Free Rad. Res., Vol. 32, pp. 497–506
Reprints available directly from the publisher
Photocopying permitted by license only

© 2000 OPA (Overseas Publishers Association) N.V.
Published by license under
the Harwood Academic Publishers imprint,
part of The Gordon and Breach Publishing Group.
Printed in Malaysia.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

Antioxidants and Gadolinium Chloride Attenuate Hepatic Parenchymal and Endothelial Cell Injury Induced by Low Flow Ischemia and Reperfusion in Perfused Rat Livers

SHANNON M. BAILEY[a] and LESTER A. REINKE[b,*]

[a]*Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA;* [b]*Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA*

Accepted by Prof. M.B. Grisham

(Received 15 August 1999; In revised form 11 September 1999)

The objective of this study was to determine whether Kupffer cells contribute to parenchymal and endothelial cell damage induced by ischemia–reperfusion in perfused rat livers. Parenchymal and endothelial cell injury were determined by measuring activities of lactate dehydrogenase (LDH) and purine nucleoside phosphorylase (PNP), respectively, in the effluent perfusate of livers subjected to 60 min of low flow ischemia followed by 30 min of reperfusion. Upon reperfusion, LDH and PNP activities increased significantly within the first 10 min of reperfusion and remained elevated over control values throughout the duration of reperfusion. Pretreatment with gadolinium chloride, an inhibitor of Kupffer cell function, significantly decreased LDH and PNP efflux during reperfusion by approximately 60% and 50%, respectively. When Kupffer cells were stimulated by vitamin A pretreatment, PNP efflux was doubled during reperfusion. Vitamin E pretreatment attenuated LDH and PNP release by approximately 70% during reperfusion compared to enzyme release in untreated livers. Moreover, the water-soluble antioxidants superoxide dismutase and desferrioxamine reduced reperfusion injury, whereas catalase had no effect on enzyme release. These results demonstrate that superoxide anions released from Kupffer cells are involved in oxidative damage to endothelial cells as well as hepatocytes during the early stages of hepatic reperfusion.

Keywords: Reperfusion injury, low flow ischemia, antioxidants, gadolinium chloride, endothelial cells, rat liver

Abbreviations: AA, arachidonic acid; Fe^{2+}, ferrous iron; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; IL-6, interleukin-6; LDH, lactate dehydrogenase; LPO, lipid peroxidation; NADPH Ox, NADPH oxidase; PLA_2, phospholipase A_2; PGE_2, prostaglandin E_2; PNP, purine nucleoside phosphorylase; $O_2^{\bullet-}$, superoxide anion; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α

INTRODUCTION

Restoration of the oxygen supply after a prolonged period of hypoxia/ischemia can

* Corresponding author. Tel.: (405)271-6593. Fax: (405)271-7477. E-mail: lester-reinke@ouhsc.edu.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

exacerbate rather than prevent tissue injury. This paradoxical phenomenon is commonly referred to as reoxygenation or reperfusion injury and is believed to contribute to liver dysfunction from organ preservation and transplantation[1,2] and shock.[3] Even though the exact biochemical mechanisms responsible for reperfusion injury are not known, accumulating evidence indicates that reactive oxygen species are generated upon the re-introduction of oxygen to ischemic liver tissue and play a major role in producing the cell damage associated with ischemia and reperfusion.[4,5]

Previous studies have focussed on the role of the Kupffer cell as the critical mediator of ischemia–reperfusion injury.[6–9] Kupffer cells reside in the hepatic sinusoids, and upon appropriate stimulation, produce a number of biologically active substances including reactive oxygen and nitrogen species, cytokines, proteases,and eicosanoids, which can damage tissue. [10] Previous studies demonstrated that reperfusion of ischemic liver stimulated the phagocytic activity of Kupffer cells[11] which could trigger the release of toxic mediators leading to liver injury. Indeed, studies have provided evidence that oxygen radical intermediates are generated by Kupffer cells following an episode of ischemia–reperfusion.[8,9] Furthermore, Bremer *et al.*[9] demonstrated that inactivation of Kupffer cells with gadolinium chloride inhibited free radical formation and protected hepatocytes from reperfusion injury. Thus, Kupffer cells appear to play a major role in reperfusion-induced injury to parenchymal cells.

Because endothelial cells are in direct contact with sinusoidal Kupffer cells, they may be particularly susceptible to Kupffer cell-mediated reperfusion injury. Hepatic endothelial cells have been shown to be susceptible to reperfusion injury following cold ischemic storage of livers[12] and to hypoxia–reoxygenation injury using endothelial cell cultures.[13] Moreover, endothelial cell damage[14] was observed in livers subjected to a model of warm low flow ischemia–reperfusion where lethal cell injury was observed in hepatocytes.[9,15] However, direct evidence demonstrating the involvement of Kupffer cells in endothelial cell injury following warm low flow ischemia–reperfusion is still lacking.

Therefore, the first objective of this present study was to test the hypothesis that Kupffer cells are a contributing factor to endothelial cell damage following low flow ischemia and reperfusion. To test this hypothesis, rats were treated with gadolinium chloride, an inhibitor of Kupffer cell function, or vitamin A, a stimulator of Kupffer cell function, and endothelial cell injury was determined by measuring the release of the endothelial cell-specific enzyme purine nucleoside phosphorylase (PNP). Lactate dehydrogenase (LDH) efflux was also measured to assess damage to the parenchymal cells. To further evaluate the role of reactive oxygen species in hepatic reperfusion injury the effects of the water-soluble antioxidants, superoxide dismutase and catalase were examined, as well as the iron chelator desferrioxamine. Finally, studies were undertaken to determine whether α-tocopherol pretreatment could modify endothelial and parenchymal cell injury induced by ischemia and reperfusion.

MATERIALS AND METHODS

Chemicals

Desferrioxamine mesylate, superoxide dismutase from bovine erythrocytes, catalase from bovine liver, (+)-α-tocopherol acetate, and all trans-retinol acetate were obtained from Sigma Chemical Company (St. Louis, MO, USA). Gadolinium chloride was obtained from Aldrich (Milwaukee, WI, USA). Other chemicals and enzymes were of the highest quality commercially available.

Animals and Treatments

Male Sprague–Dawley rats (160–180 g) were group-housed in a temperature and humidity

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

controlled environment, maintained on a 12 h light–dark schedule, and fed laboratory rat chow and water *ad libitum* for one week before they were used for experiments. The rats were fasted for at least 16 h before experiments. For the fast, the rats were housed individually in suspended cages, but had free access to water. Gadolinium chloride (30 mg/kg body weight) dissolved in 0.9% NaCl was administered by i.v. tail vein injection under isoflurane anesthesia 24 h before liver perfusion experiments to destroy Kupffer cells in the liver.[9] Retinol acetate (vitamin A, 250,000 IU/kg body weight/day) was administered by i.p. injection for 8 days prior to experiments to enhance Kupffer cell function.[6,16] The vitamin A solution was prepared in α-tocopherol-stripped corn oil and kept at 4°C. Control rats received an equal volume injection of α-tocopherol-stripped corn oil. Corn oil had no effect on enzyme release measurements; thus results with control oil-treated rats were combined with untreated controls for analysis. Rats were pretreated with approximately 300 units of α-tocopherol acetate (vitamin E) orally for 4 days before liver perfusion experiments to increase vitamin E levels in liver. This study was approved by the Institutional Animal Care and Use Committee and followed the standard procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH, Bethesda, MD, USA).

Liver Perfusion: Low Flow Ischemia and Reperfusion Protocol

Livers were perfused with Krebs–Henseleit bicarbonate buffer, pH 7.4, saturated with 95% O_2 : 5% CO_2 and maintained at 37°C as described previously.[17,18] The livers were perfused with a blood-free perfusion buffer in order to avoid complicating factors from blood constituents, such as circulating neutrophils which could become activated during ischemia, superoxide dismutase, or other compounds with antioxidant properties. Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) before surgery and livers were perfused via a cannula placed in the portal vein, and the effluent perfusate was directed out of the liver via a cannula inserted in the vena cava. The oxygen concentration in the effluent perfusate was monitored using a Teflon-shielded, Clark-type oxygen electrode, and the oxygen consumption of the liver was calculated from the difference between influent and effluent oxygen concentrations, and expressed as a function of the perfusion flow rate, and liver wet weight. For perfusion, the livers were placed in a plexi-glass chamber and maintained at an environmental temperature of 37°C. Oxygen delivery to the liver was controlled by varying the perfusion rate of the oxygen-saturated buffer to the tissue. Livers were perfused at a perfusion flow rate of 3–4 ml/min/g liver for 40 min, after which the perfusion flow rate was reduced by 75% and maintained for 60 min to initiate low flow ischemia.[9,15,19] After 60 min of low flow ischemia the flow rate was increased over a 1 min period to the original control flow rate where it was maintained for 30 min to study reperfusion injury. Control (i.e. normal flow) livers were perfused at 3–4 ml/min/g liver throughout the entire perfusion experiment. In other experiments, the perfusion buffer was supplemented with superoxide dismutase (30 Units/ml), catalase (100 Units/ml), or desferrioxamine mesylate (0.5 mM) to determine their effect on reperfusion injury to endothelial and parenchymal cells.

Lactate Dehydrogenase and Purine Nucleoside Phosphorylase Measurements

Samples of effluent perfusate were collected and placed on ice for the determination of LDH and PNP activities. LDH is released from many types of liver cells, whereas the presence of PNP in the perfusate is considered to specifically indicate damage to endothelial cells.[14,20] Samples were collected every 10 min before and during low flow ischemia, whereas samples were collected every 2 min during reperfusion to detect rapid

RIGHTSLINK

changes in enzyme release. Samples were collected every 10 min for control livers. LDH[21] and PNP[22] activities in the effluent perfusate were determined by standard enzymatic methods. Enzyme activities in the effluent perfusate were expressed as a function of the perfusion flow rate and liver wet weight and reported as units/min/g liver. Units are defined as 1.0 nmol NAD^+ produced/min at 25°C for LDH and 1.0 nmol uric acid produced/min at 37°C for PNP.

Determination of Liver Vitamin E Content

The concentration of vitamin E in livers was measured in liver extracts by high pressure liquid chromatography (HPLC) coupled with electrochemical detection as described by Lang *et al.*[23] Analysis was performed using a Waters HPLC system equipped with a Rainin Microsorb-MV 5 μM C-18 column (4.6 mm ID, 25 cm long) and a Dionex Pulsed Amperometric electrochemical detector fitted with a glassy carbon electrode. The mobile phase consisted of 20 mM $LiClO_4$, 10% methanol, and 90% ethanol. The electrochemical detector was set at an applied potential of 0.6 V and the sensitivity (nA) was adjusted on a per sample basis to obtain maximum chromatogram peak heights. The concentration of vitamin E was calculated by comparison to the peak height of an external standard of (±)-α-tocopherol dissolved in ethanol that was subjected to the same conditions used to extract liver lipids. The concentration of vitamin E in liver is reported as nmol vitamin E/g liver.

Statistical Analysis

The values are reported as the mean ± SE for the number of experiments indicated in each treatment group. Statistical differences between groups were analyzed with Student's *t*-test and one factor ANOVA with the level of significance set at $p < 0.05$. For statistical analysis, the area under the time course curves for LDH and PNP efflux (Figures 1–3) were integrated between 100–130 min of perfusion, which corresponds to the reperfusion period. Area under the curve analysis was done using SigmaPlot™ for Windows transformation software.

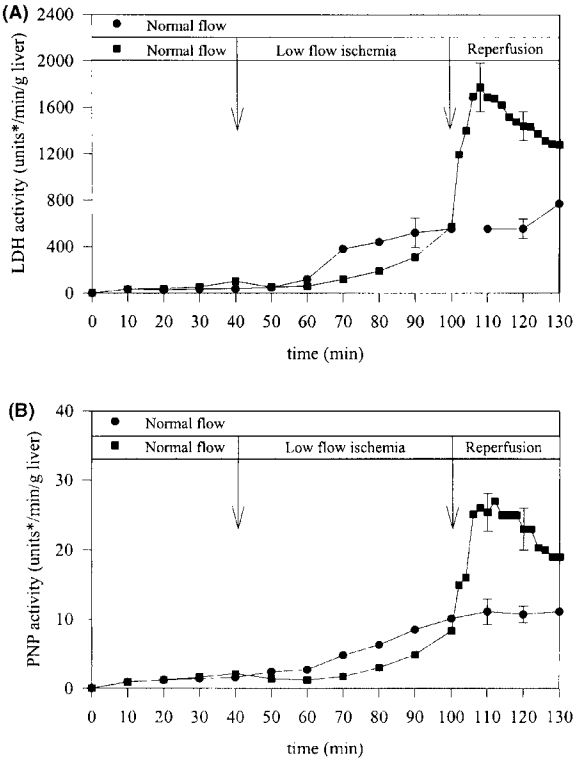

FIGURE 1 Low flow ischemia–reperfusion induces parenchymal (A) and endothelial cell (B) injury. Livers were subjected to either normal flow perfusion (●) or 60 min of low flow ischemia followed by 30 min of reperfusion (■) at 37°C. Activities of LDH and PNP were measured in the effluent perfusate as indicators of parenchymal and endothelial cell injury, respectively. The data represent the mean ± SE for 4–6 liver perfusions per group. Characteristic SE bars are presented for 2 data points per group. *One nmol product formed/min.

RESULTS

Low Flow Ischemia–Reperfusion Induces Parenchymal and Endothelial Cell Injury

Livers were perfused for 40 min with a hemoglobin-free, oxygen-saturated buffer at a flow rate of 3–4 ml/min/g liver to ensure adequate

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK

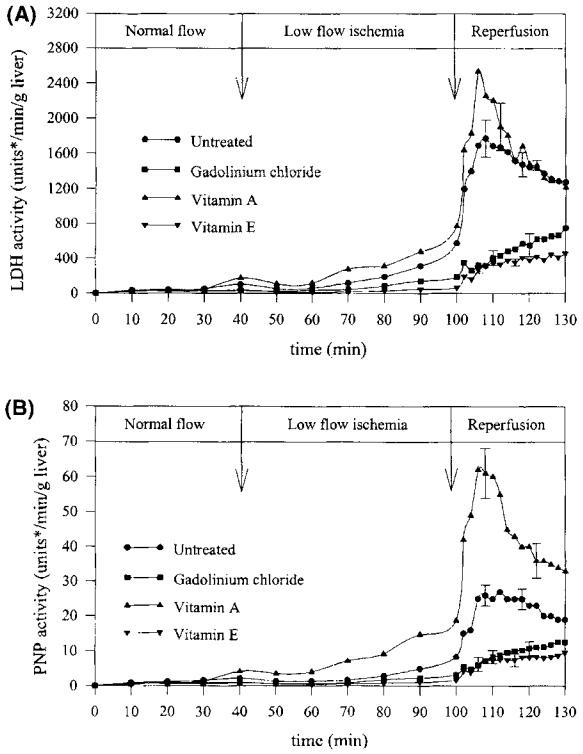

FIGURE 2 Modulation of low flow ischemia–reperfusion injury by gadolinium chloride, vitamin A, and vitamin E pretreatment. Livers isolated from untreated (●), gadolinium chloride (■), vitamin A (▲), and vitamin E-treated rats (▼) were subjected to 60 min low flow ischemia followed by 30 min of reperfusion at 37°C. LDH (A) and PNP (B) activities were measured in the effluent perfusate as indicators of parenchymal and endothelial cell injury, respectively The data represent the mean ± SE for 4–6 liver perfusions per group. Characteristic SE bars are presented for 2 data points per group. *One nmol product formed/min.

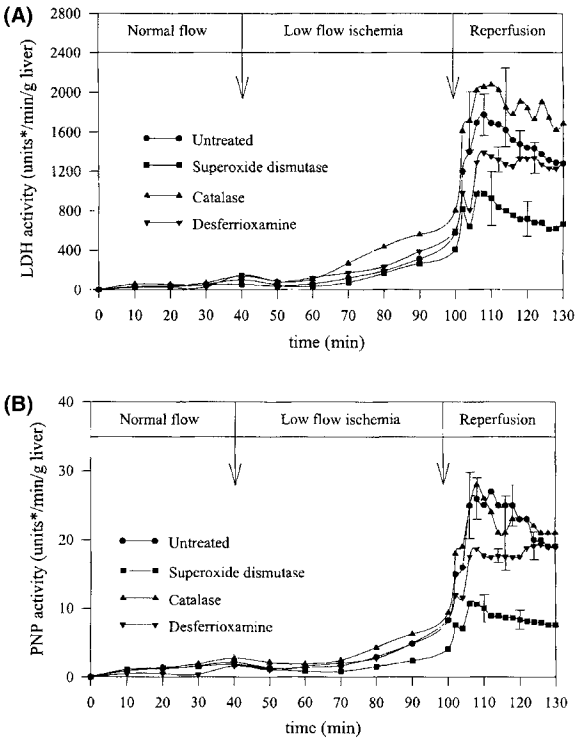

FIGURE 3 Effects of superoxide dismutase (■), catalase (▲), and desferrioxamine (▼) on low flow ischemia–reperfusion injury. Livers were perfused with buffer containing superoxide dismutase (30 Units/ml), catalase (100 Units/ml), or desferrioxamine (0.5 mM) and subjected to 60 min of low flow ischemia followed by 30 min of reperfusion at 37°C. Livers not exposed to antioxidants served as untreated controls (●). LDH (A) and PNP (B) activities were measured in the effluent perfusate as indicators of parenchymal and endothelial cell injury, respectively. The data represent the mean ± SE for 4–6 liver perfusions per group. Characteristic SE bars are presented for 2 data points per group. *One nmol product formed/min.

oxygenation of the liver before initiation of low flow ischemia.[24] Minimal release of LDH and PNP into the effluent perfusate occurred during the 40 min pre-ischemic acclimation period (Figure 1). After 40 min of normal flow, the perfusion flow rate was decreased 75% and perfusion was maintained at this low rate for 60 min to initiate low flow ischemia.[9,15,19] During low flow ischemia, LDH and PNP activities in the effluent perfusate remained low; however, there was a gradual increase in enzyme release during the last 20 min of low flow ischemia. Low flow ischemia was restricted to 60 min because preliminary studies demonstrated that longer periods of low flow ischemia (e.g. 90–180 min) caused substantial enzyme release before reperfusion. Upon restoration of flow, there was a dramatic increase in enzyme release into the effluent perfusate that peaked approximately 8–10 min into the reperfusion period and remained elevated until the end of the experiment (Figure 1). Low flow ischemia and reperfusion significantly increased LDH ($p = 0.0007$) and PNP ($p = 0.005$) efflux compared

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK

TABLE I Effect of gadolinium chloride and antioxidants on ischemia/reperfusion-induced LDH and PNP efflux

Treatment groups	Enzyme efflux rates[a]	
	LDH	PNP
I Normal flow control	17,865 ± 3104	331 ± 44
II Ischemia–reperfusion		
Untreated	42,995 ± 2673[b]	651 ± 62[b]
Gadolinium chloride	16,203 ± 3071[c]	300 ± 51[c]
Vitamin A	45,545 ± 5591	1257 ± 122[c]
VitaminE	10,237 ± 1203[c]	214 ± 51[c]
Superoxide dismutase	22,574 ± 5171[c]	255 ± 38[c]
Catalase	53,710 ± 11,444	663 ± 142
Desferrioxamine	35,241 ± 2224[c]	461 ± 58[c]

Livers were subjected to either normal flow perfusion or 60 min of low flow ischemia followed by 30 min of reperfusion as shown in Figures 1–3. Area under the time course curves for LDH and PNP efflux (Figures 1–3) were integrated between 100 and 130 min of perfusion, which corresponds to the reperfusion period. Results represent the mean ± SE of 4–6 liver perfusions per group.
[a]Units released/g liver; [b]$p < 0.05$, compared to normal flow control group; [c]$p < 0.05$, compared to untreated ischemia–reperfusion group.

to normal flow controls during 100–130 min of perfusion, which corresponds to the reperfusion period of ischemic livers (Table I). The release of PNP clearly demonstrates that reperfusion of isehemic liver damages hepatic endothelial cells.

The basal rate of oxygen consumption measured during the pre-ischemic acclimation period was 1.32 ± 0.07 μmol/min/g liver. Oxygen uptake was well-maintained in normal flow control livers and livers subjected to low flow ischemia–reperfusion. For example, the rate of oxygen consumption measured at 120 min of perfusion was 1.47 ± 0.07 μmol/min/g liver in normal flow control livers and 1.31 ± 0.06 μmol/min/g liver in ischemia–reperfused livers.

Modulation of Low Flow Ischemia–Reperfusion Injury by Gadolinium Chloride and Vitamin A

In this part of the study, rats were pretreated with gadolinium chloride or vitamin A to determine the role of Kupffer cells in liver ischemia–reperfusion injury (Figure 2). Pretreatment with gadolinium chloride, an inhibitor of Kupffer cell function,[9] significantly decreased the release of LDH and PNP into the effluent perfusate during reperfusion compared to livers from untreated rats (Figure 2). LDH efflux during reperfusion was decreased approximately 60% in livers from gadolinium chloride-treated animals compared to livers from untreated animals (Table I). Similarly, efflux of PNP during reperfusion was significantly reduced from 651 ± 62 to 300 ± 51 Units/g liver in livers from untreated and gadolinium chloride-treated rats, respectively (Table I). When rats were pretreated with vitamin A, which stimulates Kupffer cell function,[6,16] PNP efflux was approximately doubled during reperfusion (Figure 2B), whereas there was no significant difference in LDH efflux between untreated and vitamin A-treated rats (Table I). These data provide evidence that Kupffer cells are involved in reperfusion injury, and support the hypothesis that endothelial cells are sensitive to alterations in Kupffer cell activity.

Vitamin E Pretreatment Ameliorates Liver Ischemia–Reperfusion Injury

Livers from rats pretreated with vitamin E, a lipid-soluble antioxidant, exhibited a significant decrease in effluent perfusate LDH and PNP activities during low flow ischemia and reperfusion. Upon reperfusion, the characteristic burst of LDH and PNP release observed in livers from untreated rats was absent in livers from rats treated with vitamin E (Figure 2). Area under the time course curve analysis revealed that vitamin E treatment significantly decreased LDH and PNP efflux approximately 70% during reperfusion compared to untreated livers (Table I). Vitamin E treatment resulted in a 10-fold increase in the vitamin E content of liver from 12 ± 1 to 112 ± 21 nmol/g liver in untreated and vitamin E-treated rats, respectively. These results demonstrate that increases in hepatic vitamin E content protect hepatic endothelial cells and parenchymal cells from ischemia–reperfusion injury.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

Effect of Superoxide Dismutase, Catalase, and Desferrioxamine on Ischemia–Reperfusion Injury

Superoxide dismutase and desferrioxamine protected against reperfusion injury in livers subjected to 60 min of low flow ischemia (Figure 3). For example, LDH efflux was significantly decreased by 50% in livers exposed to superoxide dismutase compared to untreated livers (Table I). Similarly, PNP efflux was significantly decreased by 60% from 651 ± 62 to 255 ± 38 Units/g liver in untreated and superoxide dismutase-treated livers, respectively, during the 30 min reperfusion phase (Table I). Moreover, livers perfused with desferrioxamine exhibited a significant, although moderate decrease in LDH and PNP activities in the effluent perfusate during reperfusion (Table I). Perfusion with catalase had no effect on LDH and PNP efflux (Table I). These results suggest that superoxide anion and iron-catalyzed free radical reactions may contribute to the early damage to endothelial and parenchymal cells induced by reperfusion of ischemic liver.

DISCUSSION

The results of the present study confirm and extend earlier findings that liver is susceptible to damage as a consequence of low flow ischemia and reperfusion.[9,15,19,25] In this experimental model of low flow ischemia and reperfusion, periportal regions remain normoxic, whereas downstream pericentral regions of the liver lobule become anoxic due to inadequate delivery of oxygen.[26] Interestingly, the hepatocytes residing in the midzonal region of the liver lobule, i.e. the region of liver tissue bordering the normoxic periportal and anoxic pericentral regions, are generally the first cells to lose viability from low flow ischemia and reperfusion.[15,19] Reperfusion of ischemic liver resulted in the dramatic increase in the release of the liver enzymes LDH and PNP into the effluent perfusate (Figure 1). The release of LDH into the effluent perfusate is generally used as a marker of injury for hepatic parenchymal tissue.[9,15,19,25] In contrast, PNP is primarily localized in the sinusoidal endothelial cells in liver[20] and its release upon reperfusion indicates that endothelial cells were also damaged by ischemia and reperfusion (Figure 1B). These data suggest that ischemia renders endothelial cells as well as parenchymal cells susceptible to injury upon the re-introduction of oxygen.

Pretreatment of rats with gadolinium chloride decreased hepatic oxygen uptake by 15%–1.10 ± 0.03 μmol/min/g liver (data not shown), as reported by other investigators.[9,27] Impaired oxygen utilization might be explained by decreased requirements by Kupffer cells, and/or decreased mitochondrial cytochromes c_1 and c.[27] However, impaired oxygen utilization would be expected to exacerbate effects of low flow ischemia on tissue function, and thus cannot explain the protective effects of gadolinium chloride against liver injury (Table I). The effects of gadolinium chloride are probably best explained by decreased release of reactive oxygen intermediates or other mediators from Kupffer cells during reperfusion, as suggested in previous studies.[6,8,9] This hypothesis is strengthened by increased endothelial cell injury when Kupffer cell function was stimulated by pretreatment of rats with vitamin A (Figure 2B). Using an *in vivo* model of ischemia–reperfusion, Jaeschke and Farhood[6] observed that Kupffer cell activation with vitamin A or *P. acnes* significantly increased plasma levels of oxidized glutathione, which is an indicator of oxidative cell injury, while gadolinium chloride pretreatment prevented this effect. Indeed, Hoglen *et al.*[16] demonstrated that pretreatment with vitamin A increased rat Kupffer cell respiration, reactive oxygen species formation, and tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) production. Hence, vitamin A may increase reperfusion-induced endothelial cell injury, at least in part, by enhancing reactive oxygen species generation by Kupffer cells after restoration of oxygen. Moreover, because vitamin A does not increase the

RIGHTSLINK

steady-state concentration of PNP in liver,[28] the presence of PNP in the perfusate upon reperfusion must be due to increased release from damaged endothelial cell membranes. Thus, these observations demonstrate that Kupffer cells contribute to reperfusion injury, especially to the endothelium, in liver.

A proposed scheme for Kupffer cell-mediated ischemia–reperfusion injury is presented in Figure 4. We propose that reperfusion stimulates Kupffer cells to release reactive oxygen species (e.g. superoxide anion) which damage endothelial and hepatocyte cell membranes. In the present study, gadolinium chloride prevented reperfusion injury by inhibiting Kupffer cell function, whereas vitamin A increased injury by augmenting Kupffer cell activity. Our findings strongly suggest that superoxide anions generated by Kupffer cells were responsible for endothelial and parenchymal cell injury because reperfusion injury was reduced by superoxide dismutase, an antioxidant enzyme that would have remained in the sinusoidal space. Previous studies have demonstrated increases in spontaneous and phorbol ester-stimulated superoxide production in Kupffer cells isolated from ischemic and reperfused liver.[7,8] The protective effect of desferrioxamine, an iron chelator, also suggests that iron may have contributed to the formation of reactive oxygen species, such as hydroxyl radicals. However, sinusoidal hydrogen peroxide may not be involved because catalase was not protective against injury. Younes and Strubelt[29] also found that superoxide dismutase and desferrioxamine reduced liver cell damage induced by nitrogen hypoxia and reoxygenation. Taken together, these data indicate a role for superoxide anions generated by Kupffer cells in reperfusion injury to endothelial and parenchymal cells.

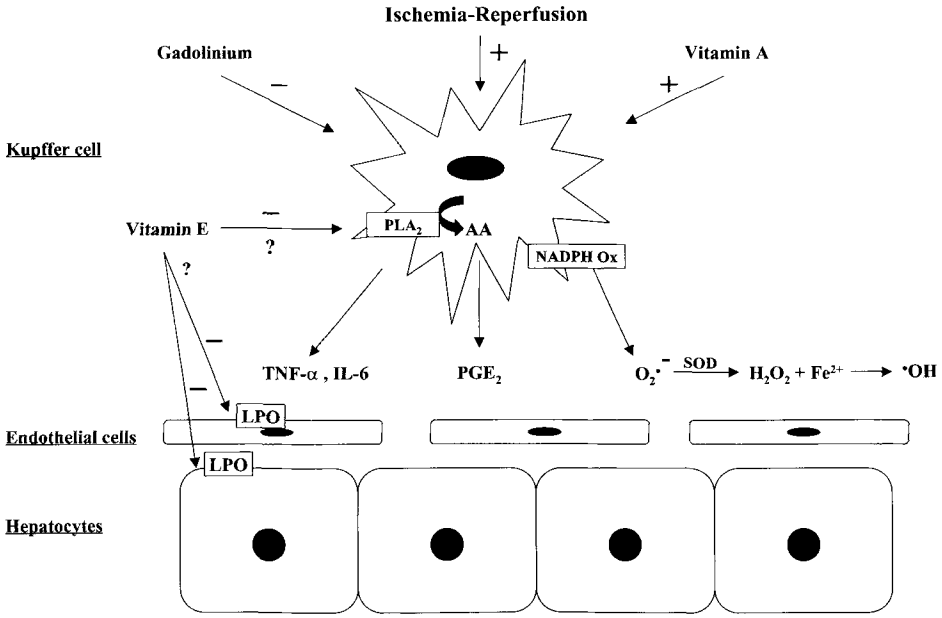

FIGURE 4 Scheme depicting the potential role of Kupffer cells in ischemia–reperfusion injury. Reperfusion-activated Kupffer cells release superoxide anions ($O_2^{\bullet -}$, cytokines (e.g. TNF-α, IL-6), and eicosanoids (e.g. PGE_2) which have deleterious effects on hepatocytes and endothelial cells. Gadolinium chloride is shown to inhibit Kupffer cell activity which is represented by the minus sign (−), whereas ischemia–reperfusion and vitamin A are shown to enhance Kupffer cell function which is represented by the plus sign (+). Vitamin E is speculated to be protective by inhibiting lipid peroxidation in hepatocytes and endothelial cells, as well as attenuating Kupffer cell-mediated production of eicosaniods. AA, arachidonic acid; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; LPO, lipid peroxidation; NADPH Ox, NADPH oxidase; PLA_2, phospholipase A_2; SOD, superoxide dismutase.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

In the present study, supplementation of liver vitamin E provided a significant protective effect against reperfusion injury to hepatocytes and endothelial cells. Previous studies have demonstrated a significant increase in malondialdehyde release during reperfusion[9,30] which was normalized by the water-soluble vitamin E analogue trolox C.[31] Moreover, others have reported that pretreatment with vitamin E inhibited the accumulation of lipid peroxides in liver tissue after *in vivo* ischemia–reperfusion.[32,33] Furthermore, Kuo *et al.*[34] demonstrated that vitamin E protects hepatocellular membrane protein thiols, especially those of actin, rather than membrane lipids from oxidative damage. Hence, in our studies vitamin E may have inhibited free radical-mediated lipid peroxidation reactions and/or protein thiol oxidation, thereby increasing the structural integrity of hepatocyte and endothelial cell membranes and making them more resistant to oxidative damage during reperfusion (Figure 4).

In addition to reactive oxygen species, Kupffer cells release a variety of inflammatory mediators including cytokines and eicosanoids, which may contribute to reperfusion injury in our isolated perfused rat liver model. Previous studies have demonstrated enhanced release of various cytokines including TNF-α and interleukins by Kupffer cells following global hepatic ischemia and reperfusion *in vivo*.[35,36] In addition, Gyenes and de Groot[37] demonstrated that primary cultures of rat Kupffer cells released significant levels of eicosanoids when exposed to reoxygenation after 4 h of hypoxia. However, at present it is not clear whether cytokines or eicosanoids or both are involved in the immediate damage observed in liver following ischemia and reperfusion. Interestingly, Sakamoto *et al.*[38] demonstrated that vitamin E pretreatment significantly decreased PGE_2 production in peritoneal macrophages due to inhibition of phospholipase A_2 and cyclooxygenase. Thus, it is possible that in our studies vitamin E may have attenuated reperfusion injury by inhibiting the production of these inflammatory eicosanoids by Kupffer cells (Figure 4). Further studies are necessary to reveal the precise mechanism of vitamin E protection in hepatic reperfusion injury.

In conclusion, the results presented in this study indicate that liver endothelial and parenchymal cells are susceptible to injury by low flow ischemia and reperfusion. Modulation of Kupffer cell function with gadolinium chloride and vitamin A revealed that Kupffer cells play a major role in reperfusion injury to these two liver cell types. Moreover, attenuation of reperfusion injury with superoxide dismutase suggests that superoxide anions released by Kupffer cells contribute to the early damage to endothelial and parenchymal cells observed during reperfusion. Finally, increased hepatic concentrations of vitamin E were highly protective against the toxic effect of Kupffer cells during reperfusion. Thus, antioxidants and treatments designed to inhibit Kupffer cell function during the early, immediate stages of reperfusion may provide benefit against the deleterious actions of infiltrating neutrophils which contribute to hepatic damage in the later stages of reperfusion injury.

Acknowledgements

The research was supported by the National Institute on Alcohol Abuse and Alcoholism (Bethesda, MD, USA) grant AA07337.

References

[1] R.G. Thurman, H. Bunzendahl and J.J. Lemasters (1993) Role of sinusoidal lining cells in hepatic reperfusion injury following cold storage and transplantation. *Seminars in Liver Disease*, **13**, 93–100.

[2] O. Chazouilleres, Y. Calmus, M. Vaubourdolle and F. Ballet (1993) Preservation-induced liver injury. Clinical aspects, mechanisms and therapeutic approaches. *Journal of Hepatology*, **18**, 123–134.

[3] R.C. Dart, D.C. Liebler and I.G. Sipes (1993) Hepatic injury and lipid peroxidation during hemorrhagic shock and resuscitation. *Life Sciences*, **53**, 1685–1690.

[4] B.J. Zimmerman and D.N. Granger (1994) Mechanisms of reperfusion injury. *American Journal of Medical Science*, **307**, 284–292.

[5] H. Jaeschke (1995) Mechanisms of oxidant stress-induced acute tissue injury. *Proceedings of the Society for Experimental Biology and Medicine*, **209**, 104–111.

RIGHTSLINK

[6] H. Jaeschke and A. Farhood (1991) Neutrophil and Kupffer cell-induced oxidant stress and ischemia–reperfusion injury in rat liver. *Americal Journal of Physiology*, **260**, G355–G362.
[7] H. Jaeschke, A.P. Bautista, Z. Spolarics and J.J. Spitzer (1991) Superoxide generation by Kupffer cells and priming of neutrophils during reperfusion after hepatic ischemia. *Free Radical Research Communications*, **15**, 277–284.
[8] Y. Shiratori, H. Kiriyama, Y. Fukushi, T. Nagura, H. Takada, K. Hai and K. Kamii (1994) Modulation of ischemia–reperfusion-induced hepatic injury by Kupffer cells. *Digestive Diseases and Sciences*, **39**, 1265–1272.
[9] C. Bremer, B.U. Bradford, K.J. Hunt, K.T. Knecht, H.D. Connor, R.P. Mason and R.G. Thurman (1994) Role of Kupffer cells in the pathogenesis of hepatic reperfusion injury. *Americal Journal of Physiology*, **267**, G630–G636.
[10] K. Decker (1990) Biologically active products of stimulated liver macrophages (Kupffer cells). *European Journal of Biochemistry*, **192**, 245–261.
[11] K.A. Lindert, J.C. Caldwell-Kenkel, S. Nukina, J.J. Lemasters and R.G. Thurman (1992) Activation of Kupffer cells on reperfusion following hypoxia: particle phagocytosis in low-flow reflow model. *Americal Journal of Physiology*, **262**, G345–G350.
[12] J.C. Caldwell-Kenkel, R.T. Currin, Y. Tanaka, R.G. Thurman and J.J. Lemasters (1991) Kupffer cell activation and endothelial cell damage after storage of rat livers: effects of reperfusion. *Hepatology*, **13**, 83–95.
[13] D.A. Samarasinghe and G.C. Farrell (1996) The central role of sinusoidal endothelial cells in hepatic hypoxia–reoxygenation injury in the rat. *Hepatology*, **24**, 1230–1237.
[14] T. Minor, W. Isselhard and K. Berghaus (1993) Parenchymal and vascular endothelial cell injury in the hypoxic and reperfused rat liver. Evidence for superoxide anion generation by perfusion with ferricytochrome c. *Biomedicine and Pharmacotherapy*, **47**, 213–218.
[15] M.B. Marotto, R.G. Thurman and J.J. Lemasters (1988) Early midzonal cell death during low-flow hypoxia in the isolated, perfused rat liver: protection by allopurinol. *Hepatology*, **8**, 585–590.
[16] N.C. Hoglen, B.A. Abril, J.M. Sauer, D.L. Earnest, R.S. McCuskey, R.C. Lantz, S.A. Mobley and I.G. Sipes (1997) Modulation of Kupffer cell and peripheral blood monocyte activity by *in vivo* treatment of rats with all-*trans*-retinol. *Liver*, **17**, 157–165.
[17] R. Scholz, W. Hansen and R.G. Thurman (1973) Interaction of mixed-function oxidation with biosynthetic processes. I. Inhibition of gluconeogenesis by aminopyrine in perfused rat liver. *European Journal of Biochemistry*, **38**, 64–72.
[18] L.A. Reinke, J.S. Tupper, P.R. Smith and D.J. Swenny (1987) Diminished pentose cycle flux in perfused liver of ethanol-fed rats. *Molecular Pharmacology*, **31**, 631–637.
[19] B.U. Bradford, M. Marotto, J.J. Lemasters and R.G. Thurman (1986) New, simple models to evaluate zone-specific damage due to hypoxia in the perfused rat liver: time course and effect of nutritional state. *Journal of Pharmacology and Experimental Therapeutics*, **236**, 263–268.
[20] P.N. Rao, T.R. Walsh, L. Makowka, R.S. Rubin, T. Weber, J.T. Snyder and T.E. Starzl (1990) Purine nucleoside phosphorylase: a new marker for free oxygen radical injury to the endothelial cell. *Hepatology*, **11**, 193–198.
[21] H.U. Bergmeyer and B. Bernt. Lactate dehydrogenase: uv assay with pyruvate and NADH. In: *Methods of Enzymatic Analysis*, edited by H.U. Bergmeyer. New York: Academic Press, Inc., 1974.
[22] P.A. Hoffe, R. May and B.C. Robertson (1978) Purine nucleoside phosphorylase from *Salmonella typhimurium* and rat liver. *Methods of Enzymology*, **51**, 517–524.
[23] J.K. Lang, K. Gohil and L. Packer (1986) Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. *Analytical Biochemistry*, **157**, 106–116.
[24] G.J. Gores, L.J. Kost and N.F. LaRusso (1986) The isolated perfused rat liver: conceptual and practical considerations. *Hepatology*, **6**, 511–517.
[25] J.J. Lemasters, C.J. Stemkowski, S. Ji and R.G. Thurman (1983) Cell surface changes and enzyme release during hypoxia and reoxygenation in the isolated, perfused rat liver. *Journal of Cell Biology*, **97**, 778–786.
[26] J.J. Lemasters, S. Ji and R.G. Thurman (1981) Centrilobular injury following low flow hypoxia in isolated, perfused rat liver. *Science*, **213**, 661–663.
[27] J. Ferreira, G. Tapia and L.A. Videla (1998) Effects of the Kupffer cell inactivator gadolinium chloride on rat liver oxygen uptake and content of mitochondrial cytochromes. *FEBS Letters*, **426**, 263–265.
[28] K.D. Lettinga, W. Gutter, C.J.F. Van Noorden, J.P.M. Schellens and W.M. Frederiks (1996) Early effects of high doses of retinol (vitamin A) on the *in situ* cellular metabolism in rat liver. *Liver*, **16**, 1–11.
[29] M. Younes and O. Strubelt (1988) The involvement of reactive oxygen species in hypoxic injury to rat liver. *Research Communications in Chemical Pathology and Pharmacology*, **59**, 369–381.
[30] Z. Zhong, J.J. Lemasters and R.G. Thurman (1989) Role of purines and xanthine oxidase in reperfusion injury in perfused liver. *Journal of Pharmacology and Experimental Therapeutics*, **250**, 470–475.
[31] M. Younes, E. Kayser and O. Strubelt (1992) Effect of antioxidants on hypoxia/reoxygenation-induced injury in isolated perfused rat liver. Pharmacology and Toxicology, **71**, 278–283.
[32] S. Marubayashi, K. Dohi, K. Ochi and T. Kawasaki (1986) Role of free radicals in ischemic rat liver cell injury: prevention of damage by α-tocopherol administration. *Surgery*, **99**, 184–191.
[33] S.M. Lee and M.G. Clemens (1992) Effect of α-tocopherol on hepatic mixed function oxidases in hepatic ischemia/reperfusion. *Hepatology*, **15**, 276–281.
[34] J.H. Kuo, H.W. Chen, R.G.R. Chou and C.K. Lii (1997) Vitamin E protection of cell morphology under oxidative stress is related to cytoskeletal proteins in rat hepatocytes. *Archives of Toxicology*, **71**, 231–237.
[35] G.A. Wanner, W. Ertel, P. Muller, Y. Hofer, R. Leiderer and M.D. Menger (1996) Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation. *Shock*, **5**, 34–40.
[36] G.A. Wanner, P. Muller, W. Ertel, C.J. Busch, M.D. Menger and K. Messmer (1998) Differential effect of cyclooxygenase metabolites on proinflammatory cytokine release by Kupffer cells after liver ischemia and reperfusion. *American Journal of Surgery*, **175**, 146-151.
[37] M. Gyenes and H. de Groot (1993) Prostanoid release by Kupffer cells upon hypoxia–reoxygenation: role of pH_i and Ca_i^{+2}. *American Journal of Physiology*, **264**, G535–G540.
[38] W. Sakamoto, K. Fujie, J. Nishihira, M. Mino, I. Morita and S.I. Murota (1991) Inhibition of PGE_2 production in macrophages from vitamin E-treated rats. *Prostaglandins Leukotrienes and Essential Fatty Acids*, **44**, 89–92.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/21/11
For personal use only.

RIGHTSLINK