© Birkhäuser Verlag, Basel, 1997

CMLS, Cell. mol. life sci. 53 (1997) 294–302
1420-682X/97/040294-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Dietary vitamin E does not protect from endotoxin-induced hepatic microvascular dysfunction

M. Rücker[a], B. Finckh[b], A. Kohlschütter[b], M. D. Menger[a] and B. Vollmar[a,*]

[a]*Institute for Clinical and Experimental Surgery, University of Saarland, D-66421 Homburg/Saar (Germany), Fax +49 6841 166553, e-mail: exbvol@med-rz.uni-sb.de*
[b]*Clinic for Paediatrics, University of Hamburg, D-20246 Hamburg (Germany)*
Received 7 November 1996; received after revision 30 December 1996; accepted 20 January 1997

Abstract. Starting from the concept that lipopolysaccharide (LPS)-associated hepatotoxicity involves the action of reactive oxygen species, the present study was conducted to test whether vitamin E, a lipophilic antioxidant, prevents LPS-induced hepatic microvascular dysfunction and liver injury. Fifty-two rats were divided into three groups and fed diets containing 0 (n = 16), 75 (n = 18) or 8000 mg (n = 18) α-tocopherol acetate/kg food for four weeks. At 1 h and 6 h after intravenous LPS-exposure (10 mg/kg *E. coli* LPS) hepatic microvascular response and liver injury were assessed by the analysis of Kupffer cell phagocytic activity, leukocyte-endothelial cell interaction and nutritive sinusoidal perfusion (intravital fluorescence epi- illumination technique) as well as bile flow, serum liver enzyme activities and tissue histomorphology. In animals fed with 75 mg vitamin E/kg (standard diet), LPS caused hepatic Kupffer cell activation (increased phagocytic activity) and hepatic microvascular leukocyte activation, with stasis in sinusoids and adherence in postsinusoidal venules (1 h) followed by leukocytic infiltration into tissue (6 h) and progredient sinusoidal perfusion failure (6 h). Hepatic microvascular injury was accompanied by reduced bile flow and enhanced liver enzyme release. Vitamin E-enriched diet (8000 mg/kg) and even vitamin E-deficient diet did not significantly affect LPS-induced hepatic microvascular cell activation and perfusion failure. Thus, we conclude, that vitamin E is not effective to protect from endotoxin-induced hepatic microvascular dysfunction.
Key words. Vitamin E; α-tocopherol acetate; scavenger; reactive oxygen species; leukocyte-endothelial cell interaction; Kupffer cell; microcirculation; liver.

Evidence for reactive oxygen species-mediated organ injury is well documented in human sepsis and in animal models with endotoxemia and systemic inflammation [1]. Accordingly, reactive oxygen species generated by phagocytic cells upon activation by endotoxin [2] have been proposed as mediators in the pathogenetic sequelae of liver damage [3–5]. This is based on a variety of in vitro, in situ and in vivo studies showing the generation and release of reactive oxygen species by Kupffer cells (KC) and leukocytes in response to endotoxin [6–8]. The oxidative stress is further documented by the accumulation of hepatic thiobarbituric acid-reactive substances, representing the products of reactive oxygen species-mediated lipid peroxidation [9–12], and by the decrease in endogenous antioxidants, such as α-tocopherol, reduced coenzyme Q_9 and reduced glutathione, in livers subjected to endotoxemia [9, 13, 14]. Moreover, the protective effect of exogenously applied antioxidants underlines the involvement of reactive oxygen species in the pathophysiological sequelae of endotoxin-associated toxicity, including liver damage [9, 10, 15].

Vitamin E functions in vivo as a chain-breaking antioxidant [16] and has been reported to protect from postischemic tissue damage of heart, kidney, stomach, liver as well as striated muscle and skin tissue both in experimental models and human trials [17–23]. In contrast to postischemic tissue damage, only a few studies report on the efficiency of vitamin E in the prevention of endotoxemia-associated hepatotoxicity [10]. Sugino and coworkers have demonstrated preservation of energy rich phosphates and prevention of oxidative stress-induced lipid peroxidation by vitamin E in endotoxemic mice [10]. Although it is well known that both activated Kupffer cells and leukocytes mediate endotoxin-induced hepatic organ dysfunction and failure [8, 24–29], there is no information available as to whether antioxidative intervention with vitamin E has the potential to prevent the manifestation of microvascular and parenchymal injury. Therefore, we studied the effect of vitamin E on endotoxin-induced non-parenchymal cell activation, hepatic nutritive perfusion failure and liver dysfunction using intravital fluorescence microscopy.

Materials and methods

Animals. Male Sprague Dawley rats received either a vitamin E-enriched diet (8000 mg α-tocopherol acetate/kg; n = 18) or a vitamin E-deficient diet (0 mg α-tocopherol acetate/kg; n = 16) ad libitum from weanling (19–23 d of age; body weight: 45 ± 1 g) for four weeks.

* Corresponding author.

These rats are referred to as E+ and E−, respectively. Control animals (C; n = 18) were fed a standard diet containing 75 mg α-tocopherol acetate/kg. Besides α-tocopherol acetate as the only vitamin E compound, the concentrations of the other nutrients were the same for the three groups. Diets were obtained from Ssniff (Soest, Germany). The rats had free access to food and water. Dietary treatment had no effect on weight gain of the animals, with 157 ± 3 g (E+) and 161 ± 4 g (E−) increase of body weight within four week (C, 149 ± 5 g). After four weeks animals (C, n = 12; E+, n = 12; E−, n = 11) were exposed to a single bolus injection of *E. coli* lipopolysaccharide (LPS, serotype 0128: B12; 10 mg/kg iv; Sigma, Deisenhofen, Germany). At either 1 h (C: n = 6; E+: n = 6; E−: n = 6) or 6 h (C: n = 6; E+: n = 6; E−: n = 5) after LPS-exposure, anesthesia and microsurgical preparation were performed for the intravital microscopic study. Sham-treated animals (sC, n = 6; sE+, n = 6; sE−, n = 5) received isotonic saline solution only, but underwent the identical experimental protocol as the LPS-exposed animals.

Model. Experiments were performed in accordance with the German legislation on the protection of animals and the 'ethical principles and guidelines for scientific experiments on animals' of the Swiss Academy of Medical Sciences. Under atropine premedication (1.25 mg/kg body weight s.c.) and pentobarbital anesthesia (50 mg/kg body weight i.p.) the animals were tracheotomized to facilitate spontaneous respiration (room air). The animals were placed on their backs on a heating pad for maintenance of body temperature at 36–37 °C. Polyethylene catheters (PE 50, ID 0.58 mm, Fa. Portex, Hythe, UK) in the right carotid artery and jugular vein allowed for the assessment of systemic hemodynamics, injection of fluorescent dyes for intravital microscopy and permanent infusion of isotonic saline solution at a rate of 5 ml/kg body weight × h. Following transverse laparotomy and cannulation of the common bile duct (PE-50) for continuous collection of bile, the animals were positioned on their left side and the left liver lobe was exteriorized and covered with a glass slide for intravital fluorescence microscopy [30, 31].

Intravital fluorescence microscopy. By use of a modified Zeiss-Orthoplan microscope with a 100 W HBO mercury lamp (Axiotech, Zeiss, Jena, Germany) attached to a blue filter system (450–490 nm/ > 520 nm, excitation/emission wavelength), hepatic microcirculation was analysed by the epi-illumination technique. The microscopic images were recorded by a CCD video camera (FK 6990, COHU, Prospective Measurements Inc., San Diego, CA, USA) and transferred to a video system (S-VHS Panasonic AG7350, Matsushita, Tokyo, Japan). Using a water immersion objective (W 20 × /0.5; Zeiss, Jena, Germany) magnification of × 730 was achieved on the video screen (PVM-2130 QM, Sony, Munich, Germany). Contrast enhancement was achieved by i.v. injection of sodium fluorescein (2 μmol/kg i.v.; Merck, Darmstadt, Germany) and used for analysis of sinusoidal perfusion [30]. The use of the green filter system (530–560/ > 580 nm) permitted the analysis of leukocyte-endothelial cell interaction after staining the leukocytes in vivo with rhodamine-6 G (2 μmol/kg iv; Merck, Darmstadt, Germany) [31]. For intravital microscopic analysis of phagocytic activity of KC, plain fluorescent latex particles (diameter 1.1 μm; Polyscience Inc., Warrington, PA) were injected intra-arterially through the carotid catheter (3×10^8/kg in 1 ml isotonic saline) [32, 33].

Quantitative video analysis. Quantitative assessment of microcirculatory parameters was performed off-line by frame-to frame analysis of the videotaped images. Within 10 acini per animal, sinusoidal perfusion failure was determined by counting the number of non-perfused sinusoids (given as percentage of all sinusoids visible) [30]. Leukocyte-endothelial cell interaction was analysed within 10 hepatic acini and 10 postsinusoidal venules per animal, including i) the number of stagnant leukocytes, located within sinusoids (given as cells/acinus), and not moving during an observation period of 20 s, (ii) the number of rolling leukocytes (given as percentage of non-adherent leukocytes), moving within postsinusoidal venules at a velocity less than two-fifths of the centerline velocity, and (iii) the number of adherent leukocytes, located within postsinusoidal venules (given as cells/mm^2 endothelial surface, calculated from diameter and length of the vessel segment studied, assuming cylindrical geometry), and not moving or detaching from the endothelial lining during an observation period of 20 s [31].

Phagocytic activity of KC was assessed by the determination of the kinetics of latex particle adherence off-line by frame-to-frame-analysis of the videotaped images. Ten to 15 observation fields per animal were analysed successively within 5 min of injection. The kinetics of adherence were quantified by the number of particles moving in sinusoids as the percentage of all particles visible in the acini during observation for 10 s [32, 33]. Since variations in the absolute number of beads per acinus were found in association with alterations of sinusoidal perfusion, all data were normalized and expressed as the percentage of particles visible in sinusoids per microscopic field.

Sampling and assays. Bile flow was measured continuously via the catheter in the common bile duct and standardized per g liver wet weight (μl/min × g). At the end of the experiments, arterial blood samples were taken for the spectrophotometric determination of aspartate aminotransferase (AST) serum activities, which served as an indicator for hepatocellular disintegration. In addition, plasma was obtained from EDTA-anticoagulated blood for determination of vitamin E (α-tocopherol) concentrations. Moreover, liver tissue sections were sampled and stored at −70 °C until extraction for

Table 1. Concentrations of α-tocopherol in arterial plasma (μmol/mg cholesterol) and hepatic tissue (μmol/g liver wet weight) from animals fed with different amounts of vitamin E (α-tocopherol acetate) concentrations.

	Diet	Sham	Diet	1 h LPS	6 h LPS
Plasma α-tocopherol (μmol/mg)					
	sC	0.43 ± 0.11	C	0.51 ± 0.03	0.32 ± 0.05
	sE+	0.74 ± 0.11‡	E+	1.35 ± 0.17‡	0.95 ± 0.30‡
	sE−	0.11 ± 0.02‡†	E−	0.09 ± 0.01‡†	0.09 ± 0.02‡†
Hepatic tissue α-tocopherol (μmol/g liver wet weight)	sC	42 ± 5	C	37 ± 3	57 ± 4
	sE+	614 ± 99‡	E+	920 ± 149‡	720 ± 202‡
	sE−	6 ± 1‡†	E−	5 ± 1‡†	6 ± 1‡†

All values are given as mean ± SEM. Animals received either a standard vitamin E diet, containing 75 mg α-tocopherol acetate/kg food (C, n = 18), a vitamin E-enriched diet (8000 mg α-tocopherol acetate/kg; E+, n = 18) or a vitamin E-deficient diet (0 mg α-tocopherol acetate/kg; E−, n = 16) for four weeks. Studies were undertaken at either 1 h or 6 h after a single LPS-exposure (*E. coli* LPS, 10 mg/kg iv). Sham-treated animals (sC, sE+, sE−) received isotonic saline solution. ‡$p < 0.01$ vs standard diet-fed animals (sC and C); †$p < 0.01$ vs vitamin E-enriched diet fed animals (sE+ and E+).

Table 2. Effect of different dietary vitamin E intake on LPS-induced changes in arterial blood pressure, systemic leukocyte count, hepatic bile flow and serum AST activities.

	Diet	Sham	Diet	1 h LPS	6 h LPS
Arterial blood pressure (mmHg)					
	sC	106 ± 9	C	80 ± 4**	106 ± 5##
	sE+	116 ± 6	E+	76 ± 6**	107 ± 5##
	sE−	121 ± 4	E−	75 ± 5**	103 ± 5##
Systemic leukocyte count ($\times 10^9$/μl)					
	sC	7.6 ± 1.0	C	2.2 ± 0.4**	5.7 ± 0.6##
	sE+	10.4 ± 1.1	E+	2.6 ± 0.1**	7.9 ± 1.2##
	sE−	7.9 ± 0.8	E−	2.3 ± 0.3**	6.2 ± 0.7##
Bile flow (μl/min × g)					
	sC	1.7 ± 0.2	C	1.5 ± 0.1	0.8 ± 0.1**##
	sE+	1.7 ± 0.2	E+	1.7 ± 0.1	0.9 ± 0.1**##
	sE−	1.4 ± 0.1	E−	1.4 ± 0.2	0.8 ± 0.2**
Serum AST activities (U/L)					
	sC	65 ± 3	C	135 ± 10**	196 ± 30**
	sE+	66 ± 2	E+	173 ± 22**	157 ± 12**
	sE−	66 ± 4	E−	130 ± 14**	201 ± 52**

All values are given as mean ± SEM. Animals received either a standard vitamin E diet, containing 75 mg α-tocopherol acetate/kg food (C, n = 18), a vitamin E-enriched diet (8000 mg α-tocopherol acetate/kg; E+, n = 18) or a vitamin E-deficient diet (0 mg α-tocopherol acetate/kg; E−, n = 16) for four weeks. Studies were undertaken at either 1 h or 6 h after a single LPS-exposure (*E. coli* LPS, 10 mg/kg iv). Sham-treated animals (sC, sE+, sE−) received isotonic saline solution. **$p < 0.01$ vs sham-treated animals with the corresponding diet, ##$p < 0.01$ vs 1 h LPS.

the determination of α-tocopherol. The tissue sections were homogenized with 0.9% saline (30 μl/mg tissue) at 4 °C. The resulting tissue homogenates as well as the plasma samples were used for quantification of α-tocopherol by high performance liquid chromatography with electrochemical detection [34]. Plasma α-tocopherol concentrations were corrected for cholesterol plasma concentrations determined enzymatically and expressed in table 1 as μmol α-tocopherol/mg cholesterol. Hepatic tissue α-tocopherol concentrations were expressed as μmol/g liver wet weight (table 1). For histomorphological examination and analysis of leukocytic tissue infiltration, hematoxylin- eosin sections for routine histology and chloracetate-esterase sections for quantitative analysis of leukocyte infiltration into tissue (cells per 50 high power fields (HPF)) were prepared from 4% formalin-fixed, paraffin-embedded tissue specimens.

Statistical analysis. All data are expressed as mean ± SEM. After disproving the assumption of normality and equal variance across groups, differences between groups were assessed using the Kruskal-Wallis one–way analysis of variance on ranks (overall differences) followed by the Student-Newman-Keuls method (pairwise multiple comparisons). Overall statistical significance was set at $p < 0.05$. Statistics were performed using the software package SigmaStat (Jandel Corporation, San Rafael, CA, USA).

Results

Vitamin E nutritional status. The mean body weights and liver weights at the end of the experiment were 235 ± 5 g and 7.7 ± 0.2 g, 231 ± 4 g and 8.4 ± 0.2 g, and 225 ± 5 g and 7.9 ± 0.2 g for the animals on the diet containing 75 mg, 8000 mg, and 0 mg α-tocopherol

acetate/kg food, respectively. These values did not differ significantly between the three groups. The concentrations of α-tocopherol in arterial plasma and hepatic tissue are depicted in table 1. After 4 weeks of dietary supplementation with vitamin E (sE+), the concentrations of α-tocopherol in arterial plasma and liver tissue were found to be significantly ($p < 0.01$) increased 1.7 and 14.6 times when compared to those values determined in standard diet-fed animals (sC: 0.43 ± 0.11 μmol/mg and 42 ± 5 μmol/g). Animals which were fed the vitamin E-deficient diet (sE−) revealed plasma and hepatic tissue α-tocopherol concentrations of 0.11 ± 0.02 μmol/mg and 6 ± 1 μmol/g, respectively ($p < 0.01$ vs sC and sE+). No differences in vitamin E levels were observed between sham-treated animals and those studied at 1 h and 6 h after LPS-exposure (table 1).

Systemic circulatory parameters. Independent of the diet the animals received, a single bolus injection of LPS caused a significant reduction in mean arterial blood pressure of ~32% at 1 h after the exposure ($p < 0.01$ vs sham; table 2). At 6 h after the LPS-exposure, mean arterial blood pressure was found to be within the physiological range with no significant differences between groups (table 2). Sham-treated animals revealed stable hemodynamic conditions, as reflected by a mean arterial blood pressure in the range of 106–121 mmHg (table 2).

As well as systemic hypotension, LPS induced an initial drop in systemic leukocyte count (1 h), which was comparable in extent in groups C, E+ and E−, when compared to sham-treated animals (table 2). LPS-induced systemic leukopenia was only transient, since values of systemic leukocyte counts were found to be normalized at 6 h after LPS-exposure (C, 5.7 ± 0.6; E+, 7.9 ± 1.2; E−, $6.2 \pm 0.7 \times 10^9$/μl). Sham-treated animals revealed unchanged systemic leukocyte counts in the range $7.6–10.4 \times 10^9$/μl (table 2).

Hepatic cellular response to LPS. Analysis of kinetics of latex particle adherence in sham-treated standard diet fed animals (sC) revealed that $79.9 \pm 5.9\%$ of the particles visible per screen and per 10 s of observation were still moving 1 min after injection. Three and five minutes after injection, this number had decreased to $39.5 \pm 16.4\%$ and $18.0 \pm 9.7\%$, respectively (fig. 1). The dietary regimen did not influence KC phagocytic activity in sham-treated animals. LPS slightly increased phagocytosis of KC in that at 1 h after the exposure adherence of latex particles was found to be enhanced with only $17.8 \pm 2.7\%$ and $3.5 \pm 2.4\%$ of particles still moving at three and five minutes after injection (fig. 1). At 6 h after LPS exposure, however, latex particle adherence was markedly delayed with 3 min-values of $49.2 \pm 4.2\%$ and 5 min-values of $42.0 \pm 5.4\%$, when compared to sham-treated animals (fig. 1). In animals fed with either a vitamin E-enriched or a vitamin E-deficient diet, LPS had similar effects on KC phagocytic activity when compared with standard diet fed animals (fig. 1).

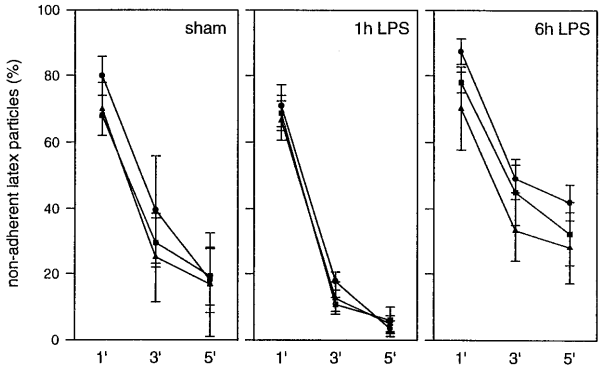

Figure 1. Kinetics of adherence of latex particles. Number of non-adherent fluorescent-labelled latex particles at 1, 3 and 5 min after intra-arterial injection, given as percentage of all particles visible in microscopic fields observed for 10 s by means of intravital fluorescence microscopy. Measurements were performed at 1 h and 6 h after LPS exposure in animals fed with either a vitamin E-enriched (■, 8000 mg α-tocopherol acetate/kg; E+, n = 6 (1 h LPS) and 5 (6 h LPS)) or a vitamin E-deficient (▲, 0 mg α-tocopherol acetate/kg; E−, n = 6 (1 h LPS) and 6 (6 h LPS)) diet. Control animals were fed a standard diet containing 75 mg α-tocopherol acetate/kg (●, C, n = 6 (1 h LPS) and 6 (6 h LPS)). Sham-treated animals received isotonic saline solution (sE+, n = 6; sE−, n = 5; sC, n = 6). Mean ± SEM.

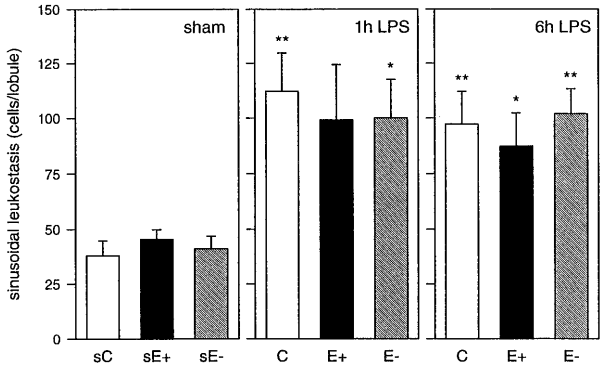

Figure 2. Number of stagnant leukocytes in sinusoids, given as cells/lobule, using intravital fluorescence microscopy at 1 h and 6 h after LPS exposure in animals fed with either a vitamin E-enriched (■; 8000 mg α-tocopherol acetate/kg; E+, n = 6 (1 h LPS) and 5 (6 h LPS)) or a vitamin E-deficient (▨; 0 mg α-tocopherol acetate/kg; E−, n = 6 (1 h LPS) and 6 (6 h LPS)) diet. Control animals were fed a standard diet containing 75 mg α-tocopherol acetate/kg (□; C, n = 6 (1 h LPS) and 6 (6 h LPS)). Sham-treated animals received isotonic saline solution (sE+, n = 6; sE−, n = 5; sC, n = 6). Mean ± SEM. *$p < 0.05$, **$p < 0.01$ vs sham-treated animals fed with the corresponding diet.

Within the hepatic microvascular network, LPS induced an immediate leukocytic accumulation and enhanced leukocytic interaction with the endothelial lining of both sinusoids and postsinusoidal venules. In standard diet-fed animals (C), the number of leukocytes stagnant within the hepatic sinusoids was found to be significantly higher, with 112 ± 17 and 97 ± 15 cells/lobule at 1 h and 6 h after LPS exposure, compared to sham-treated controls (sC, 39 ± 7 cells/lobule) (fig. 2). LPS-

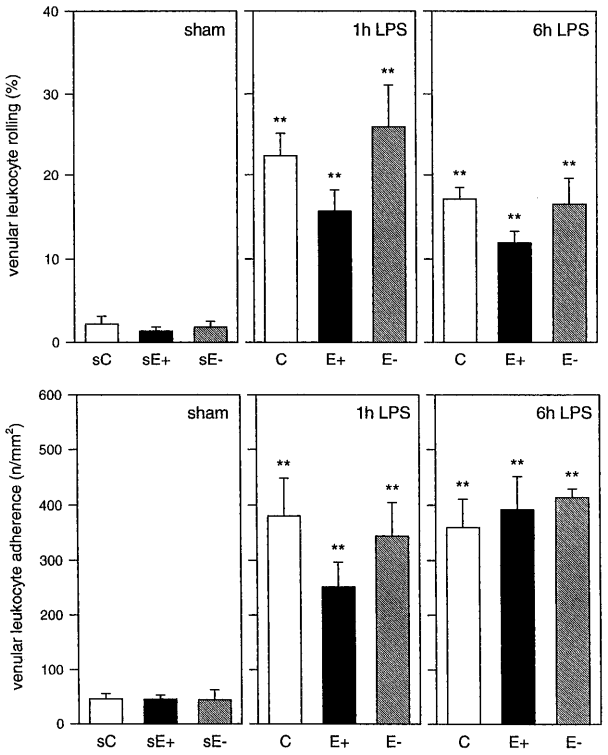

Figure 3. Number of rolling leukocytes (upper panel), given as percentage of non-adherent leukocytes, and number of adherent leukocytes in postsinusoidal venules (lower panel), given as cells/mm^2 endothelial surface, using intravital fluorescence microscopy at 1 h and 6 h after LPS exposure in animals fed with either a vitamin E-enriched (■; 8000 mg α-tocopherol acetate/kg; E+, n = 6 (1 h LPS) and 5 (6 h LPS)) or a vitamin E-deficient (▨, 0 mg α-tocopherol acetate/kg; E−, n = 6 (1 h LPS) and 6 (6 h LPS)) diet. Control animals were fed a standard diet containing 75 mg α-tocopherol acetate/kg (□, C, n = 6 (1 h LPS) and 6 (6 h LPS)). Sham-treated animals received isotonic saline solution (sE+, n = 6; sE−, n = 5; sC, n = 6). Mean ± SEM. **p < 0.01 vs sham-treated animals fed with the corresponding diet.

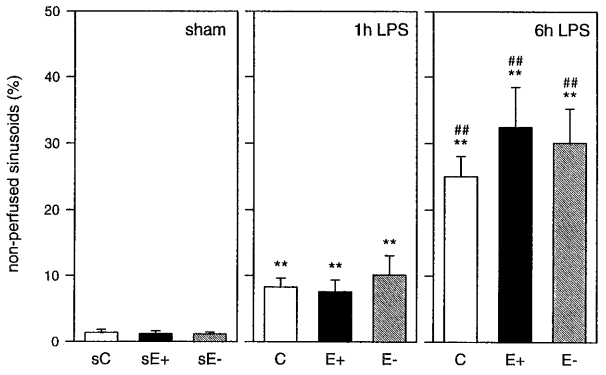

Figure 4. Sinusoidal perfusion failure, i.e. number of non-perfused sinusoids, given in as percentage of all sinusoids visible using intravital fluorescence microscopy at 1 h and 6 h after LPS exposure in animals fed with either a vitamin E-enriched (■; 8000 mg α-tocopherol acetate/kg; E+, n = 6 (1 h LPS) and 5 (6 h LPS)) or a vitamin E-deficient (▨, 0 mg α-tocopherol acetate/kg; E−, n = 6 (1 h LPS) and 6 (6 h LPS)) diet. Control animals were fed a standard diet containing 75 mg α-tocopherol acetate/kg (□, C, n = 6 (1 h LPS) and 6 (6 h LPS)). Sham-treated animals received isotonic saline solution (sE+, n = 6; sE−, n = 5; sC, n = 6). Mean ± SEM. **p < 0.01 vs sham-treated animals fed with the corresponding diet, ##p < 0.01 vs 1 h LPS.

induced sinusoidal leukostasis (1 h) remained unaffected by both vitamin E-enriched (99 ± 25 cells/lobule, p < 0.01 vs sE+, 45 ± 5 cells/lobule) and vitamin E-deficient diets (100 ± 17 cells/lobule, p < 0.01 vs sE−, 41 ± 6 cells/lobule) (fig. 2). This accounts also for the data on sinusoidal leukostasis at 6 h after LPS exposure (fig. 2).

In postsinusoidal venules, LPS exposure enhanced both the primary and secondary interaction of leukocytes with the endothelium. A proportion of rolling leukocytes of 22 ± 3% and, concomitantly, a seven- to eight-fold higher number of adherent leukocytes (381 ± 67 cells/mm^2), were observed when compared to sham-treated controls at 1 h (p < 0.01 vs sC, 2 ± 1% and 46 ± 10 cells/mm^2) (fig. 3). Neither dietary supplementation (E+) nor depletion of vitamin E (E−) could influence the LPS-induced leukocytic response, as given by the comparably enhanced endothelial interaction within postsinusoidal venules (E+, 16 ± 3% and 252 ± 45 cells/mm^2; E−, 26 ± 5% and 344 ± 60 cells/mm^2) (fig. 3). At 6 h after LPS-exposure, venular leukocyte-endothelial cell interaction was found to be in the range of the 1 h-values and, in parallel, did not differ between the vitamin E dietary groups (fig. 3). Within the hepatic venular microvasculature of sham-treated animals, leukocytes were not activated, as indicated by the negligible fraction of rolling leukocytes (sC, 2 ± 1%; sE+, 1 ± 1%; sE−, 2 ± 1%) and the low numbers of firmly adherent leukocytes (sC, 46 ± 10 cells/mm^2; sE+, 45 ± 8 cells/mm^2; sE−, 44 ± 19 cells/mm^2) (fig. 3).

Hepatic microvascular response to LPS. LPS-induced hepatic cellular response was accompanied by a progressive impairment of nutritive tissue perfusion with an increase in non-perfused sinusoids from 8.3 ± 1.4% at 1 h to 25.1 ± 3.0% at 6 h after LPS exposure in standard diet fed animals (C) compared to the average fraction of non-perfused sinusoids, constantly <2% in sham-treated animals (sC, 1.0 ± 0.5%) (fig. 4). Magnitude and time course of hepatic microvascular perfusion failure was found to be comparable in both the vitamin E-fed (E+, 7.6 ± 1.8% (1 h) and 32.4 ± 6.1% (6 h)) and the vitamin E-deficient LPS-exposed animals (E−, 10.2 ± 2.9% (1 h) and 30.1 ± 5.1% (6 h)). In sham-treated animals, dietary regimen had no influence on hepatic nutritive perfusion, with 1.2 ± 0.5% and 1.2 ± 0.3% non-perfused sinusoids in groups sE+ and sE−, respectively (fig. 4).

Hepatocellular function and integrity. Independent of the different diets fed, bile flow was found to be still unchanged at 1 h after LPS exposure, compared to sham-treated animals (table 2). However, it was significantly restricted at 6 h after LPS exposure with no differences between the dietary groups (table 2). Serum AST activities were found to be <100 U/L in all three dietary sham-treated groups. In contrast, LPS induced a

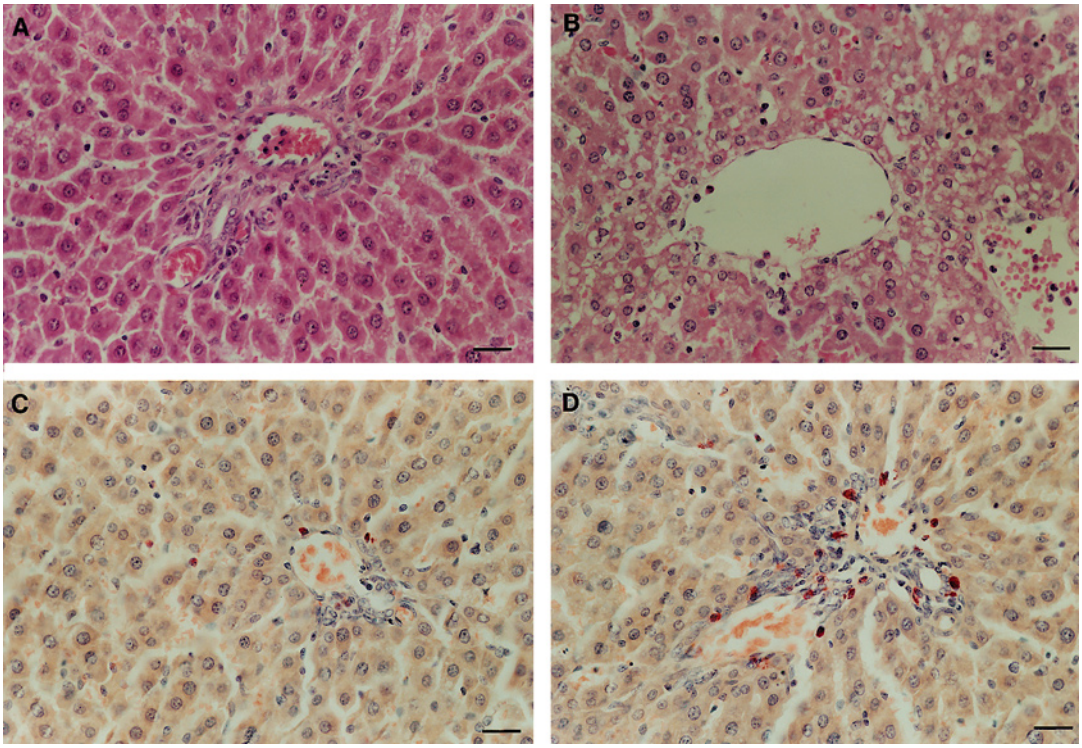

Figure 5. Light microscopic images of hematoxylin-eosin (*A*, *B*) and chloracetate esterase-stained (*C*, *D*) hepatic tissue sections of a sham-treated and LPS-exposed (6 h) animal fed with a vitamin E enriched-diet. Note the intact hepatic morphology with normal appearing hepatocytes and absence of tissue-infiltrating leukocytes in the sham-treated animals (*A*, *C*), whereas LPS-exposed liver tissue revealed hepatocellular vacuolization and leukocytic tissue infiltration (*B*, *D*). No differences in the histopathological pattern of the LPS-induced liver damage could be observed between the dietary groups. Bars represent 50 μm.

two- to threefold increase of serum AST activities at 1 h and 6 h after the exposure, indicating onset of liver damage. Thereby, supplementation or depletion of vitamin E by diet did not influence LPS-induced hepatocellular disintegration (table 2).

Histomorphology. In contrast to the intact hepatic morphology and normal-appearing hepatocytes at 1 h after either LPS or NaCl exposure in all three dietary groups (fig. 5A), routine histology of hematoxylin–eosin stained sections of livers at 6 h after LPS exposure indicated distinct tissue damage, particularly characterized by hepatocellular vacuolization (fig. 5B). Therefore, no differences were observed between the standard diet-fed animals and those with the vitamin E-enriched and vitamin E-deficient diet.

Quantitative analysis of chloracetate-esterase-stained hepatic tissue sections revealed a 2.5- to 3.5-fold increase in the number of tissue infiltrating leukocytes at 1 h and 6 h after LPS exposure in standard diet-fed animals (C, 15 ± 1 and 20 ± 2 cells/50 HPF, $p < 0.05$ vs sC, 6 ± 1 cells/50 HPF) with no significant differences when compared with the animals of group E+ (14 ± 2 and 24 ± 4 cells/50 HPF) and group E− (13 ± 2 and 24 ± 3 cells/50 HPF) (fig. 5D). In sham-treated animals, dietary regimen did not influence leukocytic tissue infiltration (sE+, 3 ± 1 cells/50 HPF; sE−, 4 ± 1 cells/50 HPF) (fig. 5C).

Discussion

Our study demonstrates in vivo that LPS induces an initial activation of Kupffer cells with enhanced phagocytic activity. Concurrently, LPS caused a decrease in circulating leukocytes due to their sequestration in the liver, as indicated both by leukocyte accumulation and adherence within the hepatic microvascular network and increased leukocytic infiltration into tissue. Since initial Kupffer cell activation and intrahepatic leukocyte accumulation and adherence precede nutritive perfusion failure and manifestation of hepatocellular injury and organ dysfunction, LPS-induced cellular responses may be considered as trigger mechanisms for the initiation/propagation of tissue injury.

KC-phagocytosis of LPS has already been shown to parallel the excessive release of a variety of inflammatory mediators. Included in this list are peptide mediators, such as tumor necrosis factor-α and interleukin-6 [33], eicosanoids and platelet activating factor [28] as well as reactive oxygen species, in particular superoxide anion [28, 35]. LPS potentially renders KC cytotoxic, which then might contribute to liver injury following LPS exposure [36, 37]. It is further reported that LPS-activated KC are toxic to hepatocytes due to diminution of hepatocyte protein synthesis [24, 28]. This modulation of hepatocyte function by KC has been suggested

to represent at least in part the mechanism of LPS-induced hepatic insufficiency [24].

Concomitantly, leukocytes play an active role in mediating some of the tissue damage observed in endotoxinemia. This is supported by the fact that LPS exerts toxic effects on hepatocytes only in the presence of leukocytes [25]. Jaeschke and coworkers demonstrated by light microscopic analysis in an endotoxin shock model in mice, that leukocytes are present in liver sinusoids several hours before there is evidence for a leukocyte-induced parenchymal cell injury [27]. Accordingly, Hewett and coworkers reported that a large number of leukocytes were observed within the liver as early as 45 min after LPS, well before the onset of severe liver injury [29]. The results of the present study confirm and extend the latter observations in that before extravasation into tissue, leukocytes interact with the endothelium, thereby presenting two distinct mechanisms, i.e. stasis in sinusoids as well as rolling and adherence in postsinusoidal venules. Leukocytes may not solely contribute to LPS-induced hepatic microvascular damage and liver dysfunction by sinusoidal plugging since sites of leukocytic accumulation in sinusoids do not necessarily correlate with sites of overt microvascular damage [38] or with individual sinusoidal perfusion failure [39]. Deleterious effects of activated leukocytes within the hepatic microvasculature, in particular of those adhering to the venular endothelium, may rather be deduced by the release of cytokines [40], arachidonic acid metabolites [41] and by proteinase-mediated mechanisms [42].

Within this scenario of LPS-induced activation of KC and leukocytes, reactive oxygen species are implicated as potential mediators in the process of liver injury [27]. Results from experimental studies, reporting protective effects of antioxidants in endotoxemic animals [10], indirectly support the hypothesis of reactive oxygen species-mediated tissue injury. In this context, α-tocopherol, the biologically active homologue of vitamin E, was tested, since it is known to form an integral part of phospholipid membranes, mainly subcellular ones such as those of nuclei, mitochondria and microsomes, affording both stability and a free radical scavenging action in the liquid phase, especially against lipoperoxyl radicals [16]. Unexpectedly, however, data from the present study indicate that dietary vitamin E administration did not afford protection against LPS-induced microvascular injury and liver dysfunction, despite the fact that the increase in α-tocopherol both in arterial plasma and hepatic tissue observed in those animals fed the vitamin E-enriched diet was marked and corresponded well with differences in α-tocopherol concentrations observed in rabbits on a high vitamin E diet as compared to standard diet-fed animals [43]. Moreover, dietary α-tocopherol depletion did not potentiate endotoxemic cell activation and hepatic microvascular dysfunction. These findings are in accordance with previous studies, demonstrating lack of protection from galactosamine/endotoxin-induced hepatocellular damage by vitamin E supplementation [44], and lack of aggravation of liver injury by α-tocopherol deficiency [45].

However, the failure of the anti-oxidant vitamin E to protect from LPS-induced Kupffer cell activation, microvascular leukocyte adherence and hepatocellular damage/dysfunction does not necessarily exclude the action of reactive oxygen species in LPS-mediated liver injury. Vitamin E acts as a lipid-soluble, chain-breaking antioxidant with pronounced scavenging potential in the intracellular but not extracellular space [16]. Thus, extracellularly released reactive oxygen species may – even under conditions of vitamin E supplementation – participate in LPS-induced liver injury. This view is supported by the fact that the extracellularly distributed radical scavenger superoxide dismutase but not vitamin E protects from endotoxin-induced hepatic injury [44].

Similar to the observations in hepatic ischemia/reperfusion injury [46], Kupffer cells and leukocytes have been described as the principal source of reactive oxygen species formation in LPS-induced vascular, i.e. extracellular, oxidative stress [14, 27]. The deleterious mechanism of action of these extracellular reactive oxygen species seems to be the inactivation of plasma antiproteases rather than lipid peroxidation [27, 47], since leukocyte-derived proteases have been shown to be mainly responsible for the damage to hepatocytes [27], while lipid peroxidation products, such as liver lipid conjugated dienes and exhaled ethane, are not found to be increased in endotoxin- or galactosamine/endotoxin-treated animals [11, 44]. Moreover, extracellularly released reactive oxygen species further activate Kupffer cells, leukocytes and microvascular endothelium, which may additionally contribute to LPS-induced liver injury by the release of cytotoxic mediators, such as leukotrienes, TNF-α, and nitric oxide. Thus, the combined usage of extracellularly distributed oxygen radical scavengers and leukocytic protease inhibitors [12], or the blockade of leukotrienes, TNF-α, and nitric oxide synthesis [48], might be a superior concept to dietary vitamin E supplementation in the prevention of endotoxemia-induced liver cell damage and hepatic dysfunction.

Acknowledgments. This work was supported by a grant from the Wilhelm Sander-Stiftung (Nr. 93.019.1) and the European Community (BMH4-CT95-0875).

1 Southorn P. A. (1988) Free radicals in medicine. II. Involvement in human disease. Mayo Clin. Proc. **63:** 390–408
2 Fox E. S., Broitman S. A. and Thomas P. (1990) Bacterial endotoxins and the liver. Lab. Invest. **63:** 733–741
3 McCord J. M. and Fridovich I. (1978) The biology and pathology of oxygen radicals. Ann. Intern. Med. **89:** 122–127

4 Fantone J. C. and Ward P. (1982) Role of oxygen-derived free radicals and metabolites in leukocyte- dependent inflammatory reactions. Am. J. Physiol. **107:** 397–418
5 Miller R. A. and Britigan B. E. (1995) The formation and biologic significance of phagocyte-derived oxidants. J. Invest. Med. **43:** 9–45
6 Bautista A. P., Mézáros K., Bojta J. and Spitzer J. J. (1990) Superoxide anion generation in the liver during the early stage of endotoxemia in rats. J. Leukoc. Biol. **48:** 123–128
7 Bautista A. P. and Spitzer J. J. (1990) Superoxide anion generation by in situ perfused rat liver: effect of in vivo endotoxin. Am. J. Physiol. **259:** G907–G912
8 Mayer A. M. S. and Spitzer J. A. (1991) Continuous infusion of *Escherichia coli* endotoxin in vivo primes in vitro superoxide anion release in rat polymorphonuclear leukocytes and Kupffer cells in a time-dependent manner. Infect. Immun. **59:** 4590–4598
9 Ogawa R., Morita T., Kunimoto F. and Fujita T. (1982) Changes in hepatic lipoperoxide concentration in endotoxemic rats. Circ. Shock **9:** 369–374
10 Sugino K., Dohi K., Yamada K. and Kawasaki T. (1987) The role of lipid peroxidation in endotoxin-induced hepatic damage and the protective effect of antioxidants. Surgery **101:** 746–752
11 Clements N. C. and Habib M. P. (1995) The early pattern of conjugated dienes in liver and lung after endotoxin exposure. Am. J. Respir. Crit. Care Med. **151:** 780–784
12 Htwe T., Suzuki M., Ouchi K., Fukuhara K. and Matsuno S. (1996) Effects of urinastatin and free radical scavengers on hepatic lipid peroxidation in endotoxemia. J. Surg. Res. **61:** 206–214
13 Sugino K., Dohi K., Yamada K. and Kawasaki T. (1989) Changes in the level of endogeneous antioxidants in the liver of experimental endotoxemia and the protective effects of antioxidants. Surgery **105:** 200–206
14 Okabe H., Irita K., Taniguchi S., Kurosawa K., Tagawa K., Yoshitake J.-I. et al. (1994) Endotoxin causes early changes in glutathione concentrations in rabbit plasma and liver. J. Surg. Res. **57:** 416–419
15 Takeyama N., Shoji Y., Ohashi K. and Tanaka T. (1996) Role of reactive oxygen intermediates in lipopolysaccharide-mediated hepatic injury in the rat. J. Surg. Res. **60:** 258–262
16 Burton G. W. and Ingold K. U. (1989) Vitamin E as an in vitro and in vivo antioxidant. Ann. N. Y. Acad. Sci. **570:** 7–22
17 Massey K. D. and Burton K. P. (1989) α-Tocopherol attenuates myocardial membrane-related alteration resulting from ischemia and reperfusion. Am. J. Physiol. **256**: H192–H199
18 Takenaka M., Tatsukawa Y., Dohi K., Ezaki H., Matsukawa K. and Kawasaki T. (1981) Protective effects of α-tocopherol and coenzyme Q_{10} on warm ischemic damages of the rat kidney. Trans-plantation **32:** 137–141
19 Yoshikawa T., Yasuda M., Ueda S., Naito Y., Tanigawa T., Oyamada H. et al. (1991) Vitamin E in gastric mucosal injury induced by ischemia-reperfusion. Am. J. Clin. Nutr. **53:** 210S–214S
20 Marubayashi S., Dohi K., Ochi K. and Kawasaki T. (1986) Role of free radicals in ischemic rat liver cell injury. Prevention of damage by α-tocopherol administration. Surgery **99:** 184–191
21 Rabl H., Khoschsorur G., Colombo T., Petritsch P., Rauchenwald M., Koltringer P. et al. (1993) A multivitamin infusion prevents lipid peroxidation and improves transplantation performance. Kidney Int. **43:** 912–917
22 Herbaczyńska-Cedro K., Klosiewicz-Wasek B., Cedro K., Wasek W., Panczenko-Kresowska B. and Wartanowicz, M. (1995) Supplementation with vitamins C and E suppresses leukocyte oxygen free radical production in patients with myocardial infarction. Eur. Heart J. **16:** 1044–1049
23 Rabl H., Khoschsorur G. and Petek W. (1995) Antioxidative vitamin treatment: Effect of lipid peroxidation and limb swelling after revascularization operations. World J. Surg. **19:** 738–744
24 Keller G. A., West M. A., Cerra F. B. and Simmons R. L. (1985) Multiple systems organ failure. Modulation of hepatocyte protein synthesis by endotoxin activated Kupffer cells. Ann. Surg. **201:** 87–95
25 Holman J. M. and Saba T. M. (1988) Hepatocyte injury during postoperative sepsis: activated neutrophils as potential mediators. J. Leukoc. Biol. **43:** 193–201
26 Biliar T. R., Curran R. D., West M. A., Hofmann K. and Simmons R. L. (1989) Kupffer cell cytotoxicity to hepatocytes in coculture requires L-arginine. Arch. Surg. **124:** 1416–1421
27 Jaeschke H., Farhood A. and Smith C. W. (1991) Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. Am. J. Physiol. **261:** G1051–G1056
28 Monden K., Arii S., Itai S., Sasaoki T., Adachi Y., Funaki N., et al. (1991) Enhancement and hepatocyte-modulating effect of chemical mediators and monokines produced by hepatic macrophages in rats with induced sepsis. Res. Exp. Med. **191:** 177–187
29 Hewett J. A., Schultze E., VanCise S. and Roth R. A. (1992) Neutrophil depletion protects against liver injury from bacterial endotoxin. Lab. Invest. **68:** 347–361
30 Vollmar B., Glasz J., Leiderer R., Post S. and Menger M. D. (1994) Hepatic microcirculatory perfusion failure is a determinant of liver dysfunction in warm ischemia-reperfusion. Am. J. Pathol. **145:** 1421–1431
31 Vollmar B., Menger M. D., Glasz J., Leiderer R. and Messmer K. (1994) Impact of leukocyte– endothelial cell interaction in hepatic ischemia-reperfusion injury. Am. J. Physiol. **267:** G786–G793
32 Vollmar B., Glasz J., Post S. and Menger M. D. (1994) Depressed phagocytic activity of Kupffer cells after warm ischemia-reperfusion of the liver. J. Hepatol. **20:** 301–304
33 Vollmar B., Rüttinger D., Wanner G. A., Leiderer R. and Menger M. D. (1996) Modulation of Kupffer cell activity by gadolinium chloride in endotoxemic rats. Shock **6:** 434–441
34 Finchk B., Kontush A., Commentz J., Hübner C., Burdelski M. and Kohlschütter A. (1995) Monitoring of ubiquinol-10, ubiquinone-10, carotenoids, and tocopherols in neonatal plasma microsamples using high-performance liquid chromatography with coulometric electrochemical detection. Anal. Biochem. **232:** 210–216
35 Decker K. (1990) Biologically active products of stimulated liver macrophages (Kupffer cells). Eur. J. Biochem. **192:** 245–261
36 Alexander P. and Evans R. (1971) Endotoxin and double strand RNA render macrophage cytotoxic. Nature New Biol. **232:** 76–78
37 Ferluga J. and Allison A. C. (1978) Role of mononuclear infiltrating cells in pathogenesis of hepatitis. Lancet **2:** 610–611
38 Ferguson D., McDonagh P. F., Biewer J., Paidas C. N. and Clemens M. G. (1993) Spatial relationship between leukocyte accumulation and microvascular injury during reperfusion following hepatic ischemia. Int. J. Microcirc. Clin. Exp. **12:** 45–60
39 Vollmar B., Richter S. and Menger M. D. (1996) Leukocyte stasis in hepatic sinusoids. Am. J. Physiol. **270:** G798–G803
40 Lloyd A. R. and Oppenheim J. J. (1992) Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune system. Immunol. Today **13:** 169–172
41 Tiegs G. and Wendel A. (1988) Leukotriene-mediated liver injury. Biochem. Pharmacol. **37:** 2569–2573
42 Mavier P., Preaux A.-M., Guigui B., Lescs M.-C., Zafrani E.-S. and Dhumeaux D. (1988) In vitro toxicity of polymorphonuclear neutrophils to rat hepatocytes: evidence for a proteinase-mediated mechanism. Hepatology **8:** 254–258
43 Wiseman S. A., Van Den Boom M. A. P., De Fouw N. J., Wassink M. G., Op Den Kamp J. A. F. and Tijburg L. B. M. (1995) Comparison of the effects of dietary vitamin E on in vivo and in vitro parameters of lipid peroxidation in the rabbit. Free Rad. Biol. Med. **19:** 617–626
44 Wendel A., Tiegs G. and Werner C. (1987) Evidence for the involvement of a reperfusion injury in galactosamine/endotoxin-induced hepatitis in mice. Biochem. Pharmacol. **36:** 2637–2639

45 Barrow L., Patel H. R. and Tanner M. S. (1992) α-Tocopherol deficiency fails to aggravate toxic liver injury but liver injury causes α-tocopherol retention. J. Hepatol. **16:** 332–337
46 Jaeschke H. and Farhood A. (1991) Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am. J. Physiol. **260:** G355–G362
47 Weiss S. J. (1989) Tissue destruction by neutrophils. N. Engl. J. Med. **320:** 365–376
48 Thiemermann C., Ruetten H., Wu C. C. and Vane J. R. (1995) The multiple organ dysfunction syndrome caused by endotoxin in the rat: attenuation of liver dysfunction by inhibitors of nitric oxide synthase. Br. J. Pharmacol. 11**6:** 2845–2851

.