Cholesterol Supplementation Prevents Necrosis and Inflammation But Enhances Fibrosis in Alcoholic Liver Disease in the Rat

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Based on studies that show a role for the low-density lipoprotein (LDL)-receptor in arachidonic acid delivery and eicosanoid synthesis in macrophages, the present study investigated the effect of cholesterol supplementation on pathological changes and thromboxane (TX) synthesis in alcoholic liver injury. Male Wistar rats were intragastrically fed ethanol with either corn oil or fish oil for 1 month. Control rats received isocaloric amounts of dextrose instead of ethanol. An additional group of rats fed either ethanol or dextrose with fish oil or corn oil were supplemented with 1% cholesterol. At the time of killing, all rats had the following evaluated: liver histopathology, lipid peroxidation, liver and plasma thromboxane levels, plasma endotoxin and messenger RNA (mRNA) levels of LDL-receptor, tumor necrosis factor α (TNF-α), cyclooxygenase (Cox)-1 and -2, and transforming growth factor β (TGF-β). Rats fed ethanol with either fish oil or corn oil developed fatty liver, necrosis, inflammation, and central vein collagen deposition. Cholesterol supplementation enhanced the degree of fibrosis but prevented necrosis and inflammation. These alterations in pathological changes by cholesterol were accompanied by absent TNF-α and Cox-2 mRNAs, decreased thromboxane levels, decreased lipid peroxidation, and increased TGF-β mRNA. Cholesterol enrichment of the diet thus decreases proinflammatory components, but enhances fibrosis in ethanol-fed rats. (HEPATOLOGY 1997;26:90-97.)

Our studies that showed a role for different types of dietary fat and the requirement for linoleic acid in the pathogenesis of experimental alcoholic liver disease (ALD) led us to investigate the mechanism(s) by which linoleic acid promoted liver injury. We hypothesized that the metabolism of linoleic acid to arachidonic acid and its subsequent conversion to arachidonic acid metabolites led to the observed ethanol-induced decrease of arachidonic acid in the livers of ethanol-fed rats. Our studies showed that increased production of thromboxane B2 (TXB2) by nonparenchymal liver cells and increased levels of thromboxane in plasma correlated with the presence of liver injury. The stimulus for enhanced thromboxane production by the nonparenchymal liver cells was most likely endotoxin.

It has recently been shown that monocytes and other cells use the classical low-density lipoprotein (LDL)-receptor pathway to deliver arachidonic acid for the production of eicosanoids through the cyclooxygenase (Cox) pathway. Thus dietary cholesterol, by modulating the LDL-receptor concentrations and possibly eicosanoid production, could influence the severity of alcohol-induced liver injury. The LDL-receptor has been shown to be up-regulated in the livers of ethanol-fed rats and could, therefore, contribute to the increased eicosanoid production in these rats.

Prostaglandin and thromboxane synthesis is dependent on Cox, the central enzyme in the prostaglandin synthetic pathway. It is now evident that, in many cell lines, tissues, and organs, there exists two forms of Cox: a constitutive enzyme, designated Cox-1, and an inducible enzyme, designated Cox-2. Under normal physiological conditions, tissue prostanoid synthesis depends on the availability of arachidonic acid and enzymatic activity of Cox-1. Cox-2 is inducible by proinflammatory stimuli such as cytokines, endotoxin, and lipid peroxidation. We have recently shown that up-regulation of Cox-2 in liver is associated with pathological changes in the intragastric feeding rat model for ALD, and that the up-regulation Cox-2 messenger RNA (mRNA) was accompanied by increased thromboxane A2 production in liver. Furthermore, the Kupffer cells were identified as the major cell type in which Cox-2 was up-regulated. We therefore hypothesized that provision of dietary cholesterol, can, by regulating LDL-receptor concentrations and delivery of arachidonic acid to cells, modulate the production of vasoactive eicosanoids, and, therefore, the severity of liver injury. In experimental animals such as the hamster and the rabbit, dietary cholesterol increases liver cellular cholesterol and suppresses both hepatic cholesterol synthesis and hepatic LDL receptor expression. In the rat, dietary cholesterol also increases liver cellular cholesterol, but its effect on the LDL-receptor is variable- and species-dependent. The present study evaluated the effect of cholesterol administration on pathological changes in two dietary models of alcoholic liver injury (corn oil-ethanol and fish oil-ethanol) that produce different severities of liver damage. Rats fed ethanol with corn oil produce moderate liver injury; those fed fish oil and ethanol show more severe liver injury. To evalu-

Abbreviations: ALD, alcoholic liver disease; TXB2, thromboxane B2; LDL, low-density lipoprotein; Cox, cyclooxygenase; mRNA, messenger RNA; TNF-α, tumor necrosis factor α; CD, corn oil plus dextrose; CE, corn oil plus ethanol; Chol, cholesterol; FD, fish oil plus dextrose; FE, fish oil plus ethanol; HDL, high-density lipoprotein; TGF-β, transforming growth factor β; PCR, polymerase chain reaction.

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Received October 22, 1996; accepted January 6, 1997.

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ate the inter-relationship between dietary cholesterol supplementation, LDL-receptor mRNA, thromboxane production, and liver pathology, we evaluated the above parameters in ethanol-treated and control groups. We also evaluated mRNA levels for tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)), Cox-1 and Cox-2, lipid peroxidation, and plasma endotoxin, as well as related changes in these parameters to pathological changes.

**MATERIALS AND METHODS**

**Animal Model.** Male Wistar rats weighing between 225 and 250 g were fed a liquid diet via permanently implanted gastric canulæ as described previously.\(^{1,6}\) The rats were administered their total nutrient intake by intragastric infusion. Eight groups of rats (5 rats/group) were fed freshly prepared diets with either fish oil or corn oil as the source of fatty acids, which contributed 35% of calories. The fatty acid composition of the diets has been described previously.\(^{2,3}\) Vitamins and minerals were given as described previously.\(^{1,6,14}\) The experimental groups were: corn oil plus dextrose (CD), corn oil plus ethanol (CE), corn oil plus dextrose plus cholesterol (CD+Chol), fish oil plus dextrose (FD), fish oil plus ethanol (FE), fish oil plus dextrose plus cholesterol (FD+Chol), and fish oil plus ethanol plus cholesterol (FE+Chol).

The liquid diet was infused at the rate of 180 mL/kg body weight/d to achieve adequate weight gain (1 mL = 1 kcal). Ethanol was infused to maintain blood alcohol levels between 150 and 300 mg/dL. The amount was initially 10 g/kg/d, and was increased up to 16 g/kg/d as tolerance developed. The cholesterol-supplemented group received 1% cholesterol (wt/wt) and 0.5% cholic acid. The cholesterol-enriched diet was made by dissolving cholesterol into the diet. All diets were stored at 4°C under nitrogen to prevent autoxidation. Lipid peroxide measurements were performed to monitor in vitro peroxidation. All animals received humane treatment in compliance with the National Institutes of Health criteria for care of laboratory animals. All rats in the different groups were killed at 1 month.

**Histopathological Analysis Including Sirius Red Staining for Collagen.** A small sample of liver was obtained by biopsy or at killing, and then formalin-fixed. Hematoxylin-eosin staining was used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat), 1 = <25% of cells containing fat, 2 = 25-50%, 3 = 51-75%, 4 = >75%. Necrosis was evaluated as the number of necrotic foci per square millimeter; 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 groups when assessing the histology.

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) employing NIH Image version 1.22 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 pericentral vein collagen deposition. The coefficient of variation of parameters measured was determined by assessing a single central vein on six occasions (<5%). At least five central veins were evaluated for each animal.

**Plasma Lipid Measurements.** Plasma lipids were measured using an automated analyzer (Hitachi 747, Boehringer Mannheim Corp., Indianapolis, IN). Total cholesterol and triglycerides were measured using enzymatic analysis.\(^{31,22}\) High-density lipoprotein (HDL)-cholesterol was measured after precipitation of LDLs and very-low-density lipoproteins with manganese ions.\(^{25}\) LDL-cholesterol was calculated according to the following formula: LDL-cholesterol = total cholesterol – triglycerides/5.\(^{24}\)

**Blood Alcohol Measurements.** Blood was collected from the tail vein, and ethanol concentration was measured using an alcohol dehydrogenase kit from Sigma Chemical Co. (St. Louis, MO).

**Liver-Conjugated Dienes and Plasma 8-Isoprostane Measurements as Indicators of Lipid Peroxidation.** Lipid was extracted according to the method of Bligh and Dyer,\(^{25}\) and conjugated dienes were measured by the method of Recknagel and Glende.\(^{26}\) 8-Isoprostane in plasma was measured using an immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). The blood sample was obtained from the aorta, immediately centrifuged, and the plasma stored at −70°C until analysis. We have previously shown that 8-isoprostane levels in plasma correlate extremely well with liver-conjugated diene levels in controls and ethanol-fed rats.\(^{14}\)

**Plasma Endotoxin.** Blood samples were collected in endotoxin-free vials (Sigma Chemicals) and centrifuged at 400g for 15 minutes at 4°C. Samples were then diluted 1:10 in pyrogen-free water and heated to 75°C for 30 minutes to remove endotoxin-inhibitors from plasma. The Limulus Amoebocyte Lysate test (Kinetic-QLC, Whittaker Bioproducts, Walkersville, MD) was used for endotoxin measurements. Samples were incubated at 37°C for 10 minutes with limulus amoebocyte lysate. The substrate solution was subsequently added and the incubation continued for 20 minutes. The reaction was stopped with 23% acetic acid, and the samples were read spectrophotometrically at 410 nm.

**Liver and Plasma Thromboxane Metabolites.** TXB2, the chemically stable hydrolysis product of TXA2, undergoes two major pathways of metabolism.\(^{27,28}\) 2,3-dinor-TXB2 and 11-dehydro-TXB2 are the major metabolites originating via \(\beta\)-oxidation and dehydrogenation reactions. Because the measurement of TXB2 in plasma and tissues is subject to artefactual increases, it has been suggested that measurements of the longer-lived circulating metabolites, 2,3-dinor-TXB2 and 11-dehydro-TXB2 represent better markers of TXB2 production.\(^{14}\) Blood for thromboxane analysis was collected in heparinized syringes, separated immediately, and frozen until measurements were performed. TXB2, 2,3-dinor-TXB2, and 11-dehydro-TXB2 were extracted from plasma on SEP-PAK C18 cartridges (Waters Associates, Milford, MA) and eluted with ethyl acetate. Eluates were assayed for the thromboxane metabolites using enzyme-immunoassay kits (Cayman Chemical). For measurement of the liver eicosanoids, a section of liver (approximately 1 g) was rapidly homogenized in 10 mL of ice-cold methanol for 30 seconds. After centrifugation, the supernatant was dried and resuspended in 0.1 mol/L of potassium phosphate buffer and purified by elutriation through an octadecyl silyl SEP-PAK C18 cartridge. The 80% methanol eluent was assayed for TXB2, 2,3-dinor-TXB2, and 11-dehydro-TXB2.

**RNA Extraction From Liver Tissue and Analysis of LDL-Receptor mRNA by Northern Blot Analysis and TNF-\(\alpha\), Cox-1, Cox-2, and TGF-\(\beta\) mRNA by Reverse-Transcription PCR.** To examine the expression of the different mRNAs, total RNA was extracted according to the guanidinium isothiocyanate method.\(^{29}\) The total RNA concentration of each sample was determined from absorbance at 260 nm, and the quality of each RNA preparation was determined by agarose-formaldehyde gel electrophoresis and ethidium bromide staining. LDL-receptor mRNA was evaluated by Northern blot analysis, and the other mRNAs were evaluated by reverse-transcription polymerase chain reaction (PCR).

We reverse-transcribed 0.5-1 \(\mu\)g of total RNA by adding 30 \(\mu\)L of a master mix with reverse-transcriptase buffer (0.6 mmol/L MgCl2, 15 mmol/L KCl, 10 mmol/L Tris HCl [pH 8.3]), 40 pmol of downstream primer, 0.5 mmol/L dNTP mixture, 1 \(\mu\)U/mL RNase inhibitor, and 13.3 U/mL Moloney murine leukemia virus reverse transcriptase (BIBCO-BRL, Grand Island, NY) (final concentrations indicated). Samples were incubated, first for 60 minutes at 42°C and then at 75°C for 10 minutes, and then chilled on ice. Then, 2 \(\mu\)L of each sample was added to 20 \(\mu\)L of 1.5 mmol/L MgCl2, 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.3), 0.2 mmol/L of each primer, 5 \(\mu\)mol/L of each primer, and 5 \(\mu\)mol/L of each.
The blood alcohol levels were (mean ± SD unless otherwise stated. Differences between groups were evaluated using ANOVA and multiple comparisons with the Student-Newman-Keuls method.

**RESULTS**

The average weight gain over the 1-month period was not different in the various experimental groups. There was also no significant difference in blood alcohol levels among the ethanol-fed groups. The blood alcohol levels were (mean ± SE): CE, 229 ± 46 mg/dL; CE + Chol, 241 ± 38 mg/dL; FE, 253 ± 49 mg/dL; and FE + Chol, 218 ± 59 mg/dL.

**Histopathology.** As observed previously, both CE and FE groups developed fatty liver, necrosis, and inflammation (Table 1). The severity of liver injury, particularly necrosis and inflammation, was greater in FE rats than in CE rats (Fig. 1). The degree of centrilobular collagen deposition was also significantly higher in the CE and FE groups compared with their respective controls (Table 2, Fig. 2). Cholesterol feeding also increased collagen in FD rats, and a similar tendency was noted in CD rats. Of note is that the amount of collagen in the FD rats was greater than in CD rats (P < .01). Cholesterol supplementation had no effect on the degree of fatty liver, but completely prevented the development of necrosis and inflammation in both CE and FE rats (Fig. 3). In contrast to the effect of cholesterol on necroinflammatory changes in ethanol-fed rats, cholesterol increased the degree of fibrosis in all groups. The most dramatic increases were seen in the fish oil-ethanol-cholesterol-supplemented groups (Fig. 2C). Thus, cholesterol’s general profibrogenic effect in both ethanol-fed and control animals should be noted.

**Plasma Lipid Measurements.** Total serum cholesterol and the HDL cholesterol fraction were both significantly increased (P < .01) after ethanol feeding in corn oil and fish oil groups (Table 2). Cholesterol supplementation led to an approximately twofold increase in plasma cholesterol, and part of this increase was accounted for by an increase in HDL-cholesterol concentrations. Ethanol administration also led to an increase in levels of plasma triglycerides (P < .01), and cholesterol supplementation had no additional effect.

**Plasma Endotoxin.** Ethanol administration as previously described caused an increase in plasma endotoxin levels in both corn oil- and fish oil-fed groups (Table 3). Cholesterol supplementation did not significantly alter plasma endotoxin levels in these groups.

**Lipid Peroxidation.** Ethanol administration led to an increase in both liver-conjugated dienes and plasma isoprostane levels (P < .01) (Table 3). The highest levels were seen in the FE group, although the FD group also showed.

**FIG. 1.** A liver section from a rat fed fish oil and ethanol for 1 month. There is evidence of fatty infiltration, necrosis, and inflammation. Note the presence of two necroinflammatory foci. (Hematoxylin-eosin, original magnification ×400.)

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Fat (foci/mm²)</th>
<th>Inflammation (cells/mm²)</th>
<th>Collagen (% lumenal area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>0</td>
<td>0.2 ± 0.1</td>
<td>0.06 ± 0.03*</td>
</tr>
<tr>
<td>CE</td>
<td>3.7 ± 0.5*</td>
<td>4.9 ± 1.1*†</td>
<td>0.24 ± 0.04*</td>
</tr>
<tr>
<td>CD + Chol</td>
<td>0</td>
<td>0</td>
<td>0.17 ± 0.06*</td>
</tr>
<tr>
<td>CE + Chol</td>
<td>3.7 ± 0.5*</td>
<td>0</td>
<td>0.31 ± 0.03*</td>
</tr>
<tr>
<td>FD</td>
<td>0.0</td>
<td>0.3 ± 0.1</td>
<td>0.46 ± 0.12*†</td>
</tr>
<tr>
<td>FD + Chol</td>
<td>4.0 ± 0.0*‡</td>
<td>26.8 ± 10.2*†</td>
<td>0.78 ± 0.10*‡</td>
</tr>
<tr>
<td>FE + Chol</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>1.30 ± 0.27*</td>
</tr>
</tbody>
</table>

Abbreviations: CD, corn oil + dextrose; CE, corn oil + ethanol; FD, fish oil + dextrose; FE, fish oil + ethanol; Chol, cholesterol.

* P < .01 vs. appropriate dextrose-fed control (e.g., CE vs. CD).
† P < .01 vs. appropriate comparison cholesterol-supplemented group (e.g., CE vs. CE + Chol).

| Location of the predicted PCR products was confirmed by using a 100–base pair ladder (GIBCO-BRL) as a standard size marker. For quantitation, the expression of the products was quantitated using densitometric scan analysis. The index of the various mRNA signals was standardized against that of the β-actin signal from the same RNA.

Varying the number of PCR cycles demonstrated linearity between the PCR product assessed densitometrically and the number of PCR cycles. Each experiment included a negative control (sample RNA that had not been subjected to reverse transcription). This sample did not yield a PCR product, confirming the absence of extraneous genomic DNA or PCR product contaminating the samples.

Statistical Analysis. All data are expressed as means ± SD unless otherwise stated. Differences between groups were evaluated using ANOVA and multiple comparisons with the Student-Newman-Keuls method.
<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Plasma Lipids (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CD</td>
<td>76 ± 16</td>
</tr>
<tr>
<td>CE</td>
<td>102 ± 11*</td>
</tr>
<tr>
<td>CD + Chol</td>
<td>151 ± 29†</td>
</tr>
<tr>
<td>CE + Chol</td>
<td>197 ± 17‡</td>
</tr>
<tr>
<td>FD</td>
<td>69 ± 10</td>
</tr>
<tr>
<td>FE</td>
<td>87 ± 12</td>
</tr>
<tr>
<td>FD + Chol</td>
<td>189 ± 18‡</td>
</tr>
<tr>
<td>FE + Chol</td>
<td>192 ± 16‡</td>
</tr>
</tbody>
</table>

NOTE. Five rats per group.

Abbreviations: C, corn oil; D, dextrose; E, ethanol; F, fish oil; Chol, cholesterol.

* P < .01 vs. dextrose-fed control (CD, FD).
† P < .01 vs. dextrose-fed controls (CD, FD).
‡ P < .01 vs. ethanol-fed rats (CE, FE).

increased levels of lipid peroxidation compared with the CD group. Cholesterol supplementation led to a decrease in lipid peroxide levels in both ethanol-fed groups (CE and FE) (P < .01). Additionally, cholesterol supplementation decreased lipid peroxidation in fish oil-dextrose-fed rats (P < .01).

**Plasma and Liver TXB₂ and Metabolites.** TXB₂ and thromboxane metabolites were measured only in the corn oil-fed groups and not in the fish oil groups, because these metabolites in the latter group are n-3 fatty acid-derived and not as easily measured as the arachidonic acid metabolites. Furthermore, the physiological actions of the n-3 fatty acid-derived eicosanoids are not well characterized. Ethanol administration led to a significant increase in TXB₂ and its metabolites in plasma and liver (Table 4). Cholesterol supplementation significantly decreased levels of TXB₂ and thromboxane metabolites in both liver and plasma.

![Liver section images (A, B, C)](Fig. 2)
mRNA Levels of LDL-Receptor, TNF-α, Cox-1, Cox-2, and TGF-β1 and β-Actin. A faint band for LDL-receptor mRNA was detected in CE and FE groups and was not detected in either the control dextrose-fed groups or cholesterol-supplemented groups (Fig. 4). TNF-α mRNA has previously been shown to be present in CE and FE rats, a finding that was confirmed in the present study. TNF-α mRNA was absent in the cholesterol-supplemented ethanol-fed groups (Fig. 4). Cox-2 has also previously been shown to be up-regulated in the rats that exhibit necroinflammatory changes, i.e., CE and FE rats. The presence of Cox-2 in these groups was confirmed in the present study (Fig. 4). Cholesterol supplementation was associated with the absence of Cox-2 mRNA in both the CE and FE groups. Cox-1 and β-actin mRNA levels were not altered by either ethanol or cholesterol administration. The present study showed that the degree of fibrosis was enhanced by dietary cholesterol supplementation. Because of the observed relationship between TGF-β1 and fibrosis in ethanol-fed rats, we evaluated TGF-β1 mRNA in all groups. As shown in Fig. 5, the highest level of TGF-β1 expression is seen in the FE-Chol group. Cholesterol supplementation also increased TGF-β1 mRNA in the CE group, although the increase was not significant.

DISCUSSION

We have previously shown that arachidonic acid metabolites, especially TXA2 and its metabolites, are probably important in the pathogenesis of experimental ALD. Various investigators have shown that arachidonic acid, which is metabolized by the Cox pathway in cells such as macrophages, gains access to cells via the LDL receptor. We therefore hypothesized that the increase in Cox products, such as TXB2, seen in the intragastric-feeding rat model, could be modulated by altering hepatic LDL-receptor concentrations by dietary cholesterol supplementation.

As shown by others, ethanol administration in the present study resulted in an increase in plasma total cholesterol and HDL cholesterol. The increase in plasma cholesterol was probably secondary to increased hepatic synthesis of cholesterol. Seitz et al. showed that the increase in cholesterol synthesis in the livers of ethanol-fed rats was accompanied by an increase in mRNA levels for hydroxy-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis. Other investigators have also confirmed that ethanol increases the concentrations of unesterified cholesterol in rat liver.

Supplementation of both corn oil and fish oil diets with cholesterol increased plasma cholesterol, and part of this increase was caused by an increase in HDL cholesterol. In experimental animals such as the hamster and rabbit, dietary cholesterol increases plasma and liver cellular cholesterol, and suppresses hepatic cholesterol synthesis and hepatic LDL-receptor expression. Our findings in the rat, similar to those seen by other investigators, also showed increased levels of cholesterol in plasma, but not to the same extent as seen in rabbits or hamsters. The observation that LDL-receptor was detectable, albeit as a faint band, only in ethanol-fed rats and not in cholesterol-fed rats or in controls, is consistent with the observation of an ethanol-induced increase in LDL-receptor mRNA in rats. Up-regulation of the hepatic LDL-receptors in ethanol-fed rats and the increased delivery of arachidonic acid to cells may, in part, explain the increase in arachidonic acid metabolite concentrations in these animals.

The prevention of necrosis and inflammation in cholesterol-fed rats was accompanied by the absence of TNF-α mRNA and Cox-2 mRNA, and a significant decrease in lipid peroxidation. We have previously shown that TNF-α mRNA expression correlates with the presence of necrosis and inflammation in ethanol-fed rats and with increased levels of plasma endotoxin. In cholesterol-supplemented rats, TNF-α mRNA was down-regulated even though plasma endotoxin levels were increased. The critical role played by lipoproteins in the binding of endotoxin to lipopoly saccharide-binding protein and its receptor may, in part, explain the protective effect of hypercholesterolemia. Furthermore, down-regulation of the LDL-receptor may also be relevant because mice that are LDL-receptor−deficient show decreased in vivo production of cytokines in response to endotoxin. Minimally oxidized LDLs are also known to decrease lipopoly saccharide-induced TNF-α expression in macrophages. Ethanol administration is known to induce lipid oxidation in lipoproteins. Another possible explanation for the reduction in TNF-α mRNA could be the decrease in lipid peroxidation in cholesterol-fed rats. Oxidant stress enhances

**Table 3. Measurements of Plasma Endotoxin and 8-Isoprostane and Liver Lipid Peroxidation (A232) in the Different Experimental Groups**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Plasma Endotoxin (pg/mL)</th>
<th>Plasma 8-Isoprostane (pg/mL)</th>
<th>A232</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>6.0 ± 2.2</td>
<td>19 ± 9</td>
<td>0.11 ± 0.08</td>
</tr>
<tr>
<td>CE</td>
<td>52.8 ± 10.7*</td>
<td>269 ± 34*</td>
<td>0.54 ± 0.12*</td>
</tr>
<tr>
<td>CD + Chol</td>
<td>5.4 ± 3.0</td>
<td>14 ± 10</td>
<td>0.10 ± 0.07</td>
</tr>
<tr>
<td>CE + Chol</td>
<td>59.3 ± 8.4*</td>
<td>117 ± 29*†</td>
<td>0.34 ± 0.09†</td>
</tr>
<tr>
<td>FE</td>
<td>4.2 ± 1.6</td>
<td>172 ± 39</td>
<td>0.46 ± 0.09</td>
</tr>
<tr>
<td>FE + Chol</td>
<td>69.0 ± 21.5*</td>
<td>414 ± 93*</td>
<td>0.70 ± 0.17*</td>
</tr>
<tr>
<td>FE + Chol</td>
<td>4.0 ± 1.7</td>
<td>89 ± 18*</td>
<td>0.26 ± 0.10*</td>
</tr>
<tr>
<td>FE + Chol</td>
<td>72.5 ± 24.7*</td>
<td>107 ± 21†</td>
<td>0.32 ± 0.08†</td>
</tr>
</tbody>
</table>

NOTE: Five rats per group.  
* P < .01 vs. appropriate comparison dextrose-fed control (e.g., CE vs. CD).  
† P < .01 vs. appropriate comparison ethanol-fed group (e.g., CE-Chol vs. CE).  
‡ P < .01 vs. FD.

Fig. 3. Liver section from a rat fed fish oil, ethanol, and cholesterol, showing the presence of fatty liver only. Note the absence of necroinflammatory changes. (Hematoxylin-eosin, original magnification ×155.)
Table 4. Measurement of Plasma and Liver Concentrations of Thromboxane Metabolites in Plasma and Livers of Rats Fed Corn Oil Diets

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>TXB₂ (pg/mL)</th>
<th>2,3 dimor TXB₂</th>
<th>11-dehydro-TXB₂</th>
<th>TXB₂ (pg/mg protein)</th>
<th>2,3 dimor TXB₂</th>
<th>11-dehydro-TXB₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>36 ± 8</td>
<td>66 ± 9</td>
<td>64 ± 9</td>
<td>57 ± 11</td>
<td>52 ± 8</td>
<td>61 ± 7</td>
</tr>
<tr>
<td>CE</td>
<td>686 ± 103*</td>
<td>678 ± 124*</td>
<td>611 ± 98*</td>
<td>160 ± 31*</td>
<td>169 ± 33*</td>
<td>161 ± 21*</td>
</tr>
<tr>
<td>CD + Chol</td>
<td>39 ± 10</td>
<td>62 ± 11</td>
<td>74 ± 8</td>
<td>63 ± 9</td>
<td>50 ± 12</td>
<td>53 ± 9</td>
</tr>
<tr>
<td>CE + Chol</td>
<td>239 ± 43†</td>
<td>197 ± 69†</td>
<td>198 ± 59†</td>
<td>89 ± 17</td>
<td>73 ± 12</td>
<td>87 ± 10</td>
</tr>
</tbody>
</table>

Abbreviations: C, corn oil; D, dextrose; E, ethanol; Chol, cholesterol.

* P < .01 vs. control (CD) group.
† P < .01 vs. CE group.

release of TNF-α by macrophages, and, furthermore, treatment with free-radical scavengers contributes to down-regulation of TNF-α secretion during endotoxemia. Our observations that down-regulation of TNF-α mRNA was associated with decreased lipid peroxidation is consistent with the effects of inhibition of lipid peroxidation on TNF-α release and cytotoxicity. The mechanism(s) by which cholesterol inhibits lipid peroxidation is unknown, but it has been suggested that the presence of increased amounts of cholesterol in cell membranes reduces oxidative damage by inhibiting the free-radical propagation reaction.

Another enzyme whose expression is also regulated by lipid peroxidation is Cox-2. Cox-2 induction by lipopolysaccharide, TNF, and lipid peroxidation leads to enhanced formation of proinflammatory eicosanoids such as TXA₂. The down-regulation of Cox-2 in cholesterol-fed rats could be a result of both decreased lipid peroxidation and decreased TNF-α expression. The decrease in liver and plasma TXB₂ in the cholesterol-supplemented group occurred in association with down-regulation of Cox-2 expression. Consistent with our findings that cholesterol-fed rats had lower expression of Cox-2 is the observation that enrichment of cells with cholesterol makes them less responsive to the inductive effects of serum on Cox-2 synthesis and activity. Other reasons for the decrease in thromboxane metabolites in liver and plasma in cholesterol-supplemented rats include the fact that enrichment of cells with cholesterol decreases phospholipase A₂ activity, thereby reducing arachidonic acid availability. Cholesterol enrichment also inhibits Cox, the enzyme responsible for the conversion of arachidonic acid to the prostanoids. Although the measurements of thromboxane in liver reflects production of this eicosanoid by all hepatic cell types, the major site of Cox-2 up-regulation in alcoholic liver injury is the Kupffer cell. Thus, the Kupffer cell is probably the major contributor to the increased production of thromboxanes. Furthermore, we have also previously described increased production of TXB₂ in Kupffer cell–enriched supernatant from rats fed corn oil and ethanol. Additional studies in Kupffer cells of ethanol-fed rats, evaluating arachidonic acid delivery by the LDL-receptor pathway, will yield

Fig. 4. Reverse-transcription PCR analysis of Cox-2, TNF-α, Cox-1, and β-actin mRNAs in liver samples from the various experimental groups. One microgram of RNA was subjected to reverse transcription as detailed in Materials and Methods. Cox-1 and β-actin are detected in all groups; TNF-α and Cox-2 were detected only in CE and FE groups. LDL-receptor mRNA (LDL-R) by Northern blot analysis was detected in CE and FE rats; LDL-R mRNA was down-regulated by cholesterol treatment (CI-chol, FE-chol).

Fig. 5. TGF-β₁ mRNA levels in the different treatment groups. TGF-β₁ mRNA (normalized for β-actin) were increased (P < .01) in ethanol-fed rats (CE, 2.6 ± 0.6 vs. CD 1.0; FE, 3.9 ± 0.9 vs. FD 1.8 ± 0.4). Cholesterol supplementation led to increased TGF-β₁ mRNA in both ethanol-fed groups, but the increase was significant only in the CE-cholesterol group (P < .05). C, corn oil; D, dextrose; E, ethanol; and Chol, cholesterol.
critical information relating this pathway to alcoholic liver injury.

The enhanced deposition of central vein collagen in cholesterol-fed rats is surprising in view of the absence of necrosis and inflammation. It is widely believed that necroinflammatory changes are necessary precursors of alcohol-induced liver fibrosis.\textsuperscript{50-52} Cholesterol feeding has been shown to enhance liver fibrosis in some species. Rabbis fed cholesterol together with fish oil for 14 weeks develop perivenulare fibrosis.\textsuperscript{53} The mechanisms involved in the pathogenesis of fibrosis in that model were not evaluated. Buyens et al. also showed that cholesterol feeding to rabbits led to the development of centrilobular liver fibrosis, which was accompanied by activation of hepatic stellate cells.\textsuperscript{54} Wanless et al., who demonstrated hepatic sinusoidal fibrosis in cholesterol-fed rabbits, hypothesized that the mechanism of injury could involve either accumulation of bile salts or generation of cholesterol oxidation products.\textsuperscript{55} Hypercholesterolemia has also been shown to accelerate the progression of fibrosis in other organs such as the kidney.\textsuperscript{56-57} One hypothesis for the acceleration of fibrosis by cholesterol is the up-regulation of TGF-\(\beta_1\) gene expression.\textsuperscript{58} The results of the present study, which show up-regulation of TGF-\(\beta_1\) in livers of cholesterol-fed rats, are consistent with this hypothesis. We and others have previously shown that macrophages are the major sources of TGF-\(\beta_1\) in the intragraftic-feeding rat model.\textsuperscript{59,60} The stimulus for up-regulation of TGF-\(\beta_1\) in cholesterol-fed rats is unknown.

In summary, dietary cholesterol supplementation led to the prevention of necrosis and inflammation in the intragraftic-feeding rat model for ALD, but increased the deposition of collagen around the central vein in liver. The absence of necrosis and inflammation was accompanied by down-regulation of TNF-\(\alpha\) mRNA, and decreased levels of lipid peroxidation. The decrease in plasma and liver levels of the proinflammatory eicosanoids, TXA\(_2\) and its metabolites, in cholesterol-fed animals was accompanied by down-regulation of Cox-2. Cholesterol-enrichment of the diet also led to enhanced fibrosis, which was probably related to increased TGF-\(\beta_1\) expression. The modulation of different pathological processes by cholesterol in experimental ALD suggests that the processes of inflammation and fibrosis may be regulated independently. Furthermore, hypercholesterolemia may influence the progression of clinical ALD in humans.

REFERENCES

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