Cinnamon Extract Protects against Acute Alcohol-Induced Liver Steatosis in Mice

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Abstract

Acute and chronic consumption of alcohol can cause increased intestinal permeability and bacterial overgrowth, thereby increasing portal endotoxin levels. This barrier impairment subsequently leads to an activation of hepatic Kupffer cells and increased release of reactive oxygen species as well as of tumor necrosis factor-α (TNFa). Recent studies have suggested that cinnamon extract may have antiinflammatory effects. In the present study, the protective effects of an alcoholic extract of cinnamon bark was assessed in a mouse model of acute alcohol-induced steatosis and in RAW 264.7 macrophages, used here as a model of Kupffer cells. Acute alcohol ingestion caused a >20-fold increase in hepatic lipid accumulation. Pretreatment with cinnamon extract significantly reduced the hepatic lipid accumulation. This protective effect of cinnamon extract was associated with an inhibition of the induction of the myeloid differentiation primary response gene (MyD)88, inducible nitric oxide (NO) synthase (iNOS), and plasminogen activator inhibitor 1 mRNA expression found in livers of alcohol-treated animals. In vitro prechallenge with cinnamon extract suppressed lipopolysaccharide (LPS)-induced MyD88, iNOS, and TNFa expression as well as NO formation almost completely. Furthermore, LPS treatment of RAW 264.7 macrophages further resulted in degradation of inhibitor kB; this effect was almost completely blocked by cinnamon extract. Taken together, our data show that an alcohol extract of cinnamon bark may protect the liver from acute alcohol-induced steatosis through mechanisms involving the inhibition of MyD88 expression.

Introduction

Alcoholic liver disease (ALD) is still among the leading causes of morbidity and mortality in the world. Fat accumulation in the liver, originally thought to be a benign nonprogressive histological state, is one of the first characteristics of the early phase of ALD. Furthermore, results of recent studies suggest that steatosis may play a critical role not only in the onset of ALD but also in the progression of the disease to later stages (e.g. fibrosis and cirrhosis) (1,2). Therefore, therapies protecting against the onset of ALD may also be beneficial for the later stages of the disease. Controlled i.g. administration of ethanol using a binge-drinking mouse model (3) produces pathological changes in the liver that resemble the early alterations (e.g. steatosis) that occur in humans. Utilizing this model, a treatment regimen of potential therapeutic value for humans can be tested.

There is evidence that acute and chronic alcohol consumption can cause increased intestinal translocation of bacterial endotoxin, subsequently leading to an activation of hepatic Kupffer cells and hepatic lipid accumulation [for review, see (4)]. Indeed, acute and chronic alcohol ingestion can result in increased portal endotoxin levels (5,6). Furthermore, it has been shown that mice deficient in the endotoxin receptors CD14 or toll-like receptor (TLR)4 and the tumor necrosis factor-α (TNFa) receptor 1 are protected against alcohol-induced liver injury (7,8). The results of McKim et al. (9) and Kono et al. (10) further indicate that the production of superoxide by NADPH oxidase and the free radical of nitric oxide (NO) formed by the inducible form of NO synthase (iNOS) are key factors in the development of ALD. Recently, it has been shown that the effects of TNFa after acute and chronic alcohol ingestion on hepatocytes are partly mediated through an insulin-dependent induction of the expression of plasminogen activator inhibitor (PAI)-1 (3). Thus, a therapy protecting against endotoxin-induced activation of Kupffer cells and the subsequent effects of TNFa (e.g. insulin resistance and induction of PAI-1) could be useful in humans to prevent ALD. Supporting this hypothesis, previous studies have shown that early alcoholic liver injury in rodents is decreased significantly with the use of antibiotics, antioxidants (e.g. green tea), and insulin sensitizers (e.g. metformin and pioglitazone) (3,11,12).

Cinnamon extract derived from cinnamon bark has been shown to improve symptoms associated with the metabolic syndrome in rodents and humans (e.g. improve glucose metabolism...
and lipid profile) (13–15). In a model of fructose-induced insulin resistance, the concomitant treatment of rats with cinnamon extract fed a diet rich in fructose enhanced muscular insulin signaling (13). Furthermore, the results of Azumi et al. (16) indicate that water-based extracts of cinnamon bark might bind endotoxin, thereby protecting against endotoxin-induced organ damage. However, mechanisms involved in the protective effects of extracts derived from cinnamon have not yet been clarified. The purpose of the present study was to test the hypothesis that an alcoholic extract of cinnamon bark protects against early alcohol-induced liver injury in a mouse model of acute alcoholic steatosis and to determine potential mechanisms involved in this protective effect.

Materials and Methods

Cinnamon extract. A commercially available alcoholic extract of cinnamon bark (1:5) was obtained from Maros (Germany). In pilot experiments, lipopolysaccharide (LPS)-binding capacity was assessed using a commercially available Limulus Amebocyte Lysate assay (Charles River) following the instructions of the manufacturer. In brief, LPS was mixed with cinnamon extract or polymyxin B and incubated for 30 min at room temperature. After 30 min, the samples were heated at 70°C for 20 min and the absorbance was measured at 405 nm after adding Limulus Amebocyte Lysate reagent, a chromogen substrate. Levels of LPS detected were not affected by cinnamon extract at concentrations used in in vitro experiments, whereas the incubation with polymyxin B reduced the detected LPS concentration in the samples by ~90–95% (Supplemental Table 1). However, the cinnamon extract used in the present study exerted a marked antioxidant effect as assessed by a commercially available kit (Randox) (Supplemental Table 2).

Animals and treatments. Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Procedures were approved by the local Institutional Animal Care and Use Committee. Six-week-old C57BL/6J mice (Janvier) consumed food (17.2 MJ/kg; 8.4 MJ from carbohydrates, 6.1 MJ from protein, 2.5 MJ from fat, 0.5 MJ from fiber; SSNIFF) and tap water ad libitum. The alcoholic extract of cinnamon bark or maltose, 6.1 MJ from protein, 2.5 MJ from fat, 0.5 MJ from fiber; SSNIFF) mice (Janvier) consumed food (17.2 MJ/kg diet: 8.4 MJ from carbohydrates, 6.1 MJ from protein, 2.5 MJ from fat, 0.5 MJ from fiber; SSNIFF) and tap water ad libitum. The alcoholic extract of cinnamon bark or vehicle (70% ethanol) was given in drinking water [0.0005 L (0.5 mL) medium was subsequently removed and replaced with fresh serum-free medium containing 0.05 mg LPS/L serum-free media (L 4524; Sigma) in the presence and absence of cinnamon extract. Cells were incubated for 18 h, rinsed with PBS, and either lysed with Trizol (Invitrogen) for later RNA isolation or Dignum A buffer to obtain cytosolic fractions (see below). Cinnamon extract in concentrations used in the cell culture experiments did not affect cell viability as assessed by trypan blue exclusion.

Immunoblots. To prepare cytosolic fractions, RAW 264.7 macrophages were homogenized in Dignum A buffer [1 mol/L HEPES, 1 mol/L MgSO 4, 2 mol/L KCl, 1 mol/L dithiothreitol] containing a protease inhibitor mix (Roche). Proteins (20–75 μg protein per well) were separated in an 8% SDS-polyacrylamide gel. Proteins were transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences) using a semidy electrophore. The resulting blots were then probed with antibodies against phospho-inhibitor 38 (Ser9), IκBα (both Cell Signaling Technology), and iNOS (Affinity Bioreagents) and bands were visualized using Super Signal Western Dura kit (Pierce). To ensure equal loading, all blots were stained with Ponceau red; signals were normalized to β-actin, which was detected using a commercially available antibody (Cell Signaling Technology).

Hepatic triglyceride determination. Mouse liver tissue was homogenized in 2× PBS. Tissue lipids were extracted with methanol/chloroform (1:2), dried in an evaporating centrifuge, and resuspended in 5% fat-free bovine serum albumin. Colorimetric assessment of triglyceride levels was conducted using a commercially available test kit (TR 210, Randox). Values were normalized to protein concentration in liver homogenate using the Bradford assay (Bio-Rad Laboratories).

Oil Red O staining. To determine hepatic lipid accumulation, frozen sections of liver (10 μm) were stained with Oil Red O (Sigma) for 10 min, washed, and counterstained with hematoxylin for 45 s (Sigma). Densitometric analysis of staining was performed as described previously in detail (3). Briefly, an image acquisition and analysis system (Zeiss) incorporating a Zeiss microscope was used to capture and analyze the Oil Red O-stained tissue sections at 400× magnification. The extent of staining in the liver lobule was defined as the percent of the field area within the default color range determined by the software. To determine means, data from 5 fields per section of each tissue section were pooled.

Detection of nitrite. To determine nitrite levels in cell culture media, we used a commercially available Griess reagent kit (Promega). In brief, 30 μL of cell supernatant was mixed with 100 μL of Griess reagent and absorbance was measured at 550 nm.

Endotoxin assay. Endotoxin was determined as described previously in detail (17). Briefly, plasma samples were heated at 70°C for 20 min. Endotoxin plasma levels were determined using a commercially available endpoint Limulus Amebocyte Lysate assay (Charles River) following the instructions of the manufacturer (Supplemental Table 4).

RNA isolation and real-time RT-PCR. Total RNA was extracted from RAW 264.7 macrophage-like cells as well as liver tissue samples using Trizol (Invitrogen). RNA concentrations were determined spectrophotometrically and 1 μg total RNA was reverse transcribed using a Moloney murine leukemia virus RT and oligo(dT) primers followed by a DNase digestion step (Fermentas). PCR primers for TNFα, PAI-1, myeloid differentiation primary response gene (MyD88), iNOS, and β-actin were designed using the software Primer 3 (Whitehead Institute for Biomedical Research) (Supplemental Table 3). Sybr Green Universal PCR Master mix (Applied Biosystems) was used to prepare the PCR mix. Primers were added to a final concentration of 300 nmol/L. The amplification reactions were carried out in an iCycler (Bio-Rad Laboratories) with initial hold steps (95°C for 2 min) and 45 cycles of a 3-step PCR (95°C for 15 s, 60°C for 15 s, 72°C for 30 s). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. The comparative C T method was used to determine the amount of target gene normalized to an endogenous reference (β-actin) and relative to a calibrator (2 −ΔΔC T). The purity of PCR products was verified by melting curves and gel electrophoresis.

Statistical analyses. Results are reported as means ± SEM. One-way ANOVA with Bonferroni's post hoc test (Bonferroni’s multiple compar-
ison test) was used for the determination of significance among treatment groups. Post hoc tests were conducted when the means were significantly different in 1-way ANOVA. Logarithmic transformation of raw data were done in cases of unequal variances. Results of all analyses were considered to be significantly different at $P < 0.05$.

Results

Cinnamon extract prevents hepatic steatosis. Lipid staining and triglyceride content in livers from maltose dextrin-treated mice was minimal regardless of other treatments and did not differ from those in livers from naive control mice; results of maltose dextrin-treated mice are shown to represent these groups (Fig. 1A, upper left panel, and Fig. 1B). In contrast, ethanol treatment caused a significant $>20$-fold accumulation of lipids in mouse livers 12 h after ingestion. Pretreatment with alcoholic extract of cinnamon bark prior to ethanol ingestion significantly blunted alcohol-induced liver steatosis by $\sim 45\%$. (Fig. 1A, lower right panel, and Fig. 1B). Cinnamon extract exerted the same effects on ethanol-induced hepatic triglyceride accumulation in this group (Fig. 1C). Because this model represents the early phase of ALD, groups did not differ in plasma ALT (data not shown).

Cinnamon extract prevents hepatic induction of MyD88, iNOS, and PAI-1 but does not affect TLR4 expression. Expression of TLR4 did not differ between groups regardless of treatment (Table 1). However, acute alcohol exposure resulted in a significant $>5$-fold increase in hepatic MyD88 mRNA expression in mice compared with the maltose dextrin controls (Table 1). Treatment with cinnamon extract before ethanol ingestion almost completely blocked the alcohol-induced induction of MyD88. Pretreatment with cinnamon extract also significantly blocked the alcohol-induced expression of iNOS and PAI-1 mRNA in livers of mice (Table 1).

Cinnamon extract blocks MyD88 but does not affect TLR4 expression in vitro. To further determine the mechanisms underlying the protective effects of alcoholic cinnamon extract on acute alcohol-induced liver steatosis, we assessed the effects of cinnamon extract on LPS-stimulated RAW 264.7 macrophages. Eighteen hours after LPS exposure, TLR4 mRNA expression did not differ among untreated RAW 264.7 macrophages, RAW 264.7 macrophages incubated with vehicle (70% ethanol), or LPS (0.05 mg/L media) (Table 2). Furthermore, pretreatment of cells with different concentrations of cinnamon extract (1–4 $\mu$L/mL media) did not affect TLR4 mRNA expression of cells. In contrast, mRNA expression of MyD88 was significantly increased by $\sim 4$-fold in RAW 264.7 macrophages stimulated with LPS compared with untreated cells or vehicle-treated cells (Table 2). Preincubation with cinnamon extract blocked the
MyD88 100
6
% of control
determining TNF
Cinnamon extract exerted similarly protective effects when
cells with cinnamon extract attenuated this effect of LPS.
resulted in a significant;
naı̈ ve cells and cells treated with vehicle only. Pretreatment of;
with LPS (0.05 mg/L media) and vehicle for 18 h resulted in an
expression compared with naı̈ ve cells and cells treated with
mRNA, and NO formation in
RAW 264.7 macrophages stimulated with LPS for 18 h. Expression of iNOS
mRNA and protein concentration as well as nitrite release were
minimal in untreated and vehicle-treated cells, respectively. In
RAW 264.7 macrophages challenged with LPS (0.05 mg/L media) for 18 h, iNOS mRNA expression and iNOS protein levels were significantly higher by ~33-fold and ~28-fold, respectively, when compared with untreated cells (Table 2; Fig.
2A,B). Concomitant exposure of RAW 264.7 macrophages to
Cinnamon extract and LPS significantly blunted the LPS-induced
iNOS mRNA expression and elevation of protein levels. Protec-
tive effects of the cinnamon extract were similar when determin-
ing nitrite concentration in cell culture supernatant 18 h after the
challenge of RAW 264.7 macrophages with LPS (Table 2).

Cinnamon extract blocks iNOS and nitrite release in vitro.
To determine whether the protective effects of alcoholic cinna-
om extract on LPS-induced expression of MyD88 were also
affecting iNOS, mRNA expression and protein levels of iNOS
as well as release of nitrite were determined in RAW 264.7
macrophages stimulated with LPS for 18 h. Expression of iNOS
mRNA and protein concentration as well as nitrite release were
minimal in untreated and vehicle-treated cells, respectively. In
RAW 264.7 macrophages challenged with LPS (0.05 mg/L
media) for 18 h, iNOS mRNA expression and iNOS protein
levels were significantly higher by ~33-fold and ~28-fold, re-
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ing nitrite concentration in cell culture supernatant 18 h after the
challenge of RAW 264.7 macrophages with LPS (Table 2).

Cinnamon extract attenuates IkB activation and TNFα
expression in vitro. Phosphorylation of IkB was minimal in
untreated RAW 264.7 macrophages and cells treated with vehi-
cle only for 18 h (Fig. 3). Treatment of RAW 264.7 macrophages
with LPS (0.05 mg/L media) and vehicle for 18 h resulted in an
~7-fold increase of phosphorylation of IkB compared with
naive cells and cells treated with vehicle only. Pretreatment of
cells with cinnamon extract attenuated this effect of LPS.
Cinnamon extract exerted similarly protective effects when
determining TNFα mRNA in RAW 264.7 cells 18 h after being
challenged with LPS. Specifically, incubation of cells with LPS
resulted in a significant ~17-fold induction of TNFα mRNA
expression compared with naive cells and cells treated with
vehicle only (Table 2). Concomitant treatment of cells with
Cinnamon extract (1–4 μL/mL media) significantly blocked
LPS-induced expression of TNFα mRNA in RAW 264.7 cells.

Discussion
Therapies preventing the first stage of alcohol-induced liver
damage (e.g. lipid accumulation) are still limited. Animal-based
models resembling conditions of the early stages of ALD in
humans (e.g. steatosis and steatohepatitis) have been found not
only to be useful tools to investigate the pathophysiology un-
derlying the effects of alcohol on the liver but also to evaluate
new therapeutic strategies. Because acute and chronic alcohol-
induced liver disease seem to share similar mechanisms [for

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TLR4</th>
<th>MyD88</th>
<th>iNOS</th>
<th>PAI-1</th>
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</thead>
<tbody>
<tr>
<td>Water + maltose dextrin</td>
<td>100 ± 19</td>
<td>126 ± 15</td>
<td>106 ± 14</td>
<td>91 ± 30</td>
</tr>
<tr>
<td>Cinnamon extract + maltose dextrin</td>
<td>100 ± 34</td>
<td>187 ± 33</td>
<td>646 ± 13</td>
<td>231 ± 35</td>
</tr>
<tr>
<td>Water + ethanol</td>
<td>100 ± 13</td>
<td>197 ± 22</td>
<td>328 ± 42</td>
<td>167 ± 25</td>
</tr>
<tr>
<td>Cinnamon extract + ethanol</td>
<td>100 ± 20</td>
<td>354 ± 28</td>
<td>922 ± 10</td>
<td>317 ± 79</td>
</tr>
</tbody>
</table>

*Data are means ± SEM, n = 5–7. Means in a row with superscripts without a common letter differ, P < 0.05.

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LPS</th>
<th>LPS + 1 μL</th>
<th>LPS + 2 μL</th>
<th>LPS + 3 μL</th>
<th>LPS + 4 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100 ± 40</td>
<td>52 ± 19</td>
<td>70 ± 31</td>
<td>32 ± 6.0</td>
<td>43 ± 16</td>
</tr>
<tr>
<td>MyD88, % of control</td>
<td>100 ± 12</td>
<td>520 ± 14</td>
<td>361 ± 115</td>
<td>251 ± 74.0</td>
<td>136 ± 20.0</td>
</tr>
<tr>
<td>TLR4, % of control</td>
<td>100 ± 13</td>
<td>343 ± 37</td>
<td>280 ± 250</td>
<td>2061 ± 129</td>
<td>1870 ± 272</td>
</tr>
<tr>
<td>iNOS, % of control</td>
<td>100 ± 15</td>
<td>1898 ± 86</td>
<td>1170 ± 351</td>
<td>1315 ± 23.0</td>
<td>363 ± 23.0</td>
</tr>
<tr>
<td>TLR4, % of control</td>
<td>100 ± 3.4</td>
<td>12 ± 1.0</td>
<td>10 ± 0.6</td>
<td>9 ± 1.0</td>
<td>5 ± 1.0</td>
</tr>
<tr>
<td>NO, μmol/L</td>
<td>3 ± 0.4</td>
<td>12 ± 1.0</td>
<td>10 ± 0.6</td>
<td>9 ± 1.0</td>
<td>5 ± 1.0</td>
</tr>
</tbody>
</table>

*Data are means ± SEM, n = 4 except NO, n = 3. Means in a row with superscripts without a common letter differ, P < 0.05.

Cinnamon protects against alcoholic liver disease
How does cinnamon extract protect against alcohol-induced steatosis? Previous studies have indicated that intestinal bacterial overgrowth, increased intestinal translocation of bacterial endotoxin, the subsequent activation of Kupffer cells (e.g., formation of reactive oxygen species), and release of TNF-α play a key role in the development of acute and chronic alcohol-induced liver damage [for review, see (4)]. Furthermore, results obtained in rodent models of ALD suggest that therapies interfering with the intestinal bacterial overgrowth and Kupffer cell activation (e.g., antibiotics and antioxidants) may protect against ALD. Results of other groups such as Azumi et al. (16) using water-soluble cinnamon extracts suggest that some cinnamon extracts might possess an endotoxin-binding capacity; however, the alcoholic cinnamon extract at concentrations used in the present study did not possess any endotoxin-binding capacity (Supplemental Table 1). Because the results of Anderson et al. (20) and our own experiments indicate that cinnamon extract derived from water-based or alcoholic cinnamon bark may possess a marked antioxidative capacity (see Supplemental Table 2), we determined the effect of cinnamon extract on the alcohol-dependent induction of iNOS in livers of mice. Indeed, treatment with cinnamon extract almost completely protected mice from acute ethanol-induced induction of hepatic iNOS; however, we also found an almost complete protection of mice against acute ethanol-induced expression of hepatic MyD88, whereas TLR4 expression was not affected regardless of treatment. Taken together, our data obtained in vivo in mouse liver suggest that cinnamon extract may block the ethanol-induced activation of hepatic Kupffer cells.

To further exploit the concept that cinnamon extract might protect livers from acute alcohol-induced steatosis through blocking the endotoxin-induced expression of the MyD88 and thereby blocking the MyD88 signaling cascade in Kupffer cells, we determined the expression of TLR4, MyD88, TNF-α, and iNOS as well as iNOS protein levels and the release of nitrite in LPS-treated RAW 264.7 macrophages, a model of hepatic Kupffer cells. Similar to the results obtained in vivo, expression of TLR4 was not affected by LPS and/or cinnamon extract in RAW 264.7 macrophages. In contrast, MyD88 and all other parameters were significantly decreased in a dose-dependent way in LPS-challenged cells concomitantly treated with cinnamon extract. Because the activation of the transcription factor nuclear factor-κB (NFκB) is critical for TNF-α production and NFκB can be regulated redox-sensitive, we also determined phosphor-IκBα levels in RAW 264.7 cells. Consistent with our results found for MyD88, TNF-α, and iNOS expression as well as nitrite release, the LPS-induced increase of phosphor-IκBα levels was significantly blocked in cells concomitantly treated with cinnamon extract while stimulated with LPS. Taken together, these data show that the protective effect of alcohol extract of cinnamon bark under the conditions used in our experiments may result from an inhibition of the expression of MyD88, probably through mechanisms involving an interaction of cinnamon extract with transcription factors involved in the LPS-induced induction of MyD88 and the subsequent formation of reactive oxygen species and blockage of the NFκB activation; however, the exact mechanisms involved and the components of cinnamon bark responsible for the inhibitory effects on MyD88 mRNA expression remain to be determined. Our results further indicate that the extraction methods used to obtain the cinnamon extract (e.g., water vs. ethanol) may markedly influence its effects. These results do not preclude that alcohol extracts obtained from cinnamon bark may also exert protection through antioxidative effect or other mechanisms.

In summary, the results of the present study suggest that alcoholic extracts of cinnamon bark protect against acute alcohol-induced liver steatosis by attenuating the alcohol-dependent induction of MyD88 and the subsequent formation of reactive oxygen species and induction of TNF-α. Furthermore, these results add further weight to the concept that pharmacological
or nutritive supplementation substances interfering with the activation of Kupffer cells have the potential to protect the liver from alcohol-induced damage. Although future studies will be needed to identify the compound(s) responsible for the protective effects of alcoholic cinnamon extract and to further explore the effects of the cinnamon extract in humans, our results suggest that alcohol extracts of cinnamon may be useful therapeutically for the treatment of diseases associated with an activation of MyD88.

**Literature Cited**