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Acute Ethanol Binge Followed by Withdrawal Regulates Production of Reactive Oxygen Species and Cytokine-Induced Neutrophil Chemoattractant and Liver Injury During Reperfusion After Hepatic Ischemia

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ABSTRACT

This work tests the hypothesis that withdrawal from an acute ethanol binge regulates the production of reactive oxygen species (ROS) and chemokines by Kupffer cells, and as a result compromises or protects the liver from injury. Male Sprague–Dawley rats received an intravenous ethanol bolus (1.75 g/kg), followed by an intravenous infusion of 200–300 mg/kg/h for 12 h. At 12 h, ethanol infusion was stopped and replaced by saline. At 18 h, rats were subjected to 45 min of partial hepatic ischemia, followed by 0–24 h of reperfusion (I/R). At specific time points, Kupffer cells were isolated for superoxide anion assay and CINC (cytokine-induced neutrophil chemoattractant) and MIP-2 (macrophage inflammatory protein-2) production *in vitro*. Alanine transferase (ALT) activity, endotoxin, CINC, and MIP-2 were measured in serum samples taken at appropriate intervals. Results show that at 3 h post reperfusion, serum ALT was significantly elevated in the ethanol-treated group + I/R, compared with the saline + I/R group. ROS production by Kupffer cells at this time was also significantly increased compared with the saline + I/R group. However, ethanol withdrawal + I/R did not significantly alter CINC and MIP-2 production at 3 h of reperfusion. After 24 h, serum ALT was lower in the ethanol + I/R group than in the saline + I/R group. Superoxide anion and MIP-2 releases by Kupffer cells were not statistically significantly different between these two groups at this time. CINC production by Kupffer cells from the ethanol-treated + I/R group was significantly lower than in the saline + I/R group. Concomitantly, CINC and nuclear factor-κB (NF-κB) mRNAs and NF-κB translocation and binding in Kupffer cells in this treatment group were down-regulated. Moreover, the number of polymorphonuclear neutrophils (PMNs) sequestered in the liver was significantly lower in the ethanol + I/R group than in the saline-treated group. ROS and chemokine productions in sham animals with or without ethanol were lower than in the I/R group. These data suggest that acute ethanol binge followed by withdrawal may compromise the liver to injury during the early phase, whereas in the later phase it may be protective. Furthermore, these results support the notion that Kupffer cells are involved in hepatic injury in the early phase, whereas PMNs participate more actively during the later phase of reperfusion. *Antioxid. Redox Signal.* 4: 721–731.

INTRODUCTION

OXYGEN-DERIVED RADICALS are involved in the pathogenesis of liver injury during ethanol intoxication (11, 28) and reperfusion after hepatic ischemia (22). The liver is a rich source of proinflammatory mediators, primarily due to the large number of resident macrophages or Kupffer cells in this organ (8). The extracellular releases of reactive oxygen

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species (ROS) by Kupffer cells, and to a lesser extent endothelial cells, contribute to the initiation of hepatocyte injury during ethanol intoxication and reperfusion after ischemia. Hepatic sequestration of neutrophil occurs during reperfusion after hepatic ischemia (24, 25), endotoxemia (6), and chronic ethanol intoxication (2). As a result, polymorphonuclear neutrophils (PMNs), as well as resident Kupffer cells in the liver, are considered to play an important role in the initiation of hepatic injury under these pathological conditions, because of their ability to produce potent cytotoxic mediators. Acute ethanol infusion for 3–12 h does not enhance the migration of PMNs into the liver (4). In fact, acute ethanol intoxication suppresses lipopolysaccharide (LPS)-mediated sequestration of PMNs in the liver (10).

During chronic ethanol intoxication, endotoxemia, and reperfusion after hepatic ischemia, adhesion molecules and chemotactic cytokines or chemokines mediate migration of inflammatory leukocytes in the liver. Among these chemokines are the α and β chemokines. α or CXC (cysteine–variable amino acid–cysteine) chemokines generally attract PMNs to the site of inflammation, whereas β or CC (cysteine–cysteine) chemokines are chemoattractant for mononuclear cells (31). During reperfusion after partial hepatic ischemia, enhanced neutrophil emigration into the liver is presumably due in part to the local production of α-chemokines by hepatic nonparenchymal and parenchymal cells. In the rat, cytokine-induced neutrophil chemoattractant factor (CINC) and macrophage inflammatory protein-2 (MIP-2) are implicated in the sequestration of PMNs in the liver (5). These chemokines are produced by macrophages and hepatocytes in response to endotoxin, ethanol, tumor necrosis factor-α (TNFα), and interleukin-1β (15, 34, 37).

We have previously demonstrated that acute ethanol intoxication enhances the production of ROS in a time-dependent manner (4). Withdrawal of ethanol after an acute ethanol binge for 12 h also enhances the release of ROS and induces mild hepatic injury (4). This condition does not enhance neutrophil migration into liver (4). During the early phase of reperfusion after hepatic ischemia, Kupffer cells (but not hepatic PMNs) are primed for enhanced ROS production (25). Thus, this work examines the effect of the combined impact of ethanol withdrawal and reperfusion after partial hepatic ischemia on free radical release, hepatic injury, and chemokine release. This work is based on the rationale that, in humans, ethanol consumption for several hours followed by withdrawal and trauma (as a result of accident or assaults) may compromise or even protect the host from enhanced morbidity and even mortality.

MATERIALS AND METHODS

Preparation of experimental animals

Acute ethanol binge followed by withdrawal: The day before the experiments, arterial and venous catheters were implanted in male Sprague–Dawley rats (250–300 g; Charles River Breeding Laboratories, Cambridge, MA, U.S.A.) using aseptic surgical techniques. A group of rats received an ethanol bolus [20% (vol/vol) in sterile saline intravenously] at a dose of 1.75 g/kg of body weight followed by continuous infusion at a rate of 200–300 mg/kg/h for 12 h. Control rats received a similar volume of saline. Infusion was stopped at 12 h, and the animals were allowed to recover from ethanol for another 6 h. This phase is termed the ethanol withdrawal phase.

Partial hepatic ischemia followed by reperfusion: At 6 h after the infusion was stopped, the rats were subjected to partial hepatic ischemia followed by reperfusion. This procedure has been described previously (25, 26). In brief, after the rats were anesthetized with pentobarbital (35 mg/kg i.p.), an abdominal incision was made to expose part of the liver. The blood vessels supplying the median and left lateral hepatic lobes were occluded with an atraumatic Glover bulldog clamp for 45 min. Reperfusion was initiated by removing the clamp. The abdominal incision was surgically closed, and the animals were allowed to recover. Other groups of saline- and ethanol-treated rats were subjected to sham surgery. These procedures were performed under aseptic conditions. Blood samples were collected 1 min before the initiation of ischemia, at 0–24 h of reflow or reperfusion. These samples were used for the determination of serum alanine transaminase (ALT), endotoxin, and chemokines. Figure 1 shows the outline of the experimental protocol described above.

Measurement of serum ALT, endotoxin, and chemokines

Serum ALT and endotoxin were measured using diagnostic kits from Sigma (St. Louis, MO, U.S.A.). Chemokines (MIP-2 and CINC) were measured using enzyme-linked immunosorbent assay (ELISA) kit and antibody pairs from BioSource (Camarillo, CA, U.S.A.).

Isolation of Kupffer cells and neutrophils

At 3 and 24 h after reperfusion, the rats were anesthetized with pentobarbital (35 mg/kg i.p.) and subsequently subjected to liver perfusion with Hanks' balanced salt solution containing collagenase (250 μg/ml), $CaCl_2$ (550 μg/ml), and bovine serum albumin (1 mg/ml) for 15 min at 37°C. The liver was cut into small pieces, suspended in Gey's buffer, and shaken for 5 min. They were further digested in Hanks' con-

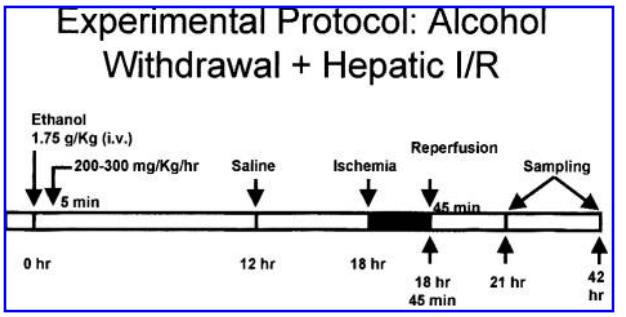

FIG. 1. Experimental protocol used in this study. Rats were given an intravenous bolus and infusion of ethanol for 12 h. At this time, ethanol was replaced with saline (ethanol withdrawal phase). Rats were subjected to hepatic ischemia for 45 min at 6 h of ethanol withdrawal. Reperfusion was induced for 24 h. I/R, ischemia/reperfusion.

taining Pronase E (20 mg/g of liver). The cell suspensions were subjected to centrifugal elutriation to isolate the Kupffer cells and PMNs sequestered in the liver. Kupffer cells were collected at 45 ml/min fraction. PMNs were separated from Kupffer cells by density gradient centrifugation (Nycoprep, 1.077 Animal, Accurate Chemicals). Using this method, cell viability was >95%.

Superoxide anion assay on isolated cells

Superoxide anion assay on isolated cells was performed according to the procedures described previously (6). Isolated Kupffer cells and hepatic PMNs in Hanks' balanced salt solution were each layered onto a 35-cm sterile petri dish (Costar) at a final cell density of 0.75–1.0 $\times$ 10^6 cells/plate. Ferricytochrome *c* (50 µmol; Sigma) was added to the reaction mixture. Superoxide dismutase (SOD; 300 units/well) was added to the negative controls. Each assay was performed in triplicate. Superoxide anion was measured based on a change in absorbance (difference in absorbance with or without SOD) against a cell-free blank. Delta absorbance was converted to nanomoles using the molar extinction coefficient of 21.1 mM^{-1} cm^{-1}. Superoxide anion is expressed in nanomoles per 10^6 cells per hour.

Primary culture of Kupffer cells

Isolated Kupffer cells were suspended in Dulbecco minimum essential medium (Sigma) containing 10% fetal bovine serum and antibiotics. They were cultured for 24 h in a humidified environment containing 7.5% CO_2. At the end of the incubation period, culture supernatants were assayed for CINC and MIP-2 using commercially available antibody pairs (CINC) and ELISA kits (MIP-2) from BioSource.

Reverse transcription–polymerase chain reaction

To determine the regulation of chemokine mRNA and TNF signaling protein mRNA in Kupffer cells, these cells were suspended in RNAlater (Ambion, Austin, TX, U.S.A.) and stored at 4°C until assayed. Cells in RNAlater were washed in phosphate-buffered saline and their RNAs extracted using TRI-chloroform mixture (Sigma). From this preparation, first single-stranded cDNA was transcribed using the cDNA kit (Invitrogen, Carlsbad, CA, U.S.A.). cDNA was amplified in a PCR reaction containing 2.5 U of Taq DNA polymerase (Sigma), 0.08 m*M* dNTP, and 0.1 p*M* primers KC and RANTES. Primers for CINC and TNF signaling proteins (CytoXpress Multiple cDNA amplification kit) were obtained from BioSource International. Thermocycling conditions for PCR were 94°C (1.5 min) for denaturation, followed by 35 cycles at 94°C for 30 s, annealing at 60°C for 45 s plus another 45 s at 72°C, and a final 7 min at 72°C (Bio-Rad GeneCycler, Bio-Rad Laboratories, Hercules, CA, U.S.A.). β-Actin mRNA (457 bp) was used as housekeeping gene. PCR products were electrophoresed on 2% agarose (Invitrogen). The gels were stained with ethidium bromide (5 µg/ml) and analyzed using GelDoc 8000 (UVP).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of Kupffer cells were obtained by homogenizing cell lysates with buffer A containing 10 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* dithiothreitol and 1 m*M* phenylmethylsulfonyl fluoride in a Dounce homogenizer. The cell lysate was kept in ice for 10 min and centrifuged at 850 *g* for 10 min at 4°C. The supernatant was removed, and the cell pellet was resuspended in buffer A containing 0.1% Triton, incubated for 10 min at 4°C, and spun at 850 *g* for 10 min. The cell pellet was washed twice with buffer A and was resuspended in 100 µl of buffer C [20 m*M* HEPES, pH 7.9, 25% glycerol (vol/vol), 0.42 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol, and 1 m*M* phenylmethylsulfonyl fluoride]. Nuclear proteins were recovered after centrifugation at 20,000 *g* for 15 min at 4°C. ^{32}P-end-labeled double-stranded nuclear factor-κB (NF-κB) consensus oligonucleotide (0.1 ng/10,000 cpm; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added to the nuclear extracts and incubated for 20 min at 20°C. The samples were electrophoresed on 4% polyacrylamide gel. Radioactivity was measured using an Ambis Radioactive Imaging System (San Diego, CA, U.S.A.). The gels were dried and subjected to autoradiography. These procedures were based on the methods described previously (7, 17).

Statistics

Data presented in this article represent means ± SEM of five to seven rats per treatment group. Statistical significance at $p < 0.05$ was assessed by ANOVA followed by Student–Neuman–Keuls multiple comparisons test. Different letters indicate statistical significance. Thus, columns with the same letters were not statistically significantly different, whereas those with different letters were.

RESULTS

Serum ALT

Figure 2 shows that ethanol withdrawal after an acute ethanol binge slightly elevated serum ALT level compared with that of the parallel control group. At 0 h of reperfusion after 45 min of partial hepatic ischemia [ischemia/reperfusion (I/R)], serum levels of ALT were increased in both ethanol withdrawal and saline-treated groups. At 3–5 h of

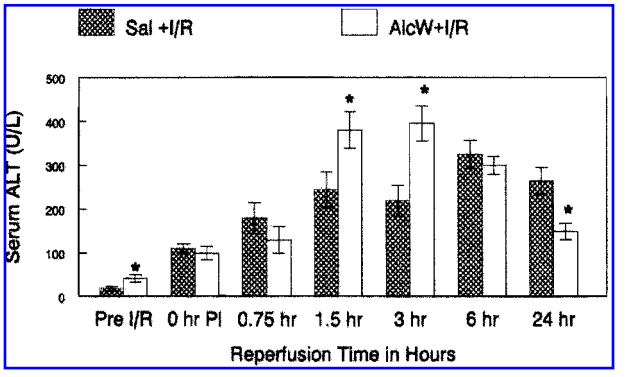

FIG. 2. Effect of ethanol withdrawal on serum ALT activity during reperfusion after hepatic ischemia (I/R). The asterisks indicate statistical significance at $p < 0.01$ vs. saline + I/R, $n = 7$ per group (Student's *t* test). AIcW, alcohol withdrawal.

reperfusion, the level of this enzyme was significantly elevated in the ethanol plus I/R exposed rats compared with the saline-treated I/R control. After 24 h, serum ALT level was significantly lower in the ethanol plus withdrawal-treated rats than the saline control. Serum ALT at 24 h was <25 U/L in rats treated with ethanol (plus withdrawal) or saline-treated rats subjected to sham surgery (Fig. 2).

Serum endotoxin

At 0 h of reperfusion after hepatic ischemia, serum endotoxin concentration in both ethanol- (68 ± 10 pg) and saline-treated rats (73 ± 8 pg/ml) increased fourfold compared with the preischemic level (<15 pg/ml). Serum endotoxin level remained above 60 pg/ml through 24 h of reperfusion in both treatment groups (saline and ethanol) subjected to I/R. In ethanol and saline + sham animals, serum endotoxin remained the same.

Serum chemokines

CINC and MIP-2 were measured in sera during reperfusion after hepatic ischemia. Figure 3 shows that CINC (<150 pg/ml) was present in sera before partial hepatic ischemia was initiated. Serum CINC remained elevated during the first 3 h and was significantly reduced at 24 h of reperfusion, compared with saline + I/R (Fig. 3). Serum MIP-2 was present in sera in very small amount (<25 pg/ml). This value did not significantly increase during reperfusion in the ethanol + I/R and saline + I/R groups. The values obtained in these groups were similar to those that were subjected to sham surgery with or without ethanol (Fig. 3).

Superoxide anion release

Figure 4 shows that at 3 h of reperfusion after hepatic ischemia, Kupffer cells were primed for enhanced basal release of superoxide anion. Superoxide release (6.5 ± 0.5 nmol/10^6 cells/h) was significantly increased in Kupffer cells from rats that were subjected to ethanol infusion followed by withdrawal and I/R. This value was significantly higher compared with that of the parallel group previously exposed to saline and I/R (4.2 ± 0.7 nmol/10^6 cells/h) (Fig. 4A).

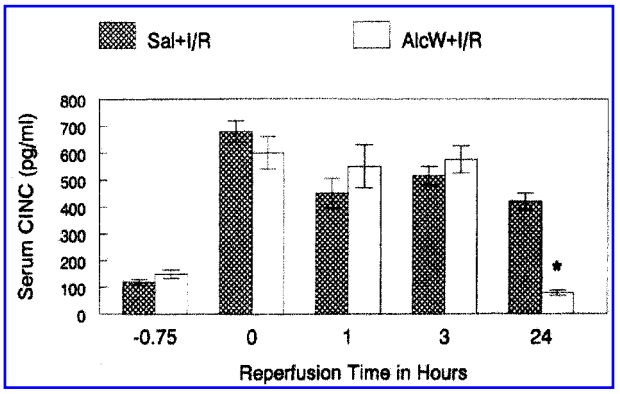

FIG. 3. Effect of ethanol withdrawal on serum CINC during reperfusion after hepatic ischemia (I/R). The asterisk indicates statistical significance at $p < 0.01$ vs. saline + I/R, $n = 7$ per group (Student's t test).

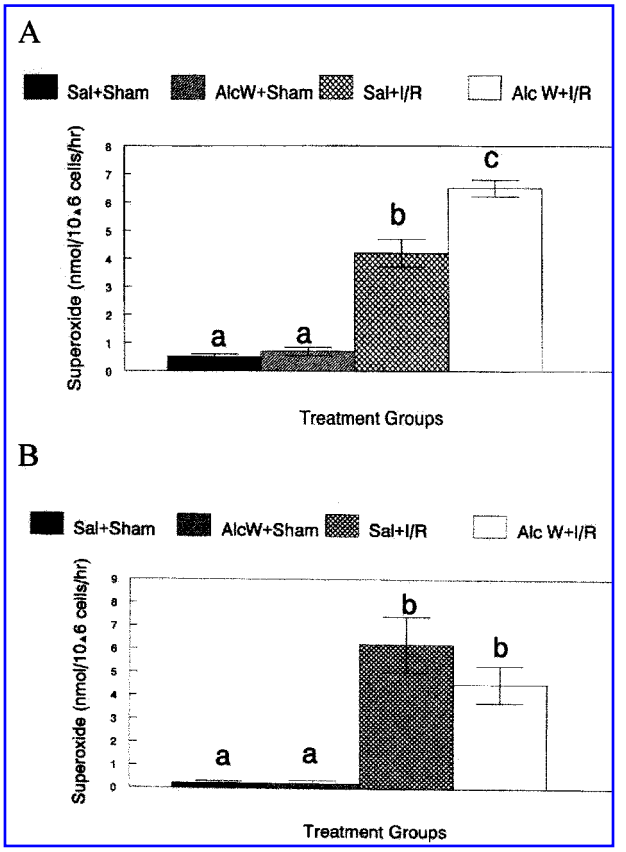

FIG. 4. Effect of ethanol withdrawal on superoxide anion production by Kupffer cells at 3 h (A) and 24 h (B) of reperfusion after hepatic ischemia. Kupffer cells were isolated at 3 h of reperfusion. Superoxide anion was measured by SOD-inhibitable reduction of cytochrome c in the absence of stimuli. Data were analyzed by ANOVA followed by Student–Neuman–Keuls multiple comparisons test. Different letters indicate statistical significance at $p < 0.05$. ($n = 7$ per group).

At 24 h after reperfusion, basal superoxide anion releases by Kupffer cells from saline + I/R and ethanol +I/R groups were still elevated (5.5 ± 0.5 nmol and 4.7 ± 0.9 nmol, respectively) (Fig. 4B). These values were not statistically different from each other. Spontaneous superoxide production by Kupffer cells from rats subjected to sham surgery with or without ethanol was <0.5 nmol/10^6 cells/h (Fig. 4B).

CINC production by Kupffer cells

Kupffer cells were isolated from four treatment groups described above. They were cultured for 24 h in the absence of stimuli to determine spontaneous production of CINC *in vitro*. Figure 5 shows that Kupffer cells from saline + I/R (3,300 ± 500 pg/10^6 cells/24 h) released a large amount of CINC compared with those obtained from rats subjected to ethanol withdrawal and I/R (750 ± 65 pg). The amount of CINC produced by Kupffer cells from ethanol withdrawal and

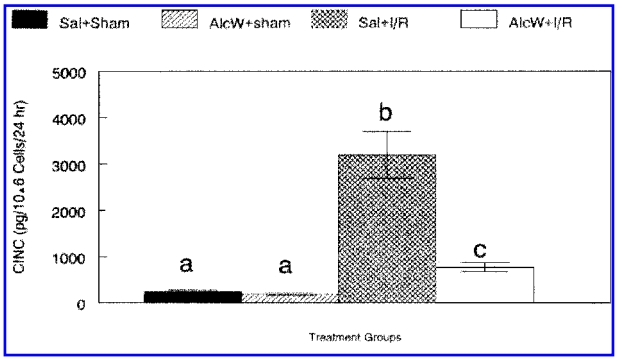

FIG. 5. Effect of ethanol withdrawal on CINC production by Kupffer cells at 24 h of reperfusion after hepatic ischemia. Kupffer cells were isolated at 24 h of reperfusion. CINC in 24-h culture supernatant was measured by ELISA. Data were analyzed by ANOVA followed by Student–Neuman–Keuls multiple comparisons test. Different letters indicate statistical significance at $p < 0.01$ ($n = 7$ per group).

saline-treated rats subjected to sham surgery was <300 pg (Fig. 5).

CINC mRNA expression was also analyzed in freshly isolated Kupffer cells. Results show that CINC mRNA (0.8 ± 0.06 CINC mRNA/β-actin mRNA, mean ± SEM from four independent experiments/group) was endogenously expressed in normal Kupffer cells. After hepatic I/R, CINC mRNA expression (1.5 ± 0.09; $p < 0.001$ vs. all treatment groups) was significantly enhanced. After ethanol withdrawal followed by I/R, CINC mRNA (0.52 ± 0.02) was down-regulated. Ethanol withdrawal alone did not have any significant effect on CINC mRNA expression (0.68 ± 0.032) in Kupffer cells. Representative gels are shown in Fig. 6.

MRNA expression for TNF signaling proteins

Figure 7A shows the effect of alcohol withdrawal and I/R on mRNA expression for TNF signaling proteins in Kupffer cells. Ethanol withdrawal + I/R was associated with the down-regulation of mRNAs for NF-κB, LICE, and TNFα compared with saline + I/R. Figure 7B shows representative gels from three independent experiments.

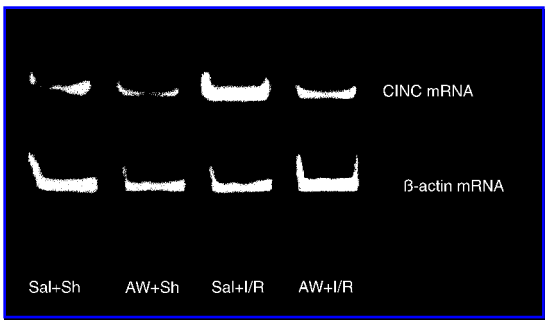

FIG. 6. Effect of ethanol withdrawal on CINC mRNA expression in Kupffer cells at 24 h of reperfusion after hepatic ischemia. Representative gels from from independent experiments are shown. Sh, sham; Aw, alcohol withdrawal.

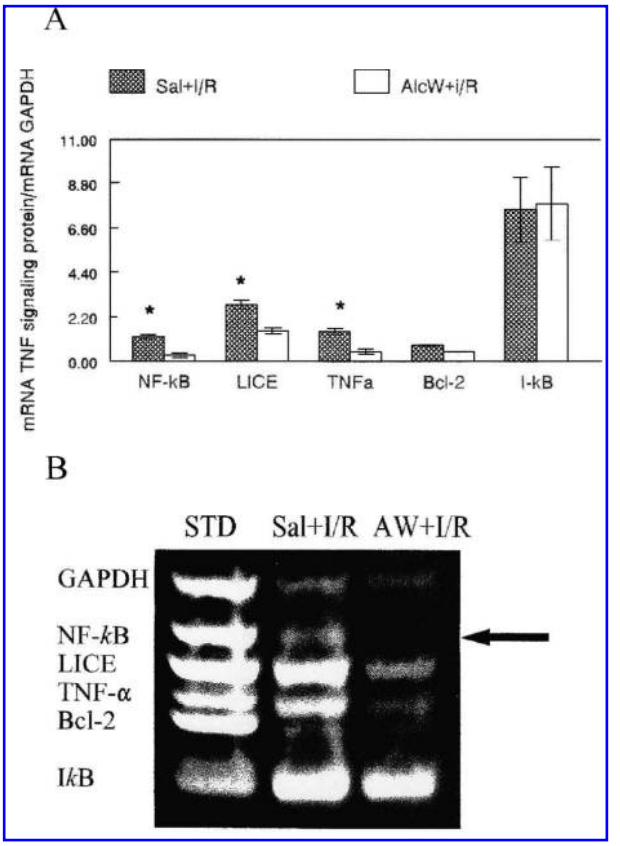

FIG. 7. Effect of ethanol withdrawal on mRNA expression for TNF signaling proteins in Kupffer cells at 24 h of reperfusion after hepatic ischemia. The expression of mRNA for TNF signaling proteins was analyzed using the CytoXpress Multiple cDNA amplification kit from BioSource. Units are based on the ratio of mRNA for TNF signaling proteins versus mRNA for glyceraldehyde-3-phosphatedehydrogenase(GAPDH) (**A**). Data were analyzed by Student's *t* test. The asterisks indicate statistical significance at $p < 0.05$ versus alcohol withdrawal + I/R ($n = 3$ per group). Lower panel **(B)** shows representative gels from three independent experiments.

NF-κB binding

Figure 8A shows that nuclear NF-κB binding (to the NF-κB consensus oligonucleotide) in the Kupffer cells from rats treated with saline and subjected to I/R was significantly enhanced. In the ethanol withdrawal + I/R group, NF-κB binding was suppressed by >55%. A small amount of NF-κB binding in the saline + sham and ethanol withdrawal + sham groups was detected (Fig. 8A). Representative EMSA gels from saline + I/R and ethanol + I/R groups are shown in Fig. 8B.

Superoxide release by hepatic neutrophils

Basal superoxide production by hepatic PMNs after 24 h of reperfusion was <0.2 nmol/10^6 cells/h. In the presence of phorbol 12-myristate 13-acetate (PMA), superoxide release was significantly increased to 20 ± 3 nmol/10^6 cells/h in the saline + I/R group. In the ethanol withdrawal + I/R group,

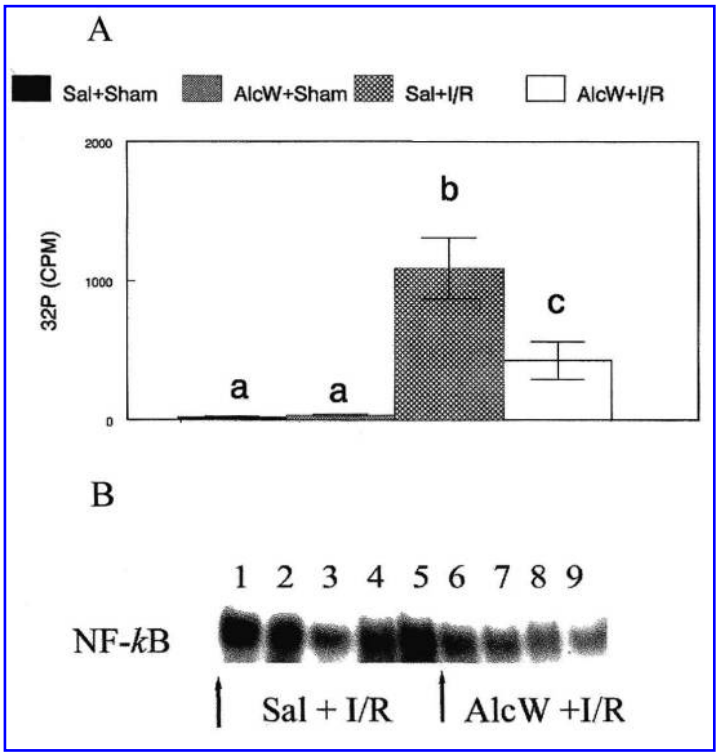

FIG. 8. Effect of ethanol withdrawal on NF-κB binding of nuclear extracts from Kupffer cells to NF-κB consensus oligonucleotide at 24 h after hepatic ischemia. Nuclear extracts from Kupffer cells were obtained after 24 h of hepatic ischemia or sham surgery and were subjected to EMSA (**A**). Data were analyzed by ANOVA followed by Student–Neuman–Keuls multiple comparisons test. Different letters indicate statistical significance at $p < 0.05$ (n = 4–5 per group). ^{32}P was measured using a Phosphorimager. Lower panel (**B**) shows individual EMSA from saline + I/R (lanes 1–5) and alcohol withdrawal + I/R (lanes 6–9).

this spontaneous superoxide release by PMNs was 19 ± 2.25 nmol/10^6 cells/h (Fig. 9). PMA-induced superoxide anion releases by blood PMNs from saline and ethanol (+ withdrawal)-treated rats subjected to sham surgery were 12 ± 1.5 and 10 ± 2 nmol/10^6 cells/h.

In the absence of liver injury, the number of PMNs sequestered in the liver was <2 × 10^6/liver. Figure 10 shows that after 24 h of reperfusion, 150 ± 25 × 10^6/liver PMNs were isolated from the livers of saline-treated + I/R group. The number of PMNs was significantly less in the ethanol withdrawal + I/R group (20 ± 5 × 10^6/liver) (Fig. 10). These values were still higher than in those rats subjected to sham surgery with or without ethanol (Fig. 10).

Histopathological alterations in the liver

Although hepatic injury at 3 h of reperfusion as assessed by elevated serum ALT was observed, profound pathological changes were not seen in the ischemic hepatic lobes of both

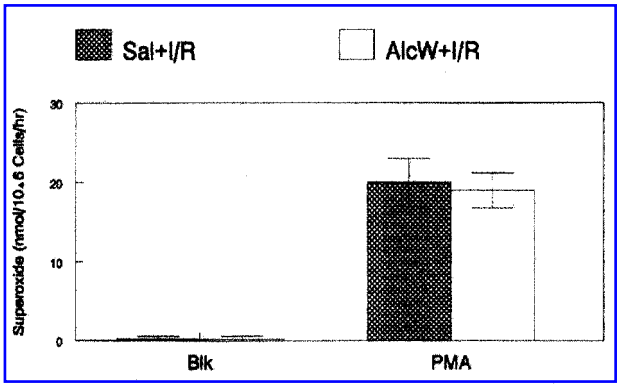

FIG. 9. Effect of ethanol withdrawal on superoxide anion production by hepatic PMNs at 24 h of reperfusion after hepatic ischemia. Hepatic PMNs, from rats subjected to saline + I/R and ethanol withdrawal + I/R, were isolated 24 h after reperfusion. Superoxide anion release was measured by SOD-inhibitable reduction of cytochrome c in the presence or absence of 1 μM PMA. Data were analyzed by ANOVA followed by Student–Neuman–Keuls multiple comparisons test (n = 7 per group).

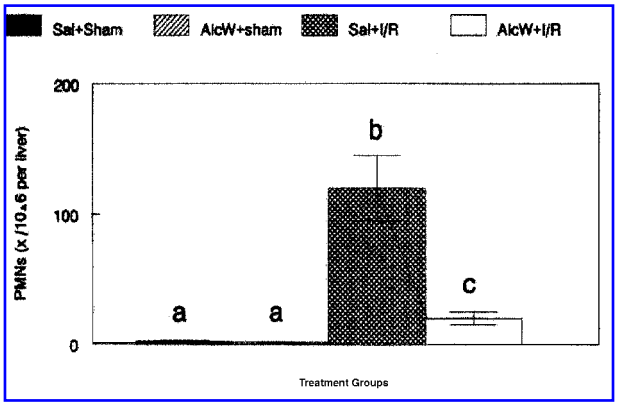

FIG. 10. Effect of ethanol withdrawal on neutrophil sequestration in the liver at 24 h of reperfusion after hepatic ischemia. These data refer to the number of PMNs isolated from the livers after pronase digestion and centrifugal elutriation. Data were analyzed by ANOVA followed by Student–Neuman–Keuls multiple comparisons test. Different letters indicate statistical significance at $p < 0.05$ (n = 7 per group).

treatment groups. However, Fig. 11A shows severe necrosis and neutrophil infiltration in the hepatic sinusoids and parenchyma of the ischemic hepatic lobes obtained from saline + I/R rats after 24 h of reperfusion. In contrast, there was mild neutrophil influx and necrosis in the ischemic lobes of the livers obtained from rats subjected to ethanol withdrawal and I/R (Fig. 11B).

DISCUSSION

These results demonstrate that withdrawal after an acute ethanol binge elicited differential effects on hepatic injury in a time-dependent manner. At 3–5 h of reperfusion, hepatic injury was manifested more profoundly in rats previously subjected to ethanol binge and withdrawal. Concomitantly, Kupffer cells were also primed for enhanced production of oxygen-derived radicals. These radicals are known to play a major role in hepatic injury in a number of pathological conditions, *i.e.,* ethanol intoxication, sepsis and endotoxemia, and hepatic ischemia and reperfusion.

The mechanism by which Kupffer cells are primed for enhanced ROS release is due in part to complement activation (26). Enhanced activation of complement by endotoxin may play an important role in this process during reperfusion after hepatic ischemia. As shown in results, superoxide anion release by Kupffer cells was enhanced during the early phase of I/R. ROS formation was greater in Kupffer cells from the ethanol + I/R group. Another important factor during the early phase of reperfusion during withdrawal is glucose

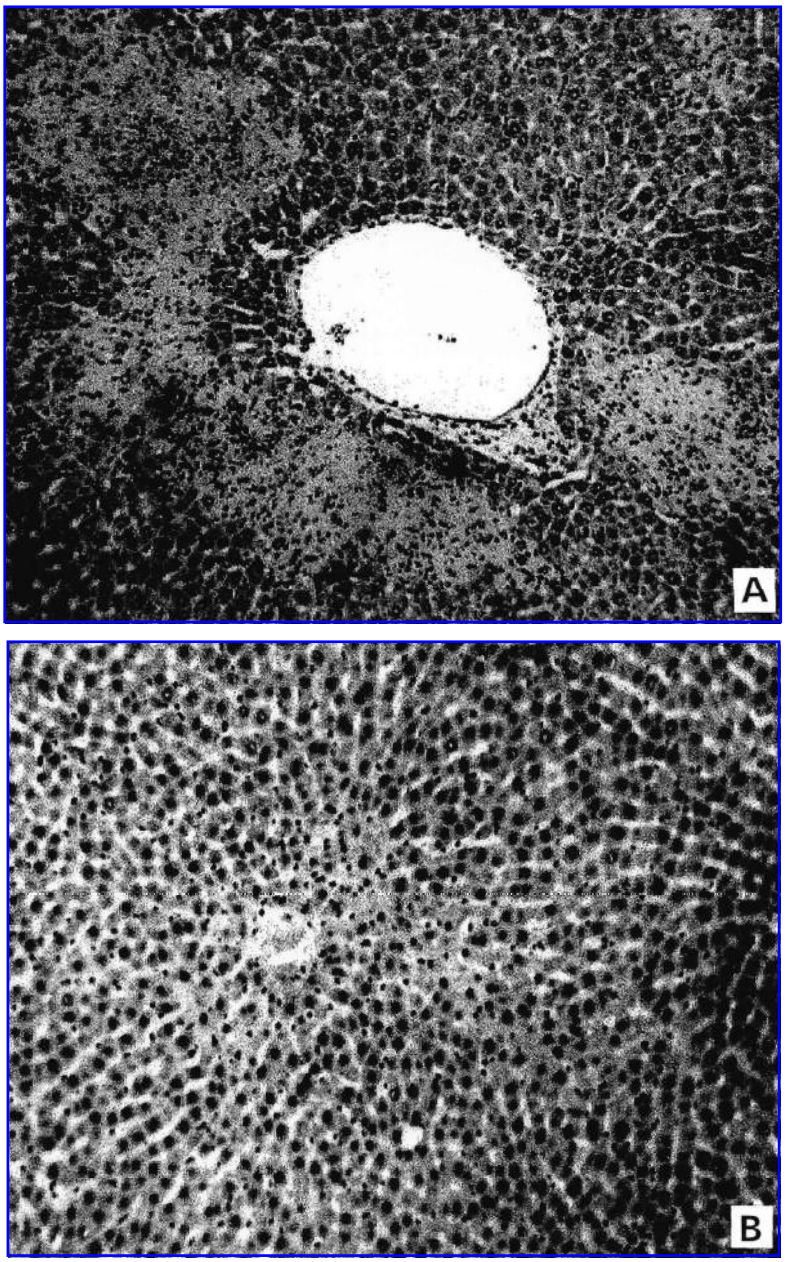

FIG. 11. Histological sections of livers from rats treated with saline (A) or ethanol followed by withrawal (B) and subjected to hepatic ischemia and reperfusion. Hematoxylin and eosin stain.

uptake. We have shown previously that acute ethanol withdrawal is associated with enhanced glucose uptake and concomitant up-regulation of ROS formation (4). Enhanced glucose use by phagocytic cells is also associated with free radical formation through the hexose monophosphate shunt. The up-regulation of ROS production under this condition is not associated with TNF production. Thus, at this time point (3 h of reperfusion or 9 h of ethanol withdrawal), the combined impact of enhanced glucose use and complement product may contribute to further increases in superoxide release by Kupffer cells. Ethanol-induced hypoxia (18) may also contribute to the priming of Kupffer cells for enhanced ROS production during the early phase of reperfusion after hepatic ischemia. Others have also demonstrated activation of Kupffer cells for enhanced ROS (1) and early acetaminophen-induced centrilobular injury during ethanol withdrawal (33). Enhanced release of ROS under this condition may compromise the liver to injury. Results show that serum ALT was greater in the ethanol + I/R group at 3–5 h of reperfusion. At this time, significant sequestration of PMNs into the liver was not observed. Although elevated serum CINC was observed during the early phase of reperfusion, Kupffer cells are likely to play a more important role in hepatic injury than PMNs at this point.

CINC and MIP-2 are members of the CXC chemokine subfamily that primarily attract PMNs to the site of inflammation or injury. Chemokines are also implicated in liver injury during reperfusion after hepatic ischemia (13). Long-term exposure to ethanol has also been shown to up-regulate MIP-2 and CINC production in the liver (2, 37). Results show that serum CINC was elevated during the first 5 h of reperfusion in saline and ethanol-treated rats. After 24 h, serum CINC was lower in the ethanol + I/R group than in the saline + I/R group. Concomitantly, PMN migration to the liver after 24 h of production by Kupffer cells was significantly lower than in the saline + I/R group. ROS production by Kupffer cells at this time was also slightly lower in the ethanol + I/R than in the saline + I/R group. Thus, ethanol intoxication (or binge) for 12 h followed by withdrawal still induced an attenuating effect much later. The attenuating effect of acute ethanol has been shown in numerous studies. In most cases however, ethanol has to be present to induce liver injury as observed during the early phase of reperfusion after hepatic ischemia. These results suggest that significant increases in CINC may contribute to enhanced migration of PMNs in the liver after 24 h of reperfusion. CINC is produced by a number of cells, including Kupffer cells and hepatocytes. Results show that at 24 h of I/R (saline), Kupffer cells were primed for enhanced CINC production at the protein and mRNA levels. In the ethanol withdrawal + I/R group, CINC in the circulation was reduced. We have shown that ethanol infusion attenuates LPS-induced TNFα, chemokine, and ROS production in the rat (6, 10, 16). Under these conditions, ethanol concentration is maintained at 170 mg/dl. However, in another study, the presence of ethanol in the circulation is not required to elicit its down-regulatory activity on Kupffer cell activation. Single oral ingestion of ethanol also attenuates HIV-1 gp120-induced chemokine production by murine splenocytes and Kupffer cells (36). The attenuating effect of ethanol in this condition is observed at 24 h after HIV-1 gp120 treatment, whereby ethanol in the circulation is not present. The long-lasting effect of ethanol on immunocompetent cells has also been demonstrated after ethanol has been removed from the circulation (30). In human studies, acute ethanol consumption also suppresses chemokine and cytokine production by mononuclear cells treated with LPS *ex vivo* (32, 35). The mechanism for ethanol-mediated attenuation of LPS-induced chemokine or cytokine production is attributed to the ability of ethanol to inhibit the translocation and activation of NF-κB in monocytes and macrophages (32). This is also supported by the current results that show ethanol withdrawal plus hepatic I/R was associated with the down-regulation of mRNA expression for NF-κB and CINC in Kupffer cells. CXC chemokines, *i.e.,* KC, IL-8, and CINC, are considered redox-responsive chemokines because their production may be regulated by free radicals that act as secondary messengers for the activation of nuclear transcription factors (12, 29). NF-κB is an important transcription factor that regulates the synthesis and secretion of various cytokines and chemokines in response to immunological stimuli (12, 21). This study shows that after 24 h of reperfusion in rats subjected to ethanol withdrawal, there was a slight down-regulation of superoxide release by Kupffer cells compared with those that received saline + I/R. The observed down-regulation may contribute in part to reduced nuclear translocation and binding of NF-κB, CINC mRNA expression, and secretion during the later phase of I/R during ethanol withdrawal. Thus, based on these observations, the initial insult by ethanol during ethanol infusion (in spite of the withdrawal phase) may be sufficient to induce its down-regulatory activity on Kupffer cells. Acute ethanol intoxication is likely to suppress inflammation, whereas chronic alcohol consumption exacerbates such condition after a secondary endotoxin challenge (3).

Figure 12 shows a schematic diagram of the proposed inflammatory response during reperfusion after hepatic ischemia and alcohol binge followed by withdrawal. The down-regulatory action of ethanol on inflammatory response may involve acetaldehyde production. Cytochrome P450–2E1 is the only ethanol-metabolizing system in Kupffer cells. Acetaldehyde is a known by-product of ethanol metabolism. It has been shown that preexposure of Kupffer cells to acetaldehyde suppresses NF-κB activation (27). This may explain in part the ethanol-induced down-regulation of NF-κB translocation and binding in Kupffer cells. Figure 12 also shows the interaction between Kupffer cells, neutrophils, and hepatocytes and the role of chemokines, adhesion molecules, free radicals, and chemokine receptors in liver injury.

Taken together, these data demonstrate that ethanol withdrawal may compromise the liver to injury during the early phase of reperfusion. The potentiating action of ethanol on injury has been reported elsewhere (9, 14, 19). However, ethanol withdrawal was protective during the later phase of hepatic I/R. This protection may be due in part to reduced production of CINC, which could result in less migration of PMNs into the liver following reperfusion. This aspect is critical because PMNs are implicated in injury during the later phase of reperfusion after hepatic ischemia (23, 24). During ethanol withdrawal and trauma, the most usual causes of morbidity and mortality are not the injuries, but infections associated with wounds. These conditions are observed in patients

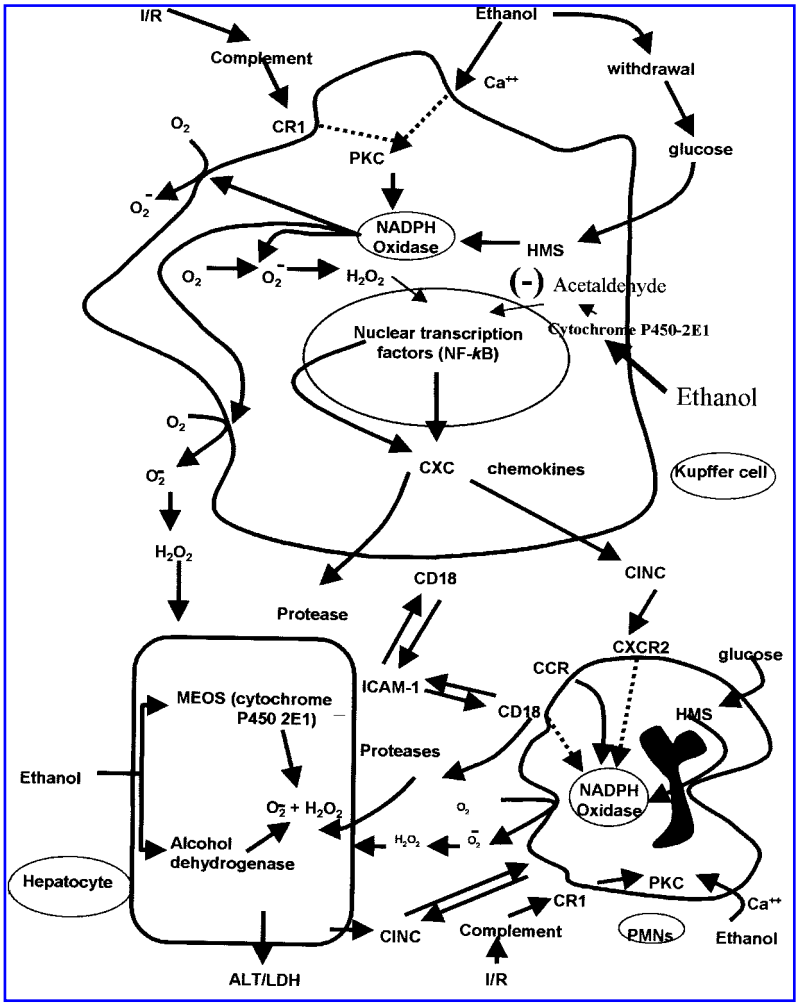

FIG. 12. Schematic diagram of the proposed inflammatory response during reperfusion after hepatic ischemia and alcohol binge followed by withdrawal. Arrows indicate a positive or up-regulatory activity. (−) on the arrow indicates down-regulatory activity. Abbreviations used: CR, complement receptor; HMS, hexose monophosphate shunt; ICAM-1, intercellular adhesion molecule-1; LDH, lactate dehydrogenase; PKC, protein kinase C.

with penetrating abdominal trauma (20). Furthermore, these results highlight the significance that acute and sustained exposure to ethanol (binge) followed by withdrawal, in the absence of trauma or physical injury, may have a potent antiinflammatory effect on the immune system. As a result, the susceptible individual may develop an enhanced propensity to develop acute infections. Free radical formation and chemotaxis by phagocytic cells, *i.e.,* macrophages, neutrophils, may allow the persistence of pathogenic microorganisms. These processes are important for the elimination of infectious agents. Although injury or trauma is minimized by ethanol, immune su5ppression, associated with attenuated innate immune responses by Kupffer cells and neutrophil, may still compromise the host to infectious diseases.

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ABBREVIATIONS

ALT, alanine transferase; CC, cysteine-cysteine; CINC, cytokine-induced neutrophil chemoattractant; CXC, cysteine–variable amino acid–cysteine; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; I/R, ischemia/reperfusion; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein-2; NF-κB, nuclear transcription-κB; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear neutrophils; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor.

REFERENCES

1. Abril ERL, Earnest DL, Jolley CS, Chen G, and Lantz RC. Enhanced Kupffer cell function characterizes the recovery period following binge type alcohol intake in rats. In: *Cells of the Hepatic Sinusoids, Vol. 5,* edited by Knook DL, Wisse E, and Wake K. Leiden, The Netherlands: Kupffer Cell Foundation, 1995, pp 75–76.
2. Bautista AP. Chronic alcohol intoxication induces hepatic injury through enhanced macrophage inflammatory

protein-2 production and intercellular adhesion molecule-1 expression in the liver. *Hepatology* 25: 335–342, 1997.
3. Bautista AP. The role of Kupffer cells in the induction of hepatic injury during alcohol intoxication. *Alcohol Clin Exp Res* 22: 255S–259S, 1998.
4. Bautista AP and Spitzer JJ. Postbinge effects of acute alcohol intoxication on hepatic free radical formation. *Alcohol Clin Exp Res* 20: 502–509, 1996.
5. Bautista AP and Wang E. Chronic ethanol intoxication enhances production of cytokine-induced neutrophil chemoattractant and macrophage inflammatory protein-2 by hepatocytes after human immunodeficiency virus-1 glycoprotein 120 vaccination. *Alcohol* 24: 35–44, 2001.
6. Bautista AP, D'Souza NB, Lang CH, Bagwell J, and Spitzer JJ. Alcohol downregulates the superoxide anion release by hepatic phagocytes. *Am J Physiol* 260: R969–R976, 1991.
7. Bellezo JM, Britton RS, Bacon BR, and Fox ES. LPS-mediated NF-κB activation in rat Kupffer cells can be induced independently of CD14. *Am J Physiol* 270: G956–G961, 1996.
8. Benaceraff B. Functions of Kupffer cells. In: *The Liver, Morphology, Biochemistry, Physiology, Vol. 2,* edited by Rouille CE. New York: Academic Press, 1964, pp. 37–45.
9. Blomqvist S, Thorne J, Elmer O, Jonsson BA, Strand SE, and Lindahl SG. Early post-traumatic changes in hemodynamics and pulmonary ventilation in alcohol-pretreated dogs. *J Trauma* 27: 40–44, 1987.
10. Bukara M and Bautista AP. Acute alcohol intoxication and gadolinium chloride attenuate endotoxin-induced release of CC chemokines in the rat. *Alcohol* 20: 193–203, 2000.
11. Cederbaum AI. Role of lipid peroxidation and oxidative stress in alcohol toxicity. *Free Radic Biol Med* 7, 537–539, 1989.
12. Chandel NS, Tryzyna WC, McClintock DS, and Schumacker PT. Role of oxidant in NF-κB activation and TNF-α gene transcription induced by hypoxia and endotoxin. *J Immunol* 165: 1013–1021, 2000.
13. Colletti LM, Kunkel SL, Walz A, Burdick MD, Sunkel RG, Wilke CA, and Strieter RM. The role of cytokine networks in the local liver injury following hepatic ischemia/reperfusion in the rat. *Hepatology* 23: 506–514, 1996.
14. Desiderio MA. The potentiation of the response to blunt cardiac trauma by ethanol in dogs. *J Trauma* 26: 467–473, 1986.
15. Driscoll KE. Macrophage inflammatory proteins: biology and role in pulmonary inflammation. *Exp Lung Res* 20: 473–490, 1994.
16. D'Souza NB, Bagby GJ, Nelson S, Lang CH, and Spitzer JJ. Acute alcohol infusion suppresses endotoxin-induced serum tumor necrosis factor. *Alcohol Clin Exp Res* 13: 295–298, 1989.
17. Essani NA, McGuire GM, Manning AM, and Jaeschke H. Endotoxin-induced activation of NF-κB and expression of E-selectin mRNA in hepatocytes, Kupffer cells and endothelial cells in vivo. *J Immunol* 156: 2956–2963, 1996.
18. French S, Li J, and French BA. Hypoxia reperfusion injury in an alcohol binge drinking rat model. *Hepatology* 32: 908, 2000.
19. Garrison HG, Hansen AR, Cross RE, and Proctor HJ. Effect of ethanol on lactic acidosis in experimental hemorrhagic shock. *Ann Emerg Med* 13: 26–30, 1984.
20. Gentilello LM, Cobean RA, Walker AP, Moore EE, Wertz MJ, and Dellinger EP. Acute ethanol intoxication increases the risk of infection following penetrating abdominal trauma. *J Trauma* 34: 669–674, 1993.
21. Ghosh SM, May J, and Koop EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 16: 225–230, 1998.
22. Jaeschke H. Reactive oxygen and ischemia/reperfusion injury of the liver. *Chem Biol Interact* 79: 115–136, 1991.
23. Jaeschke H and Smith CW. Mechanisms of neutrophil-induced parenchymal cell injury. *J Leukoc Biol* 61: 647–653, 1997.
24. Jaeschke H, Farhood A, and Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. *FASEB J* 4: 3355–3359, 1990.
25. Jaeschke H, Bautista AP, Spolarics Z, and Spitzer JJ. Superoxide generation by Kupffer cells and priming of neutrophils during reperfusion after hepatic ischemia. *Free Radic Res Commun* 15: 277–284, 1991.
26. Jaeschke H, Farhood A, Bautista AP, Spolarics Z, and Spitzer JJ. Complement activates Kupffer cells and neutrophils during reperfusion after hepatic ischemia. *Am J Physiol* 264: G801–G809, 1993.
27. Jokelainen K, Thomas P, Lindros K, and Nanji AA. Acetaldehyde inhibits NF-kappaB activation through I-kappaB activation in rat Kupffer cells. *Biochem Biophys Res Commun* 253: 834–836, 1998.
28. Kaplowitz N and Tsukamoto H. Oxidative stress and liver disease. *Prog Liver Dis* 14: 131–159, 1996.
29. Lakshiminarayanan V, Drab-Weiss EA, and Roebuck KA. H_2O_2 and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-kappa B to the interleukin-8 promoter in endothelial and epithelial cells. *J Biol Chem* 273: 32670–32678, 1998.
30. Laso FJ, Madruga JI, San Miguel JF, Ciudad J, Lopez A, Alvarez-Mon M, and Orfao A. Long lasting immunological effects of ethanol after withdrawal. *Cytometry* 26: 275–280, 1996.
31. Luster AD. Chemokines—chemotactic cytokines that mediate inflammation. *N Engl J Med* 338: 436–445, 1998.
32. Mandrekar P, Catalano D, and Szabo G. Inhibition of LPS-mediated NF-κB activation in human monocytes. *Int Immunol* 11: 1781–1790, 1999.
33. McCuskey RS, Machen, NW, Wang X, McCuskey MK, Abril E, Earnest DL, and De Leve L. Single ethanol binge exacerbates early acetaminophen-induced centrilobular injury to the sinusoidal endothelium and alters sinusoidal blood flow. In: *Cells of the Hepatic Sinusoids,* edited by Knook DL, Wisse E, de Zanger R, and Arthur MJP. Leiden, The Netherlands: The Kupffer Cell Foundation, 2001, pp. 68–70.
34. Shiratori Y, Takada H, Hikiba Y, Nakata R, Okano K, Komatsu Y, Niwa U, Matsumara M, Shiina S, and Omata M. Production of chemotactic factor, interleukin-8 from hepatocytes exposed to ethanol. *Hepatology* 18: 1477–1482, 1993.

35. Szabo G, Chavran S, Mandrekar P, and Catalano D. Acute alcohol consumption attenuates IL-8 and MCP-1 induction in response to ex vivo stimulation. *J Clin Immunol* 19: 67–76, 1999.
36. Wang E and Bautista AP. Regulation of CXC and CC chemokine gene expression in murine splenic T-lymphocytes during acute alcohol intoxication. *FASEB J* 50:21, 2000.
37. Yamada S, Matsuoka H, Momosaka Y, Izumi H, Khono, K, Yamaguchi Y, and Eto S. Effect of long-term ethanol consumption on ability to produce CINC in the rat liver and its gender differences. *Alcohol Clin Exp Res* 23: 61S–66S, 2000.

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2. Nympha B. D'Souza El-Guindy, Elizabeth J. Kovacs, Philippe De Witte, Claudia Spies, John M. Littleton, Willem J. S. De Villiers, Amanda J. Lott, Timothy P. Plackett, Nadine Lanzke, Gary G. Meadows. 2010. Laboratory Models Available to Study Alcohol-Induced Organ Damage and Immune Variations: Choosing the Appropriate Model. *Alcoholism: Clinical and Experimental Research* **34**:9, 1489-1511. [CrossRef]
3. Rongqian Wu, Wayne W. Chaung, Mian Zhou, Youxin Ji, Weifeng Dong, Zhimin Wang, Xiaoling Qiang, Ping Wang. 2010. Milk Fat Globule EGF Factor 8 Attenuates Sepsis-Induced Apoptosis and Organ Injury in Alcohol-Intoxicated Rats. *Alcoholism: Clinical and Experimental Research* **34**:9, 1625-1633. [CrossRef]
4. Jianhua Rao, Chuanyong Zhang, Ping Wang, Ling Lu, Feng Zhang. 2010. All-trans Retinoic Acid Alleviates Hepatic Ischemia/Reperfusion Injury by Enhancing Manganese Superoxide Dismutase in Rats. *Biological & Pharmaceutical Bulletin* **33**:5, 869-875. [CrossRef]
5. Patrick Greiffenstein, Patricia E. Molina. 2008. Alcohol-Induced Alterations on Host Defense After Traumatic Injury. *The Journal of Trauma: Injury, Infection, and Critical Care* **64**:1, 230-240. [CrossRef]
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