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TITLE

ALPHA-TOCOPHEROL LIPOSOMES ALLEVIATE LPS-INDUCED HEPATOTOXICITIES

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α-Tocopherol liposomes alleviate LPS-induced hepatotoxicities

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Summary Gram-negative bacteria, in part through lipopolysaccharide (LPS) and tumor necrosis factor-alpha (TNFα), activate phagocytes to generate reactive oxygen species (ROS), which have been known to play a key role in the pathogenesis of liver injury. Accordingly, we hypothesized that the susceptibility of the liver to ROS should be reduced by augmenting its antioxidant status. Adult male Sprague-Dawley rats were pretreated with α-tocopherol liposomes (20 mg α-tocopherol/kg body weight, i.v.), plain liposomes or saline. 24 h after liposomal treatment, rats were injected intravenously with LPS (1 mg/kg, *Escherichia coli*: 0111:B4) and killed 2 h later. Livers of saline-pretreated animals challenged with LPS were damaged as demonstrated by increases in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. The hepatic injury appeared to be associated with oxidative stress-mediated mechanisms as evidenced by increases in lipid peroxidation and decreases in glutathione concentration in the liver, both indices of oxidative stress. Also, LPS injection resulted in increases in plasma TNFα and thromboxane B_2 (TXB_2) levels, as well as increases in hepatic myeloperoxidase (MPO) activity and chloramine concentration, suggestive of activation of the inflammatory response. Pretreatment of rats with plain liposomes, 24 h prior to LPS challenge, failed to protect against the LPS-induced liver injury. Although pretreatment of animals with α-tocopherol liposomes was not effective in preventing the LPS-induced inflammatory response, it conferred a partial protection against the LPS-induced changes in plasma AST and ALT activities as well as in hepatic levels of lipid peroxidation, glutathione and chloramine concentrations. These data appear to suggest that augmentation of the hepatic antioxidant status is effective in alleviating the LPS-induced liver injury.

INTRODUCTION

Septic shock may develop as a complication of infection. The incidence of septic shock has increased progressively over the past few decades with a mortality rate ranging between 40 and 60%, despite advances in antimicrobial therapy and critical care medicine. The pathophysiological changes observed in Gram-negative bacteremia resemble those often accompanying lipopolysaccharide (LPS) administration. LPS, a component of the bacterial wall of Gram-negative bacteria, has been recognized as one of the most potent bacterial products in the induction of host inflammatory responses which may lead to multiple organ failure and ultimately death.[1–5]

It has been recognized that the dominant organ of LPS entrapment following its administration is the liver. Despite the ability of the liver to detoxify LPS, marked morphological and biochemical alterations occur in the organ after its exposure to LPS. Several investigators have reported that patients or experimental animals that die from septic shock display alterations in Kupffer cells; formation of fibrin thrombi; increased neutrophils in liver sinusoids; and zonal necrosis of the liver.[3,6,7]

Neutrophils and other inflammatory cells play a major role in the pathogenesis of LPS-induced liver injury.[3,8–11] The following observations have been reported implicating neutrophils as mediators of LPS-induced liver injury:

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(i) neutrophils accumulate in the liver and associate with foci of hepatocellular necrosis during exposure to LPS; (ii) depletion of circulating neutrophils by anti-neutrophil antibodies attenuates LPS-induced liver injury; (iii) inhibition of toxic neutrophil products ameliorates liver injury; and (iv) inhibition of Kupffer cell function with methyl palmitate confers protection against LPS hepatotoxicity.[3,8–11]

In recent years, it has become apparent that LPS-induced tissue injury is linked to the phagocyte's ability to release a complex array of agents that can destroy normal cells. Among these agents are enzymes such as lysozymes, peroxidases and elastases as well as reactive oxygen species such as superoxides, hydrogen peroxide, hydroxyl radical and hypohalous acid.[2,3,8–11] Reactive oxygen species are known to injure tissues through peroxidation of membrane lipids, DNA strand breakage, and alterations of amino acids and cellular metabolism.[2,12–14] Recognizing the fact that LPS-induced liver injury is associated with the presence of reactive oxygen species, we hypothesized that the administration of α-tocopherol should attenuate such injury. α-Tocopherol is a lipophilic antioxidant which can protect against oxidant-induced tissue injury by reducing membrane lipid peroxidation and lipid peroxide formation, possibly through its action in scavenging reactive oxygen species and by exerting a stabilizing effect on membranes.[15,16]

The present study was undertaken to investigate whether the intravenous administration of liposome-entrapped α-tocopherol would attenuate the LPS-induced acute liver injury. α-Tocopherol was incorporated in liposomes because, in its free form, it is too viscous to be administered and emulsifiers used to solubilize the antioxidant are toxic to tissues.[17,18] Liposomes are artificially prepared phospholipid vesicles with amphipathic features. Hydrophilic molecules can be encapsulated in the aqueous spaces and lipophilic molecules can be incorporated in the lipid bilayers. Encapsulation of antioxidants in liposomes promotes their therapeutic potential against oxidant-induced tissue injury, presumably by liposomes facilitating the intracellular uptake and extending the half-lives of the entrapped antioxidants.[19–23] In this study, the protective effect of α-tocopherol liposomes against LPS-induced liver injury was assessed biochemically by measuring the plasma activities of ALT and AST as well as the hepatic levels of GSH and lipid peroxidation.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (*E. coli*, LPS 0111:B4) and α-tocopherol were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were obtained from Sigma Chemical Co. or BDH (Toronto, Ont., Canada).

Animals

Male Sprague-Dawley rats (approximate body weight 220–250 g) were purchased from Charles River Canada, Inc. (St Constant, Que., Canada). All animals were housed in stainless-steel cages with free access to pelleted purina laboratory chow and tap water. The animals were kept at room temperature (22–24°C) and were exposed to alternate cycles of 12 h light and darkness. Animals used in this study were treated and cared for in accordance with the guidelines recommended by the Canadian Council on Animal Care.

Preparation of α-tocopherol liposomes

α-Tocopherol liposomes were prepared from a mixture of DPPC and α-tocopherol in a 7:3 molar ratio. The lipids were dissolved in chloroform:methanol (2:1 v/v) and the lipid mixture was dried in a water bath at 40°C under a stream of helium to a thin film coating the interior surface of the glass vessel. Any traces of solvent were removed by placing the vessel under vacuum for at least 1 h. The dried lipid was hydrated with 1.0 ml of 5 mM potassium phosphate buffer, pH 6.5, containing 3 mM EDTA, and then vortexed to form multilamellar vesicles. The multilamellar vesicles were extruded (10 times) with an extruder (Lipex Biomolecules, Vancouver, BC, Canada) through two stacked polycarbonate filters of 400 nm pore size using a helium pressure of 100–200 psi. The extrusion process favours a more homogeneous vesicle size distribution. Free α-tocopherol was removed by washing the liposomes twice in 5 mM potassium phosphate buffer, pH 6.5, and pelleting at 110 000 *g* for 1 h at 5°C in a Beckman L8-70 ultracentrifuge. Liposomal vesicle size was determined with the use of a Coulter N4SD particle-size analyzer and was found to have a mean diameter of 389 ± 35 nm.

Treatment of animals

α-Tocopherol liposomes (20 mg α-tocopherol/kg body weight) or plain liposomes (32 μmoles of lipid/animal) were administered intravenously via the tail vein. 24 h after liposomal administration, rats were injected intravenously with a single dose of *E. coli* LPS (1.0 mg/kg body weight) to induce liver injury. Injections were administered between 0800–0900 h. LPS was suspended in saline and prepared shortly before use. Control animals received an equivalent volume of saline.

Experimental design

To investigate whether α-tocopherol liposomes would protect livers against the toxic effects of LPS, rats pretreated with α-tocopherol liposomes or plain liposomes were injected with a single dose of LPS and killed 2 h later. The protective effect of α-tocopherol liposomes or plain liposomes against LPS-induced liver damage was assessed biochemically by measuring the plasma AST, ALT, TNFα and TXB_2 activities as well as the hepatic MPO activity and chloramine concentration. Lipid peroxidation levels and GSH concentrations in the livers of control and treated animals were also measured.

Tissue preparations

Blood samples were collected from animals by cardiac puncture under halothane anesthesia. The collected blood was centrifuged immediately and plasma was stored at –70°C and used within 48 h for enzyme analysis. Livers were removed from animals immediately after decapitation and rinsed with ice-cold saline to remove excess blood. All subsequent steps were carried out at 0–4°C. Following rinsing, livers were quickly weighed and finely minced. Approximately 1 g of liver sample was homogenized with a Brinkmann Polytron in a sufficient volume of ice-cold 50 mM potassium phosphate buffer, pH 7.4, to produce a 20% homogenate. For the measurement of lipid peroxidation, homogenates were prepared as described previously except the homogenizing medium contained 3 mM ethylenediaminetetraacetic acid (EDTA).

Enzyme measurements

AST and ALT activities in plasma were determined by the method of Reitman and Frankel[24] with a Sigma Diagnostic kit No. 505 and enzyme activities were expressed as Sigma Frankel units/ml of plasma.[24] The activity of myeloperoxidase (MPO) in sonicated whole liver homogenates was determined as described by Suntres and Shek[25] by following the changes in optical density resulting from the decomposition of hydrogen peroxide in the presence of tetramethylbenzidine and was expressed as changes in absorbance measured at 450 nm. Activities of superoxide dismutase, catalase, glutathione reductase (GSH-R) and glutathione peroxidase (GSH-Px) in sonicated liver homogenates were determined as previously described.[26] Protein determinations were estimated by the method of Lowry et al.[27]

Determination of chloramines

Chloramine concentrations in hepatic homogenates were determined by colorimetric measurement of the triiodide ion formed by the oxidation of potassium iodide.[28]

Determination of lipid peroxidation

Liver homogenates from treated and control animals were assayed for the presence of lipid conjugated dienes as described by Suntres and Shek.[25]

Determination of reduced glutathione concentrations

Reduced glutathione, more precisely non-protein sulphydryl, concentrations in hepatic homogenates were determined as described by Suntres and Shek.[29] Briefly, tissues were homogenized in 20% (w/v) TCA and centrifuged at 10 000 rpm for 20 min in a refrigerated Sorval RC-5B centrifuge. An aliquot of the supernatant fraction in 0.3 M phosphate buffer was treated with 5,5-dithiobis-[2-nitrobenzoic acid] and the absorbance at 412 nm was measured.

Measurement of plasma TNFα and TXB_2 concentrations

TNFα was measured by an enzyme-linked immunosorbent assay employing affinity-purified rabbit anti-TNFα as capture antibody and horseradish peroxidase labelled mouse monoclonal anti-TNFα as conjugated antibody (Genzyme, Cambridge, MA, USA). For the detection of TXB_2 in plasma, a specific RIA kit (Amersham, UK) was used. The assay is based on the competition between unlabelled TXB_2 and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for TXB_2.

Measurement of neutrophils in whole blood

Blood samples were collected in tubes containing 5% EDTA. The EDTA-treated blood samples were used to determine neutrophil counts using an automated Coulter JT hematology analyzer (Coulter Electronics Inc., Oakville, Ont., Canada).

Statistical analysis

Data from control and treated groups were evaluated by one-way analysis of variance (ANOVA). If the F values were significant, the one-tailed Student's t test was used to compare the treated and the control groups. The level of significance was accepted at $P < 0.05$.[30]

RESULTS

Plasma AST and ALT activities

The measurement of hepatic enzymes released into the blood has been employed as a reliable indicator for the assessment of hepatotoxicity. Plasma AST and ALT activities in saline-pretreated animals were substantially ele-

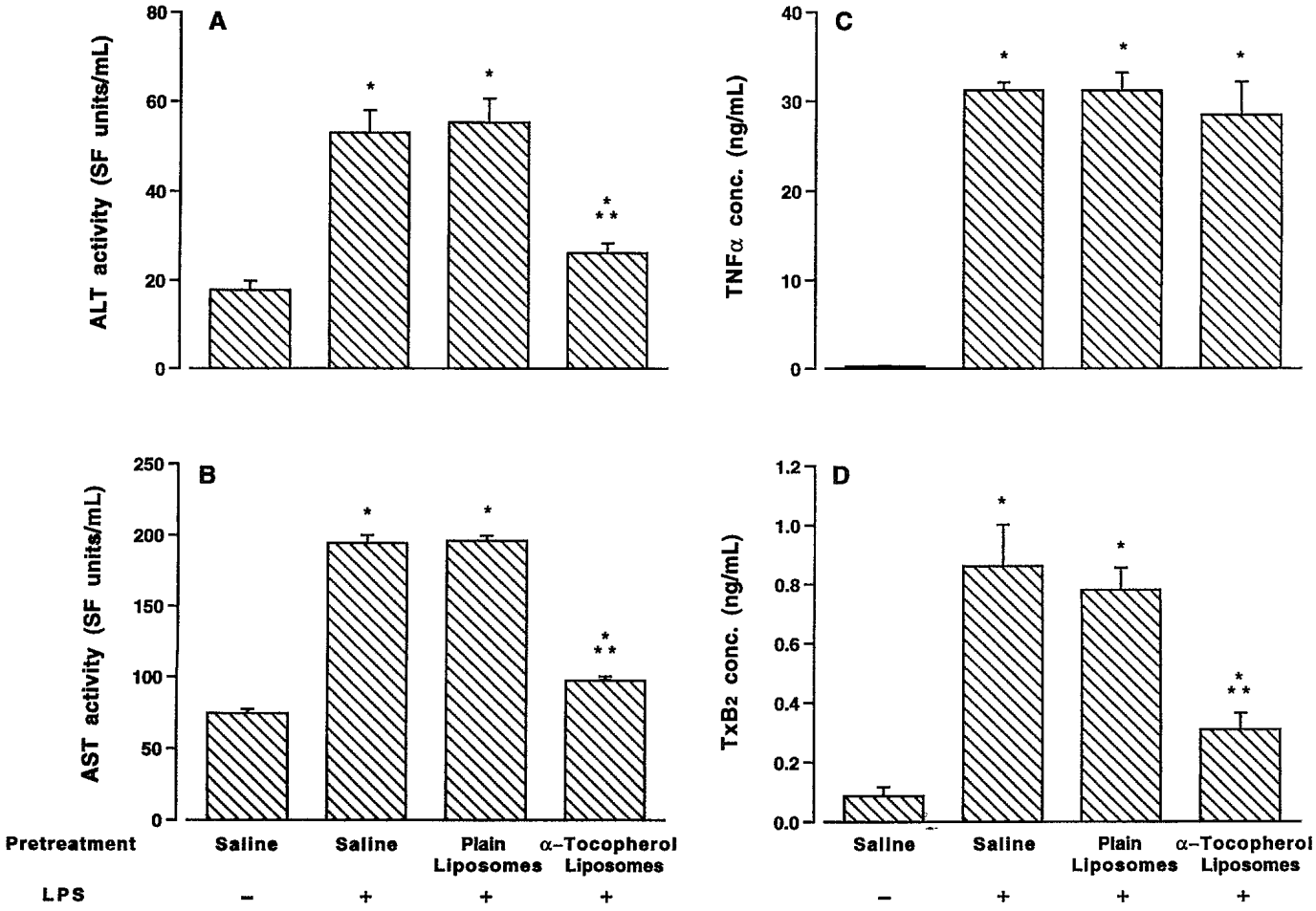

Fig. 1 Changes in plasma ALT, AST, TNFα and TXB_2 levels in LPS-injected animals receiving various pretreatments. Rats were pretreated intravenously with saline, plain liposomes or α-tocopherol liposomes as described in 'Materials and methods' and 24 h later, pretreated animals were given an i.v. injection of LPS (1 mg/kg). 2 h after LPS injection, animals were bled by cardiac puncture and their plasma used for the measurement of ALT and AST activities as well as TNFα and TXB_2 concentrations. Each vertical bar represents the mean ± SEM of five animals. *Significantly different ($P < 0.05$) from the corresponding value obtained from saline-pretreated animals without LPS challenge; **significantly different ($P < 0.05$) from the corresponding value obtained from saline-pretreated animals challenged with LPS.

vated (by 160% and 198%, respectively) following intravenous LPS administration (Fig. 1A,B). Pretreatment of animals with plain liposomes did not alter the LPS-induced changes in plasma AST and ALT activities. In contrast, the LPS-induced increases in plasma activities of AST and ALT in animals pretreated with α-tocopherol liposomes were significantly lower than that of animals in the saline-pretreated group.

Plasma TNFα and TXB_2 concentrations

Plasma TNFα and TXB_2 concentrations in saline-pretreated animals were significantly elevated following LPS challenge (Fig. 1C,D). A very similar increase in plasma TNFα concentration was also observed in LPS-challenged rats pretreated with plain liposomes or α-tocopherol liposomes. Pretreatment with α-tocopherol liposomes, however, significantly reduced the LPS-induced increase in plasma TXB_2.

Blood neutrophils and hepatic MPO and chloramine

The circulating neutrophil concentrations of LPS-injected animals were all decreased by about 50% regardless of their pretreatment with saline, plain liposomes or α-tocopherol liposomes (Fig. 2A). Since LPS is known to stimulate neutrophil infiltration in liver and other organs and MPO is an enzyme primarily localized in neutrophils, hepatic MPO activities of control and treated animals were also determined. As shown in

Figure 2B, the MPO activity in saline-pretreated animals were substantially elevated by 39% following LPS challenge, suggestive of neutrophil infiltration in the liver. A very similar increase in MPO activity was also observed in LPS-challenged rats pretreated with plain liposomes or α-tocopherol liposomes.

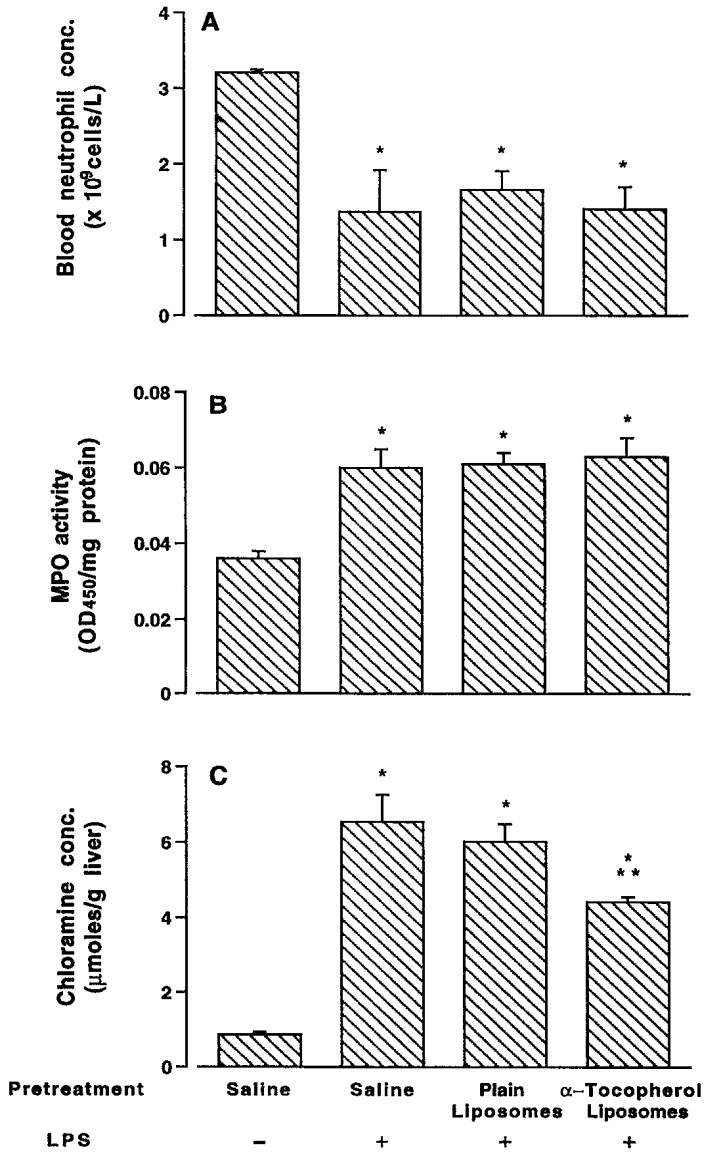

Fig. 2 Changes in blood neutrophil, hepatic myeloperoxidase (MPO) and chloramine levels in LPS-injected animals receiving various pretreatments. Rats were pretreated intravenously with saline, plain liposomes or α-tocopherol liposomes as described in 'Materials and methods' and 24 h later, pretreated animals were given an i.v. injection of LPS (1 mg/kg). 2 h after LPS injection, animals were bled and their livers harvested for the determination of MPO activity and chloramine levels. Each vertical bar represents the mean ± SEM of five animals. *Significantly different ($P < 0.05$) from the corresponding value obtained from saline-pretreated animals without LPS challenge; **significantly different ($P < 0.05$) from the corresponding value obtained from saline-pretreated animals challenged with LPS.

Chloramines, the long-lived oxidants produced by neutrophils, are known to contribute to tissue injury.[28,31] In this study, LPS administration in saline-pretreated animals resulted in a seven-fold increase in hepatic chloramine concentration compared to that of animals in the saline-treated group (Fig. 2C). Similarly, a significant increase in chloramine concentrations was also observed in livers of rats pretreated with plain liposomes 24 h prior to LPS challenge. The chloramine concentration in the livers of rats pretreated with α-tocopherol liposomes were significantly lower than those observed in saline-pretreated animals challenged with LPS.

Lipid peroxidation and GSH levels in livers

Peroxidation of membrane lipids has been implicated as a possible mechanism of acute oxidative stress-induced lethal injury.[12,32] As shown in Figure 3A, LPS produced a significant increase in lipid peroxidation levels, indicated by the formation of diene conjugates, in liver homogenates of saline-pretreated animals. Pretreatment of animals with plain liposomes did not significantly alter the LPS-induced increase in membrane lipid peroxidation. On the other hand, pretreatment of rats with α-tocopherol liposomes significantly reduced the LPS-induced lipid peroxidation.

Since GSH is known to play an important role in protecting cells from oxidant-induced tissue injury,[12,33] levels of reduced glutathione in the livers of control and treated animals were also measured. The results presented in Figure 3B revealed that GSH levels in the livers of saline-pretreated rats challenged with LPS were significantly decreased by 25% compared to the saline-pretreated group. A similar decrease in GSH levels was also observed in livers of rats pretreated with plain liposomes 24 h prior to LPS treatment. In contrast, the magnitude of LPS-induced reduction in hepatic GSH levels in rats pretreated with α-tocopherol liposomes was significantly less than that of saline-pretreated animals.

Hepatic activities of superoxide dismutase, catalase, GSH-Px and GSH-R

To assess the relative importance of the antioxidant enzyme system in LPS-induced hepatotoxicity, the effects of LPS on hepatic superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities were examined. All these hepatic enzyme activities in animals pretreated with saline, plain liposomes, or α-tocopherol liposomes were not significantly affected 2 h post-LPS injection (data not shown). It should be noted that the challenge of animals with LPS did not significantly alter the total hepatic concentrations among the groups.

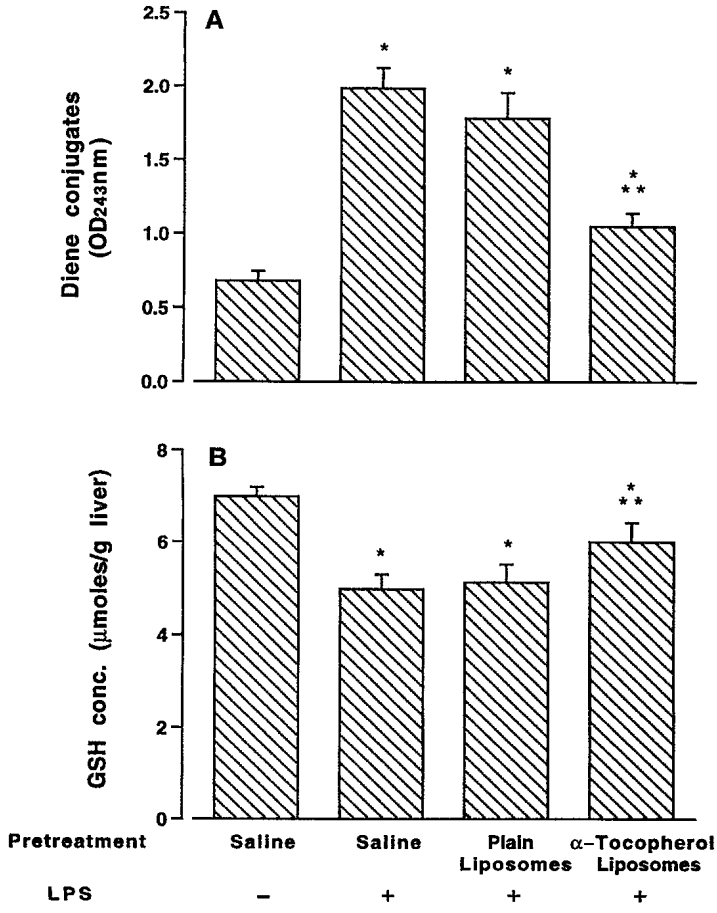

Fig. 3 Changes in lipid peroxidation levels and GSH concentrations in the livers of LPS-injected animals receiving various pretreatments. Rats were pretreated intravenously with saline, plain liposomes or α-tocopherol liposomes as described in 'Materials and methods' and, 24 h later, pretreated animals were given an i.v. injection of LPS (1 mg/kg). 2 h after LPS injection, animals were killed and their livers harvested for the determination of lipid peroxidation as measured by the formation of diene conjugates and for GSH assays. Each vertical bar represents the mean ± SEM of five animals. *Significantly different ($P < 0.05$) from the corresponding value obtained from saline-pretreated animals without LPS challenge; **significantly different ($P < 0.05$) from the corresponding value obtained from saline-pretreated animals challenged with LPS.

DISCUSSION

Systemic administration of LPS generally leads to its rapid accumulation in Kupffer cells.[3–7] The interaction of LPS with these hepatic phagocytes results in the release of mediators that play a central role in the pathogenesis of liver injury. These mediators include, but are not limited to, reactive oxygen species, cytokines such as TNFα, and degradative enzymes. LPS-induced mediators can either exert their toxic effects directly, as in the case of reactive oxygen species and degradative enzymes, or indirectly by recruiting and activating neutrophils and other inflammatory cells, known to augment tissue injuries.[3–5,34,35] Our results are in agreement with those observations where intravenous LPS administration resulted in an increase in plasma TNFα, suggestive of activation of the inflammatory response; increases in hepatic MPO activity and chloramine concentration, suggestive of neutrophil infiltration and activation; and an increase in plasma TXB_2, suggestive of perturbation of the arachidonic acid cascade.

Evidence presented in this study indicated that the liver injury observed 2 h post-LPS administration was induced, to a great extent, via oxidative stress-mediated mechanisms. LPS administration in saline-pretreated animals resulted in an increase in lipid peroxidation and reduction in GSH levels, effects alleviated by prior treatment of animals with α-tocopherol liposomes. Our results also corroborate those reported by other investigators who showed that pretreatment of animals with other antioxidants (e.g. superoxide dismutase, catalase) also prevented the LPS-induced oxidative stress-mediated hepatic injuries.[2,3,10,36–38] Furthermore, these data suggested that the LPS-induced liver injury was not due to the depletion of the antioxidant defence system, but perhaps to an overproduction of reactive oxygen species by phagocytes or possibly following the activation of the xanthine dehydrogenase/xanthine oxidase enzyme system.

Neutrophils are known to be involved in the pathogenesis of LPS-induced liver injury. Results from other studies have shown that the depletion of circulating neutrophils attenuate the LPS-induced liver injury.[3,8,39] Recruitment and activation of neutrophils in tissues is known to occur in response to the presence of inflammatory mediators.[3–5,36,37] It is conceivable, therefore, that inhibition of inflammatory mediators could prevent neutrophil recruitment in the liver as well as other tissues. Contrary to expectation, α-tocopherol liposomes failed to prevent neutrophil recruitment in the liver as indicated by a marked elevation in MPO activity in the livers of LPS-treated animals. The failure of α-tocopherol to inhibit neutrophils from migrating to and accumulating in the liver may be partly due to the inability of the antioxidant to limit the production of TNFα and other chemotactic mediators.

It has been established that activated neutrophils and macrophages can cause tissue injury via the release of several toxic metabolites, including proteases and reactive oxygen species.[3–5,31,34–37] Although α-tocopherol failed to downregulate the accumulation of neutrophils in the liver, it may dampen the toxic effects of metabolites released from phagocytic cells. Indeed, the lower chloramine levels found in the livers of animals pretreated with liposomal α-tocopherol strongly suggest

that the antioxidant may modulate the production of reactive oxygen species by neutrophils. Chloramines, long-lived oxidants produced by neutrophils, are known to participate in tissue injury by inactivating proteinase inhibitors as well as by oxidizing other cellular membrane and cytosolic components. Recently, it has been demonstrated that α-tocopherol, in addition to its antioxidant properties, has anti-inflammatory properties. α-Tocopherol is known to inhibit the generation of superoxide anion and other reactive oxygen species from granulocytes activated by certain immunochemical stimuli.[40–42]

In addition to its ability to dampen the release of reactive oxygen species from activated neutrophils, α-tocopherol can exert its protective effects by inhibiting membrane lipid peroxidation. Peroxidation of membrane lipids has been shown to be associated with sepsis and has been implicated as an early contributor to septic organ injury.[2,3,36–38] In our study, pretreatment of animals with α-tocopherol liposomes suppressed the increase in LPS-induced lipid peroxidation, a treatment effect that likely would prevent the formation and accumulation of lipid peroxides, products of peroxidized membranes known to be involved in liver and other tissue injuries. This interpretation is consistent with our other observation that liposomal α-tocopherol protected against the LPS-induced depletion of GSH which serves as a substrate in the GSH peroxidase/GSH reductase pathway to detoxify lipid peroxides. The role of GSH in protecting cells from peroxidative injury induced by several oxidants has been reported by several investigators.[14,32,33]

Perturbation of arachidonic acid metabolism is known to lead to an increased production of eicosanoids, mediators implicated in many types of shock, including endotoxin shock.[3,43,44] TXA_2, convertible to a more stable and inactive metabolite TXB_2, is one of the most potent arachidonic acid metabolites, known to exert their effects by acting as a vasoconstrictor and platelet-aggregating factor. It has been demonstrated that the inhibition of TXA_2 confers therapeutic efficacy in shock and trauma models.[45,46] It also appears that tissue levels of α-tocopherol are important in modulating the arachidonic acid cascade, as demonstrated by the observation of higher levels of TXA_2 in conditions of vitamin-E deficiency and lower levels with vitamin-E supplementation. Our present study demonstrated that pretreatment of animals with liposomal α-tocopherol significantly reduced their LPS-induced plasma TXB_2 levels after LPS injection. Although the mechanisms by which α-tocopherol modulates the arachidonic acid cascade are still under investigation, it has been reported that α-tocopherol is potentially capable of suppressing the synthesis of TXB_2 and other arachidonic acid metabolites by inhibiting phospholipase A_2 and/or cyclooxygenase and by suppressing the formation of lipid peroxides which upregulate the arachidonic acid cascade.[43,44]

Failure of α-tocopherol to influence the inflammatory response, as evidenced by its inability to lower the elevated TNFα and myeloperoxidase activities, suggests that granulocytes may exert their toxic effects by mechanisms independent of oxidative stress. It has been shown that granulocytes, once activated, can release proteases and other degradative enzymes that are known to participate in the development of LPS-induced tissue injury.[13] Since the presence of reactive oxygen species may not be the sole mechanism by which LPS exerts its damaging effects, it is not surprising that liposomal α-tocopherol failed to completely protect against LPS-induced liver injury.

Unlike α-tocopherol esters, the form most commonly used in supplements, the parenteral administration of liposomal α-tocopherol allows the delivery of the biologically active antioxidant without the need for prior activation in the gastrointestinal tract, rendering the liposomal formulation more advantageous for treating liver injury mediated by oxidative stress mechanisms. The preferential uptake of liposomes and their associated agents by cells of the reticuloendothelial system also promote their passive targeting to the liver. Moreover, another key advantage of employing the liposomal delivery system is that liposomes are virtually non-toxic and can be administered in a fairly high quantity safely to achieve the delivery of a meaningful therapeutic dose of an entrapped drug to the target site.

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