Arginine Reverses Ethanol-Induced Inflammatory and Fibrotic Changes in Liver Despite Continued Ethanol Administration

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ABSTRACT

We investigated the potential of arginine to reverse pathological changes in alcohol-induced liver injury. Four groups (six rats/group) of male Wistar rats were fed a fish oil-ethanol diet for 6 (group 2) or 8 (group 1) weeks. Rats in group 3 were fed fish oil-ethanol for 6 weeks, after which they were administered arginine with fish oil-ethanol for an additional 2 weeks. Rats in group 4 were fed fish oil-dextrose for 8 weeks. Liver samples were analyzed for histopathology, lipid peroxidation, cytochrome P4502E1 activity, nuclear factor-κB, and levels of messenger RNA for tumor necrosis factor-α, cyclooxygenase-2, and inducible nitric oxide synthase. Concentrations of endotoxin were measured in plasma. The most severe inflammation and fibrosis was detected in groups 1 and 2, as were the highest levels of endotoxin, lipid peroxidation, cytochrome P450 2E1 activity, activation of nuclear factor-κB, and mRNA levels for tumor necrosis factor-α, cyclooxygenase-2, and inducible nitric oxide synthase. Plasma nitric oxide was also increased as was nitrotyrosine in liver. After arginine was administered, there was marked improvement in the pathological changes accompanied by decreased levels of endotoxin, lipid peroxidation, activation of nuclear factor-κB, tumor necrosis factor-α, cyclooxygenase-2, inducible nitric oxide, and nitrotyrosine staining. The therapeutic effects of arginine are probably secondary to increased levels of nitric oxide but other effects of arginine cannot be excluded.

Treatment of alcoholic liver disease remains based on nutritional supplements, which include vitamins and trace elements (Fulton and McCullough, 1998; Muller et al., 1998). The role of specific pharmacological therapy remains unproven. One of the mechanisms evoked to explain alcoholic liver injury is a decrease in oxygen supply that leads to centrilobular hypoxia. (Videla et al., 1973; French et al., 1984; Tsukamoto and Xi, 1989). Nitric oxide has been proposed as one of the modulators of regional blood flow to the liver in various liver diseases (Li and Billiar, 1999). Nitric oxide also acts as an antioxidant (Joshi et al., 1999). We and others have shown that enhanced oxidant stress is associated with pathological changes in alcoholic liver injury (Cederbaum 1989; Reinke et al., 1990; Nanji et al., 1994). We have also previously shown that decreased nitric oxide production by nonparenchymal cells in the liver is associated with increased severity of liver injury in alcohol-fed rats (Nanji et al., 1995a). In those experiments, an inhibitor of nitric oxide production exacerbated alcoholic liver injury, whereas arginine, a substrate for NO, markedly attenuated the pathological changes.

We tested the hypothesis in the current study that treatment with arginine is able to reverse alcoholic liver injury even when ethanol administration was continued. The present set of experiments was designed to simulate the clinical condition in the outpatient setting in which the patient continues to drink even after alcoholic liver injury is present. At the level of the mechanism of alcohol-induced liver injury, we have proposed that elevated levels of endotoxin and lipid peroxides activate nuclear factor-κB and lead to the induction of tumor necrosis factor, cyclooxygenase-2, and other pro-inflammatory cytokines (Nanji et al., 1997, 1999). We tested the idea in the current work that treatment with arginine, leads to improvement in liver pathology together with down-regulation of the above specific pathophysiological events.

To evaluate the affect of arginine on alcoholic liver injury,

ABBREVIATIONS: FE, fish oil-ethanol; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase.

we used the intragastric feeding rat model (French et al., 1986; Tsukamoto et al., 1990). This model shows the pathological changes characteristic of alcoholic liver disease and allows the correlation between biochemical and pathological changes.

Materials and Methods

Animal Model and Treatment Groups. Four groups of male Wistar rats (six rats/group) weighing between 250 and 275 g were studied to evaluate the effects of arginine on the pathological and biochemical parameters in ethanol-fed rats. The rats were fed a liquid diet by continuous infusion through permanently implanted gastric tubes as described previously (French et al., 1986; Tsukamoto et al., 1990). The liquid diet contained fish oil as the source of dietary fat. The amount of ethanol administered was modified to maintain a blood level of ethanol between 150 and 350 mg/dl. The amount of ethanol was initially 10 g/kg/day and increased to 16 g/kg/day as tolerance developed. Each ethanol-fed rat had at least two measurements of blood alcohol. The experimental design is shown schematically in Fig. 1. Rats in group 1 were fed a fish oil-ethanol (FE) diet for 8 weeks and then killed; rats in group 2 were fed the fish oil-ethanol diet for 6 weeks and then killed. Rats in group 3 were fed the fish oil-ethanol diet for 6 weeks and then administered L-arginine (100 mg/kg of body weight/day) via intragastric tube for 2 weeks. The fish oil-ethanol diet was continued for this 2-week period during which arginine was administered. Rats in group 4 received fish-oil dextrose for 8 weeks. When the animals were killed, a sample of liver was obtained for histopathological analysis, and the remainder of the liver was rapidly excised, washed with ice-cold potassium chloride, and cut into small pieces that were transferred to plastic vials and placed in liquid nitrogen. The vials were stored at $-80°C$.

Histopathological Analysis Including Sirius Red Staining for Collagen. A small sample of liver was obtained and formalin-fixed when the rats were killed. Hematoxylin and eosin stain was used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat), 1+, <25%; 2+, 26 to 50%; 3+, 51 to 75%; and 4+, >75% of cells containing fat. Necrosis was quantified as the number of necrotic foci per square millimeter; and inflammation was scored as the number of inflammatory cells per square millimeter. At least three different sections were examined per sample of liver. The pathologist evaluating these sections was unaware of the treatment the rats had received.

For evaluation of fibrosis around the central veins, sections were stained with Sirius red and analyzed using computerized image analysis. The area of collagen deposition around each central vein was measured using a Macintosh-based morphometric analysis system (Apple Computer Inc., Brea, CA) with NIH Image version 1.52 software. The cross-sectional area of the central vein lumen was measured using the same technique. The area of collagen deposition was divided by the area of the central vein lumen to correct for the size of the lumen and provide a standardized measurement of central vein collagen. The coefficient of variation was determined by assessment of a single central vein on six occasions (<5%). Pericellular fibrosis was estimated as the number of positively staining sites on adjacent hepatocyte surfaces per 100 hepatocytes around the central vein.

Immunohistochemistry for Nitrotyrosine. Sections of liver tissue were immunostained with antiserum to nitrotyrosine. Sections were deparaffinized in xylene and rehydrated through graded ethanol concentrations. To block endogenous peroxidase activity, the sections were immersed in 3% hydrogen peroxide for 5 min at room temperature. The sections were then stained with antibody to nitrotyrosine (Upstate Biotechnology, Lake Placid, NY). Positive staining was indicated by a brown color generated with diaminobenzidine. Control sections were incubated with normal rabbit IgG.

Measurement of Blood Alcohol Levels. Blood was collected from the tail vein, and ethanol concentration was measured using the alcohol dehydrogenase kit from Sigma Chemical (St. Louis, MO). Blood was obtained at killing and at other times during the ethanol feeding period.

Measurement of Plasma Endotoxin Levels. Blood samples were collected in endotoxin-free vials (Sigma Chemical) and centrifuged at $400g$ for 15 min at 4°C. Samples were then diluted 1:10 in pyrogen-free water and heated to 75°C for 30 min to remove inhibitors of endotoxin from plasma. The limulus amoebocyte lysate test (Kinetic-QLC; Whittaker Bioproducts, Walkerville, MD) was used for measurements of endotoxin. Samples were incubated at 37°C for 10 min with limulus amoebocyte lysate. The substrate solution was added, and the incubation continued for 20 min. The reaction was stopped with 25% acetic acid. Samples were read spectrophotometrically at 410 nm.

Measurement of Nitric Oxide in Plasma. Nitric oxide in plasma was measured as previously described (Nanji et al., 1995a). Briefly, nitric oxide was generated from the nitrite and nitrate anions. The nitric oxide reacted with machine-generated ozone to form nitrogen dioxide, which generated light at 6500 to 8000 Å. The amount of light generated was concentration-dependent and was measured with a photomultiplier tube.

Determination of Thiobarbituric Acid-Reaction Substances and Conjugated Dienes. Levels of liver thiobarbituric acid-reactive substances were measured according to the method of Ohkawa et al. (1997). Conjugated dienes in the total lipid extracted from liver homogenates were quantified by measurements of optical density between 220 and 300 nm as described by Recknagel and Glende (1984).

Preparation of Liver Microsomes and Measurement of 4-Nitrophenol Hydroxylase Activity. The microsomal fraction was prepared as previously described (Nanji et al., 1995b). The term "microsomal fraction" was applied to the $100,000g$ particulate fraction derived from liver homogenates by differential centrifugation. The subcellular fraction was stored at $-80°C$ until use. 4-Nitrophenol hydroxylase activity was determined as previously described (Koop, 1986).

Determination of Nuclear Factor-κB and IKBα. Fractionation of the livers into cytosolic and nuclear extracts was as previously published (Nanji et al., 1999). Nuclear factor-κB (NF-κB) binding was determined by electromobility shift assay as described previously (Nanji et al., 1999). Briefly, equal amounts of nuclear extracts were incubated with ^{32}P-labeled NF-κB oligonucleotide, and the DNA-protein complexes were separated on polyacrylamide gel. Specificity of NF-κB binding was verified by competition assays and the ability of specific antibodies to supershift protein-DNA complexes. In the competition assay, a 100-fold excess of the unlabeled oligonucleotide was used; in the supershift assay, antiserum against the p50 submit of NF-κB was used. Scanning densitometry was used to quantitate the degree of NF-κB activation.
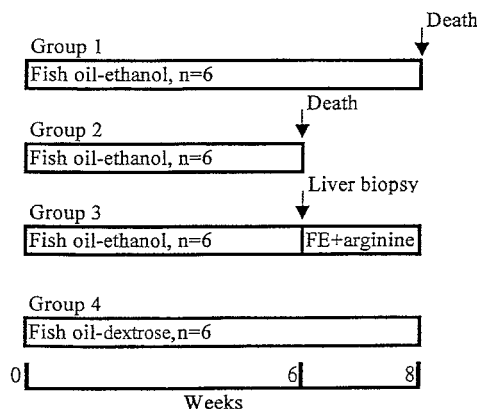

Fig. 1. Schematic of the experimental design.

Western blot analysis was done using antibody against IKBα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as previously described (Nanji et al., 1999). The antibody-reactive bands were visualized by use of enhanced chemiluminescence assay (PerkinElmer Life Sciences, Boston, MA).

RNA Extraction and Analysis of mRNAs for Cyclooxygenase-1 and -2, Tumor Necrosis Factor-α, Inducible Nitric Oxide Synthase, and β-Actin by Reverse Transcription Polymerase Reaction. To examine the expression of the above mRNAs in liver, total RNA was extracted according to the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1997). The transcription into complementary DNA and amplification was carried out as previously described. The sequences of the primer pairs have been reported previously (Nanji et al., 1997). The gels were analyzed by laser scanning densitometry as previously described (Nanji et al., 1997).

Statistical Analysis. All data are expressed as means ± S.D. unless otherwise indicated. Differences between groups were analyzed using analysis of variance or Student's t test as appropriate.

Results

There was no significant difference in weight gain before administration of the diet containing arginine (Table 1). Blood alcohol levels ranged between 150 and 410 mg/dl and were similar among the groups (Table 1).

Effect of Arginine on Liver Pathology. Feeding a FE diet for 6 (group 2) or 8 weeks (group 1) caused fatty liver, necrosis, and inflammation (Table 2; Fig. 2). Control animals fed fish oil-dextrose (group 4) showed no pathological changes (Fig. 2). Arginine administration led to a significant improvement in pathological changes; there was a decrease in the degree of fatty liver, necrosis, and inflammation (Table 2; Fig. 2). Arginine administration also led to a decrease in the amount of central vein collagen and pericellular fibrosis (Figs. 2 and 3).

Modulation of Endotoxin and Lipid Peroxidation. The effect of arginine on the known mediators of alcohol-induced lives injury, i.e., endotoxin and lipid peroxidation, was evaluated at the completion of administration of the experimental diets. Concentrations of endotoxin were significantly decreased ($p < 0.01$) after institution of the arginine-containing diet. (Table 3). Similarly, levels of conjugated dienes and thiobarbituric acid-reactive substances were significantly decreased ($p < 0.01$) after arginine administration. Part of the explanation for the arginine-induced decrease in lipid peroxidation could be the decrease in cytochrome P450 2E1 activity (Table 3). Levels of endotoxin, conjugated dienes, and thiobarbituric acid were not different among rats fed ethanol and fish oil for 6 or 8 weeks.

TABLE 1
Blood alcohol levels and weight gain before and after arginine administration

Experimental Group	Blood Alcohol Level	Weight Gain	
		Before Arginine	After Arginine
	mg/dl	*g*	
FE, 8 weeks	246 ± 51	68 ± 11	
FE, 6 weeks	231 ± 46	53 ± 8	
FE-arginine, 8 weeks[a]	260 ± 53	56 ± 10	28 ± 7
FD, 8 weeks	0	77 ± 8	

FD, rats fed fish oil and dextrose.
[a] The 8-week period includes 6 weeks of ethanol and liquid diet followed by administration of arginine and ethanol for 2 additional weeks.

Effect of Arginine on Activation of NF-KB. To evaluate the effect of arginine on activation of nuclear factor-κB, electrophoretic mobility shift assays of nuclear extracts from whole liver were carried out. Nuclear localization of nuclear factor-κB was increased in the fish oil-ethanol-fed groups (Fig. 4; Table 4). Activation was decreased in the arginine-fed group. To determine whether activation of nuclear factor-κB was a result of degradation of IKBα, IKBα was evaluated by Western blot analysis. In livers from rats fed fish oil-ethanol, IKBα was either absent or markedly decreased (Fig. 4; Table 4). IKBα levels were higher in the arginine-treated group compared with the fish oil-ethanol groups (Table 4).

The protein/DNA complex was further characterized by competition and supershift assays as previously described (Nanji et al., 1999). Briefly, antibodies against p50 used in the supershift assay demonstrated the specificity of the band and excess NF-κB oligonucleotide abrogated complex formation (data not shown).

Effect of Arginine on Tumor Necrosis Factor-α, Cyclooxygenase-2, and Inducible Nitric Oxide Synthase. We have previously proposed that lipid peroxidation and endotoxin activate nuclear factor-κB, which leads to induction of tumor necrosis factor-α, cyclooxygenase-2, and inducible nitric oxide synthase in alcoholic liver injury. Because the mRNA levels of the above are too low to be detected by Northern or ribonuclease protection assays, we used RT-PCR for mRNA analysis. We confirmed that mRNA levels of tumor necrosis factor-α, cyclooxygenase-2, and inducible nitric oxide synthase are up-regulated in fish oil-ethanol-fed rats. Arginine supplementation led to down-regulation of all three mRNAs. Cyclooxygenase-1, the constitutive isoform of cyclooxygenase, was similar in all groups (Table 4).

Effect of Arginine on Plasma Nitric Oxide and Liver Nitrotyrosine. Ethanol administration significantly increased the levels of nitric oxide in plasma (Table 3). In the ethanol-fed group treated with arginine, the nitric oxide levels were significantly lower ($p < 0.05$) than in the ethanol-fed groups (groups 1 and 2) but higher than in the dextrose-fed control group ($p < 0.05$). Intense nitrotyrosine labeling was observed in the livers of ethanol-fed rats (Fig. 5), in contrast, much less nitrotyrosine was detected in the arginine-treated and in the dextrose-fed rats (Fig. 5).

Discussion

Relationship between the Current Experimental Model and Alcoholic Livers Disease in Humans. The major problem in treatment of alcoholic liver disease in humans remains the adherence of patients to abstinence from alcohol. Many studies have been carried out to determine the effectiveness of therapy in reducing the progression of liver injury in alcoholics (reviewed in Fulton and McCullough, 1998). The current data are important in this regard because the experimental model used has striking resemblance to the clinical setting in which alcoholic liver disease occurs. The rats in the present experiments had, similar to the patient with alcoholic liver disease, manifestations of liver injury prior to the application of a therapeutic regime. This method of therapy is in contrast to our previous studies that simulated an in-hospital model where alcohol was discontinued prior to administration of therapy (Nanji et al., 1995b, 1996). The current data show that administration of arginine, even

TABLE 2
Severity of pathologic changes in the treatment groups

Treatment Group	Duration of Feeding	Fatty Liver (0–4)	Necrosis	Inflammation
	weeks		*foci/mm²*	*cells/mm²*
Group 1				
FE, 8 weeks	8	4.0 ± 0.0	1.1 ± 0.4	26.2 ± 7.8
Group 2				
FE, 6 weeks	6	4.0 ± 0.0	1.0 ± 0.4	$25.8 \pm .4$
Group 3				
FE-arginine				
Fish oil-ethanol	6	4.0 ± 0.0	0.9 ± 0.2	29.1 ± 8.7
Fish oil-ethanol + arginine	2	$1.8 \pm 0.6^{a,b}$	$0.3 \pm 0.1^{a,b}$	$9.1 \pm 2.4^{a,b}$
Group 4				
FD, 8 weeks	8	0^{b}	0^{b}	0.2 ± 0.1^{b}

[a] $p < 0.01$ vs. fish oil-ethanol in the same group (group 3).
[b] $p < 0.01$ vs. fish oil-ethanol in groups 1 and 2.
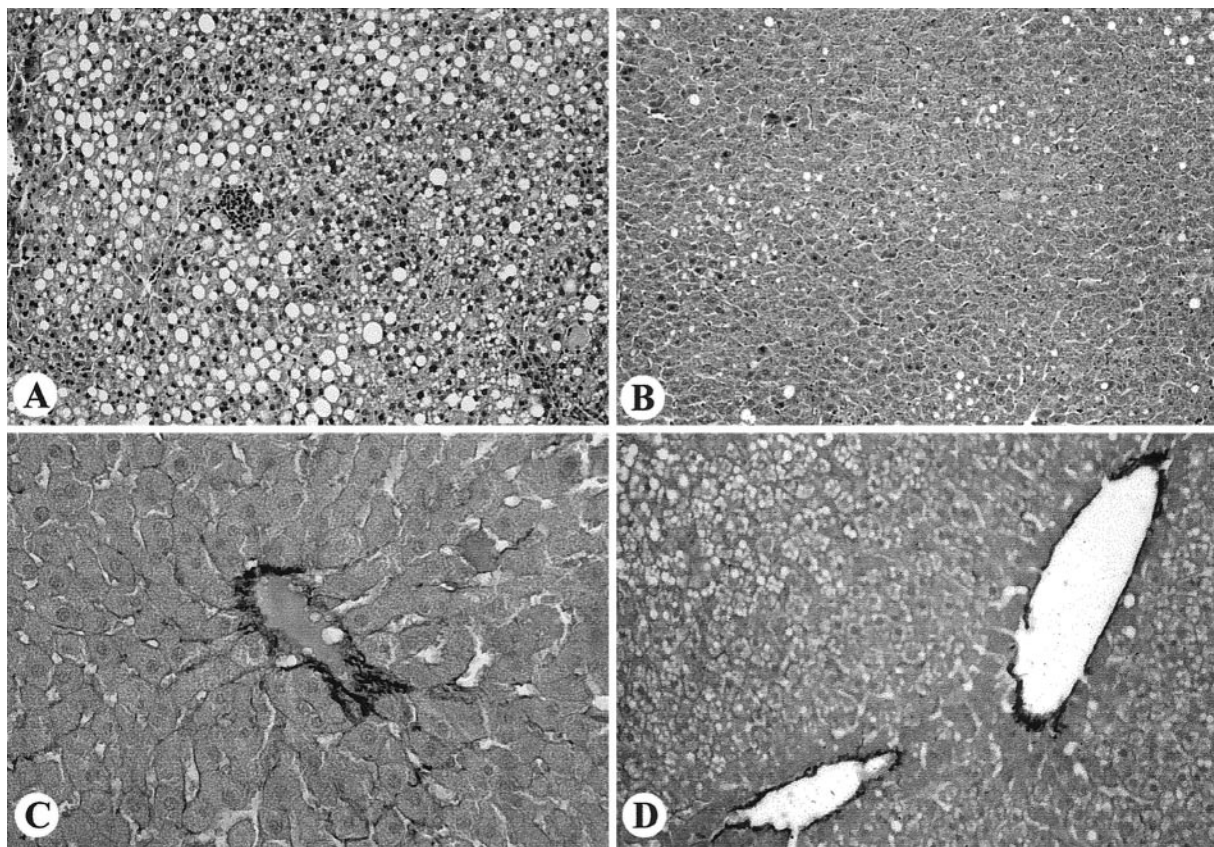
Fig. 2. A, liver from a rat fed fish oil-ethanol for 6 weeks showing the presence of fatty liver, necrosis, and inflammation (hematoxylin and eosin, 155× magnification). B, liver from a rat fed fish oil-ethanol for 6 weeks followed by fish oil-ethanol-arginine for 2 weeks. Note the reduction in the degree of fatty liver, necrosis, and inflammation (hematoxylin and eosin, 155× magnification). C, liver section from a rat fed fish oil-ethanol for 6 weeks and stained with Sirius red. The connective tissue component of the central vein and cellular fibrosis are increased (magnification, 400×). D, liver section from a rat treated with fish oil-ethanol-arginine for 2 weeks after 6 weeks of fish oil-ethanol administration. Collagen deposition and pericellular fibrosis are markedly decreased compared with fish oil-ethanol-fed rats.

when ethanol administration was continued, reduced the indices of liver pathology, which included fatty liver, necrosis, inflammation, and fibrosis.

Mechanisms by Which Arginine Down-Regulates Pathological Events Triggered by Ethanol. Levels of endotoxin and lipid peroxides increase in alcohol-induced liver injury and are believed to be responsible for the hepatotoxic effects of alcohol. (Cederbaum, 1989; Nanji et al., 1993, 1994; Reinke et al., 1990; Adachi et al., 1995). The observed differences in liver pathology in the rats fed fish oil-ethanol and those given arginine can be explained, at least in part, by differences in levels of endotoxin and lipid peroxides. Animals treated with arginine had an approximately 50% decrease in the levels of endotoxin and lipid peroxidation compared with those fed fish oil-ethanol. (Table 3). The mechanisms leading to endotoxemia in ethanol-fed rats included a reduction in the capacity of Kupffer cells to detoxify endotoxin and increased permeability of the intestinal mucosa to endotoxin (Nanji et al., 1993). The reduction in endotoxin levels in arginine-treated rats is probably related to the improvement in liver function; arginine, to the best of our knowledge has no effect on the permeability of the gut to
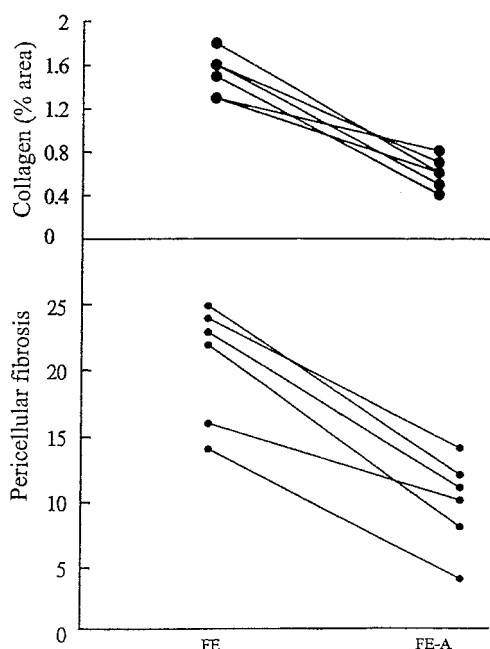
Fig. 3. Changes in the amounts of collagen and pericellular fibrosis in rats fed FE for 6 weeks followed by fish oil-ethanol-arginine (FE-A) for 2 weeks. The results of the biopsy at 6 weeks served as a baseline for comparison with the results after treatment with arginine. In the arginine-treated animals, the amount of central vein collagen (% area) decreased from 1.5 ± 0.2% to 0.6 ± 0.1% ($p < 0.01$). Pericellular fibrosis was also decreased from 21 ± 5 to 11 ± 3 ($p < 0.05$). The amount of collagen and pericellular fibrosis was not significantly different in the liver biopsies obtained after 6 weeks in comparison to values seen in animals fed fish oil-ethanol for 6 weeks (group 2) or 8 weeks (group 1).

Fig. 4. Representative electrophoretic mobility shift assay for NF-κB activation in nuclear extracts and Western blot analysis for IKBα protein in the cytosolic extracts from livers in the different experimental groups. Extracts from rats in the FE group showed increased binding activity (lane 1). Binding activity was decreased in the arginine-treated group (lane 2) (see also Table 4). Activation of NF-KB was associated with decreased IKBα protein levels in the FE rats, and the level of IKBα was higher in the arginine-treated rats compared with the FE group (Table 4). A low level of NF-κB activation was seen in rats fed fish oil-dextrose (lane 3).

endotoxin. The differences in lipid peroxidation could reflect changes in the activity of cytochrome P450 2E1, which decreased about 50 to 60% with arginine administration (Table 3). Cytochrome P450 2E1 is a major contributor to lipid peroxidation in ethanol-fed rats, and its inhibition leads to a decrease in the level of lipid peroxidation in the livers of ethanol-fed rats (Morimoto et al., 1995).

The mechanism for the decrease in cytochrome P450 2E1 activity with arginine is likely related to the effect of inhibition of the enzyme activity by nitric oxide. Nitric oxide is generated from arginine and reacts with CYP 2E1 and inhibits its activity and generation of free radicals (Gergel et al., 1997; Khatsenko, 1998).

Arginine-Induced Down-Regulation of Nuclear Factor-κB, Tumor Necrosis Factor-α, Cyclooxygenase-2, and Inducible Nitric Oxide Synthase. One pathway by which endotoxin and lipid peroxidation promote alcoholic liver injury is via activation of nuclear factor-κB, a ubiquitous transcription factor that activates many inflammatory genes. Our data confirm our previous observation that activation of nuclear factor-κB occurs in association with necroinflammatory changes in fish oil-ethanol-fed rats (Nanji et al., 1999). The decrease in the degree of nuclear factor-κB activation by arginine was accompanied by down-regulation

TABLE 3
Measurement of endotoxin, lipid peroxidation, and 4-nitrophenol hydroxylase and nitric oxide in the experimental groups

Experimental Group	Endotoxin	TBARS	Conjugated Dienes A_{232}	4-Nitrophenol Hydroxylase	Nitric Oxide
	pg/ml	*nmol/mg protein*		*nmol/min/mg*	*μm/l*
1) FE, 8 weeks	73 ± 12	1.52 ± 0.36	0.50 ± 0.15	3.2 ± 0.69	110 ± 22
2) FE, 6 weeks	76 ± 14	1.48 ± 0.32	0.46 ± 0.12	3.01 ± 0.57	101 ± 19
3) FE-arginine, 8 weeks	31 ± 8[a]	0.59 ± 0.14[a]	0.19 ± 0.09[a]	1.12 ± 0.13[b]	63 ± 10[b]
4) FD, 8 weeks	10 ± 5[a,c]	0.33 ± 0.11[a]	0.16 ± 0.07[a]	1.17 ± 0.16[b]	34 ± 8[b,c]

TBARS, thiobarbituric acid-reactive substances.
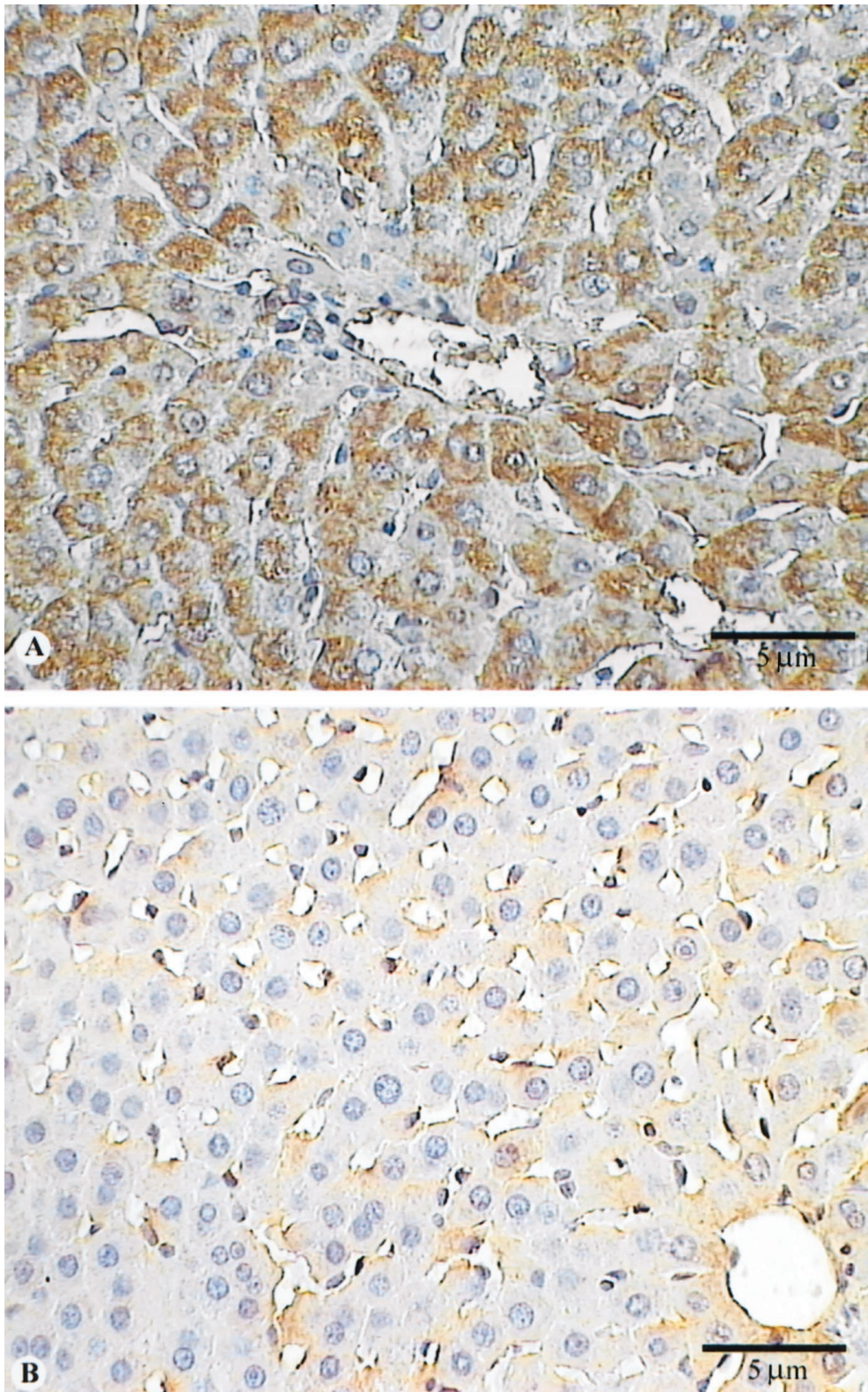[a] $p < 0.01$ vs. groups 1 and 2.
[b] $p < 0.05$ vs. groups 1 and 2.
[c] $p < 0.05$ vs. group 3.

TABLE 4
Analysis of NF-κB, IκBα, TNF-α, Cox-2, and Cox-1 in the different experimental groups

Experimental Group	NF-κB	IκB-α	TNF-α mRNA	Cox-2 mRNA	iNOS mRNA	Cox-1 mRNA
	relative units			*relative to β-Actin*		
1) FE, 8 weeks	6.4 ± 1.8	0.17 ± 0.09	7.2 ± 2.8	1.4 ± 0.6	4.8 ± 1.1	1.0 ± 0.1
2) FE, 6 weeks	5.3 ± 2.1	0.21 ± 0.06	6.9 ± 1.9	1.3 ± 0.5	4.2 ± 1.4	1.0 ± 0.2
3) FE-arginine	1.4 ± 0.8[a]	0.46 ± 0.09[a]	3.0 ± 1.8[b]	0.4 ± 0.1[b]	1.1 ± 0.4[a]	1.2 ± 0.2
4) FD, 8 weeks	1.0[a]	1.0[a]	1.0[a]	0[a]	0.6 ± 0.2[a]	1.0

[a] $p < 0.01$ vs. groups 1 and 2.
[b] $p < 0.05$ vs. groups 1 and 2.

Fig. 5. Immunohistochemical analysis of nitrotyrosine in liver specimens obtained from a rat fed fish oil-ethanol for 6 weeks (A) and fish oil-ethanol for 6 weeks followed by fish oil-ethanol-arginine for 2 weeks (B). An increase in the level of nitrotyrosine staining, particularly in the centrilobular area, was evident in all rats fed fish oil-ethanol for 6 weeks. A marked decrease in nitrotyrosine staining was seen in all of the arginine-treated rats (B). The level of staining was similarly low in fish oil-dextrose-fed rats (data not shown).

of tumor necrosis factor-α and cyclooxygenase-2. A growing body of evidence implicates nuclear factor-κB, tumor necrosis factor-α, and cyclooxygenase-2 in inflammatory liver injury (McClain et al., 1993; Dinchuk et al., 1995; Nanji et al., 1997). We therefore hypothesize that down-regulation of these pro-inflammatory stimuli by arginine and possibly nitric oxide contribute to the improvement in liver pathology.

In the present study, increased levels of mRNA for inducible nitric oxide synthase, nitric oxide, and staining for nitrotyrosine were seen in the hepatocytes of ethanol-fed rats. The increase in nitrotyrosine together with the increase in iNOS suggests that NO production in hepatocytes and formation of nitrotyrosine are intimately linked. This view is further supported by the down-regulation of inducible nitric

oxide synthase and nitrotyrosine formation in arginine-treated rats. The formation of nitrotyrosine depends on the reaction between NO and superoxide, which yields the powerful oxidant peroxynitrite (Grisham, 2000). The association between increased levels of lipid peroxidation, iNOS mRNA, and nitrotyrosine underscores the importance of the interaction between nitric oxide and free radicals in alcoholic liver injury.

One assumption for the mechanism involved in the protective effect of arginine in liver injury is that it acts as a precursor for nitric oxide (Nanji et al., 1995a; Cottart, 1999; Vallance and Chan, 2001). However, this hypothesis is controversial since the enzyme nitric oxide synthase is saturated with substrate at physiological levels (Boger and Bode-Boger, 2001). Several explanations have been provided in an attempt to resolve this "arginine paradox" (Boger and Bode-Boger, 2001). Firstly, because the endothelial isoform of nitric oxide is located in the caveolae, additionally arginine may be necessary to be fully utilized in this microenvironment (Feron and Kelly, 2001). Secondly, increased arginine may be required to overcome the inhibitory effects of endogenous inhibitors of nitric oxide synthase. Finally, the effect of arginine could be independently mediated through its vasodilatory and antioxidant effects (Cottart et al., 1999). Additionally, arginine inhibits inflammation and fibrogenesis (Boger and Bode-Boger, 2001).

The exact isoform of nitric oxide synthase responsible for protection against liver toxicity is not known. Studies using specific nitric oxide synthase inhibitors and knockout models have yielded divergent results on the role of the individual isoforms in liver toxicity (Clemens, 1999; Mashimo and Goyal, 1999). Despite these shortcomings, many of our findings could be ascribed to a protective role for nitric oxide. Nitric oxide inhibits free radical productions and decreases inflammatory changes by inhibiting nuclear factor-κB activity (Grisham, 2000). Inhibition of hepatic stellate cell function and smooth muscle proliferation by nitric oxide may account for the reversal of fibrosis (Rockey and Chung, 1995; Failli et al., 2000). Additionally, nitric oxide inhibits cyclooxygenase-2 activity causing a reduction in formation of vasoactive prostanoids (Clancy et al., 2000). Although levels of nitric oxide in arginine-treated rats were lower than in rats administered than fish oil-ethanol but still higher than in control rats, the most likely reason for the potential protective effect of nitric oxide is related to the site of synthesis. Nitric oxide production in arginine-supplemented rats is through endothelial nitric oxide synthase (Cottart et al., 1999). However, other mechanisms, outlined previously, may account for improvement in pathologic changes in addition to the nitric oxide-mediated effects.

In conclusion, our results show that arginine administration, probably through the generation of nitric oxide, leads to improvement in pathological changes such as fatty liver, necrosis, inflammation, and fibrosis. These improvements were accompanied by down-regulation of nuclear factor-κB, pro-inflammatory cytokines cyclooxygenase-2, and inducible nitric oxide synthase. It will be important to further characterize the activity of the various nitric oxide synthase isoforms in the different cell types in liver in this model to further clarify the therapeutic role of arginine and other nitric oxide-donors in alcoholic liver disease. Since nitric oxide, in addition to having antioxidant effects, can also

behave as a pro-oxidant (Joshi et al., 1999), the amount of nitric oxide-donor used in the treatment of inflammatory liver diseases needs to be carefully titrated.

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