	1A	2A	3A	4A
+Kupffer cells	B	IB	2B	3B	4B
Intermittent hypoxia	C	1C	2C	3C	4C
Preconditioning	D	ID	2D	3D	4D
+Kupffer cells intermittent hypoxia	BC	1BC	2BC	3BC	4BC
+Kupffer cells preconditioning	BD	1BD	2BD	3BD	4BD

(at room temperature). Briefly, after centrifugation to remove particulates, samples were twofold diluted with Calibrator Diluent RD5–3. Assay Diluent RD1W (50 µl/well) was added in 96-well microtiter plate which was mixed by gently tapping for 1 min. After covering with adhesive strip, the plate was incubated for 2 h at room temperature. Five washes with provided wash buffer were performed. After the last wash, remaining wash buffer was aspirated. Either mouse JE/MCP-1 or MIP-2 (100 µl/well) conjugates were added to each well. The plate was covered with adhesive strip and incubated for 2 h, at room temperature. Repeated five washes were performed and 100 µl of substrate solution were added to each well. The plate, protected from light, was incubated for 30 min at room temperature. One hundred microliters of stop solution were added and optical density was determined at 450 nm.

Statistical analysis

Each experimental value (±SEM) resulted from independent measurements. Data were evaluated statistically by ANOVA and Student's *t*-test. $P < 0.05$ was considered significant.

Results

Hypoxia–reoxygenation led chemokines transcription by hepatocytes

In our model, from numerous tested chemokines, only MCP-1 and MIP-2 mRNA formation were observed during 1-h hypoxia ($P < 0.01$ and 0.05, respectively, compared with nonstressed hepatocytes), but it was greater during the ensuing reoxygenation. The MCP-1 mRNA activation was statistically significant after 6, and 10-h reoxygenation ($P < 0.05$ and $P < 0.01$, respectively) but did not increase after 24-h reoxygenation (Fig. 1). The MIP-2 mRNA activation was statistically significant only after 24-h reoxygenation ($P < 0.05$) (Fig. 1). During the first hours of reoxygenation, MCP-1 and MIP-2 mRNA levels were not higher than during hypoxia (results not shown).

Hepatocyte MCP-1 and MIP-2 transcription was stimulated by TNF-α and coculture with Kupffer cells. When hypoxia–reoxygenation (H/R) stress was performed in the presence of TNF-α (1.17×10^{-9} M), MCP-1, and MIP-2 mRNA levels were increased, especially after 1-h hypoxia followed by 10-h reoxygenation ($P < 0.05$) (Fig. 2). MCP-1 and MIP-2 mRNA transcription levels were increased in cocultured hepatocytes/Kupffer cells subjected to the same stress conditions, but later than with TNF-α (after 1-h hypoxia followed by 24-h reoxygenation) ($P < 0.01$ for MCP-1 and $P < 0.05$ for MIP-2; Fig. 2). Kupffer cells alone were cultured under the same stress conditions and neither MCP-1 nor MIP-2 significant transcription was measured (results not shown).

MCP-1 and MIP-2 secretion during hypoxia and reoxygenation

As only MCP-1 and MIP-2 mRNA formation were detected under our stress conditions, we have focused secretion

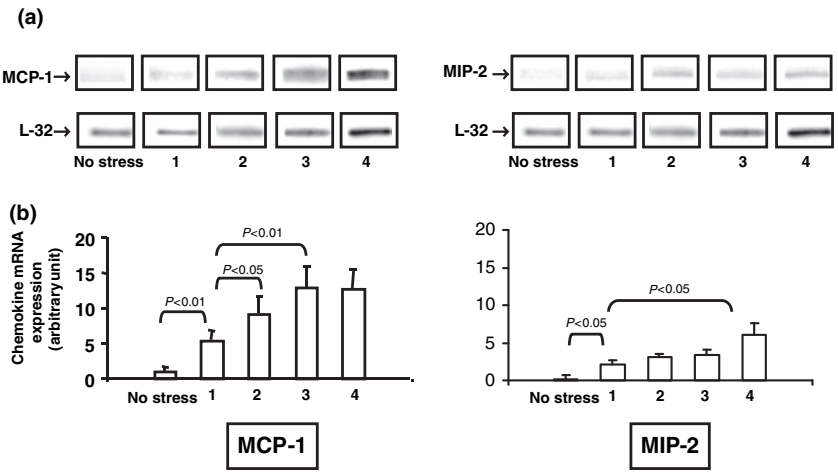

Figure 1 RNase protection assay for detection of chemokine mRNA levels in primary cultured hepatocytes. (a) RNA isolated from hepatocytes was hybridized with probes for monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) and the control gene L32. The figure shows a representative result from five independent experiments. (b) Densitometric analysis of the gel was performed and the chemokine:L32 ratio was calculated. Values are given as mean ± SEM of five independent experiments. No stress, cultured hepatocytes subjected to 24-h ambient air; 1, cultured hepatocytes subjected to 1-h hypoxia; 2, cultured hepatocytes subjected to 1-h hypoxia followed by 6-h reoxygenation; 3, cultured hepatocytes subjected to 1-h hypoxia followed by 10-h reoxygenation; 4, cultured hepatocytes subjected to 1-h hypoxia followed by 24-h reoxygenation.

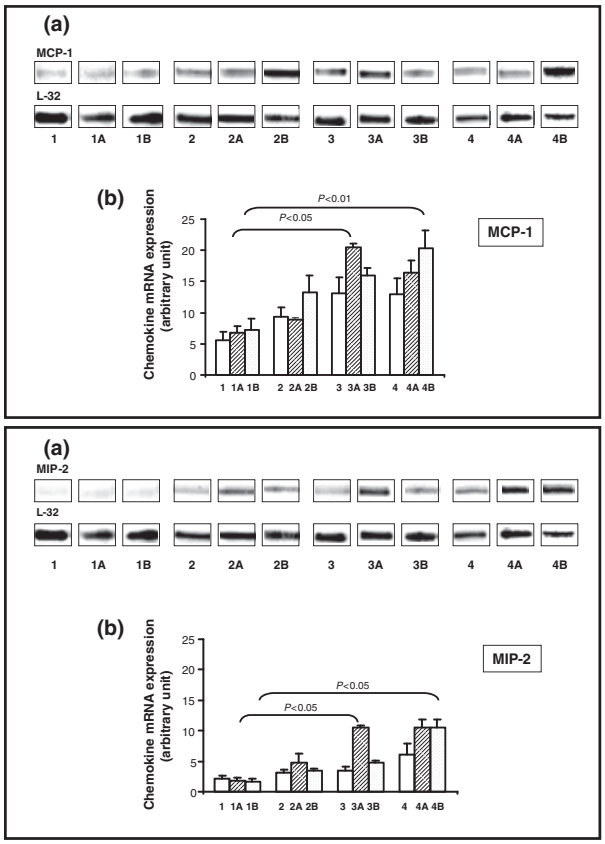

Figure 2 Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) mRNA transcription increase in primary cultured hepatocytes treated by TNF-α (1.17×10^{-9} m) and in cocultured hepatocytes/Kupffer cells. (a) RNase protection assay was performed as described in Fig. 1. The figure shows a representative result from five independent experiments. (b) Densitometric analysis of the gel was performed and the chemokine:L32 ratio was calculated. Values are given as mean ± SEM of five independent experiments. □ results shown in Fig. 1; ▨ TNF-α (1.17×10^{-9} m); ▩ coculture with Kupffer cells. 1, 1A, 1B, 1-h hypoxia; 2, 2A, 2B, 1-h hypoxia followed by 6-h reoxygenation; 3, 3A, 3B, 1-h hypoxia followed by 10-h reoxygenation; 4, 4A, 4B, 1-h hypoxia followed by 24-h reoxygenation.

analysis on these chemokines. During 1-h hypoxia, neither MCP-1 nor MIP-2 significant secretion was observed and levels were comparable with those measured without hypoxic stress. After 1-h hypoxia followed by 6-h reoxygenation, MCP-1 level increased significantly ($P < 0.05$) and the secretion peak was observed after 10-h reoxygenation ($P < 0.01$). After longer reoxygenation, MCP-1 secretion decreased. When hepatocytes were incubated with TNF-α (1.17×10^{-9} M), level rises were significantly more important and increased by a 1.7 factor after 1-h hypoxia followed by 10-h reoxygenation ($P < 0.01$). The significant rise of MIP-2 secretion was obtained after 1-h hypoxia followed by 24-h reoxygenation ($P < 0.01$), and likewise, incubation with TNF-α increased MIP-2 secretion by a 1.7 factor (Fig. 3).

Intermittent hypoxia and preconditioning did not reduce H/R-induced MCP-1 and MIP-2 transcription

Neither in cultured hepatocytes (Fig. 4) nor in cocultured hepatocytes/Kupffer cells (Fig. 5), MCP-1 and MIP-2 mRNA transcription increased by H/R stress were reduced by intermittent hypoxia or preconditioning. Although MCP-1 and MIP-2 mRNA transcription levels were slightly decreased after 24-h reoxygenation in coculture, these results were not significantly reduced (Fig. 5).

Discussion

Prolonged ischemia followed by reperfusion is necessary in surgery and/or transplantation, but causes damage in liver cells resulting in apoptosis and necrosis. I/R injury results in varying different liver cells failure which can develop various degrees of recovery. Warm ischemia affects particularly hepatocytes and sinusoidal endothelial cells. Depending upon the extent of injury, cells may either recover completely or be eliminated by apoptosis, or if severely injured, manifest processes leading to necrosis [1].

Although apoptosis and necrosis have common triggering mediators, they differ in the mechanisms of injury. The respective part of necrosis and apoptosis in I/R liver dysfunction is always much debated [36] but both processes can occur in parallel. Their intracellular cascade of events are different but can share some steps such as TNF-α and ROS secretion.

In the intact liver, Kupffer cells are located in the hepatic sinusoids and lie between or on top of the endothelial cells. The communication between these macrophages and hepatocytes occurs by inflammatory mediators' production (cytokines, nitric oxide, and ROS), but Wiss [37] showed direct cell contact through cytoplasmic extensions. I/R involves Kupffer cell secretion of TNF-α and IL-1 which lead to chemokine release by hepatocytes [32,33,38,39]. Kupffer cells themselves can release chemokines (especially MIP-2) when they are subjected to I/R [40]. The chemokines are a family of low molecular weight proteins involved in leukocyte activation and migration which exert their effects by interaction with specific receptors. In liver, they are released not only during inflammatory injury (infection, drug intoxication) but they can also be important factors for hepatocyte proliferation and hepatic regeneration after partial hepatectomy [41–43].

During endotoxic shock [41] or cytokine stimulation [6] in rat, hepatocytes generated CXC chemokines. MCP-1 plays an important role not only in recruiting cells of monocyte/macrophage lineage which contribute to the

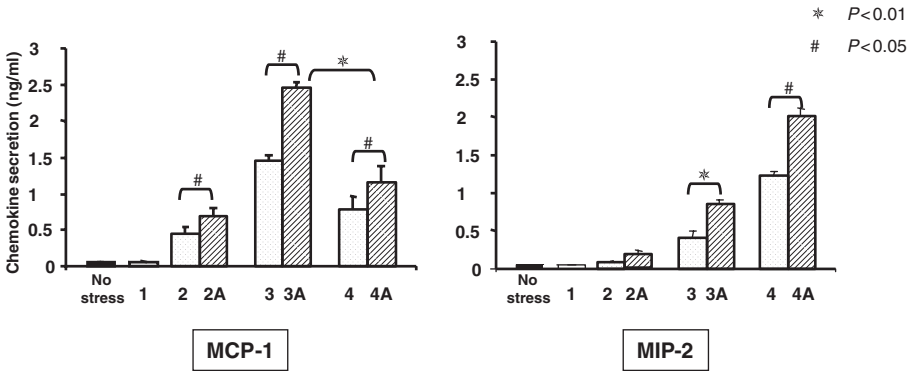

Figure 3 Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) secretion by hepatocytes subjected to hypoxia-reoxygenation stress. Chemokine levels were measured by enzyme-linked immunosorbent assay method. ■ No stress: cultured hepatocytes subjected to 24-h ambient air; □ 1, cultured hepatocytes subjected to 1-h hypoxia; ▦ incubation without TNFα; ▨ TNF-α (1.17×10^{-9} m). 2, 2A, 1-h hypoxia followed by 6-h reoxygenation; 3, 3A, 1-h hypoxia followed by 10-h reoxygenation; 4, 4A, 1-h hypoxia followed by 24-h reoxygenation. Conditions 'no stress' and '1' were performed with TNF-α (1.17×10^{-9} m) without change (results not shown). All results were significantly higher than those in 'no stress' and '1' conditions. Values are given as mean ± SEM of five independent experiments.

disease process [42] but also in modulating microcirculation [44]. MIP-2 is a well-known important mediator involved in hepatic injury through neutrophil accumulation [41,42,45]. These generated chemokines upregulate adhesion molecules [4,13], and provide a chemotactic gradient for leukocyte (especially neutrophil) transmigration [32,46]. The effects of neutrophils are primed during the initial I/R period and play a central role in the ensuing hepatic injury.

The first aim of the present study was to demonstrate that oxygen pressure variations were able to induce chemokines production by hepatocytes, isolated from other liver cells. From tested chemokines, only MCP-1 and MIP-2 transcription were observed. In our *in vitro* model, we have shown the MCP-1 and MIP-2 mRNA transcription in hepatocytes alone and already during hypoxia. Reoxygenation increased these transcriptions more quickly for MCP-1 (6 h compared with 24 h for MIP-2). Lentsch *et al.* [13] reported a significant MIP-2 mRNA transcription increase after 9-h reperfusion following 90-min ischemia, in an *in vivo* I/R model. Yamaguchi *et al.* [42] observed a peak of serum MCP-1 concentration, 6 h after liver ischemia. On human hepatocyte lines, Thornton *et al.* [32] demonstrated that hepatocytes were able to express IL-8 (human equivalent to mice MIP-2) mRNA activity when they were stimulated by proinflammatory cytokines (TNF-α and IL-1).

In our model, to specify the TNF-α activating part, cultured hepatocytes were incubated with this cytokine (1.17×10^{-9} M), and subjected to stress conditions. mRNA transcription of chemokines was significantly increased after 10-h reoxygenation. When hepatocytes were cocultured with Kupffer cells and subjected to the same stress conditions, chemokine mRNA transcription increase required 24 h. Nakamitsu *et al.* [5] have shown in an I/R model that although cytokine mRNA rates were increased rapidly, Kupffer cells released the highest level of TNF-α approximately after 24-h reperfusion. In our *in vitro* model, we observed a similar response: TNF-α, present from the beginning of the stress, led to increase of MCP-1 and MIP-2 mRNA transcription faster (10 h) than Kupffer cell activation (24 h). Although Kupffer cells are known to release chemokines [47], in our experimental model, only hepatocytes released these chemokines. This coculture model with direct contact between cells did not absolutely mimic *in vivo* situation, but it was used before to specify cellular regulation [48–51]. The secretion of chemokines in culture medium was increased by TNF-α. This result was in agreement with previous studies [7,38].

Excessive blood loss during surgery can hinder postoperative recovery. Intermittent clamping of the portal triad was better tolerated than prolonged continuous ischemia, but simple inflow occlusion (Pringle maneuver) was often preferred by surgeons when it was possible. Isozakis *et al.* [19] suggested that applying the Pringle maneuver intermittently rather than continuously led to better postoperative recovery. Significant improvement of postoperative liver function has been observed in rats and humans after intermittent ischemia or preconditioning [5,52,53]. In previous study, we have shown that intermittent ischemia *in vivo* and intermittent hypoxia in cultured hepatocytes significantly reduced apoptosis through a reduction of $JNK_1/SAPK_1$ activation and a downregulation of caspase-3 activity [29].

The second purpose of the present study was to verify if the surgical processes (described above) could modify

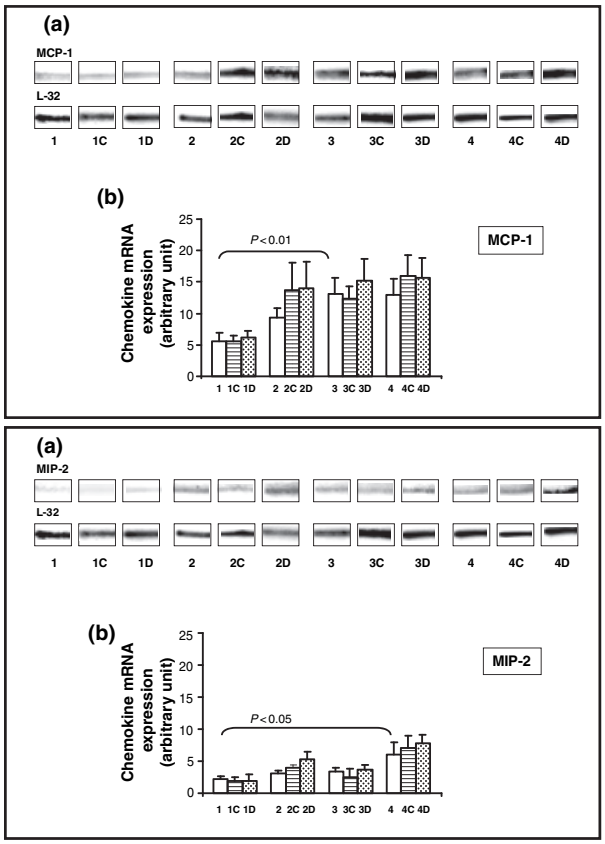

Figure 4 Intermittent hypoxia and preconditioning did not reduce monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) mRNA transcription levels in primary cultured hepatocytes. Hepatocytes were subjected to 1-h hypoxia followed by 6, 10 and 24-h reoxygenation, or to 15-min hypoxia followed by 5-min reoxygenation, four times before reoxygenation, or to preconditioning (10-min hypoxia followed by 15-min reoxygenation) before 1-h hypoxia followed by 6, 10 and 24-h reoxygenation. (a) RNase protection assay was performed as described in Fig. 1. The figure shows a representative result from five independent experiments. (b) Densitometric analysis of the gel was performed and the chemokine:L32 ratio was calculated. Values are given as mean ± SEM of five independent experiments. □ results shown in Fig. 1; ▤ intermittent hypoxia; ▨ preconditioning. 1, 1C, 1D, 1-h hypoxia; 2, 2C, 2D, 1-h hypoxia followed by 6-h reoxygenation; 3, 3C, 3D, 1-h hypoxia followed by 10-h reoxygenation; 4, 4C, 4D, 1-h hypoxia followed by 24-h reoxygenation.

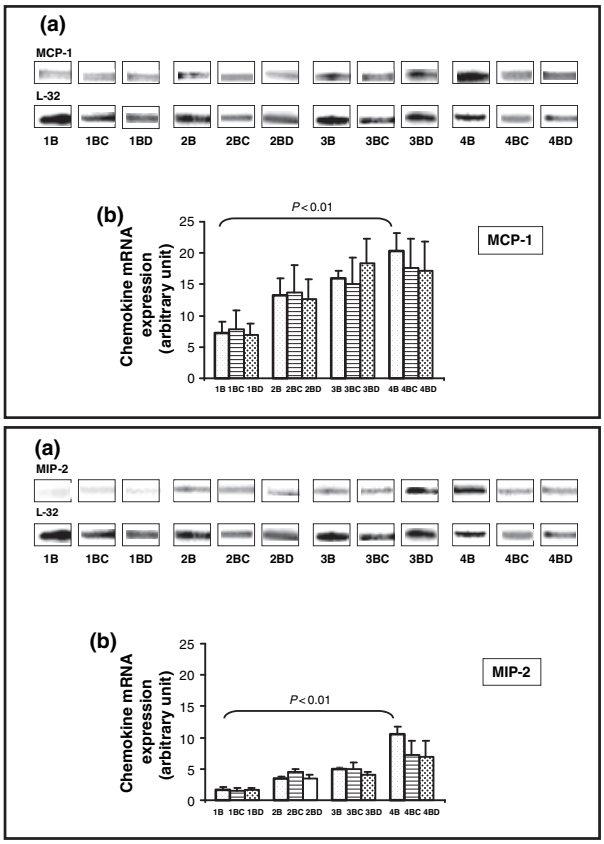

Figure 5 Intermittent hypoxia and preconditioning did not reduce monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) mRNA transcription levels in cocultured hepatocytes/Kupffer cells. Cells were subjected to 1-h hypoxia followed by 6, 10 and 24-h reoxygenation, or to 15-min hypoxia followed by 5-min reoxygenation, four times before reoxygenation, or to preconditioning (10-min hypoxia followed by 15-min reoxygenation) before 1-h hypoxia followed by 6, 10 and 24-h reoxygenation. (a) RNase protection assay was performed as described in Fig. 1. The figure shows a representative result from five independent experiments. (b) Densitometric analysis of the gel was performed and the chemokine:L32 ratio was calculated. Values are given as mean ± SEM of five independent experiments. ▩ Coculture with Kupffer cells; ▤ coculture with Kupffer cells and intermittent hypoxia; ▨ coculture with Kupffer cells and preconditioning: 1B, 1BC, 1BD, 1-h hypoxia; 2B, 2BC, 2BD, 1-h hypoxia followed by 6-h reoxygenation; 3B, 3BC, 3BD, 1-h hypoxia followed by 10-h reoxygenation; 4B, 4BC, 4BD, 1-h hypoxia followed by 24-h reoxygenation.

the transcription of these chemokines. When isolated hepatocytes were subjected to intermittent hypoxia or preconditioning protocols, MCP-1 and MIP-2 mRNA levels were comparable with those observed during continuous H/R. When the same stress conditions were performed on cocultured hepatocytes/Kupffer cells, chemokine transcription was slightly but not significantly decreased.

Our results were compatible with those of Peralta *et al.* [23], which showed that the beneficial effect of liver preconditioning was not mediated by a modulation of liver neutrophil infiltration. Their work was performed on an I/R model where all liver cell types were subjected to stress, and endothelial cells were known to release TNF-α through endothelin upregulation. Rüdiger *et al.* [34] have shown that preconditioning and intermittent ischemia protected against hepatocyte apoptosis, and even that intermittent ischemia was more efficient during long ischemic surgical procedures. We and others [26,29,54]

have highlighted the preconditioning and/or intermittent ischemia protective role through downregulation of caspase-3 activity, and apoptosis. These results have been confirmed in complementary studies on cultured hepatocytes [29].

In conclusion, our study shows, for the first time, that intermittent hypoxia and preconditioning did not influence hepatocyte MIP-2, and MCP-1 mRNA transcription. The known protective effect of both surgical procedures would rather appear to be involved in countering the process of apoptosis.

Acknowledgement

The authors thank Dr G. de Souza for his helpful advice and C. Petit for his technical assistance.

References

1. Kohli V, Selzner M, Madden JF, Bentley RC, Clavien PA. Endothelial cell and hepatocyte deaths occur by apoptosis after ischemia-reperfusion injury in the rat liver. *Transplantation* 1999; **67**: 1099.
2. Gujral JS, Bucci T, Farhood A, Jaeschke H. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats : apoptosis or necrosis? *Hepatology* 2001; **33**: 397.
3. Jaeschke H, Smith C. Mechanisms of neutrophil-induced parenchymal cell injury. *J Leukoc Biol* 1997; **61**: 647.
4. Jaeschke H. Mechanisms of reperfusion injury after warm ischemia of the liver. *J Hepatobiliary Pancreat Surg* 1998; **5**: 402.
5. Nakamitsu A, Hiyama E, Imamura Y, Matsuura Y, Yokoyama T. Kupffer cell function in ischemic and nonischemic livers after hepatic partial ischemia/reperfusion. *Surg Today* 2001; **31**: 140.
6. Thornton AJ, Ham J, Kunkel SL. Kupffer cell-derived cytokines induce the synthesis of a leukocyte chemotactic peptide, interleukin-8, in human hepatoma and primary hepatocyte culture. *Hepatology* 1992; **15**: 1112.
7. Mawet E, Shiratori Y, Hikiba Y, *et al.* Cytokine-induced neutrophils chemoattractant release from hepatocytes is moduled by Kupffer cells. *Hepatology* 1996; **23**: 353.
8. Cascieri MA, Sringer M. The chemokine/chemokine-receptor family: potential and progress for therapeutic intervention. *Curr Opin Chem Biol* 2000; **4**: 420.
9. Martinez-Mier G, Toledo-Pereyra L, Ward PA. Adhesion molecules in liver ischemia and reperfusion. *J Surg Res* 2000; **94**: 185.
10. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia-reperfusion injury in rat liver in vivo. *FASEB J* 1990; **4**: 3355.
11. Jaeschke H, Smith C, Clemens MG, Ganey PE, Roth RA. Mechanisms of inflammatory liver injury: adhesion molecules and cytotoxicity of neutrophils. *Toxicol Appl Pharmacol* 1996; **139**: 213.
12. Suzuki S, Toledo-Pereyra L, Rodriguez FJ. Role of neutrophils during the first 24 hours after liver ischemia and reperfusion injury. *Transplant Proc* 1994; **26**: 3695.
13. Lentsch AB, Kato A, Yoshidome H, McMasters KM, Edwards MJ. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC. *Hepatology* 1998; **27**: 1172.
14. Hogaboam CM, Bone-Larson CL, Steinhauser ML, *et al.* Exaggerated hepatic injury due to acetaminophen challenge in mice lacking C-C chemokine receptor 2. *Am J Pathol* 2000; **156**: 1245.
15. Yamaguchi Y, Matsumura F, Takeya M, *et al.* Monocyte chemoattractant protein-1 enhances expression of intracellular adhesion molecule-1 following ischemia-reperfusion of the liver in rats. *Hepatology* 1998; **27**: 727.
16. Yamaguchi Y, Matsumura F, Liang J, *et al.* Neutrophil elastase and oxygen radicals enhance monocyte chemoattractant potein-expression after ischemia/reperfusion in rat liver. *Transplantation* 1999; **68**: 1459.
17. Marra F, DeFranco R, Grappone C, *et al.* Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. *Am J Pathol* 1998; **152**: 423.
18. Afford SC, Fisher NC, Neil DA, *et al.* Distinct patterns of chemokine expression are associated with leukocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis. *J Pathol* 1998; **186**: 82.
19. Isozaki H, Adam R, Gigou M, Szekely AM, Shen M, Bismuth H. Experimental study of the protective effect of intermittent hepatic pedicule clamping in the rat. *Br J Surg* 1992; **79**: 310.
20. Czonka C, Szilvassy Z, Fülöp F, *et al.* Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. *Circulation* 1999; **100**: 2260.
21. Steenbergen C, Perlman M, London RE, Murphy E. Mechanism of preconditioning. Ionic alterations. *Circ Res* 1993; **72**: 112.
22. Belghiti J, Noun R, Malafosse R, *et al.* Continuous versus intermittent portal triad clamping for liver resection. *Ann Surg* 1999; **229**: 369.
23. Peralta C, Bartrons R, Riera L, *et al.* Hepatic preconditioning preserves energy metabolism during sustained ischemia. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G163.
24. Hardy KJ, McClure S, Subwongcharoen S. Ischaemic preconditionning of the liver: a preliminary study. *Aust N Z J Surg* 1996; **66**: 707.
25. Nilsson B, Friman S, Gustafsson BI, Delbro DS. Preconditioning protects against ischemia/reperfusion injury of the liver. *J Gastrointest Surg* 2000; **4**: 44.
26. Clavien PA, Yadav S, Sindram D, Bentley RC. Protective effects of ischemic preconditioning for liver resection performed under inflow occlusion in humans. *Ann Surg* 2000; **232**: 155.

27. Peralta C, Closa D, Hotter G, Gelpi E, Prats N, Rosello-Catafau J. Liver ischemic preconditioning is mediated by the inhibitory action of nitric oxid on endothelin. *Biochem Biophys Res Commun* 1996; **229**: 264.
28. Bulkley GB. Preconditioning for protection form ischemic injury: discriminating cause from effect from epiphenomenon. *Ann Surg* 2000; **232**: 163.
29. Crenesse D, Laurens M, Gugenheim J, *et al.* Intermittent ischemia reduces warm hypoxia-reoxygenation-induced $JNK_1/SAPK_1$ activation and apoptosis in rat hepatocytes. *Hepatology* 2001; **34**: 972.
30. Berry MN, Halls H, Grivell MH. Techniques for pharmacological and toxicological studies with isolated hepatocytes suspensions. *Life Sci* 1992; **51**: 1.
31. Seglen PO. Preparation of rat liver cells. *Exp Cell Res* 1973; **82**: 391.
32. Thornton AJ, Strieter R, Lindley I, Baggiolini M, Kunkel SL. Cytokine-induced gene expression of a neutrophil chemotactic factor/IL-8 in human hepatocytes. *J Immunol* 1990; **144**: 2609.
33. Coletti LM, Kunkel S, Walz A, *et al.* The role of cytokine networks in the local liver injury following hepatic ischemia/reperfusion in the rat. *Hepatology* 1996; **23**: 506.
34. Rüdiger HA, Kang K, Sindram D, Riehle HM, Clavien PA. Comparison of ischemic preconditioning and intermittent and continuous inflow occlusion in the murine liver. *Ann Surg* 2002; **235**: 400.
35. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156.
36. Clavien PA, Rudiger H, Selzner M. Mechanism of hepatocyte death after ischemia: apoptosis versus necrosis. *Hepatology* 2001; **33**: 1555.
37. Wisse E. On the fine structure and function of rat liver Kupffer cells. In: Carr I, Dames WT, eds. *Reticuloendothelial Syst*. New York, 1980: 360.
38. Coletti LM, Kunkel S, Walz A, *et al.* Chemokine expression during hepatic ischemia/reperfusion-induced lung injury in the rat. The role of epithelial neutrophil activating protein. *J Clin Invest* 1995; **95**: 134.
39. Koo DJ, Chaudry I, Wang P. Kupffer cells are responsible for producing inflammatory cytokines and hepatocellular dysfunction during early sepsis. *J Surg Res* 1999; **83**: 151.
40. Mosher B, Dean R, Harkema J, Remick D, Palma J, Crockett E. Inhibition of kupffer cells reduced CXC chemokine production and liver injury. *J Surg Res* 2001; **99**: 201.
41. Zang P, Xie M, Zagorski J, Spitzer JA. Attenuation of hepatic neutrophil sequestration by anti-CINC antibody in endotoxic rats. *Shock* 1995; **4**: 262.
42. Yamaguchi Y, Matsumura F, Liang J, *et al.* Neutrophil elastase and oxygen radicals enhance monocyte chemoattractant protein-expression after ischemia/reperfusion in rat liver. *Transplantation* 1999; **68**: 1459.
43. Ren X, Carpenter A, Hogaboam C, Colletti L. Mitogenic properties of endogenous and pharmacological doses of macrophage inflammatory protein-2 after 70% hepatectomy in the mouse. *Am J Pathol* 2003; **163**: 563.
44. Bajt ML, Farhood A, Laeschke H. Effects of CXC chemokines on neutrophil activation and sequestration in hepatic vasculature. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1188.
45. Ohkubo K, Masumoto T, Horiike N, Onji M. Induction of CINC (interleukin-8) production in rat liver by non-parenchymal cells. *J Gastroenterol Hepatol* 1998; **13**: 696.
46. Zhang PBA, Spitzer JA. Generation of a CD11b/c upregulating and chemotactic factor by hepatocytes of endotoxic rats – nonidentity with interleukin-8. *Shock* 1994; **2**: 332.
47. Hisama N, Yamaguchi Y, Ishiko T, *et al.* Kupffer cell production of cytokine-induced neutrophil chemoattractant following ischemia/reperfusion injury in rats. *Hepatology* 1996; **24**: 1193.
48. Hoebe KHN, Witkamp RF, Fink-Gremmels J, Van Miert ASJPAM, Monshouwer M. Direct cell-to-cell between Kupffer cells and hepatocytes augments endotoxin-induced hepatic injury. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G720.
49. Billiar TR, Curran RD, Ferrari FK, Williams DL, Simmons RL. Kupffer cells: hepatocyte cocultures release nitric oxide in response to bacterial endotoxin. *J Surg Res* 1990; **48**: 349.
50. West MA, Billiar TR, Curran RD, Hyland BJ, Simmons RL. Evidence that rat Kupffer cells stimulate and inhibit hepatocyte protein synthesis in vitro by different mechanisms. *Gastroenterology* 1995; **96**: 1572.
51. Lelbach A, Scharf JG, Ramadori G. Regulation of insulin-like growth factor-1 and insulin-like growth factor binding protein-1, -3 and –4 in cocultures of rat hepatocytes and Kupffer cells by interleukin-6. *J Hepatol* 2001; **35**: 558.
52. Hewitt G, Halliday I, McCaigue M, Campbell G, Rowlands B, Diamond T. Mortality, endotoxaemia and cytokine expression after intermittent and continuous hepatic ischemia. *Br J Surg* 1995; **82**: 1424.
53. Harada M, Yogita S, Tashiiro S, *et al.* Effect of intermittent liver ischemia on outcome in patients with hepatocellular carcinoma on liver cirrhosis. *J Med Invest* 1999; **46**: 205.
54. Yadav SS, Sindram D, Perry DK, Clavien PA. Ischemic preconditioning protects the mouse liver by inhibition of apoptosis through a caspase-dependent pathway. *Hepatology* 1999; **30**: 1223.