Polyphenols from *Camellia sinensis* attenuate experimental cholestasis-induced liver fibrosis in rats

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**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats (250–300 g) were fed a chow diet (Purina, St. Louis, MO) containing 0 or 0.1% of an extract of *C. sinensis* (Taiyo Kagaku, Yokkaichi, Mie, Japan) containing 85% polyphenols by weight, starting 3 days before surgery. Preliminary studies showed that average daily food consumption was ~90 g/kg body wt, which was not altered by

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addition of polyphenols. Figure 1 shows the structures and relative contents of different polyphenols in the C. sinenesis extract used. The major polyphenol, –50%, was epigallocatechin gallate. Rats underwent bile duct ligation (BDL) and transection or sham operation under ether anesthesia, as described elsewhere (53). Briefly, the common bile duct was located through a midline abdominal incision, double ligated near the liver, and transected between ligatures. Some rats were given gadolinium chloride (GdCl3; 20 mg/kg body wt iv 24 h before BDL and repeated once every 3 days afterward) to destroy Kupffer cells selectively (1). All animals received humane care in compliance with institutional guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Clinical chemistry and histology. Blood samples were collected from the tail vein at times indicated in the figure legends. Serum alkaline phosphatase, alanine transaminase, and bilirubin were measured using analytic kits from Sigma (St. Louis, MO). On the day of death, each rat was anesthetized with pentobarbital sodium (75 mg/kg ip), the abdomen was opened, and the portal vein was cannulated with a 20-gauge cannula. The liver was rinsed using a syringe containing 10 ml normal saline, followed by slow infusion of 5 ml 10% buffered formaldehyde (VWR International, West Chester, PA). After 48 h in fixative, paraffin sections were prepared and stained with hematoxylin-eosin or 0.1% Sirius red (Polysciences, Warrington, PA) and Fast green FCF (Sigma) (30). Areas in sections stained for collagens by Sirius red were quantified by image analysis using a Universal Imaging Image-IAT image acquisition and analysis system (West Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Thornwood, NY) and ×4 objective lens. Detection thresholds were set for the red color of stained collagen based on an intensely labeled point and a default color threshold range that was assigned. The degree of labeling in each section was determined from the area within the color range divided by the total cellular area.

Immunohistochemistry for 4-hydroxynonenal, PCNA, and α-smooth muscle actin. 4-Hydroxynonenal is a product of lipid peroxidation (10). To detect 4-hydroxynonenal adduct formation in the liver, some sections were deparaffinized with xylene and taken through a graded series of alcohol and water mixtures to rehydrate the tissue. Hydrated sections were exposed to mouse anti-4-hydroxynonenal monoclonal antibodies (Alpha Diagnostic, San Antonio, TX) at a 1:200 dilution in 0.1 M phosphate buffer-Tween for 30 min at room temperature. Peroxidase-conjugated anti-mouse IgG antibody (DAKO, Carpinteria, CA) was then applied, and 3,3’-diaminobenzidine chromagen was added as the peroxidase substrate. After the immunostaining procedure, a light counterstain of Meyer’s hematoxylin was applied so that 4-hydroxynonenal-labeled cells could be identified easily. Immunohistochemistry for PCNA was performed with monoclonal primary antibodies against PCNA (DAKO) at a dilution of 1:100 in PBS-Tween containing 1% bovine serum albumin at room temperature for 60 min. Staining for α-smooth muscle actin (α-SMA) was performed with monoclonal primary antibodies against α-SMA (DAKO) at a dilution of 1:200 at room temperature for 10 min.

AP-1 and NF-κB determination using EMSA. Measurement of AP-1 and NF-κB by EMSA was performed, as described in detail elsewhere (59). Briefly, nuclear extracts from liver tissue were incubated with 1 μg poly(dI-dC) in a buffer containing 1 mM HEPES (pH 7.6), 40 mM MgCl2, 0.1 M NaCl, 8% glycerol, 0.1 mM DTT, and 0.05 mM EDTA. 32P-labeled DNA probes (2 μl) with the consensus sequences for AP-1 or NF-κB [200,000 counts per min (cpm)/μl, Cerenkov counting] containing 0.4 ng of double-stranded oligonucleotides were added with or without a 250-fold excess of the cold oligonucleotide as competitor. Mixtures were incubated for 20 min and separated through a 5% polyacrylamide (29:1 cross-linking) gel, and autoradiography was followed by visualization.

RNase protection assay for procollagen α1(I), TNF-α, and transforming growth factor-β mRNA. Total RNA was isolated from liver tissue using RNA STAT 60 (Tel-Test). RNase protection assays were performed using the RiboQuant multiprobe assay system (Pharmingen, San Diego, CA) or an individual probe. Briefly, with the use of a multiprobe template set (rCK-1) for TNF-α and transforming growth factor (TGF)-β or a single-probe template for procollagen-α1(I), 32P-labeled RNA probes were transcribed with T7 polymerase followed by phenol-chloroform extraction and ethanol precipitation. Twenty micrograms of total RNA per sample were hybridized to 3.4 × 106 cpm of probe overnight at 56°C and digested with RNase followed by proteinase K treatment, phenol-chloroform extraction, and ethanol and ammonium acetate precipitation. Samples were then resolved on 5%
acrylamide-bisacrylamide (19:1) urea gels. After being dried, gels were visualized by autoradiography (15).

**Statistical analysis.** ANOVA and the Student-Newman-Keuls post hoc tests were used, and $P < 0.05$ was selected before the study to indicate significance.

**RESULTS**

**Polyphenols decrease liver injury and cell proliferation after cholestasis.** In untreated rats fed a standard chow diet, serum alanine transaminase (ALT) levels averaged 39 U/l (Fig. 2) and were not significantly altered by feeding of polyphenols or sham operation (data not shown). After BDL, ALT increased to 760 U/l after 1 day, indicating liver injury. ALT levels decreased afterward and reached a new steady-state level of $\sim$120 U/l after 1 wk (data not shown). When rats were fed *C. sinenesis* polyphenols, ALT after BDL was decreased by 45% (Fig. 2). Three weeks after BDL, ALT levels were 176 U/l in the rats fed control diet and 59 U/l in the rats fed a diet containing polyphenols ($P < 0.05$). These data show that polyphenols protect against hepatocellular injury at both early and late stages of cholestasis. Serum alkaline phosphatase (ALP), which mainly reflects cholangiocyte injury, also increased after BDL from $\sim$65 U/l before operation to 430 U/l 1 day afterward. Total bilirubin also increased from 0.7 mg/dl in untreated rats to 58 mg/dl 1 day after BDL. Treatment with *C. sinenesis* polyphenols did not significantly alter ALP release or total bilirubin levels after BDL (data not shown), indicating that polyphenols did not ameliorate cholestasis.

Histology revealed normal liver architecture after sham operation (Fig. 3). After BDL, focal necrosis developed within 2 days in livers of rats receiving a control diet. Necrotic areas accounted for 8.2% of liver sections in the bile duct-ligated group (data not shown). Polyphenols decreased necrotic areas to 2.6% ($P < 0.05$). Bile duct dilation and proliferation began 2 days after BDL and were well developed after 2 wk. *C. sinenesis* polyphenols minimized these pathological changes (Fig. 3). Immunohistochemical staining for PCNA was used to identify proliferating cells. After sham operation, total PCNA labeling was 0.3 cells/high power field ($\times$40 objective), which increased to 14.3 cells/high power field after BDL (Fig. 4). Dietary poly-
phenols blunted cell proliferation after BDL to 4.5 cells/high power field, a 68% decrease (Fig. 4). Proliferating cells were predominantly cholangiocytes, which increased to 10.5 cells/high power field from a basal level of 0.05 cells/high power field. BDL also caused proliferation of parenchymal and nonparenchymal cells. Proliferation of parenchymal cells increased 14-fold, and proliferation of cells other than parenchymal cells and cholangiocytes increased 52-fold (Fig. 4). Dietary polyphenols tended to decrease proliferation of parenchymal cells and cholangiocytes increased 52-fold (Fig. 4). Dietary polyphenols blunted proliferation of cholangiocytes by 78%, and proliferation of cells other than cholangiocytes and parenchymal cells was blunted by 77% (Fig. 4). Dietary polyphenols tended to decrease proliferation of parenchymal cells, but the difference was not statistically significant (Fig. 4).

**Polyphenols decrease hepatic fibrosis after BDL.** To evaluate the effects of polyphenols on liver fibrosis after BDL, liver sections were stained with Sirius red for collagen. No fibrosis was observed in livers from sham-operated rats (Fig. 5, top left). In rats fed a control diet, hepatic fibrosis developed within 2 wk after BDL (data not shown) and was severe after 3 wk (Fig. 5, top right). When rats fed C. sinenesis polyphenols were subjected to BDL, histology revealed decreased fibrosis (Fig. 5, bottom left). Image analysis revealed that Sirius red stained an area of 1% of liver sections from sham-operated rats (Fig. 6). Sirius red staining increased to 15.0% after 3 wk following BDL (P < 0.05). Treatment with C. sinenesis polyphenols suppressed this increase in Sirius red staining after BDL to 4% of the measured areas (Fig. 6; P < 0.05 compared with standard chow diet).

Collagen gene expression was evaluated by RNase protection assay for procollagen-α(1) mRNA. Procollagen-α(1) mRNA was barely detectable in livers from sham-operated rats (Fig. 7A). Three weeks after BDL, however, procollagen-α(1) mRNA increased 30-fold (Fig. 7, A and B). Treatment with C. sinenesis polyphenols blocked the increase in procollagen-α(1) mRNA expression by 76% (Fig. 7, A and B, P < 0.05).

**Role of Kupffer cells in cholestatic liver injury.** A previous study (41) showed that destruction of Kupffer cells attenuated liver fibrosis caused by carbon tetachloride. To investigate whether Kupffer cells play an important role in cholestatic liver injury, rats were treated with GdCl₃, a selective toxicant for Kupffer cells, before BDL. Suppression of Kupffer cells with GdCl₃ neither blunted ALT release (data not shown) nor attenuated liver fibrosis after BDL (Fig. 5, bottom right, and Fig. 6).

**Polyphenols decrease 4-hydroxynonenal adduct formation after BDL.** To investigate whether cholestasis causes oxidative stress to the liver, formation of 4-hydroxynonenal adducts, a product of lipid peroxidation (10), was detected immunohistochemically in liver sections. 4-Hydroxynonenal staining was barely detectable in livers from sham-operated rats, as expected (Fig. 8, top). By contrast, after 3 wk following BDL, 4-hydroxynonenal adducts accumulated in the liver (Fig. 8, middle). 4-Hydroxynonenal-positive areas in liver sections increased to 17% (Fig. 9; P < 0.01). Treatment with C. sinenesis polyphenols decreased 4-hydroxynonenal staining to 3.5% of the measured liver area (Fig. 8 and 9; P < 0.01).

**C. sinenesis polyphenols suppress α-smooth muscle actin formation after BDL.** Activated stellate cells are the major source of matrix proteins in diseased liver (14). Accordingly, we evaluated α-SMA, an indicator of stellate cell activation, by immunohistochemical staining after BDL. In livers from sham-operated rats, small amounts of α-SMA were detected in the smooth muscle and endothelium of blood vessels. After BDL, α-SMA increased markedly in perisinusoidal cells (Fig. 10, middle). α-SMA-positive areas, estimated by image analysis, increased from 0.3 to 13.9%, consistent with stellate cell activation in cholestatic livers. Feeding of C. sinenesis polyphenols suppressed this increase in α-SMA by 80% (P < 0.01), indicating inhibition of stellate cell activation (Fig. 10, bottom).

**Effects of experimental cholestasis and C. sinenesis polyphenols on NF-κB, activating protein-1, TNF-α, and TGF-β.** Activation of transcription factors activating protein-1 (AP-1) and NF-κB and expression of cytokines TGF-β and TNF-α are important in the fibrotic response (13). Accordingly, we examined the effects of
polyphenols on these profibrotic responses to cholestasis. NF-κB/DNA complexes were barely detectable in livers from sham-operated rats but increased 3.7-fold after BDL, consistent with our previous study (62). Polyphenols suppressed this activation (data not shown). A small amount of AP-1/DNA complex was detected in livers from sham-operated rats (Fig. 11A). Three weeks after BDL, however, AP-1/DNA complex levels increased 1.8-fold, indicating activation of AP-1 (Fig. 11A). Polyphenols largely suppressed this activation (Fig. 11A).

Minimal amounts of TGF-β1 and -β3 mRNA but no TGF-β2 mRNA were detected in livers from rats after sham operation. After BDL, TGF-β1, -2, and -3 mRNA increased by fourfold or more. Polyphenol treatment suppressed these increases in TGF-β expression (Fig. 11, B and C). Similarly, TNF-α mRNA was barely detectable in livers from sham-operated rats (Fig. 11B) but was increased after BDL (Fig. 11B). Polyphenol treatment suppressed this increase in TNF-α mRNA expression (Fig. 11B).

DISCUSSION

Plant-derived polyphenols minimize cholestatic liver injury. Current therapy for cholestasis, such as ursodeoxycholic acid, does not prevent fibrosis (46). Therefore, new strategies to prevent cholestatic-induced liver injury and fibrosis are needed. Previous studies suggest that oxidative stress occurs during cholestasis and likely plays a role in cholestatic-induced liver injury (3, 35, 48). Accordingly, antioxidant therapy represents a potential strategy to prevent liver injury and fibrosis. Previous studies show that antioxidants, including N-acetylcysteine, vitamin E, silymarin, and quercetin, decrease lipid peroxidation and partially ameliorate liver injury after BDL, but the effects of antioxidants on fibrosis remain controversial (4, 33, 35, 36, 47).

Leafy plant tissues exist in a prooxidant environment of high oxygen and bright light, with consequent formation of singlet oxygen and other reactive oxygen species.
species. Not surprisingly, plants such as *C. sinenesis* (Chinese green tea) contain high levels of polyphenols (Fig. 1), which are excellent scavengers of reactive oxygen species (12, 17). These polyphenols are more potent antioxidants than vitamins C and E (60). Polyphenol-rich extracts from *C. sinenesis* inhibit lipid peroxidation in vitro, in experimental animals, and in humans (12, 17, 20, 29, 43, 45). Accordingly, we assessed the effect of *C. sinenesis* polyphenols in a rat model of cholestasis induced by BDL.

In confirmation of previous work from several laboratories (34, 47, 53), BDL caused hepatic ALT release (Fig. 2), cell necrosis (Fig. 3), cholangiocyte proliferation (Fig. 4), and fibrosis (Figs. 5 and 6). Polyphenols substantially decreased liver injury, as reflected by ALT release and necrosis, and dramatically amelio-

**Fig. 7.** Polyphenols inhibit increased procollagen-α1(I) mRNA expression after BDL. Livers were harvested 3 wk after BDL, perfused with normal saline, and frozen in liquid nitrogen. RNase protection assays were performed, as described in MATERIALS AND METHODS. Values are means ± SE (n = 3–4 in each group). A: representative autoradiogram of Proc Ralph-α1(I) mRNA, GAPDH, sham operation with control diet (sham); sham operation with polypol containing diet (sham + polyphenols), BDL with control diet, and BDL with polyphenol-containing diet (BDL + polyphenols). B: densitometric analysis of collagen-α1 mRNA. *P < 0.05 vs. sham operation; †P < 0.05 vs. BDL with control diet.

**Fig. 8.** Polyphenols inhibit 4-hydroxynonenal formation after BDL. Livers were harvested 3 wk after BDL, and sections were stained immunohistochemically to assess 4-hydroxynonenal adduct formation. Shown are representative images: top, sham operation with control diet; middle, BDL with control diet; bottom, BDL with *C. sinenesis* polyphenol-containing diet. Bar is 100 μm.
strategy to decrease fibrosis in human cholestatic diseases.

Oxygen radical scavenging and polyphenol protection against fibrosis. Polyphenols may protect against fibrosis by a variety of mechanisms. However, polyphenol treatment did not decrease total serum bilirubin levels in bile duct-ligated rats. Thus polyphenols do not ameliorate fibrosis by decreasing cholestasis. An alternative mechanism for protection against fibrosis is polyphenol-mediated scavenging of free radicals produced during cholestasis. In support of this hypothesis, polyphenols decreased the accumulation of 4-hydroxynonenal adducts in rat livers after BDL (Figs. 8 and 9), indicating decreased lipid peroxidation (10). Oxidant-induced cell death may also cause exaggerated repair leading to fibrosis. Therefore, prevention of cell death may decrease subsequent fibrosis. In support of this hypothesis, polyphenols decreased ALT release and necrosis of parenchymal cells (Fig. 2), as well as minimizing fibrosis (Figs. 5 and 6). However, fibrosis also occurred in the areas where focal necrosis was not evident, suggesting that other profibrotic mechanisms may be involved.

Another possibility is that polyphenols prevent activation of Kupffer cells, thus decreasing formation of inflammatory and fibrogenic mediators. The role of Kupffer cells in fibrosis is controversial. Destruction of Kupffer cells attenuated liver fibrosis caused by carbon tetrachloride (41). By contrast, in a rat model of reversible biliary obstruction, inactivation of Kupffer cells impaired collagen metabolism and inhibited the resolution of fibrosis (42). Kupffer cells release many mediators that activate stellate cells, including TNF-α, TGF-β, human growth factor, PDGF, and reactive oxygen species (2, 13). PDGF and TNF-α are mitogenic factors for stellate cells (13, 37), although TNF-α can stimulate apoptosis of fibrogenic cells and thus inhibit fibrosis (13). TNF-α production and NF-κB activation increase during cholestasis (7, 11). Activation of NF-κB, probably due to oxidative stress, could lead to expression of TNF-α. A recent report from this laboratory (25) shows that hydrophobic bile acids can activate Kupffer cells and increase production of TNF-α. In addition, we showed here that activation of NF-κB and expression of TNF-α and TGF-β were increased by
Cholangiocytes may play an important role in cholestasis-induced fibrosis and cirrhosis. Cholangiocytes synthesize and secrete a number of proinflammatory and fibrosis-related mediators, including IL-6, endothelin-1, monocyte chemotactic protein-1, PDGF, TNF-α, and reactive oxygen species (8, 31, 32, 51, 58). These mediators enable the cholangiocytes to communicate extensively with other liver cells, including hepatic stellate cells, inflammatory cells, and portal fibroblasts. Ductular reaction to liver injury is considered the pacemaker of portal fibrosis (9). In a variety of cells, including cholangiocytes, reactive oxygen species stimulate proliferation (5, 28, 39, 52). By scavenging oxygen radicals, polyphenols may inhibit proliferation of cholangiocytes and their production of fibrogenic and inflammatory mediators. In support of this hypothesis, cholangiocytes proliferated dramatically after BDL as expected, and this effect was attenuated by polyphenols (Fig. 4).

Activation of hepatic stellate cells appears to be a critical step in hepatic fibrogenesis that is regulated by several factors, including cytokines and oxidative stress (14, 21, 24). Previously, the antioxidant N-acetylcysteine was shown to inhibit stellate cell activation (22). Therefore, polyphenols may prevent fibrosis by inhibiting oxidant-dependent activation and proliferation of stellate cells. In support of this hypothesis, α-SMA, a marker of stellate cell activation, dramatically increased after BDL (Fig. 10), an effect that was largely blocked by polyphenols. Consistent with this observation, a recent study (44) shows that epigallocatechin gallate, one of the major polyphenols from C. sinensis, inhibits proliferation of the human hepatic stellate cell line LI90, probably by suppressing MEK and Akt phosphorylation. Activated stellate cells also produce TGF-β, which, in turn, causes further proliferation of stellate cells and strongly stimulates production of collagen (13, 18). Oxidative stress not only increases production of TGF-β but also activates latent TGF-β (6, 27). Additionally, H2O2 activates transcriptional factors such as AP-1 and NF-κB (16) that are involved in stellate cell activation and synthesis of TGF-β (16, 24). Consistent with these earlier findings, accumulation of 4-hydroxynonenal adducts, activation of AP-1, and increases in TGF-β mRNA were all observed in the present study after BDL (Figs. 8 and 11), supporting the hypothesis that oxidative stress stimulates TGF-β production, leading to collagen gene and protein expression (Fig. 7). Moreover, polyphenol treatment largely prevented accumulation of 4-hydroxynonenal adducts, blocked activation of AP-1 and production of TGF-β, and prevented procollagen-α1(I) mRNA and α-SMA protein expression (Figs. 8 and 11). Together, our data indicate that polyphenols suppress fibrosis after BDL, at least in part, by scavenging reactive oxygen species, thus inhibiting hepatocellular necrosis, cholangiocyte proliferation, stellate cell activation, and fibrogenic cytokine TGF-β formation.

Fig. 11. Polyphenols suppress activator protein-1 (AP-1) activation and increased TNF-α and transforming growth factor (TGF)-β mRNAs were measured by RNase protection assays, as described in MATERIALS AND METHODS. A: representative autoradiogram of AP-1/DNA complex. B: representative autoradiogram of TGF-β and TNF-α mRNA. L32, ribosomal protein L32. C: densitometric analysis of TGF-β1 mRNA. *P < 0.05 vs. sham operation; **P < 0.05 vs. BDL with control diet.

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