Comparative Protection Against Liver Inflammation and Fibrosis by a Selective COX-2 Inhibitor and a Nonredox-type 5-LO Inhibitor

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Running Title: Role of COX-2 and 5-LO in hepatic inflammation and fibrosis

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Number of text pages: 33
Number of tables: 3
Number of figures: 4
Number of references: 44

Number of words:
Abstract: 215
Introduction: 539
Discussion: 1201

List of Abbreviations: LO, lipoxygenase; CCl₄, carbon tetrachloride; MCP-1, monocyte chemoattractant protein 1; PG, prostaglandin; LT, leukotriene; FLAP, five lipoxygenase-activating protein; IL, interleukin; MTT, 3-[4,5-dimethylthiazoyl-2-yl]-2,5-diphenyltetrazolium bromide

Recommended section: Gastrointestinal, Hepatic, Pulmonary, and Renal
ABSTRACT

In this study, we examined the relative contribution of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO), two major pro-inflammatory pathways up-regulated in liver disease, to the progression of hepatic inflammation and fibrosis. Separate administration of SC-236, a selective COX-2 inhibitor, and CJ-13,610, a 5-LO inhibitor, to carbon tetrachloride (CCl₄)-treated mice significantly reduced fibrosis as revealed by the analysis of Sirius Red-stained liver sections without affecting necro-inflammation. Conversely, combined administration of SC-236 and CJ-13,610 reduced both necro-inflammation and fibrosis. These findings were confirmed in 5-LO-deficient mice receiving SC-236, which also showed reduced hepatic monocyte chemoattractant protein 1 (MCP-1) expression. Interestingly, SC-236 and CJ-13,610 significantly increased the number of non-parenchymal liver cells with apoptotic nuclei (TUNEL-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.
INTRODUCTION

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 Patrano, 2001). On the other hand, emerging information has appreciated the contribution of another pathway of arachidonic acid metabolism, the 5-lipoxygenase (5-LO), in developing and sustaining inflammation (Funk, 2001; Samuelsson et al., 1987). Through the 5-LO pathway, arachidonic acid is converted into leukotrienes (LTs), including LTB₄, a potent chemotactic agent for leukocytes, and cysteinyl-LTs (LTC₄/LTD₄/LTE₄), which are potent mediators of allergic and hypersensitivity reactions (Funk, 2001; Samuelsson et al., 1987). 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 (Planagumà et al., 2005; Núñez et al., 2004; Yamamoto et al., 2003; Titos et al., 2000; Titos et al., 2003; Titos et al., 2005). Indeed, COX-2 expression is up-regulated in patients with chronic liver disease and closely correlates with progressive fibrosis in patients with hepatitis C infection (Núñez et al., 2004; Mohammed et al., 2004; Cheng et al., 2002a). 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 (Planagumà et al., 2005; Nanji et al., 1997; Yu et al., 2006). Recently, Yu and coworkers have shown that the expression of a human COX-2 transgene in murine liver
causes hepatitis (Yu et al., 2007). Importantly, COX-2 inhibition reduces cell growth and triggers apoptosis in hepatic stellate cells and exerts anti-fibrogenic actions \textit{in vivo} (Planagumà et al., 2005; Yamamoto et al., 2003; Cheng et al., 2002b). 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 (Titos et al., 2000; Uemura et al., 1994). 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; Titos et al., 2003). Finally, blockade of the 5-LO pathway with a five lipoxygenase-activating protein (FLAP) inhibitor protects the liver from experimental necroinflammatory damage and fibrosis (Titos et al., 2003; Titos et al., 2005).

Although both COX-2 and 5-LO appear to be implicated in liver injury, at present it is uncertain the exact contribution of each pathway to the initiation and progression of liver inflammation and fibrosis. 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 CCl$_4$-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.
METHODS

Materials. Male 129S2/SvPasCrl mice were purchased from Charles River (Saint Aubin les Elseuf, France). 5-LO knock-out mice (129-Allox5tm1Fun) were from The Jackson Laboratory (Bar Harbor, ME). Murine Raw 264.7 macrophages were purchased from European Collection of Cell Cultures (ECACC, Salisbury, UK). Carbon tetrachloride (CCl4), olive oil, polyethylene glycol, hydrogen peroxide, MTT, DMSO, LPS, ionophore A23187, PMA, Direct Red 80 (Sirius Red), picric acid, thiocarbazide, proteinase K, ethidium bromide and DMEM were purchased from Sigma (St. Louis, MO). Tris was from Merck (Darmstadt, Germany). Isoflurane was from Abbott Laboratories (Abbott Park, IL). AA-861 was from Biomol (Plymouth Meeting, PA). MK-571 and LTB4 and PGE2 enzyme immunoassay (EIA) kits were purchased from Cayman Chemical (Ann Arbor, MI). RNAqueous and DNA-free kits were from Ambion Inc. (Austin, TX). Penicillin-streptomycin, trypsin-EDTA, RPMI-1640 without phenol red and 1-Kb Plus DNA ladder were from Invitrogen (Carlsbad, CA). L-glutamine was from Biological Industries (Kibbutz Beit Haemek, Israel). DPBS was purchased from BioWhittaker (Cambrex Co., East Rutherford, NJ). FBS was from Biowest (Nuaillé, France). In Situ Cell Death Detection kit (POD) and DAB substrate were from Roche (Basel, Switzerland). Agarose was from Serva (Heidelberg, Germany). The High-Capacity archive kit, interleukin-6 (IL-6), monocyte chemoattractant protein 1 (MCP-1) and β-actin Assays-on-demand were from Applied Biosystems (Foster City, CA).

Animal Studies. 129S2/SvPasCrl mice were assigned to four different groups (9 mice each) that received placebo, a COX-2 inhibitor (SC-236), a nonredox-type 5-LO Inhibitor (CJ-13,610) or a combination of SC-236+CJ-13,610. SC-236 was given in the drinking water at a dose of 6 mg/kg (Kishi et al., 2000). To achieve this dose, SC-236 was dissolved in a stock solution of 5% Tween-20 and 95% PEG and diluted in distilled water to achieve a final SC-236 concentration of 0.045 mg/ml. CJ-13,610 was given in the diet at a dose of 10 mg/kg. CJ-
13,610-containing diet was prepared by PMI Nutrition International (Brentwood, MO). After 1 week of drug administration, mice received i.p. injections of CCl$_4$ (1 ml/kg in olive oil, twice a week) for eight weeks. Thereafter, animals were sacrificed and liver samples were either fixed in 10% formalin or snap-frozen in N$_2$.

Additional studies were performed in 5-LO deficient (129-Alox$^{5\text{tm1Fun}}$, n=15) and wild-type (129S2/SvPasCrl, n=14) mice (Jackson Laboratories, Bar Harbor, ME), that were divided in four experimental groups: wild-type+placebo, wild-type+SC-236, 5-LO-deficient+placebo and 5-LO-deficient+SC-236. Mice were sacrificed after six 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 Clínic 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. Briefly, liver sections were incubated for 10 minutes with thiosemicarbazide (0.5%) and stained in Sirius Red F3B (0.1%) in saturated picric acid for 1 hour and subsequently washed with acetic acid (0.5%). Sections were visualized under a microscope (Nikon Eclipse E600, Kawasaki, Japan) and 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, Basel, Switzerland). Enzymatic labeling was performed after blocking the endogenous peroxidase in 3% H₂O₂ for 20 minutes and following proteinase K treatment (20 µg/ml in HCl-Tris) for 20 minutes. The fluorescent signal was converted into a chromogenic signal by adding DAB. Finally, slides were counterstained with hematoxylin-eosin. The number of non-parenchymal TUNEL-positive cells was counted in a total of 20 fields/tissue section under the microscope (X200 magnification).

Analysis of eicosanoids. PGE₂ and LTB₄ concentrations in cell supernatants were determined in unextracted samples by EIA (Cayman Chemical, Ann Arbor, MI).

Cell incubations. Raw 264.7 cells were grown in 150 cm² flasks in DMEM supplemented with 10% FBS, 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 per well in complete medium and incubations were performed 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) in order to induce a sustained expression of COX-2 mRNA and protein, as previously described for macrophages (Barrios-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 dual COX/5-LO inhibitor, licofelone (1, 10 and 30 µM) for 15 minutes. Thereafter, cells were exposed to ionophore A23187 (2-5 µM) for additional 15 minutes in order to stimulate the production of arachidonic acid metabolites (Kouzan et al., 1985). To test the effects on 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 minutes and subsequently stimulated with ionophore A23187 (2-5 µM) for additional 15 minutes. 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 6-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 hours at 37°C and then stimulated with PMA (50 nM) and ionophore (2 µM) for additional 4 hours. 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 previous studies demonstrating effective inhibition of either COX-2 or 5-LO activity (López-Parra et al., 2005, Fischer et al., 2004 and Titos et al., 2003). The concentrations of CP-105,696 and MK-571 were selected from previous publications demonstrating selective blockade of LTB4 and LTD4 receptors, respectively (Huang et al., 2004 and Titos et al., 2000). The licofelone concentration was 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 hour at 37°C. Cell viability was tested by adding 100 µl of MTT (5 mg/ml stock solution) to each well for 3 hours and subsequent lysis with isopropyl alcohol and shaking for 20 minutes. The absorbance at 570 nm was measured in a multi-well
plate reader (BMG-Labtech, Offenburg, Germany) and cell number was calculated from a standard curve.

**Gene Profiling by reverse transcription (RT)-PCR.** RNA was isolated using the RNAqueous kit (Ambion, Austin, TX). RNA concentration was assessed in a UV-spectrophotometer and its integrity was tested in a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples were digested with DNase and retro-transcribed with the High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA). PCR amplification of COX-1, COX-2, 5-LO, FLAP, LTC₄ synthase (LTC₄S), LTA₄ hydrolase (LTA₄H) and 12/15-LO was performed with specific oligonucleotides (Table 1). Specificity of primers was confirmed in the GenBank database using the Basic Local Alignment Search Tool (BLAST) and by direct sequencing of the amplified PCR products in an ABI Prism 3130xl Genetic Analyzer using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). PCR products were analyzed by electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining.

**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). Ready-to-use primer 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 the 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 $2^{-\Delta\Delta Ct}$ described in the Comparative Ct Method (User Bulletin # 2; http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).
Statistical analysis of the results was performed using the analysis of variance and unpaired Student's $t$-test. Results are expressed as mean ± SEM and differences were considered significant at a $P$ value < 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 (Figure 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 necro-inflammatory damage (Figure 1A). In contrast, a significant reduction in necroinflammatory injury was observed following the simultaneous administration of SC-236 and CJ-13,610 (Figure 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 (Figure 1B). The administration of SC-236 significantly reduced the percentage of fibrosis area (Figure 1B). A similar reduction of fibrosis area was observed with CJ-13,610, either alone or in combination with SC-236 (Figure 1B). As shown in Figure 1C, these anti-fibrogenic actions were associated with an induction of non-parenchymal cell apoptosis. Indeed, an increased number of TUNEL-positive nuclei were observed in non-parenchymal liver cells from mice receiving SC-236 and CJ-13,610, either separately or in combination (Figure 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 (Figure 2, left panels). Consistent with these findings, hepatic MCP-1 expression, 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 SC-236 (Figure 2, middle panels). Hepatic fibrosis was reduced to a similar extent by SC-236 in both wild-type and 5-LO-deficient mice (Figure 2, right panels). 5-LO-deficient mice were not resistant to CCl4-induced fibrosis (Figure 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 Figure 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 pro-inflammatory products derived from COX and 5-LO pathways, respectively (Figure 3, B and C). SC-236 inhibited in a concentration-dependent manner PGE2 biosynthesis (Figure 3B), whereas CJ-13,610 markedly blocked LTB4 formation (Figure 3C). SC-236 and CJ-13,610 only affected macrophage viability at concentrations higher than those necessary to inhibit PGE2 and LTB4 formation (Figure 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 Figure 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 is a nonredox-type 5-LO inhibitor of recent development poorly characterized in macrophages, we compared its inhibitory properties with those of a well-
known inhibitor (i.e. AA-861) (Titos et al., 2003). As shown in Table 3, CJ-13,610 inhibited 5-LO activity, estimated by LTB$_4$ production, to a similar extent that AA-861.

We next explored the effects of drugs on macrophage IL-6 expression, since COX-2 and 5-LO are known to regulate cytokine secretion in these cells (Marcouiller et al., 2005; Sipe et al., 1992). As shown in Figure 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 (Figure 4A). AA-861 reproduced the inhibitory actions seen with CJ-13,610 (Figure 4A). SC-236 abrogated the inhibitory effect on IL-6 expression exerted by CJ-13,610 but not that exerted by AA-861 (Figure 4A). The modulation of IL-6 expression by 5-LO appeared to be mediated by LTB$_4$ since CP-105,696, a BLT1 receptor antagonist, but not MK-571, a cys-LT1 receptor antagonist, significantly reduced IL-6 expression (Figure 4B). Of note, licofelone significantly up-regulated IL-6 mRNA expression (Figure 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 pro-inflammatory 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 (Núñez et al., 2004; Mohammed et al., 2004; Cheng et al., 2002a; Uemura et al., 1994) and in experimental models including CCl₄-induced liver injury, alcoholic liver disease and diet-induced steatohepatitis (Planagumà et al., 2005; Titos et al., 2000; Titos et al., 2003; Titos et al., 2005; Nanji et al., 1997; Yu et al., 2006). Moreover, our results underscore previous studies showing protective effects of selective COX-2 and FLAP inhibitors in the liver (Planagumà et al., 2005; Yamamoto et al., 2003; Titos et al., 2005; Endoh et al., 1996).

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 since previous studies have shown that necro-inflammatory liver injury, assessed by transaminases levels, is reduced following 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 remain, at present, unknown. 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, this could not be confirmed in COX-2 deficient mice since 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 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 to inhibition of a single pathway has not been completely proven, but dual inhibitors may prevent the observed shunting of the arachidonic acid metabolism towards the 5-LO pathway following 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, 2001). Moreover, dual inhibitors have an excellent gastrointestinal profile, much better than conventional non-steroidal anti-inflammatory drugs and equivalent to selective COX-2 inhibitors (Laufer 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 a potent chemotactic activity for monocytes, lymphocytes and mesenchymal cells, including hepatic stellate cells (Muller, 2001; Marra et al., 1999). Mounting evidence indicate 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 pro-inflammatory 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 following 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 IL-6 expression, a primary pro-inflammatory 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 pro-inflammatory cytokines in monocytes and macrophages (Marcoullier et al., 2005, Sipe et al., 1992). Interestingly, a similar inhibitory effect 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 (Marcoullier et al., 2005, Sipe et al., 1992).

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 pro-inflammatory 5-LO and COX-2-derived products but also the induction of apoptosis in non-parenchymal 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 non-
parenchymal 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 5-LO inhibitors (Planagumà et al., 2005; Titos et al., 2003). 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 necro-inflammatory damage (Titos et al., 2005; Duffield 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 actions via activation of PPARγ, a transcription factor that mediates antiinflammatory and antifibrogenic effects in the liver (López-Parra et al., 2005; Marra et al., 2000). 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 nonredox-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 mounting 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 Clària, 2003), but also in the progression of liver inflammation and fibrosis. Our findings suggest that
inhibition of these two pro-inflammatory pathways represents a potential strategy for prevention of necro-inflamatory liver injury and fibrogenesis.
ACKNOWLEDGMENTS

SC-236, CJ-13,610 and CP-105,696 were kindly provided by Pfizer (St. Louis, MO).
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FOOTNOTES

Supported in part by grants from the Ministerio de Educación y Ciencia (MEC) (SAF 06/03191) and Instituto de Salud Carlos III (ISCIII) (Ciberehd). R. Horrillo is supported by Generalitat de Catalunya-European Social Funds (2006FI-00091). A. González-Pérez is supported by MEC. N. Ferré and M. López-Parra are under Juan de la Cierva (MEC) and ISCIII contracts, respectively.

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LEGENDS FOR FIGURES

Figure 1. Effects of selective inhibition of the COX-2 and 5-LO pathways in CCl₄-treated mice. (A) Effects on necroinflammatory liver injury. Representative photomicrographs (X100 magnification) of liver sections stained with hematoxylin-eosin from eight-week CCl₄-treated mice receiving either placebo (n=9), the selective COX-2 inhibitor, SC-236 (SC) (n=9), the 5-LO inhibitor, CJ-13,610 (CJ) (n=9), or a combination therapy of SC-236 and CJ-13,610 (SC+CJ) (n=9). The hepatocellular damage observed in hematoxylin-eosin-stained liver sections was analyzed by a registered pathologist and scored as described in the Material and Methods section. (B) Effects on liver fibrosis. Representative photomicrographs (X100 magnification) of Sirius Red-stained liver sections from eight-week CCl₄-treated mice receiving either placebo, SC-236 (SC), CJ-13,610 (CJ) or a combination therapy of SC-236 and CJ-13,610 (SC+CJ). (C) Effects on non-parenchymal liver cell apoptosis. Representative images (X200 magnification) of apoptotic non-parenchymal liver cells assessed by the TUNEL assay in eight-week CCl₄-treated mice receiving either placebo, SC-236 (SC), CJ-13,610 (CJ) or a combination therapy of SC-236 and CJ-13,610 (SC+CJ). TUNEL⁺-nuclei in liver sections are denoted by arrows. Results are expressed as mean ± SEM. *, P<0.05, **, P<0.01 and ***, P<0.001 versus Placebo group.

Figure 2. Effects of selective COX-2 inhibition in wild-type (A) and 5-LO-deficient mice (B) treated with CCl₄. Necroinflammation (Left panels) was analyzed in hematoxylin/eosin-stained liver sections from mice that received placebo or the selective COX-2 inhibitor, SC-236, during the six weeks of CCl₄ treatment. (Insets) Representative photomicrographs (X100 magnification) of liver sections stained with hematoxylin/eosin. MCP-1 mRNA expression (Middle panels) was determined by reverse transcription real time quantitative PCR in liver samples from mice treated with placebo or SC-236. Fibrosis (Right panels) was assessed by...
Sirius Red staining of liver sections from mice that received placebo or SC-236. (Insets) Representative photomicrographs (X100 magnification) of liver sections stained with Sirius Red. Data are representative of 29 mice. Results are expressed as mean ± SEM. *, P<0.05 and **, P<0.01 versus Placebo.

Figure 3. Modulation of eicosanoid biosynthesis in murine macrophages. (A) Representative RT-PCR analysis of COX-1, COX-2, 5-LO, FLAP, LTC₄ synthase (LTC₄S), LTA₄ hydrolase (LTA₄H) and 12/15-LO mRNA expression in un-stimulated murine Raw 264.7 cells. A 1-Kb Plus DNA ladder was used as a size standard. (B) Effects of SC-236 on PGE₂ production. Raw 264.7 cells were exposed to LPS (500 ng/ml) for 24 hours and then incubated with vehicle (0.2% DMSO) or SC-236 (0.1, 1 and 10 µM) for 15 minutes and subsequently stimulated with ionophore A23187 (2 µM) for an additional 15 minutes in a 5% CO₂ atmosphere at 37°C. PGE₂ levels were determined by EIA. (C) Effects of CJ-13,610 on LTB₄ production. Raw 264.7 cells were incubated with vehicle (0.2% DMSO) or CJ-13,610 (0.1, 1 and 10 µM) for 15 minutes and subsequently stimulated with ionophore A23187 (5 µM) for additional 15 minutes at 37°C. LTB₄ was determined by EIA. (D) Effects of SC-236 and CJ-13,610 on cell viability. Raw 264.7 cells were treated with vehicle (0.2% DMSO), SC-236 or CJ-13,610 for 1 hour at 37°C and cell number was evaluated by the MTT assay as described in Materials and Methods. (E) Effects of the dual COX/5-LO inhibitor licofelone on PGE₂ and LTB₄ production. To assess the effects on PGE₂ production, cells were exposed to LPS (500 ng/ml) for 24 hours and then incubated with licofelone (1, 10 and 30 µM) for 15 minutes and subsequently stimulated with ionophore A23187 (2 µM) for an additional 15 minutes in a 5% CO₂ atmosphere at 37°C. To assess the effects on LTB₄ production, cells were directly incubated with licofelone for 15 minutes and subsequently stimulated with ionophore A23187 (5 µM) for an additional 15 minutes. PGE₂ and LTB₄ levels were
determined by EIA. Results are expressed as the mean ± SEM of 3-4 different experiments performed in duplicate. †, P<0.05 with respect to untreated cells. *, P<0.05, **, P<0.01 and ***, P<0.001 with respect to cells not exposed to the inhibitor (0 µM concentration).

Figure 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 hours and subsequently exposed to PMA (50 nM) and ionophore A23187 (2 µM) for an additional 4 hours in a humidified 5% CO₂ atmosphere at 37°C. IL-6 mRNA expression was determined by reverse transcription real time quantitative PCR. (B) IL-6 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 hours and stimulated with PMA and ionophore A23187 as described in A. (C) Data from cells incubated with vehicle (0.2% DMSO) or the dual COX/5-LO inhibitor ML-3000 (30 µM). Results are the mean ± SEM of 3-6 experiments. *, P<0.05, **, P<0.01 and ***, P<0.001 with respect vehicle.
**Table 1.** List of primers for RT-PCR.

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<th>Gene</th>
<th>GenBank accession No</th>
<th>Sequence primers</th>
<th>Expected product size (bp)</th>
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<td>COX-1</td>
<td>NM_008969</td>
<td>5'-TCCCGGGGCTGATGCTCTTCTTC-3' 5'-CACCAGTGCCTCAACCCCATAGTC-3'</td>
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<td>COX-2</td>
<td>NM_011198</td>
<td>5'-CAAGCAGTGGGAAAGGCTCCA-3' 5'-GGCACTTGCATTGATGGTGCT-3'</td>
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<tr>
<td>5-LO</td>
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<td>5'-CCCCGAAGCTCCCCAGTGACC-3' 5'-TCCCGGGCCTTAGTGTTGATA-3'</td>
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<td>FLAP</td>
<td>NM_009663</td>
<td>5'-GGACCGGGACTCTTGTGCTGTTA-3' 5'-GCCGGGAATCTGCTGCTTAC-3'</td>
<td>339</td>
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<tr>
<td>LTC4S</td>
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<td>LTA4H</td>
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<tr>
<td>12/15-LO</td>
<td>NM_007440</td>
<td>5'-CCCACCGCGATTTTCCACG-3' 5'-AGTCCTCAGCAGCCACTCATCA-3'</td>
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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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<td>PGE₂</td>
<td>LTB₄</td>
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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±0.6</td>
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</tbody>
</table>

Raw 264.7 cells were incubated with licofelone (30 µM) or a combination of SC-236 (1 µM) and CJ-13,610 (1 µM). Results are expressed as percentage (%) inhibition of PGE₂ or LTB₄ formation with respect to vehicle. *, P<0.01 for licofelone versus SC-236+CJ-13,610.
Table 3. Comparison of the inhibitory effects between CJ-13,610 and AA-861 on LTB₄ biosynthesis.

<table>
<thead>
<tr>
<th>% inhibition LTB₄ biosynthesis</th>
<th>0.1 µM</th>
<th>1 µM</th>
<th>10 µM</th>
<th>15 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJ-13,610</td>
<td>91.5 ± 2.2</td>
<td>93.4 ± 1.9</td>
<td>90.7 ± 3.1</td>
<td>ND</td>
</tr>
<tr>
<td>AA-861</td>
<td>ND</td>
<td>95.0 ± 1.0</td>
<td>88.2 ± 2.2</td>
<td>95.8 ± 1.3</td>
</tr>
</tbody>
</table>

Results are expressed as percentage (%) inhibition of LTB₄ formation with respect to vehicle.

ND, not determined
Figure 1
Figure 2

A

Wild-type mice

Necroinflammatory Injury (Score)

Placebo SC-236

MCP-1 mRNA Expression (Relative Units)

Placebo SC-236

Sirius Red (% positive staining)

Placebo SC-236

B

5-LO knockout mice

Necroinflammatory Injury (Score)

Placebo SC-236

MCP-1 mRNA Expression (Relative Units)

Placebo SC-236

Sirius Red (% positive staining)

Placebo SC-236

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Figure 3

A

B

C

D

E

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Figure 4

A

IL-6 mRNA Expression (Relative Units)

SC-236   -   +   -   -   +   +
CJ-13,610 -   -   +   -   +   -
AA-861   -   -   -   +   -   +

B

IL-6 mRNA Expression (Relative Units)

CP-105,696 -   +   -
MK-571   -   -   +

C

IL-6 mRNA Expression (Relative Units)

Licofelone -   +