Curcumin prevents alcohol-induced liver disease in rats by inhibiting the expression of NF-κB-dependent genes

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Nanji, Amin A., Kalle Jokelainen, George L. Tipoe, Amir Rahemtulla, Peter Thomas, and Andrew J. Dannenberg. Curcumin prevents alcohol-induced liver disease in rats by inhibiting the expression of NF-κB-dependent genes. Am. J. Physiol. Gastrointest. Liver Physiol. 284: G321–G327, 2003.-Induction of NF-κB-mediated gene expression has been implicated in the pathogenesis of alcoholic liver disease (ALD). Curcumin, a phenolic antioxidant, inhibits the activation of NF-κB. We determined whether treatment with curcumin would prevent experimental ALD and elucidated the underlying mechanism. Four groups of rats (6 rats/group) were treated by intragastric infusion for 4 wk. One group received fish oil plus ethanol (FE); a second group received fish oil plus dextrose (FD). The third and fourth groups received FE or FD supplemented with 75 mg·kg⁻¹·day⁻¹ of curcumin. Liver samples were analyzed for histopathology, lipid peroxidation, NF-κB binding, TNF-α, IL-12, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-2, cyclooxygenase-2, inducible nitric oxide synthase (iNOS), and nitrotyrosine. Rats fed FE developed fatty liver, necrosis, and inflammation, which was accompanied by activation of NF-κB and the induction of cytokines, chemokines, COX-2, iNOS, and nitrotyrosine formation. Treatment with curcumin prevented both the pathological and biochemical changes induced by alcohol. Because endotoxin and the Kupffer cell are implicated in the pathogenesis of ALD, we investigated whether curcumin suppressed the stimulatory effects of endotoxin in isolated Kupffer cells. Curcumin blocked endotoxin-mediated activation of NF-κB and suppressed the expression of cytokines, chemokines, COX-2, and iNOS in Kupffer cells. Thus curcumin prevents experimental ALD, in part by suppressing induction of NF-κB-dependent genes.

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36), raising the possibility that it might be useful in preventing ALD.

The principal goal of the present study was to determine whether treatment with curcumin could suppress the expression of NF-κB-dependent genes and prevent ALD. The intragastric feeding rat model for ALD was used, because it is well established and permits the correlation of histological and biochemical parameters (8, 9, 38). Evidence is presented that curcumin prevented ALD, at least in part, by inhibiting lipid peroxidation, activation of NF-κB, and expression of pro-inflammatory mediators. Consistent with previous findings (37, 39), the Kupffer cell appears to be centrally involved in this process.

MATERIALS AND METHODS

Animal model and isolation of Kupffer cells. Male Wistar rats (Harlan Sprague Dawley, Indianapolis, IN) weighing between 225 and 250 g were fed a liquid diet by continuous infusion through permanently implanted gastric tubes as previously described (9, 38). Ethanol and diet were administered continuously by a single gastric cannula. This was achieved by joining two tubes, one carrying ethanol from one syringe pump and the other carrying diet from a second pump, so that ethanol and diet could be varied at will. The infusion through permanently implanted gastric tubes as between 225 and 250 g were fed a liquid diet by continuous infusion through permanently implanted gastric tubes as previously described (9, 38). Ethanol and diet were administered continuously by a single gastric cannula. This was achieved by joining two tubes, one carrying ethanol from one syringe pump and the other carrying diet from a second pump, so that ethanol and diet could be varied at will. The dose of ethanol was increased slowly, as tolerated development, to maintain blood alcohol levels in the range of 150–300 mg/dl. The starting dose was 8 g·kg⁻¹·d⁻¹; the final dose was 16 g·kg⁻¹·d⁻¹. Each ethanol-fed rat had 4–6 measurements of blood alcohol; the blood sample was obtained at 10 AM.

Four groups (6 rats/group) of rats were treated for 4 wk before being killed. Rats in the first group were administered fish oil plus ethanol (FE). This diet is known to cause ALD in

Determination of NF-κB binding activity and levels of IkBα in liver tissue and Kupffer cells. The livers were Fractionated, and nuclear extracts were used to determine NF-κB binding activity as described previously (16, 17, 23). Liver cytosols were used to determine amounts of IkBα. Nuclear fractions were obtained from Kupffer cells as described previously (12) by using an adaptation of the method of Schriever et al. (35). As in previous studies (23), specificity of NF-κB binding was confirmed by competition assays and the ability of a specific antibody to supershift protein-DNA complexes. In competition assays, the addition of a 100-fold excess of unlabeled competitor consensus oligonucleotide prevented binding. Supershift experiments confirmed the presence of the p50 subunit in the binding complex. Western blot analysis for IkBα was conducted using 50 μg of cytosolic protein. Samples were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel. After transfer, the nitrocellulose membrane was incubated with an anti-IkBα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500. Membranes were incubated with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG). Antibody-reactive bands were visualized by the use of an enhanced chemiluminescence assay using reagents from New England Nuclear Life Science Products (Wilmington, DE).

Analysis of mRNAs for COX-1, COX-2, iNos, TNF-α, IL-12, MCP-1, MIP-2, and β-actin by RT-PCR. To examine the expression of COX-1, COX-2, iNos, TNF-α, IL-12, MCP-1, MIP-2, and β-actin in liver tissue and Kupffer cells, total RNA was isolated according to the guanidium isothiocyanate method (6). Integrity of RNA was assessed by agarose gel electrophoresis and ethidium bromide staining. RT-PCR was performed essentially as described previously (26). The sequences of primer pairs (5’ and 3’) and PCR conditions have been reported previously (21, 22, 28, 29). To normalize signals from the different RNA samples, we amplified 2 μl of the same reverse-transcriptase reaction with β-actin-specific primers. PCR products and molecular weight markers were subjected to electrophoresis on 1% agarose gels and visualized by means of ethidium bromide staining.

Immunohistochemical analysis. Sections were immunostained with antiserum to iNos and nitrotyrosine using a DAKO Envision kit. 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. Sections were preincubated with 10% normal horse serum for 1 h to reduce nonspecific binding of the antiserum. The sections were then incubated overnight at 4°C with antibodies to iNos (Transduction Laboratories, San Diego, CA) and nitrotyrosine (Upstate Biotechnology, New York, NY). Antibodies were diluted at 1:100 in 0.05 M Tris-HCl buffer containing 2% bovine serum albumin. Sections were washed three times in PBS, then incubated with peroxidase-labeled polymer conjugated to goat anti-rabbit IgG in Tris-HCl buffer for 30 min at 37°C. Finally, sections were washed and the peroxidase was visualized by immersion in 0.05% dianisobenzidine containing 0.03% hydrogen peroxide in Tris-HCl buffer (pH 7.5) for 3 min. Sections were rinsed in distilled water and counterstained with hematoxy-
Curcumin inhibits alcohol-induced liver injury and inflammation. The degree of fatty liver also decreased in curcumin-treated rats. Consistent with the improved histology, treatment with curcumin was associated with a corresponding reduction in levels of ALT and lipid peroxidation. Neither histological nor biochemical evidence of liver injury was detected in rats that received FD or FD-curcumin diets.

**Effect of curcumin on activation of NF-κB in liver.** Electromobility shift assays were carried out to determine NF-κB binding activity in the livers of rats in the different groups. As shown in Fig. 3, the FE diet led to increased NF-κB binding activity compared with the FD diet. Interestingly, curcumin prevented the activation of NF-κB in rats fed the FE diet consistent with its ability to inhibit lipid peroxidation and liver injury. To determine whether activation of NF-κB was a consequence of degradation of IκBα, we determined levels of IκBα in the cytosolic fraction of the liver. Very low levels of IκBα were detected in the FE group (Fig. 3). By contrast, higher levels of IκBα were detected in the FD and FD-curcumin groups, indicating that curcumin was able to prevent the degradation of IκBα.

Table 1. Pathological changes, ALT activity, and lipid peroxidation in the experimental groups

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Fatty Liver, 0–4</th>
<th>Necrosis, foci/mm²</th>
<th>Inflammation, cells/mm²</th>
<th>ALT activity, 5–35 U/l</th>
<th>A232, conjugated dienes</th>
<th>TBARS, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish oil-dextrose</td>
<td>0</td>
<td>0</td>
<td>0.04 ± 0.02</td>
<td>14 ± 6</td>
<td>0.34 ± 0.07</td>
<td>0.31 ± 0.09</td>
</tr>
<tr>
<td>Fish oil-dextrose + curcumin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15 ± 3</td>
<td>0.29 ± 0.08</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>Fish oil + ethanol</td>
<td>4.0 ± 0.0†</td>
<td>1.1 ± 0.5‡</td>
<td>21.7 ± 8.8‡</td>
<td>78 ± 13‡</td>
<td>1.4 ± 0.29‡</td>
<td>1.70 ± 0.22‡</td>
</tr>
<tr>
<td>Fish oil + ethanol + curcumin</td>
<td>2.0 ± 0.8*†</td>
<td>0</td>
<td>32 ± 6†</td>
<td>0.58 ± 0.09§</td>
<td>0.69 ± 0.04§</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 rats. ALT, alanine aminotransferase; A232, conjugated dienes; TBARS, thiobarbituric acid-reacting substances. †P < 0.01 vs. fish oil-ethanol; ‡P < 0.01 vs. dextrose-fed groups; §P < 0.01 vs. other groups; $P < 0.05 vs. dextrose-fed groups.
levels of IκBα were detected in the other groups including the FE-curcumin group.

Effect of curcumin on TNF-α, IL-12, MCP-1, MIP-2, COX-2, and iNOS mRNAs in liver. Various NF-κB-responsive genes including TNF-α, IL-12, MCP-1, MIP-2, COX-2 and iNOS are overexpressed in experimental ALD (21, 22, 23). Because the levels of these mRNAs are generally too low to detect by Northern blot or ribonuclease protection assays, RT-PCR was carried out. As predicted from previous studies (21, 22, 23), we detected markedly elevated levels of these pro-inflammatory mediators in the livers of rats fed FE vs. FD diet (Fig. 4). Remarkably, treatment with curcumin resulted in a normalization of levels of each of the above pro-inflammatory mediators. Nitric oxide, a free radical product of iNOS, can potentially damage the liver. It is noteworthy, therefore, that there was a marked reduction in nitrotyrosine immunoreactivity in the livers of rats treated with FE-curcumin vs. FE (Fig. 5). Levels of COX-1 mRNA, the constitutive isoform of COX, were similar in all groups (Fig. 4).

Curcumin inhibits endotoxin (LPS)-mediated activation of NF-κB-responsive genes in Kupffer cells. Endotoxin-mediated activation of gene expression in Kupffer cells has been linked to the pathogenesis of ALD (37, 39). Hence, it was of interest to determine whether the effects observed in whole liver could be reproduced in isolated Kupffer cells. Under basal conditions, we did not detect significant NF-κB binding activity or iNOS, COX-2, MIP-2, MCP-1, IL-12, and TNF-α message (data not shown). As shown in Fig. 6, treatment with endotoxin stimulated NF-κB binding and induced the expression of each of the above pro-inflammatory mediators. This inductive effect of endotoxin was blocked by curcumin, consistent with our findings in whole liver (Fig. 4).

Discussion

Treatment of alcohol-induced liver disease remains limited to supportive measures (10, 20). Undoubtedly, the development of effective therapy to prevent or treat ALD will depend on elucidating the underlying mechanisms that contribute to liver injury. Several lines of evidence suggest that the induction of NF-κB-dependent gene expression in Kupffer cells contributes to alcohol-induced liver injury (15, 16, 27, 39). In support of this hypothesis, gene therapy has been used to inhibit the activation of NF-κB and thereby prevent experimental ALD (39). Given the current limitations of this approach (42), it would be highly desirable if a pharmacological strategy could be developed to suppress the activation of NF-κB and prevent ALD.

In the present study, we found that alcohol-induced liver injury was associated with increased amounts of lipid peroxidation and the induction of multiple NF-κB-dependent genes including TNF-α, IL-12, MCP-1, MIP-2, COX-2, and iNOS. Each of these genes has been implicated in the pathogenesis of liver injury (7, 22, 23, 26, 27, 39). In addition to preventing alcohol-induced necroinflammatory changes, treatment with curcumin prevented lipid peroxidation and the expression of the above NF-κB-dependent genes. Although curcumin is known to inhibit the activation of NF-κB and suppress inflammation (30, 33, 36), this is the first time it has been shown to prevent ALD. Whether curcumin can also be used to treat established ALD is uncertain and requires further investigation. Because curcumin can be given safely to humans (32, 34), the results of this study have potentially important therapeutic implications for individuals at risk for ALD. Clinical trials will be necessary to evaluate this question.

Another important issue concerns the locus of action of curcumin. Although NF-κB appears to be a key mediator of the inflammatory response in Kupffer cells, it functions as a survival factor in hepatocytes under...
certain physiological conditions, e.g., liver regeneration (42). Consequently, suppression of NF-κB activity can have beneficial or deleterious effects depending on the condition being studied (11). Several investigators have presented evidence that endotoxin-activated Kupffer cells produce toxic mediators, such as proinflammatory cytokines and reactive oxygen species, leading to ALD (23, 27, 37). Hence, we investigated whether curcumin blocked endotoxin-mediated induction of NF-κB-dependent gene expression in isolated Kupffer cells. Curcumin caused dose-dependent suppression of endotoxin-mediated induction of NF-κB binding activity (Fig. 6). It also blocked the expression of the same panel of genes in endotoxin-treated Kupffer cells observed in the livers of alcohol-treated rats (Fig. 7).

![Image](https://example.com/image1.png)

**Fig. 5.** Immunostaining for iNOS and nitrotyrosine in rats treated with FE compared with FE-C. A: strong staining for iNOS was present in the hepatocytes in the FE group. B: intensity of staining was decreased in the hepatocytes of FE-C rats. C and D: degree of staining for nitrotyrosine paralleled the increased staining for iNOS in FE rats (C) and was decreased by treatment with curcumin (D).

![Image](https://example.com/image2.png)

**Fig. 6.** Curcumin inhibits NF-κB binding activity and the expression of TNF-α, IL-12, MCP-1, MIP-2, COX-2, and iNOS in endotoxin-treated Kupffer cells. Isolated Kupffer cells were treated with vehicle or the indicated concentrations of curcumin for 1 h before treatment with LPS (100 ng/ml). At 15 min after adding LPS, the Kupffer cells were harvested and washed three times with PBS. Electrophoretic mobility shift assays were performed to assess NF-κB binding activity. Levels of mRNA for cytokines, chemokines, COX-2, and iNOS were assessed by RT-PCR. Treatment with curcumin inhibited NF-κB binding activity and the expression of TNF-α, IL-12, MCP-1, MIP-2, COX-2, and iNOS in endotoxin treated cells.

![Image](https://example.com/image3.png)

**Inflammatory Liver Injury**

![Image](https://example.com/image4.png)
rats (Fig. 4). Interestingly, lower concentrations of curcumin were required to maximally suppress levels of iNOS, COX-2, and MIP-2 vs. IL-12 and MCP-1 in endotoxin-treated Kupffer cells. Currently, we do not have an explanation for this difference in response to curcumin. Our data do not permit us to exclude the possibility that curcumin had direct effects on other cell types in the liver, e.g., hepatocytes, in addition to Kupffer cells. In fact, we found that treatment with curcumin led to a reduction in levels of iNOS and nitrotirosine in hepatocytes. We also cannot exclude the possibility that oxidant stress is increased secondary to activation of NF-κB (39, 42). Nonetheless, the benefits of suppressing the proinflammatory effects of NF-κB activation in the Kupffer cell appear to outweigh any potentially detrimental effects involving other cell types.

Reduction in the severity of fatty liver in the curcumin-treated rats should also be noted. Studies in ethanol-fed rats have shown that inhibition of TNF-α leads to a decrease in the amount of fat storage in the liver (37). Other mechanisms, such as induction of enhanced fatty acid catabolism, occur in the livers of rats after curcumin treatment (1). A decrease in hepatic fat accumulation in ethanol-fed rats is also observed in response to antioxidant therapy (14). Thus multiple mechanisms are probably involved in the reduction of the degree of fat accumulation observed in curcumin-treated rats in the present study.

The pathogenesis of ALD is complex. In the present study, we showed that curcumin, a dietary phenolic antioxidant, was highly effective in preventing experimental ALD. In addition to preventing alcohol-induced liver injury, it blocked lipid peroxidation, the activation of NF-κB, and the expression of proinflammatory cytokines and chemokines, iNOS and COX-2 (Fig. 7). Developing compounds that target specific molecules, such as COX-2, is useful for treating certain inflammatory conditions, such as arthritis. By contrast, this study suggests that agents that prevent the activation of a transcription factor, i.e., NF-κB, will suppress expression of a series of proinflammatory molecules and thereby prevent ALD. Ultimately, clinical trials will be needed to determine whether agents, such as curcumin, that inhibit the activation of NF-κB will be effective in preventing and possibly treating alcohol-induced liver injury in humans.

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REFERENCES


25. Nanji AA, Khwaja S, Tahan SR, and Sadrzadeh SMH. Plasma levels of a novel noncytoxogenase-derived prostanoid (8-isoprostane) correlate with severity of liver injury in experi-


