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L-CARNITINE ALLEVIATES ALCOHOL-INDUCED LIVER DAMAGE IN RATS: ROLE OF TUMOUR NECROSIS FACTOR-ALPHA

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Abstract — Aims: Excessive alcohol intake induces hepatic fatty infiltration, which has been suggested to sensitize the liver to further damage. To test this hypothesis, L-carnitine, a constitutional lipotropic compound, was administered to rats chronically treated with ethanol by liquid diet feeding for 10 weeks. **Results:** Ethanol administration caused marked steatosis, mild inflammation and elevated plasma alanine aminotransferase and tumour necrosis factor alpha (TNF-α) concentrations. Dietary supplementation with L-carnitine significantly reduced all these parameters as well as the hepatic concentration of thiobarbituric acid reactive substances, an indicator of lipid peroxidation products. Pretreatment with L-carnitine also significantly blunted ethanol-induced stimulation of TNF-α release by isolated Kupffer cells. **Conclusions:** This study provides direct support for the notion that steatosis sensitizes the liver to further damage and suggests an involvement of TNF-α in this process.

INTRODUCTION

Fatty infiltration, the first manifestation of alcohol-induced liver injury, is usually followed by inflammation, focal necrosis and terminal venular sclerosis, which ultimately can develop into cirrhosis (Tsukamoto *et al*., 2001). Steatosis was formerly considered a benign and fully reversible condition. However, new evidence suggests that hepatic fatty infiltration may in fact be an important pathogenic factor in the development of alcoholic liver disease (ALD) (Stewart *et al*., 2001). Both hepatic and extrahepatic factors, including peripheral lipolysis, enhanced hepatic fatty acid synthesis and reduced fatty acid oxidation, act together in the development of alcohol-induced fatty liver (Feinman and Lieber, 1999). On the other hand, several lipotropic compounds, including *S*-adenosyl-*L*-methionine (Lieber, 2002), glycine (Yin *et al*., 1998), taurine (Kerai *et al*., 1999) and L-carnitine (Sachan *et al*., 1984; Bahcecioglu *et al*., 1999) have been shown to alleviate steatosis.

L-carnitine (β-hydroxy-(γ-N-trimethylamino) butyrate, carnitine), is an essential factor in fatty acid metabolism. It is obligatory in the inward transport of long-chain fatty acids for intramitochondrial β-oxidation (Bremer, 1983; Ramsay and Arduini, 1993) and its lipotropic effect has been extensively studied (Seccombe *et al*., 1987; Rauchova *et al*., 1998). Carnitine has also proven effective in reducing plasma lipids and liver triglycerides in livers of rats chronically treated with ethanol (Sachan *et al*., 1984; Bertelly *et al*., 1993; Bahcecioglu *et al*., 1999).

However, the exact mechanism by which carnitine reduces steatosis is still unsettled. Recent findings suggest that one mechanism may involve Kupffer cells, the resident liver macrophages. Thus carnitine and its acyl derivatives can modulate eicosanoid synthesis by peritoneal macrophages, and decrease their response to chemotactic stimuli (Elliott *et al*., 1990), suggesting that mediator synthesis by Kupffer cells could be affected as well. Alcohol exposure can activate Kupffer cells, either directly or indirectly via enhanced release of gut-derived endotoxins (lipopolysaccharides, LPS), to produce biologically active substances, such as free radicals, pro-inflammatory cytokines, chemokines and prostanoids (Thurman, 1998). There is also new evidence that suggests that among these mediators tumour necrosis factor alpha (TNF-α) and other pro-inflammatory cytokines can affect both peripheral and hepatic lipid metabolism (Memon *et al*., 1993; Raina *et al*., 1995).

The aim of this study was to investigate the mechanism of the protective action of carnitine on liver pathology induced by chronic ethanol exposure. For this purpose rats were given ethanol in a liquid low-carbohydrate–high-fat diet for 10 weeks. Rodents fed this diet develop massive fatty infiltration, rendering the model useful for studies on the pathogenic role of steatosis. For some of the animals the diet was supplemented with a moderate dose of carnitine. To specifically investigate the involvement of Kupffer cells in the action of carnitine, the production of the pro-inflammatory cytokine TNF-α was investigated in Kupffer cells isolated from carnitine pretreated rats.

MATERIALS AND METHODS

Animals and diet

Male Wistar rats, initially weighing 150–170 g, were individually housed in stainless steel wire cages. The animals were fed a modified high-fat–low-carbohydrate liquid diet based on the commercial Lieber-DeCarli diet (LD 101A; Purina Mills, Richmond, IN, USA) as described previously (Lindros and Järveläinen, 1998). Corn oil was added to increase the caloric content of (unsaturated) fat from 35 to 44%, with a corresponding decrease in the caloric content of carbohydrate (maltodextrose) from 11 to 5.5% (or from 47 to 11% in the control diet). The percentage of calories supplied as ethanol was 34.5%. Casein (technical grade; Sigma, St Louis, MO, USA), vitamins and minerals were added to equal the composition of the Lieber-DeCarli diet. Both diets contained 16% protein and 0.4% carboxymethylcellulose (CMC; Metsä Specialty Chemicals Oy, Äänekoski, Finland) to

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increase viscosity. The metabolically inert CMC is widely used as a thickener in a variety of dairy and other food industry products. Animals were fed the alcohol diet *ad libitum* for 10 weeks. The daily intake of ethanol-consuming rats was recorded, and control animals were pair-fed an equicaloric amount of the control diet the subsequent day. Half of the animals received diet supplemented with carnitine (2% of diet dry weight). There were four groups: controls ($n = 4$), controls + carnitine ($n = 4$), ethanol ($n = 8$) and ethanol + carnitine ($n = 8$). The concentration of ethanol in the diet was gradually increased to the final concentration (5% w/v) during the week 1 of diet treatment. To monitor blood ethanol levels, blood samples (50 µl) were collected from the tail vein at weekly intervals at 08.00 hours and ethanol was assayed by head-space gas chromatography (Hu *et al*., 1995).

At termination, rats were anaesthetized with sodium pentobarbital [60 mg/kg intraperitoneally (i.p.)], the chest was opened and blood samples collected by heart puncture. Plasma was separated and stored at –20°C. Liver pieces were collected in buffered formaline solution for histology and rapidly frozen for biochemical assays.

A separate study involving the effect of carnitine pretreatment on short-term ethanol challenge was performed on 12 male Wistar rats weighing 270–290 g maintained on free access to drinking water and laboratory chow (Purina Mills, Richmond, IN, USA). Eight rats, four of which had been pretreated with carnitine (200 mg/kg in drinking water) for the last 5 days, were intubated with ethanol solution [5 g/kg, 20% (w/v) in saline] 3 and 24 h before termination. Four controls were intubated with saline. From these animals Kupffer cells were isolated. Both studies had been approved by the Committee for Animal Experimentation of the National Public Health Institute in Helsinki, Finland.

Analytical procedures and pathological evaluation

The plasma concentration of total carnitine was measured enzymatically (Schafer and Reichmann, 1989) and alanine aminotransferase (ALT) activity by using a commercial kit (Boehringer Mannheim, Germany). The concentration of TNF-α in plasma and culture media was measured by using a commercial ELISA kit (R&D Systems, NY, USA) and nitric oxide (NO, the stable metabolite nitrate) in plasma by using the Griess reagent (Eigler *et al*., 1995) and a microplate photometer (Multiskan RS; Labsystems, Finland). The concentration of lipid peroxidation products in hepatocytes was estimated by assay of thiobarbituric acid reactive substances (TBARS; mainly malondialdehyde) (Ohkawa *et al*., 1979).

For determination of triglycerides, liver tissue (about 100 mg) was homogenized and sonicated in 1 ml 95% methanol, mixed with 2 ml chloroform, the organic phase was washed with NaCl solution and dried under nitrogen. The residual was dissolved in 200 µl of tetraethylammoniumhydroxide (1:28 with 95% ethanol) and incubated at 60°C for 30 min with 200 µl of 0.05 M HCl. The formed glycerol was measured enzymatically using a commercial kit (R-Biopharm; Boehringer Mannheim). The rate of *p*-nitrophenol hydroxylation, which reflects CYP2E1 activity, was measured from hepatocyte homogenates prepared with 10 mM phosphate buffer (pH 6.8) containing 0.25 M sucrose, 10 mM mercaptoethanol, and 0.2% Triton ×100 as described before (Koop *et al*., 1991).

For histopathology, sections from formaline-fixed paraffin-embedded liver specimens were stained with haematoxylin/eosin and scored blindly for the degree of fatty infiltration and inflammation. Fatty infiltration was graded from 0–5, following a protocol previously described (Nanji *et al*., 1994), with 0 depicting absence of cells with fat vacuoles and 5 that >75% of cells contained fat vacuoles. Inflammatory cell infiltration was scored from 0–5 in 10 randomly selected low-power fields. Detection of a single inflammatory cell gave 1 point, an inflammatory focus consisting of 2–5 cells 3 points, and an inflammatory focus of more than 5 cells gave 5 points.

Isolation of Kupffer cells

Three hours after the second saline or ethanol injection liver cells were isolated from rats liver by collagenase digestion (Pertoft and Smedsrod, 1987). The hepatocyte fraction was removed by two brief centrifugations (30 s × 50 ***g***) and Kupffer cells were purified by gradient centrifugation using Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden). The cells (5×10^5 cells/well in a 24-well plate) were seeded in RPMI 1640 (GIBCO Laboratories Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycine sulfate) at 37°C. Non-adherent cells were removed after 30 min of incubation by washing and replacing the medium. Viability of the remaining attached cells, as determined by Trypan blue exclusion, always exceeded 90%. Production of TNF-α by the isolated Kupffer cells during 4 h of incubation was determined in the presence or absence of LPS (*Escherichia coli*, Serotype 0111:B4; 100 ng/ml; Sigma, MO, USA). Results were expressed as pg/µg of cellular protein. Cell lysates were prepared by adding 0.1 N NaOH and total protein determined by using a BSA™ Protein Assay Kit (Rockford, IL, USA).

Statistical analysis

All results were expressed as means ± SEM and the Student's *t*-test applied. Differences were considered statistically significant if $P < 0.05$.

RESULTS

All rats gained weight during the 10-week feeding period, but the weight gain of ethanol-treated rats was somewhat lower than in controls. Rats on carnitine supplementation gained body weight slightly more but only in the ethanol-treated group. The final body weight of control rats was 329.8 ± 9.2 g, in controls + carnitine 330.7 ± 8.8 g, in ethanol-treated 270.0 ± 5.6 g ($P < 0.05$ compared to controls) and in ethanol + carnitine treated rats 300.9 ± 6.9 ($P < 0.05$ compared to ethanol-treated). Analysis of plasma carnitine levels at the end of the 10-week experiment demonstrated that there was no difference between control rats (30.39 ± 9.0 nmol/ml) and ethanol-treated rats (28.73 ± 2.4 nmol/ml) in contrast to the concentration in ethanol-treated carnitine supplemented rats (105.4 ± 15.3 nmol/ml; $P < 0.05$).

Based on diet intake, the mean daily alcohol consumption by both ethanol-treated and ethanol + carnitine-treated rats was calculated to vary between 10.4 and 13.1 g/kg body

weight. The average tail blood ethanol concentration calculated from weekly taken samples was 35.6 mM (164 mg%) and 33.8 mM (155 mg%) for ethanol- and ethanol-carnitine treated rats, respectively. Carnitine supplementation to control rats had no significant effect on any of the parameters investigated. Consequently, controls with and without extra carnitine were combined to one control group ($n = 8$).

The 10-week ethanol treatment resulted in marked micro/macrovesicular steatosis and in infiltration of mononuclear inflammatory cells (Fig. 1). Dietary supplementation with carnitine reduced steatosis by 44% ($P < 0.05$) and inflammation by 41% ($P < 0.1$). As a consequence, the total pathology score was significantly reduced (by 43%; $P < 0.05$) (Fig. 2). The effects of ethanol and carnitine on steatosis were also seen by direct assay of liver triglycerides. In livers from ethanol treated animals the concentration of triglycerides was four times higher than in controls ($P < 0.01$) but only 1.7-fold higher after simultaneous carnitine treatment ($P < 0.05$ compared to ethanol only).

The histopathological changes were reflected in plasma levels of ALT and TNF-α. The ethanol-induced increase of ALT and TNF-α was significantly ($P < 0.05$) reduced by co-treatment with carnitine (Fig. 3). In contrast, plasma levels of NO were not significantly altered by ethanol and only moderately increased ($P < 0.1$) in rats co-treated with ethanol and carnitine (Table 1).

To estimate how chronic ethanol and carnitine treatment affected lipid peroxidation, the hepatic concentration of thiobarbituric acid reactive substances (TBARS) was measured. Ethanol treatment induced a six-fold increase in TBARS (Table 1), an increase that was significantly ($P < 0.05$) blunted by carnitine supplementation. We also investigated whether carnitine would affect the activity of the ethanol-inducible CYP2E1 enzyme by assay of p-nitrophenol hydroxylation in

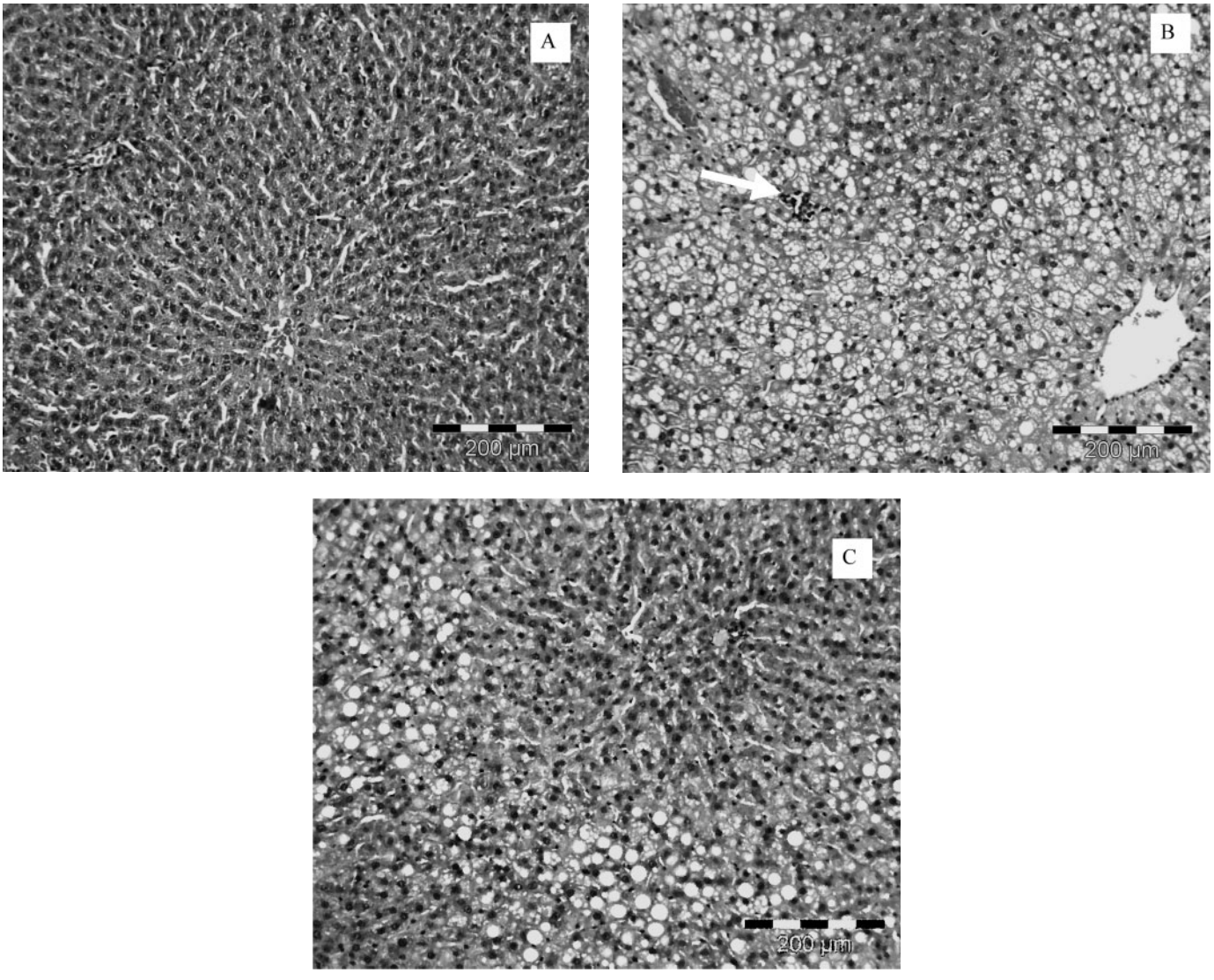

Fig. 1. Representative micrographs of liver sections taken after 10 weeks of ethanol and carnitine treatment.
(A) From a rat fed control diet. (B) From a rat fed ethanol liquid diet. Extensive panlobular mixed micro/macrovesicular steatosis is seen, as well as a focal clustering of inflammatory cells (arrow). (C) From a rat on diet with ethanol and carnitine. The steatosis is less extensive and no inflammatory foci are seen.

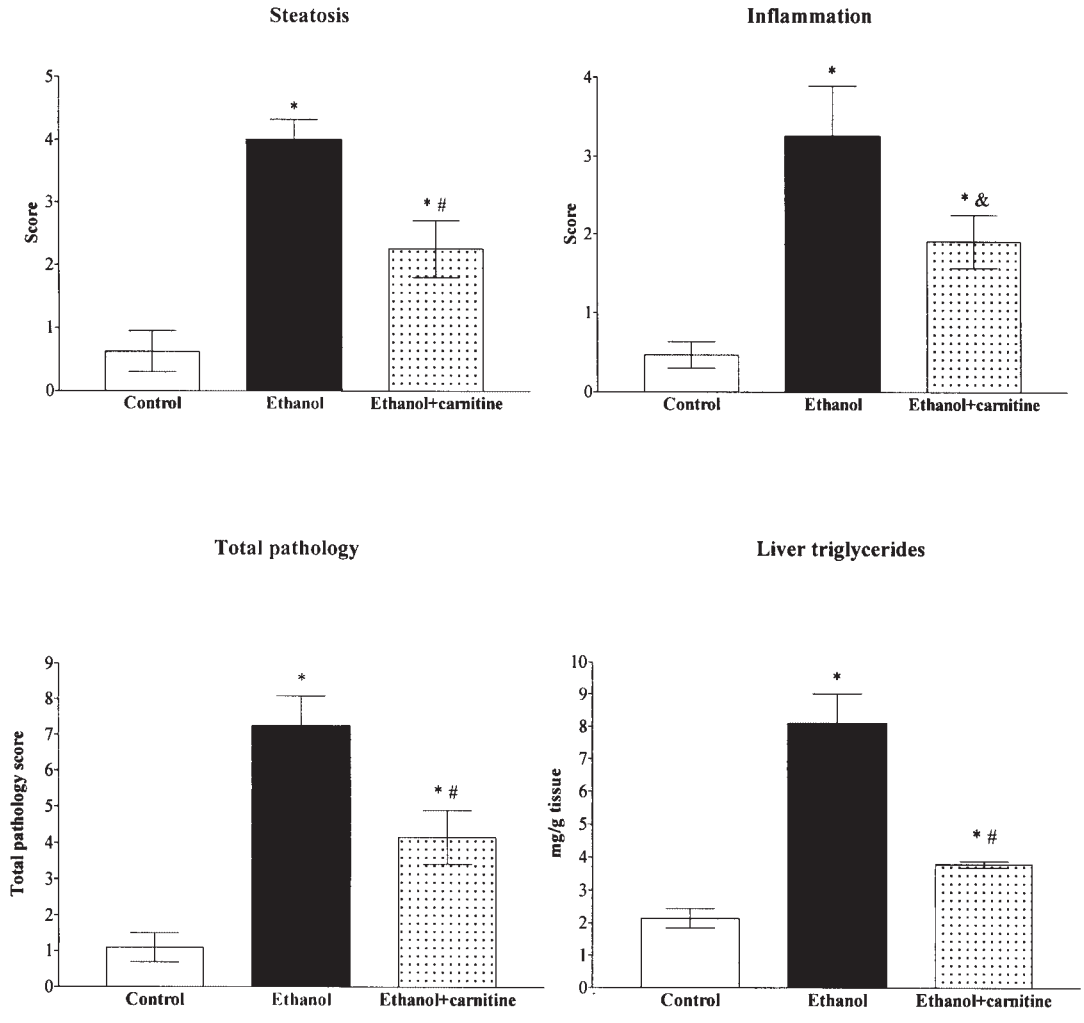

Fig. 2. Effect of dietary ethanol and carnitine treatment on steatosis, inflammation, total pathology score and triglyceride concentration. Grading of pathology was done as describedin Materials and Methods. Bars represent means ± SEM ($n = 8$). *$P < 0.05$ compared to Control; #$P < 0.05$ compared to Ethanol; &$P < 0.1$ compared to Ethanol.

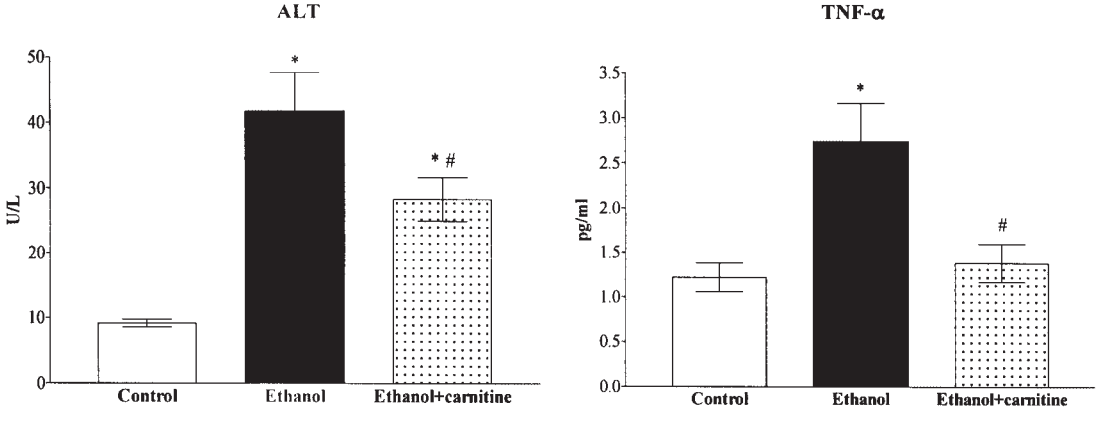

Fig. 3. Effect of dietary ethanol and carnitine treatment on plasma ALT activity and TNF-α concentration in a study. Bars represent mean ± SEM ($n = 8$). *$P < 0.05$ compared to Control; #$P < 0.05$ compared to Ethanol.

Table 1. Effect of dietary ethanol and carnitine treatment on hepatocyte thiobarbituric acid reactive substances (TBARS) concentration, p-nitrophenol hydroxylase (CYP2E1) activity and plasma nitric oxide (NO) concentration

	Control	Ethanol	Ethanol + carnitine
TBARS (nmol/mg protein)	1.18 ± 0.33	6.81 ± 2.27*	2.22 ± 0.48**
CYP2E1 (nmol/mg protein per min)	0.08 ± 0.01	0.41 ± 0.04*	0.49 ± 0.05*
NO (μM)	24.7 ± 4.2	37.1 ± 7.7	50.3 ± 10.0

Assays were performed as described in Materials and Methods. Values are given as means ± SEM ($n = 8$) are given.
*$P < 0.05$ compared to control; **$P < 0.05$ compared to ethanol.

hepatocytes. As expected, there was a significant five- to six-fold induction of CYP2E1 activity in ethanol-treated animals, but the induction was not influenced by carnitine, however.

To test whether carnitine reduced serum TNF-α levels via an effect on liver macrophages, a separate short-term ethanol experiment was performed. Rats pretreated with carnitine for 5 days were challenged with two intragastric doses of ethanol and Kupffer cells isolated 24 h later. Both basal and LPS-stimulated production of TNF-α were measured. Ethanol treatment alone significantly sensitized Kupffer cells to produce more TNF-α in the presence of LPS (Fig. 4). However, in carnitine-treated animals this effect was completely absent. In addition, in Kupffer cells from carnitine-pretreated rats the basal production of TNF-α was significantly lower compared to ethanol treatment alone.

DISCUSSION

In this study we showed that in rats treated with ethanol for 10 weeks, dietary supplementation with carnitine significantly alleviates liver pathology. Although the reduced pathology score was due mainly to diminished steatosis, reduced ethanol-induced inflammation also contributed to this effect. In addition, carnitine significantly reduced the effect of ethanol on lipid peroxidation, on plasma levels of ALT and TNF-α and on TNF-α production by Kupffer cells.

The counteracting effect of carnitine on ethanol-induced fatty infiltration is well documented (Sachan *et al*., 1984; Bertelly *et al*., 1993; Bahcecioglu *et al*., 1999), but the mechanism does not seem to be limited to its obligatory role in the transmembrane import of fatty acids for mitochondrial β-oxidation. Chronic alcohol exposure leads to aberrations in hepatic choline and methionine metabolism (Barak *et al*., 1985; Trimble *et al*., 1993). Carnitine can counteract this effect and prevent lipotrope methyl group wastage and increase production of polyamines with known immunomodulatory properties (Theoharides, 1980). Carnitine has also been suggested to interfere by affecting the rate of ethanol metabolism or blood ethanol levels (Sachan and Berger, 1986). Our results do not support this suggestion, since in the pair-feeding regimen where both ethanol-fed and ethanol-carnitine fed rats continuously metabolize ethanol, their daily intake of ethanol is similar and there was no difference in blood ethanol levels between these groups.

Much evidence supports the notion that endotoxin leaking from the gut into the circulation is a crucial pathogenic factor in alcohol-induced liver damage (ALD) (Thurman, 1998). Increased LPS levels, common in alcoholics and in experimental alcohol studies, can activate the Kupffer cells to release pro-inflammatory cytokines, including TNF-α (Adachi *et al*., 1995; Thurman, 1998). Support for an important role of LPS and Kupffer cells in ALD derives from experiments involving gut sterilization, Kupffer cell inactivation, neutralizing TNF-α

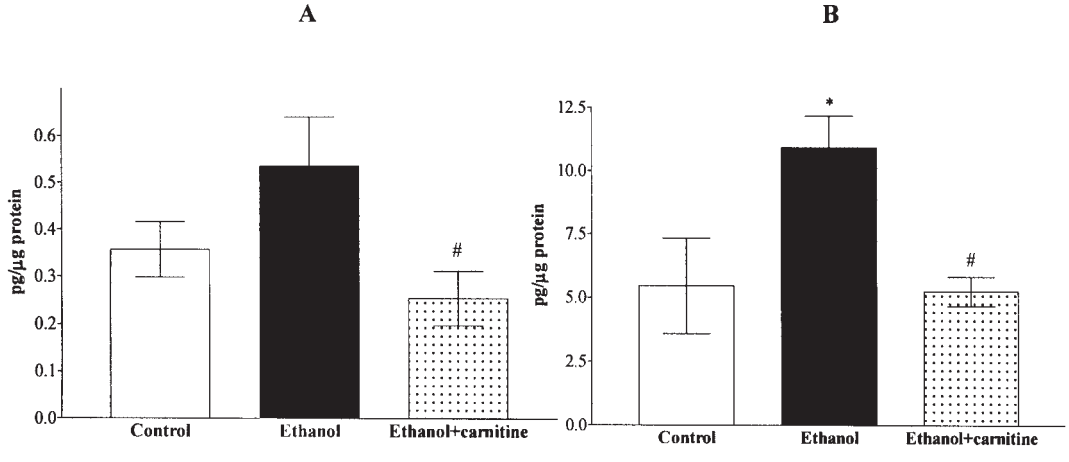

Fig. 4. Effect of carnitine pretreatment on basal (A) and LPS-stimulated (B) TNF-α production by Kupffer cells isolated from 24 h ethanol-treated rats. Carnitine, ethanol and LPS were administered as described in Materials and Methods. Results are means ± SEM ($n = 4$). *$P < 0.05$ compared to Control; #$P < 0.05$ compared to Ethanol.

antibodies and CD14 knock-out mice (Adachi *et al*., 1995; Iimuro *et al*., 1997).

Our present data suggest that carnitine also could act by affecting the responsiveness of Kupffer cells to ethanol and endotoxin. This notion is supported by both clinical and experimental evidence, suggesting that carnitine can directly modify cytokine responses. In surgical and in HIV-positive patients carnitine administration lowered circulating levels of cytokines (Delogu *et al*., 1993; De Simone *et al*., 1993) and addition of carnitine to stimulated human polymorphonuclear leukocytes reduced TNF-α production (Fattorossi *et al*., 1993). Similarly, in rats exposed to LPS or methylcholanthrene carnitine treatment alleviated the increase in serum TNF-α (Winter *et al*., 1995).

The reduced production of TNF-α by Kupffer cells could affect both steatosis and inflammation in parallel. Although carnitine may affect steatosis via mitochondrial fatty acid metabolism or via methionine/methyl group metabolism, a mechanism involving a primary role for TNF-α is a plausible alternative. Indeed, TNF receptor 1 knockout mice chronically exposed to ethanol exhibited significantly less steatosis as compared to similarly treated wild-type mice (Yin *et al*., 1999). Although in one study antibody neutralization of TNF-α reduced inflammation but not steatosis (Iimuro *et al*., 1997), several studies not involving ALD have shown that pro-inflammatory cytokines, including TNF-α, directly modulate serum and liver triglyceride levels via increased hepatic synthesis and secretion (Feingold *et al*., 1989; De Clercq *et al*., 1996). In addition, administration of endotoxin has similar effects (Feingold *et al*., 1992).

Enhanced TNF-α production can also increase the production of reactive oxygen species by macrophages (Goossens *et al*., 1995). This can stimulate lipid peroxidation, which is thought to have an important pathogenic role in alcohol-induced liver damage (Lieber, 1997; Hoek and Pastorino, 2002). We confirmed an earlier report showing that the ethanol-induced increase in lipid peroxidation is significantly blunted by carnitine (Bahcecioglu *et al*., 1999). In experimental models of oxidative stress carnitine has been demonstrated to protect against lipid peroxidation (Luo *et al*., 1999; Dayanandan *et al*., 2001; Loster and Bohm, 2001). These reports and our present data suggests the possibility that the protective action involves TNF-α.

Several laboratories including our own have presented evidence for a pathogenic role of the ethanol-inducible cytochrome (CYP2E1) enzyme in ALD (French *et al*., 1993; Fang *et al*., 1998). However, in the present study the ethanol-induced 6-fold induction of CYP2E1 was unaffected by carnitine in spite of the significantly blunted liver pathology and lipid peroxidation. Thus the protective effect of carnitine does not involve CYP2E1. Neither do our present data indicate that the effect of carnitine is mediated via NO, which has been proposed to be cytoprotective during inflammation and proinflammatory cytokine-mediated cell injury (Kim *et al*., 1997; Harris *et al*., 1999).

In summary, our results show that dietary supplementation with carnitine to ethanol-treated rats reduces liver steatosis and inflammation and inhibits TNF-α production by Kupffer cells. We propose that chronic ethanol exposure activates Kupffer cells, probably via gut-derived endotoxins, and that the hepatoprotective mechanism of carnitine involves dampening of Kupffer cell TNF-α production. This can either directly or indirectly affect both steatosis and inflammation. Further studies on the therapeutic effect of dietary carnitine supplementation to alcoholic patients seem warranted.

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