Comparative Protection against Liver Inflammation and Fibrosis by a Selective Cyclooxygenase-2 Inhibitor and a Nonredox-Type 5-Lipoxygenase Inhibitor

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ABSTRACT

In this study, we examined the relative contribution of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO), two major proinflammatory pathways up-regulated in liver disease, to the progression of hepatic inflammation and fibrosis. Separate administration of 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (SC-236), a selective COX-2 inhibitor, and CJ-13,610, a 5-LO inhibitor, to carbon tetrachloride-treated mice significantly reduced fibrosis as revealed by the analysis of Sirius Red-stained liver sections without affecting necroinflammation. Conversely, combined administration of SC-236 and 4-[3-[4-(2-methylimidazol-1-yl)phenylthio]phenyl]-3,4,5,6-tetrahydro-2H-pyran-4-carboxamide (CJ-13,610) reduced both necroinflammation and fibrosis. These findings were confirmed in 5-LO-deficient mice receiving SC-236, which also showed reduced hepatic monocyte chemoattractant protein 1 expression. Interestingly, SC-236 and CJ-13,610 significantly increased the number of nonparenchymal liver cells with apoptotic nuclei (terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive). Additional pharmacological profiling of SC-236 and CJ-13,610 was performed in macrophages, the primary hepatic inflammatory cell type. In these cells, SC-236 inhibited prostaglandin (PG) E₂ formation in a concentration-dependent manner, whereas CJ-13,610 blocked leukotriene B₄ biosynthesis. Of note, the simultaneous addition of SC-236 and CJ-13,610 resulted in a higher inhibitory profile on PGE₂ biosynthesis than the dual COX/5-LO inhibitor licofelone. These drugs differentially regulated interleukin-6 mRNA expression in macrophages. Taken together, these findings indicate that both COX-2 and 5-LO pathways are contributing factors to hepatic inflammation and fibrosis and that these two pathways of the arachidonic acid cascade represent potential targets for therapy.

The cyclooxygenase (COX) pathway is responsible for the conversion of arachidonic acid into prostaglandins (PGs), the most widely recognized mediators of inflammation (Vane and Botting, 1998). In particular, COX-2, the inducible COX isoform, is a key executor of uncontrolled inflammation and its inhibition represents a major target in the treatment of inflammatory disorders (Fitzgerald and Patrone, 2001). On the other hand, emerging information has indicated the contribution of another pathway of arachidonic acid metabolism, the 5-lipoxygenase (5-LO) pathway, in developing and sustaining inflammation (Samuelsson et al., 1987; Funk, 2001). Through the 5-LO pathway, arachidonic acid is converted into leukotrienes (LTs), including LTB₄, a potent chemotactic

ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; 5-LO, 5-lipoxygenase; LT, leukotriene; CCl₄, carbon tetrachloride; FLAP, 5-lipoxygenase-activating protein; SC-236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide; CJ-13,610, 4-[3-[4-[2-methylimidazol-1-yl]phenylthio]phenyl]-3,4,5,6-tetrahydro-2H-pyran-4-carboxamide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; AA-861, [2-(12-hydroxydodeca-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone]; MK-571, 3-[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-2-(dimethylcarbamoylthethyl)sulfanyl)methylsulfanyl] propionic acid; EIA, enzyme immunoassay; IL, interleukin; MCP-1, monocyte chemotactrant protein 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; CP-105,696, 1-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl] cyclopentane carboxylic acid; RT, reverse transcription; PCR, polymerase chain reaction; LTC₄, leukotriene C₄ synthase; LT₄A₄H, leukotriene A₄ hydrolyase.
agent for leukocytes, and cysteiny1-LTs (LTC4, LTD4, and LTE4), which are potent mediators of allergic and hypersensitivity reactions (Samuelsson et al., 1987; Funk, 2001). The fact that COX-2- and 5-LO-derived products are directly implicated in inflammation has fostered the development of drugs that directly target the formation of PGs and LTs or their binding to specific receptors. The current availability of these pharmacological tools provides new opportunities in inflammation therapy.

Recent evidence indicate that the COX-2 and 5-LO pathways have converging functions in liver inflammation, tissue remodeling, and fibrosis (Titos et al., 2000, 2003, 2005; Yamamoto et al., 2003; Núñez et al., 2004; Planagumà et al., 2005). Indeed, COX-2 expression is up-regulated in patients with chronic liver disease and closely correlates with progression of fibrosis in patients with hepatitis C infection (Cheng et al., 2002a; Mohammed et al., 2004; Núñez et al., 2004). COX-2 expression is also up-regulated in rats with carbon tetrachloride (CCL4)-induced liver injury and in experimental models of alcoholic liver disease and steatohepatitis (Nanji et al., 1997; Planagumà et al., 2005; Yu et al., 2006). Recently, Yu et al. (2007) have shown that the expression of a human COX-2 transgene in murine liver causes hepatitis. Importantly, COX-2 inhibition reduces cell growth and triggers apoptosis in hepatic stellate cells and exerts antifibrogenic actions in vivo (Cheng et al., 2002b; Yamamoto et al., 2003; Planagumà et al., 2005). Similar to COX-2, the up-regulation of 5-LO has been reported in patients with chronic liver disease and in experimental models of liver injury (Uemura et al., 1994; Titos et al., 2000). 5-LO-derived products have been shown to activate hepatic stellate cells, and inhibition of their formation induces apoptosis in Kupffer cells, the major inflammatory cell type in the liver (Titos et al., 2000, 2003). Finally, blockade of the 5-LO pathway with a 5-lipoxygenase-activating protein (FLAP) inhibitor protects the liver from experimental necroinflammatory damage and fibrosis (Titos et al., 2003, 2005).

Although both COX-2 and 5-LO seem to be implicated in liver injury, at present the exact contribution of each pathway to the initiation and progression of liver inflammation and fibrosis is uncertain. In the current study we assessed the relative contribution of COX-2 and 5-LO pathways to liver inflammation and fibrosis by administering a selective COX-2 inhibitor (SC-236) and a nonredox-type 5-LO inhibitor (CJ-13,610), either alone or in combination, to mice with CCL4-induced liver injury. Additional studies were performed in 5-LO-deficient mice, as a genetic model of 5-LO inhibition, as well as in murine macrophages, as a relevant model of inflammatory cell type.

**Materials and Methods**

**Materials.** Male 129S2/SvPasCrl mice were purchased from Charles River Laboratories (Saint Aubin les Elseau, France). 5-LO knockout mice (129-Alox5tm1Fun, n = 15) and wild-type (129S2/SvPasCrl, n = 14) mice (The Jackson Laboratory), that were divided in four experimental groups: wild-type plus placebo, wild-type plus SC-236, 5-LO-deficient plus placebo, and 5-LO-deficient plus SC-236. Mice were sacrificed after 6 weeks, and liver samples collected as described above.

All animal studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the Hospital Clinic and the European Community laws governing the use of experimental animals.

**Histological Analysis.** Liver samples were formalin-fixed, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin-eosin. Necroinflammation was scored by a registered pathologist (R.M.) unaware of the treatments as grade 0 (absent), grade 1 (spotty necrosis), grade 2 (confluent necrosis), and grade 3 (bridging necrosis). Liver fibrosis was assessed by Sirius Red staining. In brief, liver sections were incubated for 10 min with thiosemicarbazide (0.5%), followed by addition of diaminobenzidine. Finally, slides were counterstained in Sirius Red F3B (0.1%) in saturated picric acid for 1 h, and sections were incubated for 10 min with proteinase K treatment (20 μg/ml in HCl-Tris) for 20 min and after proteinase K treatment (20 μg/ml in HCl-Tris) for 20 min. The fluorescent signal was converted into a chromogenic signal by adding diaminobenzidine substrate. The microscope (Eclipse E600; Nikon, Kawasaki, Japan), and the fibrosis area quantified by morphometry using a computerized system (AnalySIS, Munster, Germany), and the fibrosis area quantified by morphometry using a computerized system (AnalySIS, Munster, Germany).

**TUNEL Assay.** Apoptosis in liver sections (5 μm) was determined by TUNEL assay (Roche Diagnostics). Enzymatic labeling was performed after blocking the endogenous peroxidase in 3% H2O2 for 20 min and after proteinase K treatment (20 μg/ml in HCl-Tris) for 20 min. The fluorescent signal was converted into a chromogenic signal by adding diaminobenzidine. Finally, slides were counterstained with hematoxylin-eosin. The number of nonparenchymal TUNEL-positive cells was counted in a total of 20 fields/tissue section under the microscope (200× magnification).

**Analysis of Eicosanoids.** PGE2 and LTβ concentrations in cell supernatants were determined in unextracted samples by ELAs.

**Cell Incubations.** Raw 264.7 cells were grown in 150 cm2 flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. All incubations were performed in cells under the 16th passage. Cells were transferred to 12-well plates at a density of 200,000 cells/well in complete medium, and incubations were performed...
formed under a humidified 5% CO₂ atmosphere at 37°C. In experiments assessing the effects of compounds on PGE₂ biosynthesis, cells were primed with LPS (500 ng/ml) to induce a sustained expression of COX-2 mRNA and protein, as described previously for macrophages (Barros-Rodiles et al., 1999). Twenty-four hours later, cells were washed and incubated with vehicle (0.2% DMSO) or increasing concentrations of the selective COX-2 inhibitor, SC-236 (0.1, 1, and 10 μM) and the cox56-LO inhibitor, licofelone (1, 10, and 30 μM) for 15 min. Thereafter, cells were exposed to ionophore A23187 (2–5 μM) for an additional 15 min to stimulate the production of arachidonic acid metabolites (Kouzan et al., 1985). To test the effect of LTB₄ biosynthesis, cells were directly incubated with vehicle (0.2% DMSO), increasing concentrations of the 5-LO inhibitors CJ-13,610 (0.1, 1, and 10 μM) and AA-861 (1, 10, and 15 μM) or licofelone (1, 10, and 30 μM) for 15 min and subsequently stimulated with ionophore A23187 (2–5 μM) for an additional 15 min. In some experiments, CJ-13,610 (1 μM) or AA-861 (10 μM) were combined with SC-236 (1 μM).

For gene expression analysis, Raw 264.7 cells were grown in six-well plates (500,000 cells/well) in serum-free medium. Twenty-four hours later, cells were incubated with vehicle (0.1% DMSO), SC-236 (3 μM), CJ-13,610 (1 μM), AA-861 (10 μM), licofelone (30 μM), the LTB₄ receptor (LTB₁) antagonist CP-105,696 (0.1 μM), or the LTD₄ receptor (Cys-LT₁) antagonist MK-571 (1 μM) for 2 h at 37°C and then stimulated with PMA (50 nM) and ionophore (2 μM) for additional 4 h. In some incubations, the compounds were combined with SC-236 (3 μM). The concentrations of SC-236, CJ-13,610, and AA-861 were selected from Tries et al. (2002), Marcouiller et al. (2005), and Vidal et al. (2007).

Cell Viability Assay. Raw 264.7 macrophages were seeded in 24-well plates (100,000 cells/well) and incubated with vehicle (0.2% DMSO), SC-236 (0.1, 1, 10, and 50 μM), or CJ-13,610 (0.1, 1, 10, and 50 μM) for 1 h at 37°C. Cell viability was tested by addition of 100 μl of MTT (5 mg/ml stock solution) to each well for 3 h and subsequent lysis with isopropyl alcohol and shaking for 20 min. The absorbance at 570 nm was measured in a multiwell plate reader (BMG Labtech, Offenbach, Germany) and cell number was calculated from a standard curve.

Gene Profiling by RT-PCR. mRNA was isolated using the RNeasy kit. RNA concentration was assessed in a UV spectrophotometer, and cell number was calculated from a standard curve. Gene GenBank Accession No. Expected Product Size

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Sequence Primers</th>
<th>Expected Product Size</th>
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<td>COX-1</td>
<td>NM_008969</td>
<td>F 5'-TCCCCGGGCGGCTGATGCTTCTTCTC-3'</td>
<td>386</td>
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<tr>
<td>COX-2</td>
<td>NM_011198</td>
<td>R 5'-CAAGCAGTGCCTCAACCCCATATGC-3'</td>
<td>459</td>
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<tr>
<td>5-LO</td>
<td>NM_008662</td>
<td>F 5'-CCCCGGAGCTACCCGAATAC-3'</td>
<td>492</td>
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<tr>
<td>FLAP</td>
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<td>R 5'-GGACCCGCCGAGCTCTGCTTCTG-3'</td>
<td>339</td>
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<tr>
<td>LTC4S</td>
<td>NM_008521</td>
<td>F 5'-GGCTGGCCATATGGAAGGACAGTAC-3'</td>
<td>141</td>
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<tr>
<td>LTA4H</td>
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<td>R 5'-TCCGGCCGAGGCCGACACACAG-3'</td>
<td>330</td>
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<tr>
<td>12/15-LO</td>
<td>NM_007440</td>
<td>F 5'-CCACCCGCGGATTTCGCCAC-3'</td>
<td>333</td>
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</tbody>
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Analysis of Gene Expression by RT and Real-time PCR. Real-time quantitative PCR was performed in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). To test the effect of primers and probe sets (TaqMan Gene Expression Assays) were used to quantify MCP-1 and IL-6 gene expression using β-actin as an endogenous control. PCR results were analyzed with Sequence Detector Software (version 2.1; Applied Biosystems). Relative quantification of gene expression was performed using the comparative Ct method. The amount of target gene, normalized to β-actin and relative to a calibrator, was determined by the arithmetic formula 2⁻ΔΔCt described in the Comparative Ct Method (User Bulletin #2; http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Statistical analysis of the results was performed using analysis of variance and unpaired Student’s t test. Results are expressed as means ± S.E.M., and differences were considered significant at P < 0.05.

Results

There were no significant differences in food intake, body weight gain, and relative liver weight among the different groups of the study (data not shown). Histological examination of hematoxylin-eosin-stained sections revealed that CCl₄-treated mice had massive and severe hepatocyte necrosis, inflammation, and ballooning at the centrilobular zone with bridging of necrosis that severely disrupted the sinusoidal and lobular architecture of the liver (Fig. 1A). In these animals, the selective COX-2 inhibitor, SC-236, or the potent 5-LO inhibitor, CJ-13,610, administered separately did not significantly modify necroinflammatory damage (Fig. 1A). In contrast, a significant reduction in necroinflammatory injury was observed after the simultaneous administration of SC-236 and CJ-13,610 (Fig. 1A). On the other hand, the examination of Sirius Red-stained sections revealed that CCl₄-treated mice had extensive collagen deposition with septa bridging portal regions (Fig. 1B). The administration of SC-236 significantly reduced the percentage of fibrosis area (Fig. 1B). A similar reduction of fibrosis area was observed with
CJ-13,610, either alone or in combination with SC-236 (Fig. 1B). As shown in Fig. 1C, these antifibrogenic actions were associated with an induction of nonparenchymal cell apoptosis. Indeed, an increased number of TUNEL-positive nuclei were observed in nonparenchymal liver cells from mice receiving SC-236 and CJ-13,610, either separately or in combination (Fig. 1C).

We next compared the effects of pharmacological inhibition of 5-LO with those exerted by the genetic inhibition of this pathway. In 5-LO-deficient mice, we confirmed that inhibition of a single pathway is not enough for preventing hepatic necroinflammation induced by CCl₄. Indeed, hepatocellular damage was significantly reduced only in 5-LO-deficient mice receiving SC-236 but not in wild-type mice receiving SC-236 or in mice lacking the 5-LO gene (Fig. 2, left panels). Consistent with these findings, expression of hepatic MCP-1, a potent chemoattractant protein that contributes to the maintenance of the inflammatory infiltrate during liver injury (Efsen et al., 2001), was only significantly down-regulated in 5-LO-deficient mice receiving either placebo, SC-236 (SC), CJ-13,610 (CJ), or a combination therapy of SC-236 and CJ-13,610 (SC+CJ). Hepatic fibrosis was reduced to a similar extent by SC-236 in both wild-type and 5-LO-deficient mice (Fig. 2, right panels). 5-LO-deficient mice were not resistant to CCl₄-induced fibrosis (Fig. 2, right panels). Given that COX-2-deficient mice exhibit decreased fertility and survival, we were unable to perform similar studies in these mice.

To characterize the pharmacological actions of COX-2 and 5-LO inhibitors at the cellular level, we set out a series of experiments in macrophages, the primary inflammatory cell type and the main source of PGs and LTs in the liver (Decker, 1990). As shown in Fig. 3A, Raw 264.7 macrophages expressed all key enzymes of eicosanoid biosynthesis, namely
COX-1, COX-2, 5-LO, FLAP, LTC4S, LTA4H, and 12/15-LO. Consistent with these findings, these cells generated PGE2 and LTB4, two proinflammatory products derived from COX and 5-LO pathways, respectively (Fig. 3, B and C). SC-236 inhibited in a concentration-dependent manner PGE2 biosynthesis (Fig. 3B), whereas CJ-13,610 markedly blocked LTB4 formation (Fig. 3C). SC-236 and CJ-13,610 only affected macrophage viability at concentrations higher than those necessary to inhibit PGE2 and LTB4 formation (Fig. 3D). In addition, we compared the effects of SC-236 and CJ-13,610 with those of the dual COX/5-LO inhibitor, licofelone. As shown in Fig. 3E, licofelone inhibited PGE2 and LTB4 formation in a concentration-dependent manner. However, the inhibitory profile of this drug on PGE2 biosynthesis was lower than that produced by the combination of SC-236 and CJ-13,610 (Table 2). Finally, given that CJ-13,610 markedly blocked LTB4 formation (Fig. 3C), SC-236 and CJ-13,610 only affected macrophage viability at concentrations higher than those necessary to inhibit PGE2 and LTB4 formation (Fig. 3D). In addition, we compared the effects of SC-236 and CJ-13,610 with those of the dual COX/5-LO inhibitor, licofelone. As shown in Fig. 3E, licofelone inhibited PGE2 and LTB4 formation in a concentration-dependent manner. However, the inhibitory profile of this drug on PGE2 biosynthesis was lower than that produced by the combination of SC-236 and CJ-13,610 (Table 2). Finally, given that CJ-13,610 markedly blocked LTB4 formation (Fig. 3C), SC-236 and CJ-13,610 only affected macrophage viability at concentrations higher than those necessary to inhibit PGE2 and LTB4 formation (Fig. 3D). In addition, we compared the effects of SC-236 and CJ-13,610 with those of the dual COX/5-LO inhibitor, licofelone. As shown in Fig. 3E, licofelone inhibited PGE2 and LTB4 formation in a concentration-dependent manner. However, the inhibitory profile of this drug on PGE2 biosynthesis was lower than that produced by the combination of SC-236 and CJ-13,610 (Table 2). Finally, given that CJ-13,610 markedly blocked LTB4 formation (Fig. 3C), SC-236 and CJ-13,610 only affected macrophage viability at concentrations higher than those necessary to inhibit PGE2 and LTB4 formation (Fig. 3D).

We next explored the effects of drugs on macrophage IL-6 expression, as COX-2 and 5-LO are known to regulate cytokine secretion in these cells (Sipe et al., 1992; Marcouiller et al., 2005). As shown in Fig. 4A, SC-236 and CJ-13,610 had opposite effects on the expression of this cytokine. On one hand, SC-236 further stimulated IL-6 expression, whereas on the other hand, CJ-13,610 significantly down-regulated its expression (Fig. 4A). AA-861 reproduced the inhibitory actions seen with CJ-13,610 (Fig. 4A). SC-236 abrogated the inhibitory effect on IL-6 expression exerted by CJ-13,610 but not that exerted by AA-861 (Fig. 4A). The modulation of IL-6 expression by 5-LO appeared to be mediated by LTB4 as CP-105,696, a BLT1 receptor antagonist, but not MK-571, a Cys-LT1 receptor antagonist, significantly reduced IL-6 expression (Fig. 4B). Of note, licofelone significantly up-regulated IL-6 mRNA expression (Fig. 4C).

**Discussion**

In this study, we provide evidence that both the COX-2 and 5-LO pathways are contributing factors in the initiation and progression of liver damage in a murine model of CCl4-induced injury and that these two proinflammatory pathways represent potential targets for therapy. Our findings are consistent with previous studies showing increased expression and enhanced formation of COX-2- and 5-LO-derived products in patients with chronic liver disease and progressive fibrosis (Uemura et al., 1994; Cheng et al., 2002a; Mohammed et al., 2004; Núñez et al., 2004) and in experimental models including CCl4-induced liver injury, alcoholic liver disease, and diet-induced steatohepatitis (Nanji et al., 1997; Titos et al., 2000, 2003, 2005; Planagumà et al., 2005; Yu et
exposed to the inhibitor (0/10000). Results are expressed as percent inhibition of PGE2 or LTB4 formation with respect to vehicle.

**TABLE 2**

Comparison of the inhibitory effects between licofelone and the combination of SC-236 and CJ-13,610 on PGE2 and LTB4 formation

<table>
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<tr>
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<th>PGE2</th>
<th>LTB4</th>
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<tr>
<td>SC-236 + CJ-13,610</td>
<td>91.5 ± 0.1</td>
<td>90.4 ± 3.1</td>
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<tr>
<td>Licofelone</td>
<td>49.9 ± 6.2*</td>
<td>90.8 ± 6.8</td>
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* P < 0.01 for licofelone versus SC-236 + CJ-13,610.

Moreover, our results underscore previous studies showing protective effects of selective COX-2 and FLAP inhibitors in the liver (Endoh et al., 1996; Yamamoto et al., 2003; Planagumà et al., 2005; Titos et al., 2005).

An interesting finding of the current study was that in addition to reducing fibrosis, the simultaneous inhibition of the COX-2 and 5-LO pathways significantly reduced necro-inflammatory liver injury. This effect was not observed after the separate administration of each inhibitor. This result is somewhat surprising because previous studies have shown that necroinflammatory liver injury, assessed by transaminases levels, is reduced after the administration of a COX-2 inhibitor to rats (Yamamoto et al., 2003). Whether species differences and/or differential drug properties may explain these divergent findings remains unknown at present. In any event, the protective effects of combined inhibition of COX-2...
and 5-LO pathways were confirmed by administering the COX-2 inhibitor to 5-LO-deficient mice. Unfortunately, these results could not be confirmed in COX-2-deficient mice because these animals exhibit markedly decreased fertility and survival. Overall, these findings open new avenues for the application of dual inhibitors with the ability to inhibit both the COX-2 and 5-LO pathways. In fact, dual inhibitors, such as the COX-5-LO inhibitor licofelone, have been shown to be efficacious in the treatment of inflammatory disorders such as arthritis, osteoarthritis, asthma, and inflammatory bowel disease (Martel-Pelletier et al., 2003; Vidal et al., 2007). Whether dual inhibitors have superior efficacy compared with inhibition of a single pathway has not been completely proven, but dual inhibitors may prevent the observed shunting of the arachidonic acid metabolism toward the 5-LO pathway after COX inhibition (Martel-Pelletier et al., 2003). In addition, dual inhibitors appear to exert some disease-modifying activity and, for example, they may stop disease progression by reducing the expression of matrix metalloproteinase-13 and IL-1β (Celotti and Durand, 2003). Moreover, dual inhibitors have an excellent gastrointestinal profile, much better than that for conventional nonsteroidal anti-inflammatory drugs and equivalent to that of selective COX-2 inhibitors (Lauffer et al., 1994; Wallace et al., 1994).

Another interesting finding of our study was that pharmacological COX-2 inhibition in 5-LO-deficient mice resulted in a reduction of hepatic MCP-1 expression. MCP-1, also known as CCL2, is a prototype of the C-C chemokine β subfamily and exhibits potent chemotactic activity for monocytes, lymphocytes, and mesenchymal cells, including hepatic stellate cells (Marra et al., 1999; Muller, 2001). Mounting evidence indicates that 5-LO products, particularly LTB₄, strongly induce expression of MCP-1 mRNA and protein and that COX-derived products modulate MCP-1 expression in an agonist-specific fashion (Efsen et al., 2001; Huang et al., 2004). Considering that increased MCP-1 expression contributes to the development of the inflammatory response in patients with active fibrogenesis (Marra et al., 1998), down-regulation of this proinflammatory chemokine may be regarded as an additional protective action associated with the simultaneous inhibition of the COX-2 and 5-LO pathways. In fact, a recent study has demonstrated that dual COX-5-LO inhibition attenuates monocyte recruitment into the arterial wall by mechanisms related to MCP-1 inhibition (Vidal et al., 2007). Along these lines, a reduction in MCP-1 expression after selective COX-2 inhibition consistently ameliorates the severity of inflammation and fibrosis in chronic pancreatitis (Reding et al., 2006).

Our findings support the concept that COX-2 and 5-LO play opposite roles in the regulation of expression of IL-6, a primary proinflammatory cytokine and a determinant factor in triggering the process of hepatic inflammation (McClain et al., 1999). Indeed, in our study we found that COX-2 inhibition amplified IL-6 expression in macrophages, whereas 5-LO inhibition down-regulated IL-6 expression in these cells. These findings are consistent with previous studies showing the potential of COX-2- and 5-LO-derived products to modulate the expression and synthesis of proinflammatory cytokines in monocytes and macrophages (Sipe et al., 1992; Marcouiller et al., 2005). Interestingly, an inhibitory effect similar to that exerted by the 5-LO inhibitor was observed with a BLT1 receptor antagonist, suggesting the direct involvement of LTB₄ in the regulation of IL-6 expression in macrophages. This finding is in agreement with the view that among the different eicosanoids, 5-LO products and, in particular, LTB₄, are important positive signals for cytokine expression and synthesis in inflammatory cells (Sipe et al., 1992; Marcouiller et al., 2005).

Our data point to the direction that the hepatic effects exerted by SC-236 and CJ-13,610 were mediated by a combination of mechanisms, involving not only inhibition of proinflammatory 5-LO- and COX-2-derived products but also the induction of apoptosis in nonparenchymal cells. Indeed, in our study we noticed that the decrease in liver fibrosis associated with COX-2 and/or 5-LO inhibition was accompanied by a parallel induction of cell death in nonparenchymal cells (i.e., hepatic stellate cells and liver macrophages). These findings are in agreement with previous studies demonstrating that hepatic stellate cells and Kupffer cells in primary culture undergo apoptosis when exposed to either COX-2 or

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**Fig. 4.** Modulation of IL-6 mRNA expression in murine macrophages. A, Raw 264.7 cells were incubated with vehicle (0.2% DMSO), SC-236 (3 μM), CJ-13,610 (1 μM), or AA-861 (10 μM) for 2 h and subsequently exposed to PMA (50 nM) and ionophore A23187 (2 μM) for an additional 4 h in a humidified 5% CO₂ atmosphere at 37°C. IL-6 mRNA expression was determined in cells incubated with vehicle (0.2% DMSO), the BLT1 receptor antagonist CP-105,696 (0.1 μM), or the Cys-LT1 receptor antagonist MK-571 (1 μM) for 2 h and subsequently exposed to PMA (50 nM) and ionophore A23187 (2 μM). Results are the mean ± S.E.M. of three to six experiments. *, P < 0.05; ***, P < 0.01; ***, P < 0.001, with respect to vehicle.
5-LO inhibitors (Titos et al., 2003; Planagumà et al., 2005). A wealth of evidence indicates that the process of hepatic stellate cell apoptosis is central to stop the progress of liver fibrosis and represents an important anti-fibrogenic strategy in the liver (Iredale et al., 1998; Friedman and Bansal, 2006). Moreover, consistent with their role in liver injury, partial depletion of Kupffer cells has been shown to prevent hepatic necroinflammatory damage (Duffield et al., 2005; Titos et al., 2005). Finally, other mechanisms independent of direct COX-2 and 5-LO inhibition may potentially be involved in the hepatoprotective actions exerted by SC-236 and CJ-13,610. For example, SC-236 and other selective COX-2 inhibitors appear to display COX-2-independent and anti-inflammatory and antifibrogenic effects in the liver (Marra et al., 2000; López-Parra et al., 2005). Another potential mechanism by which conventional 5-LO inhibitors may protect against CCl4-induced liver injury is by interfering with the metabolism of CCl4 in the hepatic cytochrome P450 or by exerting antioxidant properties. In our case, this possibility is unlikely because CJ-13,610 is a non-redox-type inhibitor of 5-LO devoid of redox and iron ligand properties (Fischer et al., 2004).

In summary, the current study uncovers the participation of both COX-2 and 5-LO pathways in maintaining the inflammatory response and subsequent fibrogenesis in a preclinical model of liver disease. Our results support the notion that the COX-2 and 5-LO pathways have converging functions, not only in cell proliferation and neo-angiogenesis (Romano and Claria, 2003), but also in the progression of liver inflammation and fibrosis. Our findings suggest that inhibition of these two proinflammatory pathways represents a potential strategy for prevention of necroinflammatory liver injury and fibrogenesis.

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References


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