Published in final edited form as:

Exp Mol Pathol. 2014 February ; 96(1): 27–35. doi:10.1016/j.yexmp.2013.10.017.

Nonselective inhibition of prostaglandin-endoperoxide synthase by naproxen ameliorates hepatic injury in animals with acute or chronic liver injury

Ralf Bahde^{1,2}, Sorabh Kapoor¹, and Sanjeev Gupta^{1,3}

¹Department of Medicine, Albert Einstein College of Medicine, Bronx, NY

²Surgical Research, Department of General and Visceral Surgery, University Hospital, Muenster, Germany

³Department of Pathology, Marion Bessin Liver Research Center, Diabetes Center, Cancer Center, Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, and Institute for Clinical and Translational Research, Albert Einstein College of Medicine, Bronx, NY

Abstract

The rising prevalence of hepatic injury due to toxins, metabolites, viruses, etc., necessitates development of further mechanisms for protecting the liver and for treating acute or chronic liver diseases. To examine whether inhibition of inflammation directed by cyclo-oxygenase pathways, we performed animal studies with naproxen, which inhibits prostaglandin-endoperoxide synthases 1 and 2 and is in extensive clinical use. We administered carbon tetrachloride to induce acute liver injury and ligated the common bile duct to induce chronic liver injury in adult rats. These experimental manipulations produced abnormalities in liver tests, tissue necrosis, compensatory hepatocyte or biliary proliferation, and onset of fibrosis, particularly after bile duct ligation. After carbon tetrachloride-induced acute injury, naproxen decreased liver test abnormalities, tissue necrosis and compensatory hepatocellular proliferation. After bile duct ligation-induced chronic injury, naproxen decreased liver test abnormalities, tissue injury and compensatory biliary hyperplasia. Moreover, after bile duct ligation, naproxen-treated rats showed more periductular oval liver cells, which have been classified as hepatic progenitor cells. In naproxen-treated rats, we found greater expression in hepatic stellate cells and mononuclear cells of cytoprotective factors, such as vascular endothelial growth factor. The ability of naproxen to induce expression of vascular endothelial growth factor was verified in cell culture studies with CFSC-8B clone of rat hepatic stellate cells. Whereas assays for carbon tetrachloride toxicity using cultured primary hepatocytes established that naproxen was not directly cytoprotective, we found conditioned medium containing vascular endothelial growth factor from naproxen-treated CFSC-8B cells protected hepatocytes from carbon tetrachloride toxicity. Therefore, naproxen was capable of ameliorating toxic liver injury, which involved naproxen-induced release of physiological

© 2013 Elsevier Inc. All rights reserved.

Author Correspondence: Sanjeev Gupta, MD, Albert Einstein College of Medicine, Ullmann Building, Room 625, 1300 Morris Park Avenue, Bronx, NY 10461; Tel: 718 430 3309; Fax: 718 430 8975; sanjeev.gupta@einstein.yu.edu; sanjvgupta@gmail.com.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cytoprotective factors in nonparenchymal liver cells. Such drug-induced release of endogenous cytoprotectants will advance therapeutic development for hepatic injury.

Keywords

Drug; injury; liver; protection; soluble factors

Introduction

The burden of liver diseases due to chronic viral hepatitis, metabolic diseases, e.g., diabetes and obesity, drugs, alcohol, environmental toxins, etc., has been rising throughout the world. Hepatic inflammation is a major component in the pathophysiology of these liver conditions although the role of anti-inflammatory drugs has not been well studied for therapeutic development. Whereas inflammation is transduced by multiple cell types and various molecular pathways driving inflammation are complex, the cyclooxygenase (COX) pathways are incriminated in many situations, including chronic liver disease in people (1–3). In experimental settings, COX pathways serve roles in liver injury, e.g., after exposure to alcohol, bacterial endotoxin, carbon tetrachloride (CCl₄), chloroform, concanavalin A, or D-galactosamine (4–7). Similarly, COX pathways were found in transgenic mice to serve roles in hepatic injury (8). Also, disease-relevant synergisms were observed in COX pathways and other inflammatory mediators, i.e., 5-lipoxygenase pathway of arachidonic acid metabolism (9).

The conversion of arachidonic acid into prostaglandins (PG) by prostaglandin-endoperoxide synthases (PTGS) 1 and 2, the former constitutive and the latter inducible, leads to multiple substrates for inflammatory mediators. Among these, major PG-derived inflammatory mediators include PGE₂, thromboxane A₂, prostacyclins, e.g., PGI₂, and other prostanoids (10). The ability to interfere with COX pathways by widely used drugs, such as naproxen, a nonselective PTGS blocker, or celecoxib, a selective PTGS2 blocker, raised interest for their uses in hepatic inflammation and/or fibrosis (11,12). However, despite presumed anti-inflammatory mechanisms of their action, studies in a rat model of acute liver inflammation also showed that in some instances naproxen or celecoxib improved outcomes not by altering activation of inflammatory cells or expression of inflammatory cytokines and chemokines (11), but by stimulating release of cytoprotective factors, such as vascular endothelial growth factor (VEGF) from hepatic stellate cells (HSC). This observation suggested an altogether different paradigm by which COX pathways could be manipulated for altering liver injury.

To develop this concept, we examined whether inhibition of PTGS1 and 2 by naproxen could be hepatoprotective in well-characterized models of CCl₄-induced acute liver injury and bile duct ligation (BDL)-induced chronic liver injury (13,14). Our consideration was that use of naproxen in suitable inhibitory doses will protect liver from acute as well as chronic injury by either direct anti-inflammatory effects on liver cells or by secondary release of cytoprotective molecules, e.g., VEGF. To demonstrate these potential hepatoprotective effects of naproxen, we performed studies at the levels of liver tests, tissue morphology, gene expression, and changes in various liver cell types. We used doses of naproxen that were previously established to inhibit PTGS1 and PTGS2 activity sufficient for 50% reduction in inflammation (ID₅₀ doses) under clinical settings (15). Moreover, cell culture assays were performed with primary rat hepatocytes and the CFSC-8 clone of HSC, which was isolated from rats with chronic liver injury (16).

Materials and methods

Drugs and Chemicals

Naproxen was purchased commercially (Sigma Chemical Co, St. Louis, MO) and was dissolved in 20% ethanol to 2 mg/ml with dilution as needed in normal saline. For cell culture studies, naproxen was diluted in Dulbecco's Minimum Essential Medium (DMEM).

Cells and cell culture

Primary hepatocytes were isolated from F344 rats by two-step collagenase perfusion, as described (11). CFSC-8B cells were originally from late Dr. M. Rojkind. Cells were cultured at 1×10^4 cells/cm² culture plastic in DMEM with 10% fetal bovine serum and antibiotics. For conditioned medium (CM), CFSC-8B cells were cultured without serum for 24 h or 48 h under hypoxia (5% O₂, 5% CO₂, 11% N₂). Cell viability was examined by thiazolyl blue (MTT) assays, as described previously (17). Primary rat hepatocytes were attached to culture dishes in serum-containing medium under normoxia (21% O₂, 5% CO₂). For cytotoxicity assays, hepatocytes were cultured for 24 h with 12 μ M CCl₄ (Sigma), followed by MTT assays.

Animal studies

Male F344 rats of 8–10 week age weighing 150–180 grams were obtained from National Cancer Institute (Bethesda, MD). The Animal Care and Use Committee at Albert Einstein College of Medicine approved protocols, according to institutional regulations and guidelines from National Institutes of Health (Bethesda, MD).

Naproxen was given i.p. in 6 or 12 mg/kg doses. For acute liver injury, rats were given 1 ml/kg CCl₄ in mineral oil (1:1, v/v) i.p. once (13). Chronic liver injury was induced by BDL in rats anesthetized with ketamine and xylazine (Fort Dodge Animal Health, Fort Dodge, IO). The common bile duct was isolated via laparotomy, ligatures were applied and 5 to 10 mm segment was resected (18).

Tissue studies

Cryostat sections of 5 μ m thickness were prepared from tissues frozen in methylbutane at -80°C . Tissue sections were fixed in 4% paraformaldehyde in phosphate buffered saline, pH 7.4 (PBS) for hematoxylin and eosin staining, and immunostaining for Ki67, cytokeratin (CK)-19, OV-6 antigen, CD45, and VEGF. For Ki67 staining, sections were permeabilized with 1% Triton X-100 in PBS at 4°C for 7 min, blocked with 3% goat serum in PBS containing 0.1% Triton X-100 for 1 h, followed by incubation for 1 h each with rabbit anti-Ki67 (1:700, Novocastra, Newcastle-upon-Tyne, UK) and peroxidase-conjugated anti-rabbit IgG (1:300, Sigma), and developed with diaminobenzidine (DAB+ kit, DAKO Cytomation, Carpinteria, CA). Tissue sections were not permeabilized for staining with anti-CD45 or anti-VEGF (1:50, Santa Cruz Biotechnology Inc., Santa Cruz, CA). For immunofluorescence, sections were blocked in 5% goat serum, 1% bovine serum albumin and 0.1% Triton X-100 in PBS, and incubated for 1 h with rabbit anti CK-19 (1:50, Santa Cruz) or mouse anti OV-6 (1:50, from H.A. Dunsford) at room temperature. Sections were stained with Alexa Fluor 488-conjugated goat anti-rabbit and goat anti-mouse IgG (1:500, Molecular Probes Inc, Eugene, OR), with DAPI-Antifade counterstaining (Molecular Probes, Invitrogen Inc., Carlsbad, CA).

To identify HSC, tissue sections were fixed in acetone at 4°C for 10 min, air-dried for 10 min, blocked in 3% goat serum for 30 min at 37°C , and incubated for 1 h with anti-desmin (1:50; DAKO) at 37°C . After washing with PBS, sections were incubated for 30 min with anti-mouse goat IgG (1:150, Sigma) at 37°C , and developed with DAB. The protocol for α -

smooth muscle actin (α -SMA) staining with monoclonal anti- α -SMA (1:400, Sigma) was identical.

For identification of fibrosis, sections fixed in ethanol were incubated with 0.1% Sirius red in aqueous solution of 1.3% picric acid (Sigma) for 20 min. Sections were treated with 0.02 N hydrochloric acid, dehydrated with graded ethanol, and mounted in Depex (Sigma). Collagen was quantitated by eluting sections by spectrophotometry at 540 nm, as described previously (19).

Morphometric analyses

Portal or lobular inflammation and necrosis were graded according to Batts and Ludwig (20): grade 0, no disease activity; grade 1, minimal portal inflammation with minimal or patchy lobular inflammation and necrosis; grade 2, mild portal inflammation with mild lobular inflammation and little hepatocellular change; grade 3, moderate portal inflammation, moderate lobular inflammation and necrosis with noticeable hepatocellular damage; and grade 4, severe portal and lobular inflammation, necrosis with prominent diffuse hepatocellular damage. The cumulative histological scores could have ranged from 0 (entirely normal) to 4 (severe inflammation and necrosis). To quantitate cells identified by staining, we examined 25 to 50 consecutive high power fields (HPF) from 3–5 animals per condition under 200x or 400x magnification. To count HSC per liver lobule, consecutive areas centered on portal radicles were counted under 400x magnification (21).

Hepatic messenger RNA studies

Total RNAs were extracted from liver and cells by Trizol Reagent (Invitrogen). Reverse transcription used RT² PCR Array First Strand Kit (SABiosciences, Frederick, MD). RT-PCR was with RT² Real-Time SYBR Green PCR master mix (SABiosciences). Array to examine 84 genes each by quantitative RT-PCR was Signal Transduction PathwayFinder Array (PARN-014A; http://www.sabiosciences.com/rt_pcr_product/HTML/PARN-014A.html). Arrays were used per manufacturer's protocols. Each analysis was with 3 replicate samples. The $2^{-\Delta\Delta}$ cycle threshold method was used for gene expression with normalization for β -actin in each sample, followed by intergroup comparisons, as described previously (22). Gene expression differences of 2-fold or higher were considered to be significant.

Serological studies

Serum samples were collected and stored at -20°C . Alanine aminotransferase (ALT), bilirubin, and alkaline phosphatase were measured in an automated clinical system.

Western blot for PTGS1 and PTGS2 expression

Liver samples were homogenized in sucrose and Tris-HCl buffer with protease inhibitors as previously described (11). Equal amounts of proteins measured by Bradford method were resolved in 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Blots were incubated with antibodies to PTGS1 (1:200; sc-1754; Santa Cruz Biotechnology Inc), PTGS2 (1:200; sc-1747-R; Santa Cruz), and β -tubulin (1:1000; Sigma), followed by with 1:5000 peroxidase-conjugated IgG (Sigma) for enzymatic chemiluminescence (NEL104; Perkin Elmer LAS Inc, Boston, MA). Densitometry used ImageJ software (National Cancer Institute, Bethesda, MD). Data were normalized to β -tubulin levels.

Experimental design

A total of 80 rats were used. To demonstrate effects of naproxen on acute liver injury, animals were first given CCl₄ followed 2 h later by 6 mg/kg or 12 mg/kg naproxen, with tissue and blood analysis after 24 h. For studies of chronic liver injury, surgery was performed for BDL, and 6 mg/kg or 12 mg/kg naproxen were given daily starting on the same day for 14 d, followed by tissue and blood analysis. Control animals were given vehicle alone. Each experimental group contained 3 to 6 rats. Healthy rats were included as controls. All cell culture studies were in triplicate conditions.

Statistical analysis

Data are shown as means±SD. Significances were analyzed by t-tests, Mann-Whitney rank sum tests, or analysis of variance (ANOVA), including Holm–Sidak pairwise comparisons by SigmaStat 3.1 (Systat Inc., Point Richmond, CA). $P<0.05$ was considered significant.

Results

We found 6 and 12 mg/kg naproxen was well-tolerated, without further morbidity or mortality in rats after either CCl₄ or BDL. These doses of naproxen, particularly 12 mg/kg, represented 50% of the published anti-inflammatory amounts (ID₅₀) of the drug (15). More detailed toxicological findings are provided below, although we confirmed by western blot that PTGS1 and PTGS2 were expressed in liver of healthy rats, as well as rats 24 h after CCl₄ or 1 week after BDL (Fig. 1). Therefore, we concluded that studies of naproxen to inhibit hepatic PTGS activity in rats, was appropriate.

Naproxen ameliorated acute liver injury

We observed significant liver test abnormalities in rats treated with CCl₄. When the extent of tissue injury was examined 6, 24 or 48 h after CCl₄, we found maximal liver injury was apparent 24 h after CCl₄. Therefore, we chose 24 h after CCl₄ for more experimental analyses. Serum ALT was 2533 ± 863 U/L in rats 24 h after CCl₄ versus 55 ± 10 U/L in healthy rats, which represented a 46-fold difference, $p<0.001$ (Fig. 2A). Serum alkaline phosphatase levels were also abnormally increased by 2.9-fold to 403 ± 68 U/L versus 138 ± 32 U/L in healthy rats, $p<0.001$ (Fig. 2B). However, total bilirubin levels did not increase after CCl₄ (Fig. 2C).

By contrast with these findings, in rats treated with CCl₄ followed by naproxen, we observed less hepatic injury, as demonstrated by significantly lower increases in serum ALT levels, which were 2.4-fold and 2.8 fold lower, after 6 mg/kg or 12 mg/kg naproxen, respectively, $p<0.05$, ANOVA. In naproxen-treated rats, we noted that serum alkaline phosphatase levels did not decline, which suggested that naproxen did not affect cholestasis after CCl₄.

Tissue analysis showed centrilobular necrosis, inflammation and induction of liver regeneration after CCl₄ (Fig. 3A–3C). The cumulative histological grade in multiple animals was 1.8 ± 0.6 in CCl₄-treated rats compared with 0 ± 0 in healthy rats. Tissue injury decreased in rats treated with either dose of naproxen. The histological grade of tissue injury after 12 mg/kg naproxen was 0.9 ± 0.3 , $p<0.05$, which was in agreement with lowering of serum ALT levels at 24 h after CCl₄. We noted less tissue infiltration with CD45+ cells after naproxen (Fig. 3B). We found only rare Ki67+ hepatocytes in healthy liver (Fig. 3C). Ki67+ cells were frequent in CCl₄-treated rats, which indicated onset of compensatory liver regeneration. By contrast, the prevalence of Ki67+ cells in naproxen-treated rats declined, indicating less need for liver regeneration, which reflected lower levels of liver injury.

Effects of naproxen on chronic liver injury

The pattern of liver injury in rats after BDL differed from CCl₄-treated rats. For instance, 14 d after BDL, serum ALT, alkaline phosphatase and total bilirubin levels were higher than in healthy rats but lower than CCl₄-treated rats: 81 ± 13 U/L, 459 ± 95 U/L, and 5.1 ± 0.3 mg/dl, respectively (Fig. 4A–C). After BDL, alkaline phosphatase levels were higher and bilirubin levels increased, which was consistent with cholestasis. Naproxen decreased liver injury after BDL since after 12 mg/kg naproxen, serum ALT levels were normal, $p < 0.05$. On the other hand, naproxen had less effects on cholestasis, as serum alkaline phosphatase levels showed lower decreases, albeit these still declined by 33% and 36% after 6 mg/kg and 12 mg/kg naproxen, respectively, $p < 0.05$. Naproxen had no effect on serum bilirubin levels after BDL, which was expected, since mechanical obstruction to bile flow is not reversible in BDL model.

Tissue samples from rats with BDL revealed significant bile duct proliferation and fibrosis (Fig. 5A–D). Hepatic collagen content increased by 2-fold, compared to healthy animals, 32 ± 7 versus 17 ± 5 μ g collagen/mg protein, $p < 0.05$. Although tissue morphology significantly improved and bile duct proliferation decreased in naproxen-treated rats, the extent of hepatic fibrosis did not change, as indicated by Sirius red and α -SMA staining, as well as liver collagen content.

On the other hand, CD45⁺ inflammatory cells, declined by >50% in naproxen-treated rats, $p < 0.05$ (Fig. 6A). In rats with BDL, we found the number of Ki67⁺ hepatocytes, especially in periportal areas (acinar zone 1), increased significantly compared with healthy rats, which reflected cholestatic liver injury (Fig. 6B). Similarly, Ki67⁺ cells increased in bile ducts, which were proliferating after biliary injury. But this was different in naproxen-treated rats, where Ki67⁺ hepatocytes as well as Ki67⁺ bile duct cells were fewer. This indicated need for less compensatory liver regeneration due to lower levels of liver injury. In agreement with this possibility, we found more OV-6⁺ cells after BDL, which incriminated activation of stem/progenitor cells in this situation (Fig. 6C). Remarkably, the number of OV-6⁺ cells in ductal or periductal areas in naproxen-treated animals was greater than after BDL alone. This indicated that naproxen promoted activation of OV-6⁺ stem/progenitor cells during biliary repair.

Next, we examined whether these results reflected paracrine signalling from liver cells. Here, VEGF was of interest in view of greater VEGF release in naproxen-treated HSC (11). We found the number of cells intensely stained with VEGF increased after BDL compared with normal animals (Fig. 7). These cells were mostly nonparenchymal in nature and were located in liver sinusoids. Moreover, VEGF⁺ cells further increased after BDL plus naproxen. VEGF was again most intensely stained in nonparenchymal liver cells, which included HSC with characteristic stellate projections. In addition, we observed mononuclear cells in sinusoids with intense VEGF staining.

Naproxen did not directly protect hepatocytes

To determine whether naproxen could be directly cytoprotective, we used cell culture models. MTT assays showed that culture of either primary rat hepatocytes or CFSC-8B cells with 1 to 10 μ M naproxen had no effect on cell viability over 24 h. By contrast, when primary rat hepatocytes were cultured with CCl₄, cell viability declined, but naproxen had no cytoprotective effects and cell viability was again unchanged (Fig. 8A). To examine cell-to-cell paracrine interactions, we then cultured primary hepatocytes with serum-free conditioned medium (CM) from CFSC-8B cells cultured under hypoxia, with or without 1 to 10 μ M naproxen. Culture of primary rat hepatocytes for 24 h with this CM increased viability by $26 \pm 10\%$ and $13 \pm 3\%$, respectively, $p < 0.05$. The CM contained increased

amounts of VEGF (not shown). To further determine whether naproxen-induced cell signalling could protect from toxin-mediated cell death, we performed CCl₄ cytotoxicity assays with primary rat hepatocytes. We found CM from CFSC-8B cells treated with naproxen was cytoprotective, and cell viability improved above controls, $p < 0.05$ (Fig. 8B). To establish whether naproxen modulated cell signalling, we analysed by qRT-PCR gene expression in CFSC-8B cells treated with 1 μ M naproxen versus untreated CFSC-8B cells cultured for 24 h under hypoxia. An array of 84 cell signalling genes showed downregulation of 44 genes (52%) in naproxen-treated CFSC-8B cells, representing pathways in apoptosis, inflammation, cell cycling and DNA damage/repair, etc. (Fig. 8C). The majority of differentially regulated genes were expressed in 2.3- to 5-fold range, excepting *Csf2* and *Gadd45a*, which were downregulated by 28.5-fold and 26.5-fold, respectively. Only 2 of 84 genes were upregulated; *Igfbp3* by 4.8-fold and VEGF by 2-fold. By contrast, culture of CFSC-8 cells with 1 μ M naproxen for 48 h led to differential regulation of only 8 of 84 genes, including lower downregulation of *Csf2*, now by only 3.6-fold, although VEGF was upregulated by 2.6-fold (Fig. 8D).

Discussion

This study provides new information in hepatoprotection via blockade of COX pathways by naproxen. Evidences for naproxen-induced benefits in acute and chronic liver injury models included improvements in liver tests, decreases in hepatic necrosis, inflammation, and compensatory hepatocellular and biliary proliferation. This effect was mediated by cell-cell signaling in liver, as naproxen stimulated HSC to induce VEGF expression in vivo as well as in vitro. Cell culture assays confirmed that CM from naproxen-treated HSC protected hepatocytes from CCl₄ cytotoxicity. This will have implications for therapeutic developments in liver disease.

The differences in CCl₄-induced acute liver injury, including necrosis, inflammation and liver regeneration, and in the BDL model of chronic liver injury, including cholestasis, bile duct proliferation and fibrosis, was in agreement with previous findings (9,12). Previously, inhibition of PTGS2 by celecoxib decreased acute liver injury in some cases (6,9); however, the role of cell-cell signaling in that process was not discovered. Moreover, in studies of liver inflammation after cell transplantation (11), naproxen did not dampen cytokine-chemokine expression. By contrast, in mice with acute liver injury induced by CCl₄, PTGS2 inhibition decreased expression of several inflammatory cytokines (9). This may have reflected differences in specific types of liver injury, and perhaps of PTGS1 and 2 versus only PTGS2 blockade in animal models. Although we found naproxen after CCl₄-induced liver injury led to less infiltration with CD45⁺ leukocytes, whether this was additionally due to lower phagocytotic activity following decreased hepatic necrosis, was not excluded. This possibility should be in agreement with lower liver regenerative activity, as indicated by fewer Ki67⁺ cells in naproxen-treated rats.

We found chronic liver injury induced by BDL improved after naproxen in several respects. In a study of PTGS2 blockade with celecoxib in rats, improvements in liver tests and cytokine profiles were observed only at seven days and not two weeks after BDL (12). In our studies, serum ALT levels were normal in naproxen-treated rats two weeks after BDL, which was consistent with hepatoprotective effects of the drug. We found no changes in collagen deposition after BDL, which was similar to previous studies of rats with BDL and treatment with the PTGS2 blocker, celecoxib (12). On the other hand, decreases in Ki67⁺ cells in naproxen-treated rats with BDL, which was similar to CCl₄-treated rats, represented less need for liver regeneration through proliferation of hepatocytes and bile ducts. These and other improvements in tissue morphology in BDL rats, despite continued expression of inflammatory cytokines, although the number of CD45⁺ cells was lower, suggested

therapeutic benefits of naproxen were due to mechanisms other than anti-inflammatory activity.

For instance, as naproxen decreased serum alkaline phosphatase levels in BDL rats, this suggested simultaneous protection to an extent of the biliary epithelium. This manifested with less need for proliferation of bile ducts in animals with BDL and naproxen treatment. The activation of OV-6+ cells in naproxen-treated animals with BDL was noteworthy, given that OV-6 is a marker of epithelial liver stem/progenitor cells (23), thus implicating their recruitment for replenishment of damaged cells. Therefore, activation of stem/progenitor cells should represent an important and more physiological repair pathway. This could have resulted from the effects of VEGF on protection of bile duct cells from injury (24). While cholangiocytes from BDL rats responded to VEGF by increased proliferation in vitro (25), whether those cells might have been enriched in OV-6+ stem/progenitor cells was unknown. In our studies, naproxen was given over a prolonged period, which differed from previous studies.

Moreover, VEGF is only one among many growth factors, e.g., hepatocyte growth factor, granulocyte-colony stimulating factor, and others, which could play further roles in oval cell proliferation (26–28). Although the balance in growth factor levels under given contexts could direct predominance of growth-promoting or growth-inhibiting effects in OV-6+ or biliary cells, it should be noteworthy that administration by gene therapy approach of recombinant VEGF alone was sufficient for activating oval cells (27). Such an ability of any drug to activate this stem/progenitor cell compartment has not previously been demonstrated.

The ability of VEGF to protect hepatocytes, and to even induce hepatic proliferation in the presence of endothelial cell-signaling (29), should be helpful for understanding effects of naproxen in vivo. Our findings indicated naproxen stimulated HSC with desmin expression, as well as greater VEGF expression, which were similar to after cell transplantation (11). However, naproxen stimulated HSC far more quickly than had cell transplantation alone, since the latter required at least three days for maximal desmin expression in HSC (21), suggesting release of additional cytokines or chemokines could possibly have interfered. The absence of an effect of naproxen on cell proliferation was verified in hepatocytes under culture conditions. On the other hand, naproxen produced significant effects on signalling mechanisms in CFSC-8B cells with increased VEGF expression in cells. This resulted in protection of hepatocytes from toxin-mediated cell death after culture with CM from naproxen-treated CFSC-8B cells. Therefore, the mechanism by which naproxen protected liver in CCl₄ and BDL models included HSC-mediated cytoprotective signalling. Furthermore, we found that VEGF was expressed in mononuclear cells in liver sinusoids, which are known to secrete VEGF (30), and such cells that include Kupffer cells thus constitute another potentially significant mechanism for cell-cell signalling after naproxen administration.

This ability of naproxen to induce endogenous VEGF represents an exciting paradigm to approach therapies in liver diseases. Prolonged naproxen use in patients with liver disease may have potential for nephrotoxicity (31). In this context, large cohort studies do indicate benefits from nonsteroidal anti-inflammatory drugs in decreasing risk of chronic liver disease (32). Therefore, development of other drugs capable of reproducing these effects of naproxen should be appropriate.

Acknowledgments

Supported in part by NIH grants R01 DK 088561, R01 DK071111 and P30-DK41296. R.B. was the recipient of a postdoctoral fellowship from German Research Foundation (DFG).

Ms. Chaoying Zhang and Ms. Gertrude Ukpong provided technical assistance.

References

1. Cheng J, Imanishi H, Iijima H, Shimomura S, Yamamoto T, Amuro Y, Kubota A, Hada T. Expression of cyclooxygenase 2 and cytosolic phospholipase A2 in the liver tissue of patients with chronic hepatitis and liver cirrhosis. *Hepatol Res.* 2002; 23:185–195. [PubMed: 12076714]
2. Mohammed NA, El Aleem SA, El Hafiz HA, McMahon RF. Distribution of constitutive (COX-1) and inducible (COX-2) cyclooxygenase in postviral human liver cirrhosis: a possible role for COX-2 in the pathogenesis of liver cirrhosis. *J Clin Pathol.* 2004; 57:350–354. [PubMed: 15047734]
3. Núñez O, Fernández-Martínez A, Majano PL, Apolinario A, Gómez-Gonzalo M, Benedicto I, López-Cabrera M, Boscá L, Clemente G, García-Monzón C, Martín-Sanz P. Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins. *Gut.* 2004; 53:1665–1672. [PubMed: 15479690]
4. Nanji AA, Miao L, Thomas P, Rahemtulla A, Khwaja S, Zhao A, Khwaja S, Tahan SR, Dannenberg AJ. Dietary saturated fatty acids down-regulate cyclooxygenase-2 and tumor necrosis factor alpha and reverse fibrosis in alcohol-induced liver disease in the rat. *Hepatology.* 1997; 26:1538–1545. [PubMed: 9397995]
5. Begay CK, Gandolfi AJ. Late administration of COX-2 inhibitors minimize hepatic necrosis in chloroform induced liver injury. *Toxicology.* 2003; 185:79–87. [PubMed: 12505447]
6. Albrecht C, Meijer DK, Lebbe C, Sägeser H, Melgert BN, Poelstra K, Reichen J. Targeting naproxen coupled to human serum albumin to nonparenchymal cells reduces endotoxin-induced mortality in rats with biliary cirrhosis. *Hepatology.* 1997; 26:1553–9. [PubMed: 9397997]
7. Mayoral R, Mollá B, Flores JM, Boscá L, Casado M, Martín-Sanz P. Constitutive expression of cyclo-oxygenase 2 transgene in hepatocytes protects against liver injury. *Biochem J.* 2008; 416:337–46. [PubMed: 18671671]
8. Yu J, Hui AY, Chu ES, Cheng AS, Go MY, Chan HL, Leung WK, Cheung KF, Ching AK, Chui YL, Chan KK, Sung JJ. Expression of a Cox-2 transgene in murine liver causes hepatitis. *Gut.* 2007; 56:991–999. [PubMed: 17148503]
9. Horrillo R, Planagumà A, González-Pérez A, Ferré N, Titos E, Miquel R, López-Parra M, Masferrer JL, Arroyo V, Clària J. Comparative protection against liver inflammation and fibrosis by a selective cyclooxygenase-2 inhibitor and a nonredox-type 5-lipoxygenase inhibitor. *J Pharmacol Exp Ther.* 2007; 323:778–786. [PubMed: 17766677]
10. Khanapure SP, Garvey DS, Janero DR, Letts LG. Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers. *Curr Top Med Chem.* 2007; 7:311–40. [PubMed: 17305573]
11. Enami Y, Bandi S, Kapoor S, Krohn N, Joseph B, Gupta S. Hepatic stellate cells promote hepatocyte engraftment in rat liver after prostaglandin-endoperoxide synthase inhibition. *Gastroenterology.* 2009; 136:2356–64. [PubMed: 19303017]
12. Yu J, Hui AY, Chu ES, Go MY, Cheung KF, Wu CW, Chan HL, Sung JJ. The anti-inflammatory effect of celecoxib does not prevent liver fibrosis in bile duct-ligated rats. *Liver Int.* 2009; 29:25–36. [PubMed: 18435714]
13. Joseph B, Bhargava KK, Trunco G, Kumaran V, Palestro CJ, Gupta S. Regulation of hepatobiliary transport activity and noninvasive identification of cytokine-dependent liver inflammation. *J Nucl Med.* 2005; 46:146–52. [PubMed: 15632045]
14. Glaser S, Lam IP, Franchitto A, Gaudio E, Onori P, Chow BK, Wise C, Kopriva S, Venter J, White M, Ueno Y, Dostal D, Carpino G, Mancinelli R, Butler W, Chiasson V, DeMorrow S, Francis H, Alpini G. Knockout of secretin receptor reduces large cholangiocyte hyperplasia in mice with extrahepatic cholestasis induced by bile duct ligation. *Hepatology.* 2010; 52:204–14. [PubMed: 20578263]
15. Huang Z, Velázquez CA, Abdellatif KR, Chowdhury MA, Reisz JA, DuMond JF, King SB, Knaus EE. Ethanesulfohydroxamic acid ester prodrugs of nonsteroidal anti-inflammatory drugs (NSAIDs): synthesis, nitric oxide and nitroxyl release, cyclooxygenase inhibition, anti-

- inflammatory, and ulcerogenicity index studies. *J Med Chem.* 2011; 54:1356–64. [PubMed: 21280601]
16. Ohayon O, Mawasi N, Pevzner A, Tryvitz A, Gildor T, Pines M, Rojkind M, Paizi M, Spira G. Halofuginone upregulates the expression of heparanase in thioacetamide-induced liver fibrosis in rats. *Lab Invest.* 2008; 88:627–33. [PubMed: 18458672]
  17. Gagandeep S, Novikoff PM, Ott M, Gupta S. Paclitaxel shows cytotoxic activity in human hepatocellular carcinoma cell lines. *Cancer Lett.* 1999; 136:109–18. [PubMed: 10211948]
  18. Ott M, Rajvanshi P, Sokhi RP, Alpini G, Aragona E, Dabeva M, Shafritz DA, Gupta S. Differentiation-specific regulation of transgene expression in a diploid epithelial cell line derived from the normal F344 rat liver. *J Pathol.* 1999; 187:365–373. [PubMed: 10398093]
  19. Junqueira LC, Bignolas G, Mourão PA, Bonetti SS. Quantitation of collagen – proteoglycan interaction in tissue sections. *Connect Tissue Res.* 1980; 7:91–96. [PubMed: 6444567]
  20. Batts KP, Ludwig J. Chronic hepatitis, and update on terminology and reporting. *Am J Surg Pathol.* 1995; 19:1409–1417. [PubMed: 7503362]
  21. Benten D, Kumaran V, Joseph B, Schattenberg J, Popov Y, Schuppan D, Gupta S. Hepatocyte transplantation activates hepatic stellate cells with beneficial modulation of cell engraftment in rats. *Hepatology.* 2005; 42:1072–1081. [PubMed: 16250034]
  22. Krohn N, Kapoor S, Enami Y, Follenzi A, Bandi S, Joseph B, Gupta S. Hepatocyte transplantation-induced liver inflammation is driven by cytokines-chemokines associated with neutrophils and Kupffer cells. *Gastroenterology.* 2009; 136:1806–17. [PubMed: 19422086]
  23. Weiss TS, Lichtenauer M, Kirchner S, Stock P, Aurich H, Christ B, Brockhoff G, Kunz-Schughart LA, Jauch KW, Schlitt HJ, Thasler WE. Hepatic progenitor cells from adult human livers for cell transplantation. *Gut.* 2008; 57:1129–38. [PubMed: 18417531]
  24. Gaudio E, Barbaro B, Alvaro D, Glaser S, Francis H, Franchitto A, Onori P, Ueno Y, Marzioni M, Fava G, Venter J, Reichenbach R, Summers R, Alpini G. Administration of r-VEGF-A prevents hepatic artery ligation-induced bile duct damage in bile duct ligated rats. *Am J Physiol Gastrointest Liver Physiol.* 2006; 291:G307–17. [PubMed: 16574985]
  25. Gaudio E, Barbaro B, Alvaro D, Glaser S, Francis H, Ueno Y, Meininger CJ, Franchitto A, Onori P, Marzioni M, Taffetani S, Fava G, Stoica G, Venter J, Reichenbach R, De Morrow S, Summers R, Alpini G. Vascular endothelial growth factor stimulates rat cholangiocyte proliferation via an autocrine mechanism. *Gastroenterology.* 2006; 130:1270–82. [PubMed: 16618418]
  26. Isfort RJ, Cody DB, Richards WG, Yoder BK, Wilkinson JE, Woychik RP. Characterization of growth factor responsiveness and alterations in growth factor homeostasis involved in the tumorigenic conversion of mouse oval cells. *Growth Factors.* 1998; 15:81–94. [PubMed: 9505165]
  27. Oe H, Kaido T, Mori A, Onodera H, Imamura M. Hepatocyte growth factor as well as vascular endothelial growth factor gene induction effectively promotes liver regeneration after hepatectomy in Solt-Farber rats. *Hepatogastroenterology.* 2005; 52:1393–7. [PubMed: 16201081]
  28. Piscaglia AC, Shupe TD, Oh SH, Gasbarrini A, Petersen BE. Granulocyte-colony stimulating factor promotes liver repair and induces oval cell migration and proliferation in rats. *Gastroenterology.* 2007; 133:619–31. [PubMed: 17681181]
  29. LeCouter J, Moritz DR, Li B, Phillips GL, Liang XH, Gerber HP, Hillan KJ, Ferrara N. Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. *Science.* 2003; 299:890–3. [PubMed: 12574630]
  30. Riazzy M, Chen JH, Steinbrecher UP. VEGF secretion by macrophages is stimulated by lipid and protein components of OxLDL via PI3-kinase and PKC ζ activation and is independent of OxLDL uptake. *Atherosclerosis.* 2009; 204:47–54. [PubMed: 18789802]
  31. Kovacevic L, Bernstein J, Valentini RP, Imam A, Gupta N, Mattoo TK. Renal papillary necrosis induced by naproxen. *Pediatr Nephrol.* 2003; 18:826–9. [PubMed: 12774222]
  32. Sahasrabudhe VV, Gunja MZ, Graubard BI, Trabert B, Schwartz LM, Park Y, Hollenbeck AR, Freedman ND, McGlynn KA. Nonsteroidal anti-inflammatory drug use, chronic liver disease, and hepatocellular carcinoma. *J Natl Cancer Inst.* 2012; 104:1808–14. [PubMed: 23197492]

Highlights

- Prostaglandin-endoperoxide synthases were inhibited to decrease liver inflammation.
- The drug, naproxen, improved outcomes in acute and chronic liver injury in animals.
- Naproxen helped by inducing release of cytoprotective molecules from liver cells.
- Naproxen activated putative progenitor cells during recovery from liver injury.
- The findings provide paradigms for therapeutic development in liver diseases.

Fig. 1. Expression of PTGS1 and PTGS2 by western blot in rats

(A) Expression of PTGS1 and PTGS2 shown in each lane in liver from individual animals.

(B) Densitometric quantitation of PTGS1 and PTGS2 levels (n=3 each). Asterisks indicate p<0.05.

Fig. 2. Effect of naproxen on CCl₄-induced acute liver injury

(A–C) Serum ALT, bilirubin and alkaline phosphatase levels in healthy animals, animals treated once with CCl₄, and animals treated with CCl₄ followed by 6 mg/kg or 12 mg/kg naproxen. Asterisks in panel A indicate naproxen improved serum ALT levels.

Fig. 3. Histological changes after acute liver injury with CCl₄ and effects of naproxen
 Shown are tissue analyses from normal rats, CCl₄-treated rats, and rats treated with CCl₄ followed by 12 mg/kg naproxen, with morphometric results in charts at bottom. (A) H&E-stained liver sections. (B) Immunostaining for CD45+ inflammatory cells. (C) Shows Ki67+ cells. Original mag., x400. Charts at bottom show morphometric results with asterisks indicating p<0.05, when normal and CCl₄-treated rats were compared, and also when CCl₄-treated rats were compared without and with naproxen.

Fig. 4. Effect of naproxen on chronic liver injury induced by BDL

Serum ALT, bilirubin and alkaline phosphatase in healthy rats, rats after BDL, and rats after BDL plus 6 mg/kg or 12 mg/kg naproxen daily for 14 d. Asterisks in panels A and C indicate improvement of serum ALT or alkaline phosphatase levels in rats after BDL plus naproxen versus BDL alone, $p < 0.05$.

Fig. 5. Effect of naproxen on BDL-induced morphological changes in liver

Shown are studies from normal rats, rats after BDL, and rats after BDL plus 12 mg/kg naproxen daily for 14 d. (A) Tissue sections stained with H&E indicating bile duct proliferation in rats after BDL and less bile duct proliferation after BDL plus naproxen. (B) Proliferation of mature CK-19+ bile duct cells after BDL with decreases after BDL plus naproxen. (C) Liver collagen with Sirius red staining. (D) Activation of α -SMA expression. No significant differences were found in Sirius red or α -SMA staining in rats with BDL alone or after BDL plus naproxen. Original mag., x400.

Fig. 6. Effect of naproxen on BDL-induced inflammation and other liver changes

Tissues from normal rats, rats after BDL, and rats after BDL plus 12 mg/kg naproxen daily for 14 d, along with morphometric results in charts at the bottom. (A) Shows CD45+ cells, which increased significantly after BDL, and declined in naproxen-treated rats with BDL. (B) Shows Ki67+ cells, which was rare in normal liver, increased after BDL, and decreased with BDL plus naproxen. (C) Shows OV-6+ cells, which increased after BDL and further increased in rats with BDL plus naproxen. Arrows in panels indicate cells stained for various markers. Original mag., x400. Asterisks in charts indicate $p < 0.05$.

Fig. 7. Effect of naproxen on VEGF expression

VEGF expression in liver increased in rats with BDL and increased further in rats with BDL plus 12 mg/kg naproxen (arrows). Inset shows magnified view of framed area in BDL plus Naproxen rat liver to show VEGF in stellate cell (arrow) and also mononuclear cell in sinusoid (arrowhead). Original mag., x400. Asterisks in chart indicate $p < 0.05$.

Fig. 8. Studies with cultured primary rat hepatocytes and CFSC-8B rat HSC cells

(A) Shows CCl₄ cytotoxicity in primary rat hepatocytes and absence of direct cytoprotection by naproxen. Asterisks indicate $p < 0.05$ versus untreated controls. (B) Effect of CM from CFSC-8B cells treated with naproxen in primary hepatocytes cultured with CCl₄ for 24 h. (C, D) Shows differences in expression of signal transduction genes by qRT-PCR in CFSC-8B cells under hypoxia with naproxen for 24 h (C) and 48 h (D) versus naproxen-untreated cells. Differentially expressed genes represented multiple pathways, including cell stress and survival, mitogenic, wnt, hedgehog, NF κ B, NFAT, p53, TGF- β , androgen, estrogen, insulin, low density lipoprotein, retinoic acid, CREB, Jak-Stat, calcium and protein kinase C, and phospholipase C pathways. The number of differentially expressed genes declined substantially during culture of CFSC-8 cells from 24 h to 48 h, likely representing

adaptation of cells to culture conditions, although Vegfa expression was upregulated under both conditions.