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Up regulation of IL-6 by ischemic preconditioning in normal and fatty rat livers: Association with reduction of oxidative stress

LORENZA TACCHINI[1,†], GAETANO CAIRO[1,†], CRISTINA DE PONTI[1], MARTA MASSIP[2], JOAN ROSELLÒ-CATAFAU[2], & CARMEN PERALTA[2]

[1]*Institute of General Pathology, University of Milano, via Mangiagalli 31, 20133 Milano, Italy, and* [2]*Experimental Pathology Department, Instituto de Investigaciones Biomédicas de Barcelona-Consejo Superior de Investigaciones Cientificas, Institut d'Investigacions Biomediques August Pi i Sunyer, Rossellò 161, 08036 Barcelona, Spain*

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Abstract

We analyzed the role of IL-6 in the protection that ischemic preconditioning (IP) exerts against hepatic ischemia reperfusion-mediated (I/R) oxidative damage, particularly in fatty livers. IP-related IL-6 up-regulation during reperfusion in steatotic and non-steatotic livers was correlated with reduced indices of liver damage, as also demonstrated by pharmacological modulation of IL-6. IP activated NF-kB and HSF during ischemia (Isc), whereas AP-1 activity was unaffected. IP blunted the activation of STAT3 and stress-responsive genes, such as NF-kB, AP-1 and heme oxygenase (HO-1) during reperfusion. The role of reduced oxidative stress in hepatoprotection of fatty livers was further demonstrated by the fact that: (i) IP prevented the decrease of glutathione levels and the increase of lipid peroxidation; (ii) the anti-oxidant GSH-ester prevented lipid peroxidation and necrosis. In conclusion, IP modulates the activity of transcription factors and triggers IL-6 production; this may prevent hepatic I/R damage in a oxidative stress-dependent way, particularly in fatty livers.

Keywords: *Reperfusion, redox reactions, oxidative stress, transcription factors*

Introduction

Normothermic ischemia-reperfusion (I/R) injury is an important determinant in the pathogenesis of the liver damage occurring during surgical procedures, such as hepatic resections and liver transplantation. In clinical transplantation, normothermic I/R injury before and during the implantation procedure may contribute to subsequent graft dysfunction or failure [1–3]. Among the damaging mechanisms, reactive oxygen species (ROS) formation and inflammatory response are the major contributors to the overall pathophysiology. Hepatic steatosis is a major risk factor after liver surgery because steatotic livers are more prone to oxidative stress and hence tolerate poorly I/R injury; therefore, steatosis accounts for the greatest number of organs rejected during the triage process applied to donor livers before transplantation [3]. Ischemic preconditioning (IP), based on prior exposure to a brief period of vascular occlusion, attenuates I/R injury in a number of organs and appears to be the most promising strategy against I/R damage [1,4,5]. IP seems particularly effective in reducing the vulnerability of fatty livers to hepatic I/R injury [6,7]. Moreover, preliminary studies of IP in fatty

†These authors contributed equally to this paper.

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livers undergoing normothermic Isc during hepatic surgery in humans have produced promising results [8]. Although several theories have been proposed to account for the protective mechanisms of IP, a clear biological basis remains to be identified. Only a full appraisal of the molecular basis of IP in both normal and fatty livers will permit the design of new clinical applications of IP in liver surgery, as well as pharmacological or genetic strategies to simulate or enhance its effectiveness.

IP seems to interfere with the two major mechanisms at the basis of I/R damage, i.e. the inflammatory response and oxidative stress, as this surgical strategy modulates the generation of pro- and anti-inflammatory cytokines, such as IL-1β and IL-10, respectively [9] and reduces the exacerbated ROS generation observed in steatotic livers undergoing I/R [10].

IL-6 is a multifunctional cytokine with an increasingly recognized hepatoprotective role [11] which could be important in I/R injury because administration of recombinant IL-6 attenuated reperfusion damage after warm Isc [12,13]. However, both up- and down-regulation of endogenous IL-6 expression have been found to correlate with the hepatoprotective effect of IP [14–18].

To investigate the molecular mechanisms underlying the protective action of IP in both normal and fatty livers, we designed a series of experiments to evaluate the effect of IP on IL-6 levels. We also analysed the activity of transcription factors, which may play an important role in the control of IL-6 expression. In addition, we analysed IL-6-dependent pathways possibly involved in hepatoprotection. The results of the present study demonstrate that IP-mediated protection is associated with a profoundly different pattern of hepatic transcriptional activity, enhanced IL-6 production and reduced oxidative stress in fatty livers.

Materials and methods

Experimental animals

Homozygous (obese, Ob) and heterozygous (lean, Ln) 16–18 weeks old Zucker rats (Iffa-Credo, L'Abresle, France), which constitute a well-characterized model of nutritionally-induced obesity, were used. Ob rats showed severe macrovesicular fatty infiltration in hepatocytes (60–70% steatosis) but no signs of inflammation [6,19]. In contrast, Ln rats showed no evidence of steatosis [6].

Treatment of animals

The animals were anaesthetized with ketamine (100 mg/kg) and xylazine (8 mg/kg) [6,20]. After a midline laparotomy, the hepatic artery and portal vein to the left and median liver lobes were occluded for the period of Isc under study. This method of partial (70%) Isc prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. Reperfusion was initiated by removal of the clamp [6,19]. Oxidative stress was induced by treating rats with the glutathione-depleting drug buthionine sulphoximine (BSO) at a dose of 90 mg/100 g of body weight.

This study was performed in accordance with the European Union regulations for animal experiments (Directive 86/609 EEC). Assignment of animals to experimental groups is described below.

Experimental design

The interventions are summarized in Table I.

1. Sham operation (SH) ($n = 12$): Ln and Ob animals (6 each) subjected to anaesthesia and laparotomy alone.
2. Isc ($n = 12$): Ln and Ob animals (6 each) subjected to 60 min of Isc [6].
3. IP ($n = 12$): Ln and Ob animals (6 each) subjected to 5 min of Isc followed by 10 min of reperfusion [6].
4. Ischemia preconditioning + Ischemia (IP/Isc): as in group 2 but with previous IP induced by 5 min of Isc followed by 10 min reperfusion.
5. Ischemia-reperfusion (R) ($n = 36$). This group is divided in the following subgroups: (5.1) Ln and Ob animals ($n = 12$, 6 each) subjected to 60 min of Isc followed by 2 h reperfusion; (5.2) Ln and Ob animals ($n = 12$, 6 each) subjected to 60 min Isc followed by 6 h reperfusion; and (5.3) Ln and Ob animals ($n = 12$, 6 each) subjected to 60 min of Isc followed by 24 h of reperfusion [6].
6. Ischemic preconditioning + I/R (IP/R): as in group 5 but with previous IP (5 min of Isc + 10 min reperfusion).
7. Ischemia-reperfusion + IL-6 administration (R/IL-6): as in group 5.2 and 5.3 but treated with rIL-6 (R&D systems, Minneapolis, MN), at a dose of 500 μg/kg (ip), 24 and 12 h before surgery [13,21].
8. Ischemic preconditioning + I/R + anti-IL-6 (IP/R/antiIL-6): as in group 5.2 and 5.3 but with previous ischemic preconditioning and treated with anti-IL-6 antibody (R&D systems Minneapolis, MN) at a dose of 16.6 μg/kg (ip) at the onset of surgery [22,23]. Control experiments with IgG were carried out.
9. Ischemia-reperfusion + glutathione ester (R/GSH): Ob animals were subjected to 60 min of Isc (I) followed by 6 or 24 h of reperfusion (R) (as groups 5.2 and 5.3, respectively) but with administration of GSH ester (5 mmol/kg, iv) 5 min before reperfusion [24,25].

Table I.   Experimental protocol set up in both fatty and normal rat livers subjected to different periods of partial Isc and reperfusion.

Group 1. SH	
Group 2. Isc	**60'I**
Group 3. IP	**5'I 10'R**
Group 4. IP/Isc	**5'I 10'R 60'I**
Group 5. R G. 5.1. R_2 G. 5.2. R_6 G. 5.3. R_{24}	**60'I** R **2h** **6h** **24h**
Group 6. IP/R IP/R_2 IP/R_6 IP/R_{24}	**5'I 10'R 60'I R 2h** **6h** **24h**
Group 7. R/IL-6 R_6/IL-6 R_{24}/IL-6	**IL-6 60'I R 6h** **IL-6 24h**
Group 8. IP/R/anti-IL-6 IP/R_6/anti-IL-6 IP/R_{24}/anti-IL-6	**Anti-IL-6 5'I 10'R 60'I R 6h** **Anti IL-6 24h**
Group 9. R/GSH R_6/GSH R_{24}/GSH	**GSH 60'I R 6h** **GSH 24h**

Biochemical determinations

ALT assay. Evaluation of hepatocyte damage was performed by enzymatic determinations of alanine aminotransferase (ALT) [26] in plasma using a commercial kit from Boehringer Mannheim (Munich, Germany).

Lipid peroxidation assay. Lipid peroxidation has been used as an indirect measurement of oxidative damage induced by ROS. Lipid peroxidation in liver samples was determined by the thiobarbiturate reaction measuring the formation of MDA. Briefly, 0.5 ml of 0.5% butylated hydroxytoluene was added to 2 ml of liver homogenate to prevent lipid autoxidation. For protein precipitation, 2 ml of 20% trichloroacetic acid was added to 2 ml of homogenate. After mixing and centrifuging, 1 ml of 0.67% thiobarbiturate-water solution was added to the supernatant and boiled for 60 min. After cooling, optical density at 530 nm was assayed [6,27,28].

Glutathione measurement. For the analysis of glutathione (GSH), liver samples were homogeneized in 1.1% KCl. Proteins were precipitated with 1 N perchloric acid. After centrifugation, samples were neutralized with 10% K_2CO_3. The amount of GSH was measured using glutathione transferase and 1-chloro-2,4-dinitrobenzene. An aliquot 50 µl of the sample were mixed with 225 µl of 0.1 M potassium phosphate buffer, pH 7.0 and 10 µl of 10 mM

1-chloro-2,4-dinitrobenzene in ethanol. The reaction was started with 5 μl of glutathione transferase solution (12 U/l) and monitored at 340–400 nm reaching the end point 5 min after enzyme addition [25,29].

IL-6 assay. Liver tissue was homogenized in 50 mM Tris, pH 7.2, 150 mM NaCl, Triton X-100 and protease inhibitors (Roche, Basel, Switzerland). The homogenate was shaken on ice for 90 min and centrifuged at 3000*g* for 15 min. IL-6 levels in the supernatant were determined using a commercial enzyme-linked immunoadsorbent assay kit (R&D Systems) [30].

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Livers were homogenized in 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 0.1 mM EGTA, 0.5 mM dithiothreithol and protease inhibitors. Homogenates were centrifuged at 300*g* at 4°C. After washing with the same buffer, the pellets were resuspended in 10 mM Hepes, pH 7.9, 0.4 M NaCl, 1.5 mM MgCl$_2$, 0.1 mM EGTA, 5% glycerol, 0.5 mM dithiothreithol and protease inhibitors. Nuclei were extracted for 30 min at 4°C with constant shaking and centrifuged at 13,600*g* for 30 min at 4°C. Aliquots of supernatants were subjected to EMSA with PAGE-purified, ^{32}P-labeled double-strand DNA containing the binding sites for the following transcription factors: NF-*k*B: [31]; Oct-1: [32]; heat shock factor (HSF): [31]; AP-1 [33]; γ-IFN activation site (GAS) elements for signal transducer and activator of transcription (STAT) [34]. For competition experiments, an excess (50×) of specific or unspecific unlabeled double-stranded sequences were added to the binding mixture. Samples were run onto a 5% polyacrylamide gel, dried and autoradiographed at −80°C with Kodak MR films. Quantitative determination was made by direct nuclear counting using an InstantImager (Packard Instruments, Milan, Italy) and the values were calculated after normalisation to activity of the constitutively expressed transcription factor Oct-1.

For supershift assays, the nuclear extracts were preincubated with 1 μg of specific antibodies (Ab) against p50, p65, STAT1, 3 and 5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) before the incubation with labeled oligonucleotide.

Northern blot analysis

Total cell RNA was electrophoresed under denaturing conditions. To confirm that each lane contained equal amounts of total RNA, the ribosomal RNA content was estimated in the ethidium bromide-stained gels by laser densitometry [35]. Filter-bound RNA was hybridised with the heme oxygenase (HO-1) [36] ^{32}P-labeled cDNA probe. Quantitative determination was made by direct nuclear counting using an InstantImager (Packard Instruments Co., Milano, Italy) and the values were calculated after normalisation to the amount of ribosomal RNA.

Immunoblot analysis

The liver was homogenised in 10 mM Hepes, pH 7.6, 3 mM MgCl$_2$, 40 mM KCl, 5% glycerol, 0.2% Nonidet P-40 and protease inhibitors and the lysate was centrifuged at 16,000*g* for 5 min. Aliquots of cytosolic supernatants containing equal amounts of proteins were electrophoresed in acrylamide-SDS gels and electroblotted to Hybond membranes (Amersham Co. Milano, Italy). After assessing transfer by means of Ponceau S staining, the membranes were saturated in 4 mM Tris–HCl, pH 7.6, 30 mM NaCl (TBS) containing 20% non-fat milk and 0.1% Tween-80 and incubated with anti-HO-1 antibody (Stressgen, Victoria, Canada, 1:1000 dilution) and an antibody to β-actin (Sigma Chemical Co., Milano, Italy) to control equal protein loading. After incubation with the appropriate secondary Ab and washing the antigens were detected using an immuno-detection kit (ECL Plus, Amersham Co., Milano, Italy) and quantitated by densitometry.

Histology

To appraise the severity of hepatic injury, hematoxylin and eosin-stained sections were evaluated by a point-counting method on an ordinal scale, as previously reported [6]. DNA fragmentation was determined using a TUNEL assay in deparaffinized liver samples using an *in situ* cell-death detection kit (Boehringer Mannheim Co., Indianapolis, IN, USA) [37]. Hepatic cells were analyzed in two different sections per animal and a total of 20 randomized, separate fields per animal were counted. TUNEL-positive nuclei were scored at 40× high-power (400 hepatic cells per field) under a light microscope. The ratio of TUNEL-positive cells to the total number was calculated [20,38]. To evaluate hepatic regeneration, the proliferating cell nuclear antigen (PCNA)-labeling index was evaluated in liver samples by immunohisto-chemistry using a commercial kit (DAKO Envision + System, peroxidase (DAB); Dako GMbh, Germany) [39].

Statistics

Data are expressed as means ± standard error of the mean. Statistical comparison was performed by analysis of variance, followed by Student–Newman–Kleus tests. An associated probability of $p < 0.05$ was considered significant.
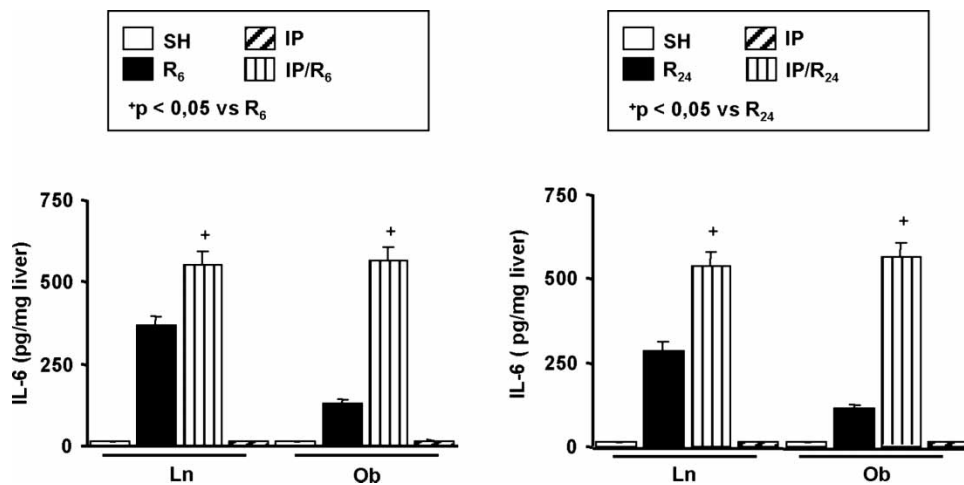
Figure 1.   Effect of IP on hepatic IL-6 levels. Liver IL-6 in Ln and Ob rats subjected to sham operation (SH group); 60 min of Isc followed by 6 and 24 h of reperfusion (R group); reperfusion with prior ischemic preconditioning (IP/R) group; ischemic preconditioning (IP group).

Results

Effect of preconditioning on IL-6 levels and liver damage indices during reperfusion

We investigated the hepatic content of IL-6 at different times of post-ischemic reperfusion. A significant increase in IL-6 levels was observed in both fatty and normal livers at 2 (not shown), 6 and 24 h of reperfusion with lower levels of IL-6 in fatty livers (Figure 1). IP increased IL-6 levels over those of non-preconditioned livers in both Ln and Ob rats and the extent of induction was greater in fatty livers.

To assess whether IL-6 expression was related to liver damage, we measured transaminase levels and assessed cell death during reperfusion. ALT levels were significantly lower in the preconditioned (IP/R) than in the non-preconditioned groups (R) livers at 2 (not shown), 6 and 24 h of reperfusion (Figure 2(A)), in agreement with previous findings [6,9]. The pretreatment with IL-6 (R/IL-6) reduced hepatic injury in both types of livers. The administration of Ab against IL-6 in the preconditioned group (IP/R/anti-IL-6) abolished the benefits of IP (IP/R) resulting in ALT levels similar to those found in the non-preconditioned group (R). A similar pattern was observed for the percentage of grade 3 necrosis (Figure 2(B)). IP and IL-6 treatment reduced the percentage of necrosis observed in both types of livers at 24 h of reperfusion. Inhibition of IL-6 action in the preconditioned group (IP/R/anti-IL6) abolished the benefits of preconditioning on grade 3 necrosis. Ob animals presented a greater percentage of necrotic cells in all groups but the extent of changes was similar to that of Ln rats. The TUNEL assay indicated that no apoptosis occurred in steatotic livers in any of the groups evaluated (results not shown). These results are in line with previous studies indicating that apoptosis does not appear to be the predominant form of hepatocyte death in reperfused steatotic livers

[19]. Conversely, in non-steatotic livers, an increase in cell TUNEL staining was observed at 24 h of reperfusion, as compared with the results obtained in the SH group (Figure 2(C)). IP and IL-6 treatment reduced this increase. The administration of Ab against IL-6 in the preconditioned group resulted in a large number of positively stained cells in the TUNEL reaction, as compared to the preconditioned group. The PCNA assay (at 6 and 24 h of reperfusion) indicated that hepatic regeneration did not occur in steatotic and non-steatotic livers in any of the groups evaluated, but PCNA-positive hepatocytes were detected in liver samples corresponding to reduced size-liver transplantation used as positive controls (not shown).

Effect of preconditioning on NF-kB, AP-1 and HSF binding activity during ischemia

To investigate the molecular basis underlying IL-6 activation by IP, we analyzed the activity of the transcription factor NF-kB, which has a recognized role in the activation of IL-6 [40]. A marked increase (about 5-fold) over basal level of binding activity present in extracts of SH samples was observed at the end of the ischemic period in normal and fatty livers which were previously subjected to preconditioning (IP/Isc) (Figure 3(A)). Conversely, IP and Isc alone did not produce any effect. The specificity of the signal for NF-kB DNA complexes was demonstrated by competition assays (Figure 3(A)). The binding activity of the constitutively expressed transcription factor Oct-1 was used to assess equal loading. To identify which NF-kB subunit was activated by the IP/Isc treatment, we performed supershift experiments using Ab for the p50 and p65 NF-kB subunits (Figure 3(B)). In both normal and fatty livers, the anti-p50 antibody shifted both the fast and slow complexes, whilst the anti-p65
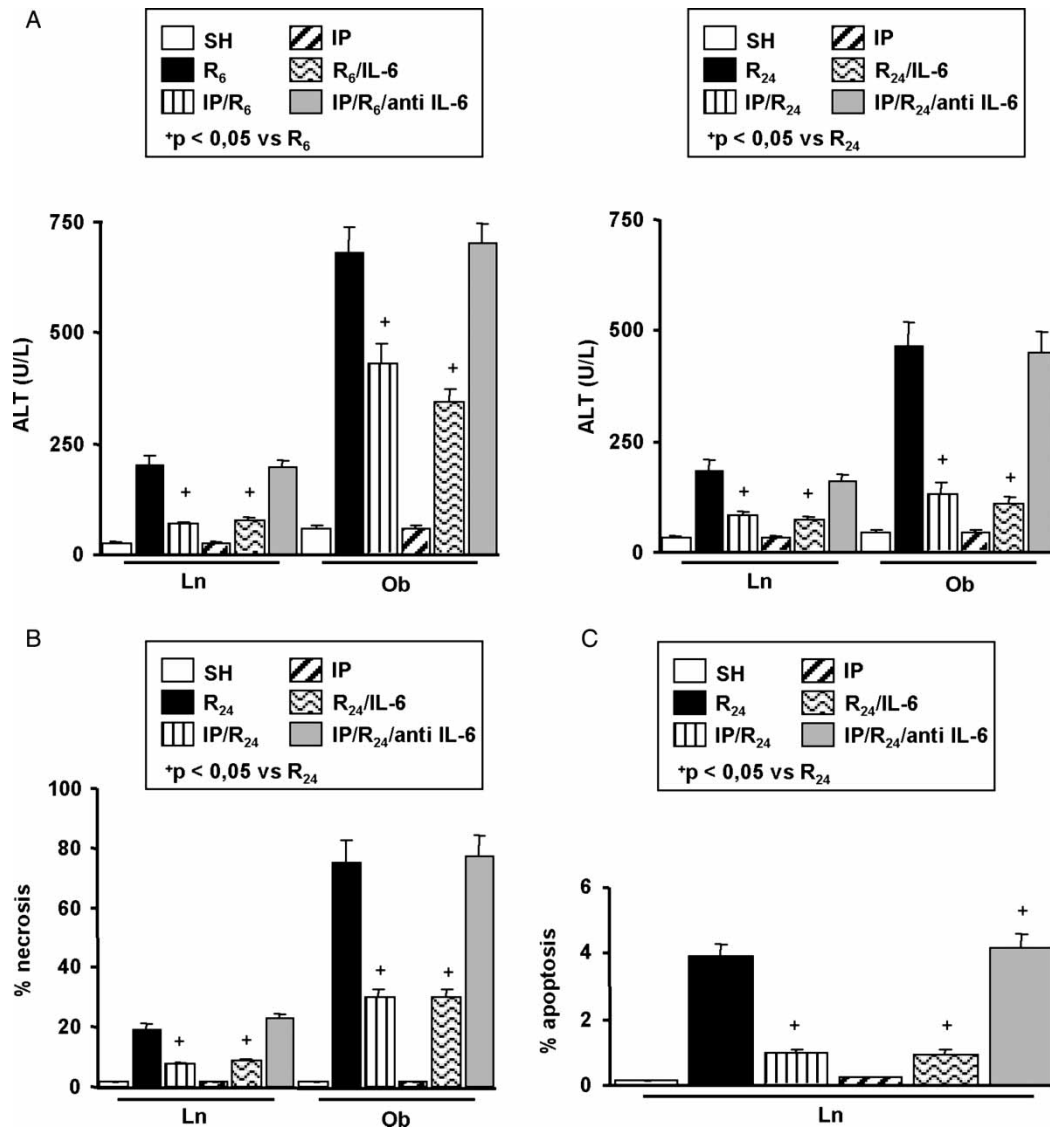
Figure 2. Effect of IP or pharmacological modulation of IL-6 on hepatic injury. (A) Plasma ALT levels were evaluated in Ln and Ob rats subjected to SH group; 60 min of Isc followed by 6 and 24 h of reperfusion (R group); reperfusion with prior IP/R group; IP group; R/IL-6 group; and IP/R/antiIL-6 group. Percentage of hepatic grade 3 necrosis (B) was evaluated in Ln and Ob rats whereas apoptosis (C) was evaluated in Ln rats. Abbreviations as in figure 2(A).

antibody immunodepleted only the slow complexes, thus showing that the two bands are composed of the p50–p50 homodimer and p50–p65 heterodimer, respectively.

We also evaluated the binding activity of the AP-1 transcription factor, which was not induced by any treatment in non-steatotic livers, but was clearly evident in livers of BSO-treated animals, used as a positive control [33] (results not shown).

Since IL-6 has been shown to be induced in concomitance with activation of the HSF [41], we also investigated the activity of this transcription factor (Figure 3(C)). In normal livers HSF binding capacity was absent in (SH) nuclear extracts and activated by Isc, in agreement with previous results [42]. However, HSF was also activated by preconditioning treatment

alone (IP), whilst the extent of activation was reduced by 50% in (IP/Isc), as compared to (Isc). A similar pattern was present in fatty livers, but the extent of induction was lower.

Effect of preconditioning on STAT binding activity during ischemia and reperfusion

Since it has been proposed that IP-mediated NF-kB activation during Isc induces the successive expression of IL-6, STAT3 and cyclin, which in turn is important for proliferation of liver cells and hence for hepato-protection [43], we next analysed the role of STAT transcription factors. STAT binding activity was remarkably induced over that of SH in both normal and fatty livers at 2 and especially at 6 h of reperfusion
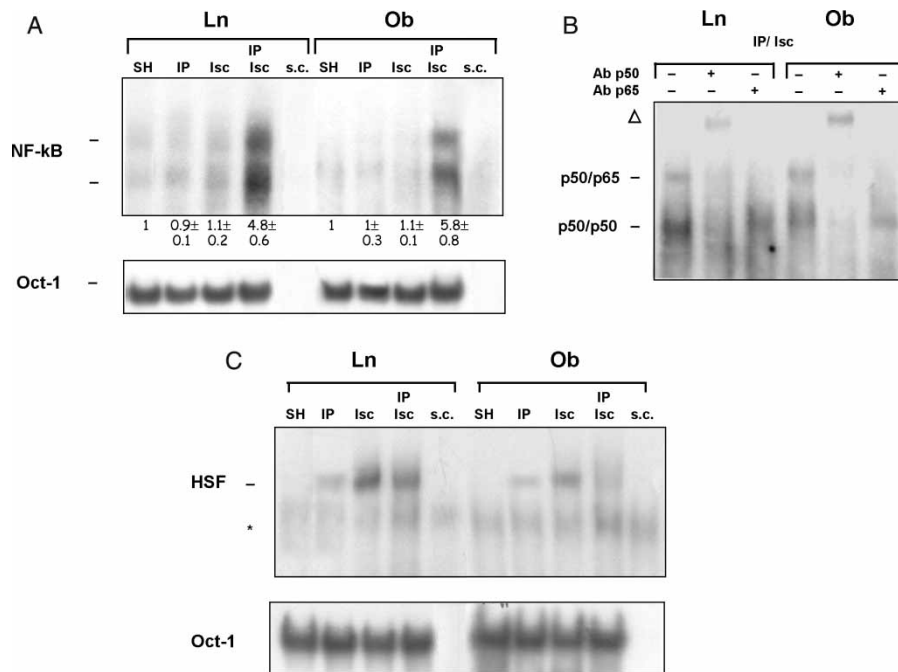
Figure 3.   Effect of IP on NF-kB and HSF DNA-binding activity. (A) EMSA analysis of NF-kB and Oct-1 DNA-binding activities in livers of Ln and Ob rats subjected to SH; IP; Isc; IP/Isc; specific competition (sc) of the IP/Isc sample. The values indicate the fold difference $\pm$ SE relative to the respective SH control. (B) Supershift assay of the nuclear extract of the IP/Isc sample with Ab against p50 and p65 subunits (Ab p50 and Ab p65). The shifted p50 band is indicated by Δ. (C) EMSA analysis of HSF and Oct-1 DNA-binding activities in livers of Ln and Ob rats: abbreviations as in Figure 3(A); specific competition of the Isc sample. Asterisk indicates a non-specific band. For each panel, one representative of four similar autoradiograms obtained with extracts of different animals is shown.

(R_2 and R_6, Figure 4(A)). No detectable binding activity was found in IP, Isc, or IP/Isc treatments, in agreement with previous findings obtained with the same DNA sequence [43] (results not shown). Preconditioning reduced by 50% the extent of induction occurring during reperfusion. Supershift assays with specific Ab against different members of the STAT family (STAT1, STAT3 and STAT5) demonstrated that the STAT3 protein is the major component of the binding complex detected by EMSA in the (R_6) and (IP/R_6) samples of both normal and fatty livers (Figure 4(B)). In summary, these results show that IP impairs the STAT3 activation of DNA binding capacity occurring during reperfusion in the livers of both Ln and Ob rats.

Effect of preconditioning on reperfusion-induced oxidative stress

The demonstration that the increased IL-6 levels induced by ischemic preconditioning were not associated with STAT3 activation, prompted us to investigate other possible mechanisms involved in hepatoprotection and therefore we evaluated whether IP abrogated oxidative stress during reperfusion.

Analysis of the binding activity of NF-kB and AP-1, which are activated by a variety of different stimuli but are mainly involved in the stress response and have a recognized role in the activation of anti-oxidant genes [4], showed that IP abolished or reduced the induction of both transcription factors occurring at 2 and 6 h of reperfusion in both Ln and Ob rat livers (Figure 5(A), compare R_2 and R_6 with IP/R_2 and IP/R_6, respectively).

We also analyzed the effect of IP on the expression of the stress-responsive HO-1 gene. IP reduced the significant increase in the levels of HO-1 occurring at 6 h of reperfusion in normal as well as fatty livers, both at the mRNA and protein levels (Figure 5(B)). These results demonstrated that IP leads to a reduced, or absent, increase of oxidative stress-related genes induced by reperfusion in both normal and fatty livers. The role of IL-6 in HO-1 expression was further confirmed by the demonstration that HO-1 protein levels in IL-6 pretreated rats (R/IL-6) were similar to those found in the preconditioned group (IP/R) while inhibition of IL-6 action in the preconditioned group (IP/R/antiIL-6) increased HO-1 protein to levels similar to those found in the non-preconditioned group (R) (Figure 5(C)).

The reduced response of stress-responsive genes may be related to a IP-mediated defense against reperfusion damage and in particular to the protection that IP confers on the increased vulnerability of fatty livers to I/R injury [6]. Since previous studies [6,9] have shown that MDA levels in Ln rats were not significantly modulated in the various experimental
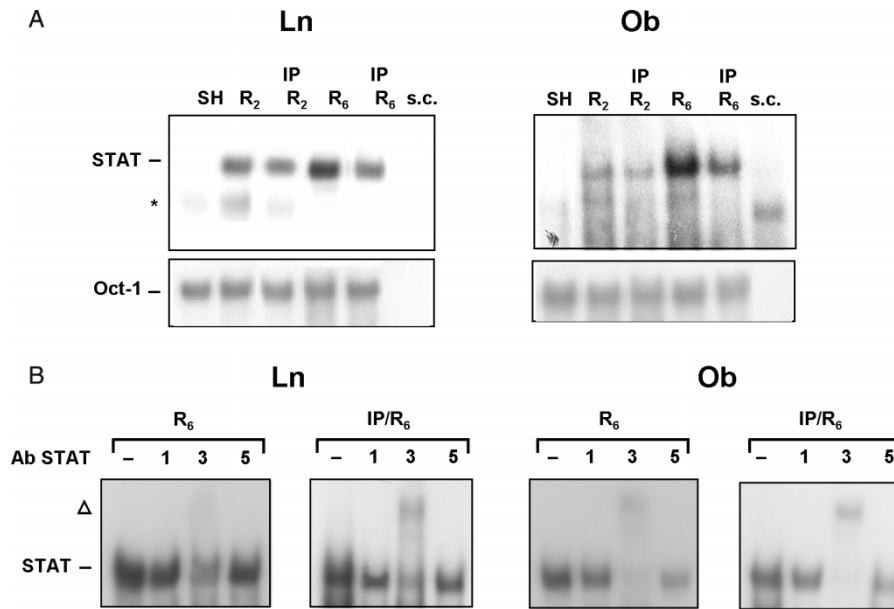
Figure 4. Effect of IP on STAT DNA-binding activity. (A) EMSA analysis of STAT and Oct-1 activities in livers of Ln and Ob rats: subjected to reperfusion for 2 and 6 h (R_2 and R_6); preconditioned reperfusion for 2 and 6 h (IP/R_2 and IP/R_6); sc of sample R_6. Asterisk indicates a non-specific band. (B) Supershift assay of the nuclear extracts of the R_6 and IP/R_6 samples with Ab against STAT1, STAT3 and STAT5 subunits. The shifted STAT3 band is indicated by Δ. For each panel, one representative of four similar autoradiograms obtained with extracts of different animals is shown.

groups examined in the present work, all the determinations reported subsequently and addressing the role of oxidative damage were performed only in Ob rats. IP reduced the strong increase in MDA levels found in fatty livers at 6 and 24 h after re-establishment of blood flow (Figure 6(A)). We also evaluated GSH levels in fatty livers at 6 and 24 h of reperfusion. Figure 6(B) shows that reperfusion leads to a significant reduction in GSH levels with respect to the SH group. The role of oxidative stress was confirmed by the finding that administration of the anti-oxidant GSH-ester resulted in significantly reduced MDA (Figure 6(A) compare second and last column) and ALT (359 ± 41 vs. 681 ± 59 at 6 h and 109 ± 12 vs. 466 ± 53 U/l at 24 h) levels during hepatic reperfusion.

To understand whether IL-6 is involved in the preconditioning-mediated prevention of oxidative stress, we evaluated MDA and GSH levels at 6 and 24 h of reperfusion (Figure 6(A) and (B)) in the livers of rats pretreated with IL-6 or anti-IL-6 Ab. MDA and GSH levels in IL-6 pretreated rats (R/IL-6) were similar to those found in the preconditioned group (IP/R). Conversely, inhibition of IL-6 action in the preconditioned group (IP/R/antiIL-6) increased MDA and GSH to levels similar to those found in the non-preconditioned group (R).

Discussion

Despite the large number of studies investigating the mechanisms of hepatoprotection by IP, knowledge of the molecular events promoting the tolerance of normal and fatty livers to reperfusion stress is still limited. In the present study, we examined several molecular mechanisms and indices of liver stress and damage at different time points to analyze both early and late processes set in motion by IP. We showed that IP is associated with higher IL-6 levels during reperfusion and reduction of oxidative stress. IL-6 has a particular role among inflammatory cytokines and its effects are considered beneficial; in particular, increasing evidence indicates a protective role of IL-6 in both normal and fatty livers [12,13]. However, both up-and down-regulation of endogenous IL-6 expression have been found to correlate with the hepatoprotective effect of IP [14–18]. In the present study, we found that differences in IL-6 levels correlated with changes in liver injury. In addition, the results obtained with the pharmacological modulation of IL-6 indicate the protective role of this cytokine in I/R processes in both types of livers and its potential role in the benefits of IP. The analysis of the activity of several transcription factors in the same experimental settings allowed us to identify molecular events possibly involved in IL-6 induction, as well as reduction of oxidative stress as a possible pathway underlying the protective effect that this cytokine may have against I/R damage in fatty livers.

A number of considerations lead us to think that the induction of hepatic IL-6 synthesis in both Ln and Ob rats depends on transcriptional events occurring during preconditioning (HSF activation) and pre-conditioned Isc (NF-kB activation). In fact, IL-6 has been shown to be induced in concomitance with activation of HSF [41], that we have shown to be
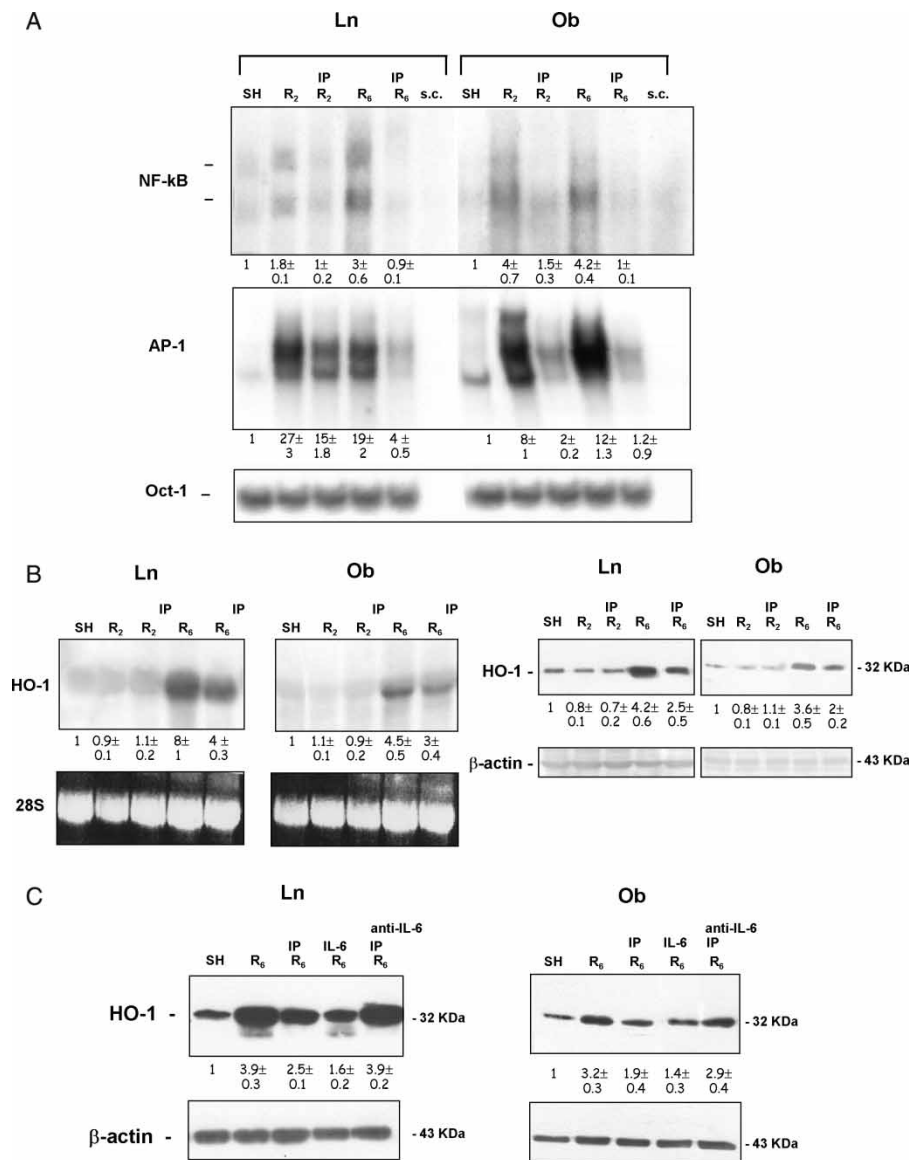
Figure 5. (A) Effect of IP on NF-kB and AP-1 DNA-binding activity during reperfusion. EMSA analysis of NF-kB, AP-1 and Oct-1 activities in livers of Ln and Ob rats. Abbreviations as in Figure 4(A). One representative of four similar autoradiograms obtained with extracts of different animals is shown. The values indicate the fold increase ± SE relative to the SH lean rat (first lane). (B) Effect of ischemic preconditioning on HO-1 expression. Northern blot (left) and immunoblotting (right) analysis of HO-1 expression in livers of Ln and Ob rats. Abbreviations as in Figure 4(A). The blots are representative of results obtained with four different animals for each treatment. The values indicate the fold variations ± SE relative to the respective SH samples. (C) Immunoblotting analysis of HO-1 protein levels in livers of Ln and Ob rats subjected to SH; reperfusion for 6 h (R_6); preconditioned reperfusion for 6 h (IP/R_6); reperfusion with prior IP/R_6/antiIL-6 group. The blots are representative of results obtained with extracts of four different animals for each treatment. The values indicate the fold variations ± SE relative to the respective SH samples.

activated by preconditioning (Figure 3). To our knowledge, this is the first time that the activation of a transcription factor during the short period of IP is reported. Such an induction may occur because of the particular characteristics of activation of this transcription factor, which are tailored to ensure a fast response [44]. Regarding NF-kB, the overexpression of the p65 subunit of NF-kB has been shown to activate the transcription of the IL-6 gene [45]; therefore, it is possible that the increase in NF-kB activity induced during IP/Isc in both types of livers

is associated with IL-6 production. Nitric oxide, adenosine, TNFα, etc. which are produced during the short period of preconditioning [1,5], are among the mediators possibly involved in the early activation of NF-kB.

How the IP-mediated modulation of cytokine production [9] and in particular the prevalence of IL-6, may impart protection? A model recently proposed to account for the protective effect of IP in the liver suggests that NF-kB activation during Isc induces the successive expression of IL-6, STAT3 and
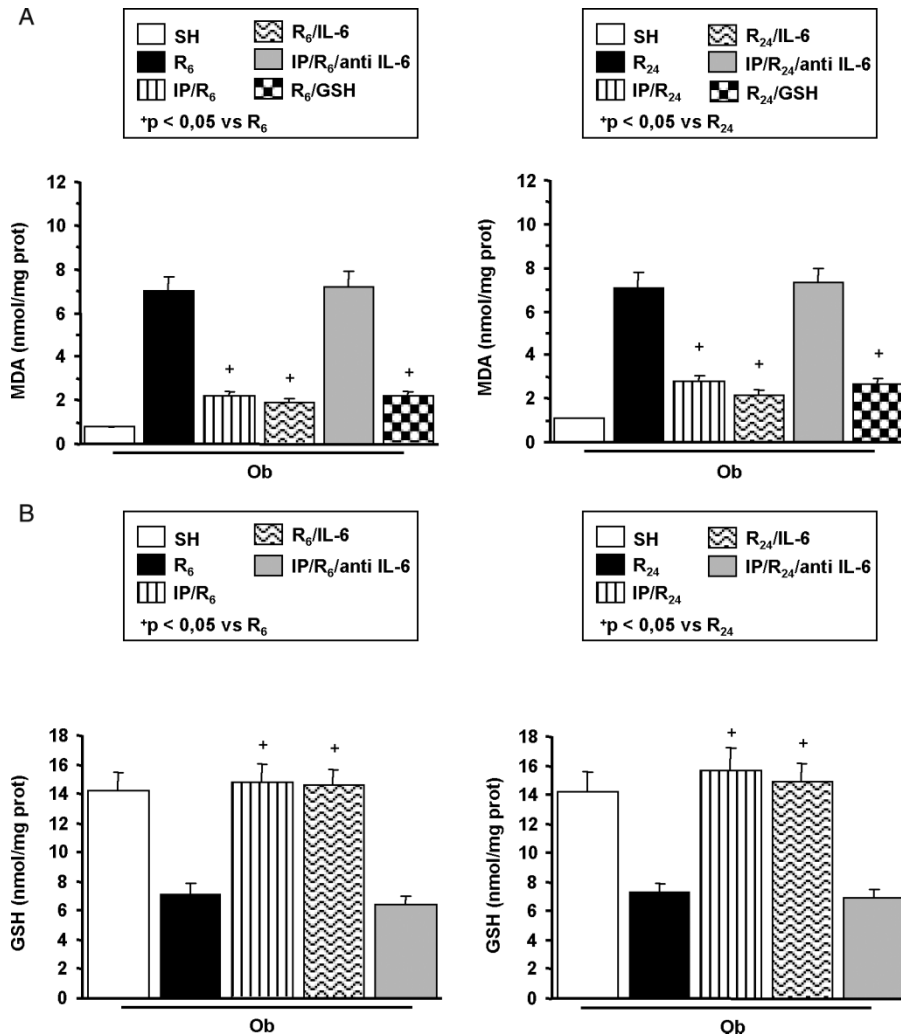
Figure 6.   Effect of IP and pharmacological modulation of IL-6 on liver lipid peroxidation and GSH levels. (A) MDA levels were evaluated in livers of Ob rats subjected to SH group; 60 min of Isc followed by 6 and 24 h of reperfusion (R group); reperfusion with prior IP/R group; IP group; R/IL-6 group; and IP/R/antiIL-6 group; R/GSH. (B) GSH levels were evaluated in livers of Ob rats. Abbreviations as in Figure 6(A).

cyclin, which in turn is important for proliferation of liver cells and hence for hepatoprotection [43,46]. Indeed, in the heart, IP-mediated activation of STAT has been demonstrated to play an important role in myocardial protection against reperfusion damage [47]. On the other hand, we found that IP abated the increase in STAT3 binding activity occurring during reperfusion. Since we have shown that STAT3 is activated also by oxidative stress caused by glutathione depletion [33], it is conceivable that, in the absence of IP, hepatic STAT3 is activated during reperfusion by oxidative stress. Thus, the impaired STAT3 activation during reperfusion in preconditioned livers may be related to the IP-dependent reduction of oxidative stress (see below). The discrepancy between our results and those of a previous study [43,46] might be explained by the different experimental protocol. In fact, Teoh et al. applied Isc for 90 min, hence seriously damaging the liver and probably causing extensive necrosis [48], resulting in turn in a

regenerative response that may benefit from increased cyclin levels. In contrast, after 60 min of Isc, as applied in our experimental model, the damage to the liver is limited [8,49] and consequently, no regeneration ensues as demonstrated by the absence of changes in PCNA levels (this study) and thymidine incorporation [50].

An alternative and likely pathway by which the higher IL-6 production induced by IP could attenuate I/R damage is to abate oxidative stress. This is particularly evident for Ob rats because in the present study we have shown that IP prevented a number of events occurring during reperfusion: (a) the induction of stress-sensitive transcription factors (AP-1 and NF-kB); (b) the modulation of the stress-responsive gene HO-1; and (c) oxidative liver damage (MDA and GSH). Notably, pharmacological modulation of IL-6 levels affected the expression of HO-1 and the accumulation of MDA and GSH, thus showing the link between IL-6 and oxidative stress. The fact that

stress responsive genes (NF-kB and AP-1 activity as well as HO-1 expression) but not markers of oxidative damage, such as MDA content, are modulated in Ln rats might indicate that reduction of oxidative stress is less involved in IP-mediated protection to non-steatotic livers.

The reduced activity of NF-kB and AP-1 during reperfusion in preconditioned livers may inhibit the late inflammatory response [51]. In agreement with this hypothesis, the attenuation of NF-kB activity after 1 h of reperfusion occurring in preconditioned non-fatty mouse livers and the subsequent reduction in the level of inflammatory cytokine expression have been implicated in the protective mechanism of IP against hepatic I/R injury [52].

In conclusion, the results of the present study characterize a possible pathway linking the inflammatory response and oxidative stress, the two major events modulated by IP. In fact, the early activation of NF-kB and HSF is possibly involved in IL-6 induction, with consequent shift in the balance of pro-and anti-inflammatory cytokines [9] and abatement of oxidative stress; in turn, this prevents the subsequent inflammatory response and liver injury. The lower IL-6 levels and the exacerbated oxidative stress observed in reperfused steatotic livers, together with the previously demonstrated induction of IL-1β [9], could explain the vulnerability of this type of livers to I/R injury. The greater effect of IP on cytokines and oxidative stress may help explain the well-known stronger protective effect of IP in Ob rats [6].

The demonstration that increased hepatic IL-6 production plays a role in IP-mediated hepatoprotection supports pioneering studies showing that exogenous IL-6 administration limits I/R injury in non-fatty livers [13]. Only long-term administration of IL-6 (10 days) protected mice with fatty livers from I/R injury [12], suggesting that a major effect of IL-6 was related to its role in slowly ameliorating hepatic steatosis. However, in this study, a much shorter IP-mediated increase of hepatic IL-6 production was sufficient to give a correlation with hepatoprotection. This indicates an additional and novel role of IL-6, probably not involving a decrease of hepatic lipid content, in improving the higher susceptibility of steatotic livers to I/R damage.

Acknowledgements

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