Prevention of Fibrosis Progression in CCl4-Treated Rats: Role of the Hepatic Endocannabinoid and Apelin Systems

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ABSTRACT
Endocannabinoids behave as antifibrogenic agents by interacting with cannabinoid CB2 receptors, whereas the apelin (AP) system acts as a proangiogenic and profibrogenic mediator in the liver. This study assessed the effect of long-term stimulation of CB2 receptors or AP receptor (APJ) blockade on fibrosis progression in rats under a non-discontinued fibrosis induction program. The study was performed in control and CCl4-treated rats for 13 weeks. Fibrosis-induced rats received a CB2 receptor agonist (R,S)-3-[2-iodo-5-nitrobenzoyl]-1-(1-methyl-2-piperidinylmethyl)-1H-indole (AM1241) (1 mg/kg b.wt.), an APJ antagonist (Ala[3]-apelin-13 sequence: Gin-Arg-Pro-Ang-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Ala (F13A) (75 μg/kg b.wt.), or vehicle daily during the last 5 weeks of the CCl4 inhalation program. Mean arterial pressure (MAP), portal pressure (PP), hepatic collagen content, angiogenesis, cell infiltrate, and mRNA expression of a panel of fibrosis-related genes were measured in all animals. Fibrosis-induced rats showed increased hepatic collagen content, reduced MAP, portal hypertension, and increased expression of the assessed messengers in comparison with control rats. However, fibrotic rats treated with either AM1241 or F13A had reduced hepatic collagen content, improved MAP and PP, ameliorated cell viability, and reduced angiogenesis and cell infiltrate compared with untreated fibrotic rats. These results were associated with attenuated induction of platelet-derived growth factor receptor β, α-smooth muscle actin, matrix metalloproteinases, and tissue inhibitors of matrix metalloproteinase. CB2 receptor stimulation or APJ blockade prevents fibrosis progression in CCl4-treated rats. The mechanisms underlying these phenomena are coincident despite the marked dissimilarities between the CB2 and APJ signaling pathways, thus opening new avenues for preventing fibrosis progression in liver diseases.

Introduction

Inflammation and remodeling are orchestrated responses ultimately directed to promoting tissue repair after organ injury. However, maintenance of the injury results in the activation of a profibrogenic cascade of events in chronic liver disease that finally leads to cirrhosis. Cirrhosis is a major determinant of morbidity and mortality and predisposes to hepatic failure and primary liver cancer. Therefore, halting the progression of fibrosis to cirrhosis has largely been considered to be a foremost goal in patients with liver disease (Friedman, 2010). Inflammation is an early event in the history of the disease. It occurs before the onset of significant clinical manifestations and becomes chronic during the evolution of the illness, particularly in patients with viral infection (Marra, 1999). This dynamic inflammatory state has been associated with liver fibrogenesis and fibrosis in experimental cirrhosis (Muñoz-Luque et al., 2008). In addition,
many inflammatory mediators have direct angiogenic activities and, in turn, angiogenesis contributes to the perpetuation and the amplification of the inflammatory state by promoting the recruitment of inflammatory cells in the neovasculature (Morales-Ruiz and Jiménez, 2005; Tugues et al., 2007). Therefore, inflammation, fibrogenesis, and angiogenesis are three closely related phenomena in chronic liver disease.

Under this scenario, suitable targets for anti-fibroproliferative therapies should include molecules that are critical in fibrosis progression and also possess inflammatory and/or angiogenesis-related properties. In this regard, two recently characterized endogenous hepatic systems are attracting increasing attention, namely the hepatic endocannabinoid and apelin systems. Endocannabinoids are lipid-related molecules participating in a wide range of physiological functions including neuroprotection, pain and motor function, energy balance and food intake, cardiovascular homeostasis, immune and inflammatory responses, and cell proliferation (Pacher et al., 2006; Reichenbach et al., 2010; Tam et al., 2011). These effects are mediated by interaction with two different types of receptors, the CB1 and CB2 receptors. Of interest, the CB1 receptor in the liver has been shown to mediate profibrogenic effects (Teixeira-Clerc et al., 2006) and has also been implicated in the pathogenesis of alcoholic and nonalcoholic liver disease (Hézode et al., 2008; Jeong et al., 2008). On the other hand, CB2 receptor agonism shows opposite antifibrogenic and anti-inflammatory effects in hepatic and nonhepatic tissue (Muñoz-Luque et al., 2008; Akhmetshina et al., 2009) and protects against liver ischemia-reperfusion injury (Horváth et al., 2011). Previous studies by our laboratory have demonstrated that the proangiogenic peptide, apelin (AP), is up-regulated in HSCs of patients with cirrhosis (Melgar-Lesmes et al., 2010). Furthermore, this peptide behaves as a paracrine mediator of fibrogenesis-related gene induction in human HSCs (Melgar-Lesmes et al., 2010), and apelin receptor (APJ) blockade has shown to be effective in reducing hepatic fibrosis and angiogenesis in rats with cirrhosis (Principe et al., 2008). Recent studies have also suggested that the apelin system is involved in inflammation and in endothelial cell proliferation (Masri et al., 2004; Daviaud et al., 2006).

In the present investigation, we sought to examine new therapeutic strategies to prevent the progression of fibrosis in injured livers of rats chronically receiving increasing doses of CCl4. We assessed the changes in messenger expression in injured livers of rats chronically receiving increasing therapeutic strategies to prevent the progression of fibrogenesis-related gene induction in human HSCs (Melgar-Lesmes et al., 2010), and apelin receptor (APJ) blockade has shown to be effective in reducing hepatic fibrosis and angiogenesis in rats with cirrhosis (Principe et al., 2008). Recent studies have also suggested that the apelin system is involved in inflammation and in endothelial cell proliferation (Masri et al., 2004; Daviaud et al., 2006).

In the present investigation, we sought to examine new therapeutic strategies to prevent the progression of fibrosis in injured livers of rats chronically receiving increasing doses of CCl4. We assessed the changes in messenger expression of a panel of genes involved in inflammation and/or tissue remodeling and the antiangiogenic, antifibrotic, and anti-inflammatory effects induced by either long-term administration of a specific CB2 receptor agonist or a selective APJ receptor antagonist in rats with experimentally induced fibrosis.
calculation of the net fibrosis area. The amount of fibrosis measured in each animal was analyzed, and the average value is presented as a percentage.

To determine the degree of hepatic apoptosis, we used the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay to detect cell death using a fluorescein-FragEL DNA Fragmentation Detection Kit (Calbiochem, San Diego, CA) according to the manufacturer’s protocol. To quantify and compare the rates of cell death between groups, a semiquantitative scoring method was used. For each sample, the number of TUNEL-positive cells was counted per 200 x high-power field. At least eight representative fields were evaluated for each experimental group, from which an average value was calculated.

Hepatic Messenger Expression of a Panel of Profibrogenic Genes in Fibrotic Rats. Liver specimens were obtained from each animal, washed in 0.1% diethyl pyrocarbonate-treated phosphate-buffered saline salt solution (140 mM NaCl, 8.5 mM Na2HPO4, and 1.84 mM NaH2PO4·H2O, pH 7.4), immediately frozen in dry ice, and stored in liquid nitrogen. Liver samples from treated and untreated animals were also fixed in 10% buffered formalin for further hematoxylin and eosin and immunostaining analysis. Total RNA was extracted from the middle liver lobe of control and fibrotic rats using a commercially available kit (RNAeasy; QIAGEN, Hilden, Germany). The RNA concentration was determined by spectrophotometric analysis (ND-100 spectrophotometer; Thermo Fisher Scientific, Waltham, MA). One microgram of total RNA was reverse-transcribed using a cDNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Specific primers and probes used for the different genes studied were designed to include intron spanning using the Universal ProbeLibrary Assay Design Center through ProbeFinder version 2.45 software (Roche Diagnostics, Indianapolis, IN; https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). A panel of selected profibrogenic genes was analyzed. The panel included the following: platelet-derived growth factor receptor β (PDGFRβ) (probe 69; left 5′-GGCGAGCGCATCTATATCT-3′ and right 5′-GGCGAGCCATGCTATATC-TATATC-3′), transforming growth factor β receptor 1 (TGFβR1) (probe 53; left 5′-AAGGGCAATATCCTGACA-3′ and right 5′-ATTGGGACATCCTACTCAAG-3′), collagen-α2 (ColIα2) (probe 95; left 5′-GAACCTTGCGAGAGAGGAGT-3′ and right 5′-ATCCA-GACCTGGTTGTGCTCTC-3′), α-smooth muscle actin (α-SMA) (probe 78; left 5′-CATCGAGAACCCTGGAGACC-3′ and right 5′-AGGCATT-TGTCACACGACAGA-3′), tissue inhibitor of metalloproteinases type 1 (TIMP1) (probe 95; left 5′-CATGGAGACCTTGCTGTTGAT-3′ and right 5′-TGGTCAATCTGCTGGTCT-3′), matrix metalloproteinase 2 (MMP2) (probe 73; left 5′-GACAAGAGCATTGAATATCACC-3′ and right 5′-GCCATCCCTTGCTCCCTC-3′), lung surfactant protein B (SP-B) (probe 51; left 5′-CGTCCATCCAGCTCAT-3′ and right 5′-ACCTGCAAAGCTCATCAGG-3′), and hyaluronan synthase 2 (HAS2) (probe 59; left 5′-CTGGATCTTCGTCCTTCC-3′ and right 5′-CTGGTATATCCATCTCAGT-3′). The mean CT of duplicate measurements was used in the initial sample. The mean CT of duplicate measurements was used to calculate ΔCT as the difference in CT for target and reference. The relative quantity of product was expressed as fold induction of the target gene compared with the reference gene according to the formula 2−ΔΔCT, where ΔΔCT represents ΔCT values normalized with the mean ΔCT of control samples.

Western Blot Analysis of Activated Caspase-3, PDGFRβ, and TIMP1. Hepatic tissue from treated and nontreated rats was individually homogenized as described previously (Muñoz-Luque et al., 2008). To detect PDGFRβ, TIMP1, and activated caspase-3, 80 μg of total denatured proteins were loaded on a 7% (PDGFRβ) and 12% (TIMP1 and caspase-3) SDS-polyacrylamide gel (Mini-PROTEAN III; Bio-Rad Laboratories, Hercules, CA). Gels were transferred for 2 h to nitrocellulose membranes of 0.45 μm for PDGFRβ and to 0.2 μm for TIMP1 and caspase-3 and blocked with 5% nonfat milk for PDGFRβ and 1% bovine serum albumin for TIMP1 and caspase-3 in TTBS buffer at room temperature for 2 h. All membranes were stained with Ponceau S Red as a control for protein loading and were then incubated overnight at 4°C with rabbit polyclonal anti-PDGFRβ (1:1000; Cell Signaling Technology, Danvers, MA), anti-TIMP1 (1:1000; Abcam, Cambridge, UK), and anti-activated caspase-3 (1:300 dilution; Abcam) for 24, 24, or 48 h, respectively. The bands were visualized by chemiluminescence (Lumi-Light Western blotting substrate; Roche Diagnostics).

Intravenous Injection of CB2 Receptor Agonist or the APJ Antagonist. Of note, intravenous injection of the CB2 receptor agonist or the APJ antagonist resulted in an approximately 50% reduction in the degree of portal hypertension. Chronic administration of the CB2 receptor agonist resulted in an approximately 50% reduction in the degree of portal hypertension. Chronic administration of the APJ antagonist did not result in a significant reduction in the degree of portal hypertension. Chronic administration of the CB2 receptor agonist or the APJ antagonist did not result in a significant reduction in the degree of portal hypertension.

Results

Liver Function Tests, Mean Arterial Pressure, and Portal Pressure in Treated and Nontreated Fibrotic Rats. Table 1 shows the biochemical tests of liver function, serum electrolytes, and systemic hemodynamics in fibrotic rats. As anticipated, in both experimental groups, fibrotic animals receiving vehicle showed the characteristic alterations of liver function tests, arterial hypotension, and significant portal hypertension. Chronic administration of the CB2 receptor agonist resulted in an approximately 50% reduction of ALT and AST levels in serum. No other significant effects were observed in the biochemical parameters measured between vehicle-treated rats and rats receiving either the CB2 receptor agonist or the APJ antagonist. Of note,
TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 10)</th>
<th>CB2 Stimulation (n = 10)</th>
<th>AM1241 (n = 10)</th>
<th>APJ Blockade (n = 10)</th>
<th>F13A (n = 7)</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>399 ± 8</td>
<td>415 ± 7</td>
<td>403 ± 12</td>
<td>379 ± 10</td>
<td>380 ± 7</td>
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<tr>
<td>AST, U/l</td>
<td>117 ± 23</td>
<td>582 ± 58†††</td>
<td>295 ± 46***</td>
<td>373 ± 72††</td>
<td>431 ± 113</td>
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<tr>
<td>ALT, U/l</td>
<td>12.8 ± 1</td>
<td>326 ± 29†††</td>
<td>199 ± 60*</td>
<td>276 ± 89††</td>
<td>282 ± 46</td>
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<tr>
<td>Lactate dehydrogenase, U/l</td>
<td>1093 ± 106</td>
<td>1127 ± 94</td>
<td>1176 ± 344</td>
<td>1016 ± 178</td>
<td>1851 ± 619</td>
</tr>
<tr>
<td>Albumin, g/l</td>
<td>36.1 ± 0.5</td>
<td>29.3 ± 1.5†††</td>
<td>30.5 ± 0.7</td>
<td>29.2 ± 0.96†††</td>
<td>30.1 ± 0.8</td>
</tr>
<tr>
<td>Serum Na⁺, mEq/l</td>
<td>142 ± 1.6</td>
<td>141 ± 1</td>
<td>142 ± 1</td>
<td>142 ± 1</td>
<td>143 ± 1</td>
</tr>
<tr>
<td>Serum K⁺, mEq/l</td>
<td>5.1 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>4.9 ± 0.1</td>
<td>4.7 ± 0.2</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>Serum osmolality, mOsmol/kg</td>
<td>292 ± 6</td>
<td>296 ± 1</td>
<td>296 ± 2</td>
<td>303 ± 3</td>
<td>302 ± 3</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>121 ± 1</td>
<td>111 ± 3†††</td>
<td>126 ± 2**</td>
<td>110 ± 3††</td>
<td>124 ± 2***</td>
</tr>
<tr>
<td>PP, mm Hg</td>
<td>5.6 ± 0.2</td>
<td>10.2 ± 0.4†††</td>
<td>7.6 ± 0.8**</td>
<td>11.3 ± 0.6†††</td>
<td>7.5 ± 0.6***</td>
</tr>
<tr>
<td>SPP, mm Hg</td>
<td>117 ± 2</td>
<td>99 ± 3†††</td>
<td>117 ± 4***</td>
<td>100 ± 4†††</td>
<td>116 ± 2*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>395 ± 15</td>
<td>399 ± 13</td>
<td>424 ± 8</td>
<td>410 ± 12</td>
<td>421 ± 8</td>
</tr>
</tbody>
</table>

a p < 0.05, compared with vehicle-treated rats (one-way ANOVA with the Newman-Reuls post hoc test and the Kruskal-Wallis test with the Dunn post hoc test when appropriate).

** p < 0.01.

*** p < 0.001.

††† P < 0.001, compared with control rats.

††† P < 0.001.

Fig. 1. A, effect of CB2 receptor activation (AM1241) and APJ blockade (F13A) on liver fibrosis. Sirius red staining of representative liver sections obtained from control rats, rats treated with vehicle, and rats receiving AM1241 (1 mg/kg per day b.wt.) or F13A (75 μg/kg per day b.wt.). Original magnification, 100×. Quantification of relative fibrosis area was assessed in 32 fields/animal. B, bars on the right show the quantitative measurement of relative fibrosis in all the animals. Results are given as means ± S.E. *** p < 0.001.

however, long-term administration of AM1241 and F13A was associated with a significant amelioration in hemodynamic function as reflected by higher MAP and SPP and lower PP in fibrotic treated rats than in fibrotic rats receiving vehicle.

Effect of CB2 Receptor Activation and APJ Blockade on Liver Fibrosis in Fibrotic Rats. Sirius red, a dye that selectively binds collagen proteins, was used to stain the collagen fibrils in the liver of CCl₄-treated rats (Jiménez et al., 1985). As shown in Fig. 1, both groups of rats had remarkable fibrosis showing initial stages of the characteristic pattern of perivenular and periportal deposition of connective tissue with development of thin portal-to-portal septa and slight evidence of architectural distortion resulting in micronodular fibrosis. However, biopsy samples obtained from fibrotic rats receiving AM1241 and from fibrotic rats receiving F13A displayed thinner septa and more preserved hepatic parenchyma than those from nontreated fibrotic animals. This result was confirmed by the morphometric analysis of all Sirius red-stained sections in which both hepatic samples of rats collected after CB2 receptor stimulation and liver biopsy samples obtained after APJ blockade showed a significant reduction in the percentage of fibrosis area compared with that in sections of the corresponding vehicle-treated fibrotic rats (Fig. 1).

Effects of CB2 Receptor Activation and APJ Blockade on Infiltrating Cells, Vessel Density, Apoptosis, and Activated Caspase-3 Expression. To assess the density of infiltrating macrophages/macrophages in the liver tissue of both vehicle and treated rats, CD68-positive cells were quantified in the parenchymal area and the periportal area. The amount of in-
filtrated cells was significantly higher in CCl₄-treated rats compared with that in controls. Chronic treatment with the CB2 receptor agonist or the APJ antagonist significantly decreased the number of CD68-positive cells in the liver of fibrotic rats (Fig. 2). Next, to evaluate whether AM1241 or F13A treatment may exert an antiangiogenic effect, anti-vWF antibody was used to quantify the number of blood vessels. There was a significant decrease in the amount of blood vessels in both AM1241- and F13A-treated animals compared with that for vehicle (Fig. 3). To explore whether antifibrogenic treatments may modify apoptosis, we performed in situ detection of nuclear DNA fragmentation by the TUNEL assay in liver sections of treated and nontreated fibrotic rats. As a positive control of the TUNEL assay, apoptosis was induced by incubation of liver sections with DNase I. No staining was observed in the negative control in which the terminal deoxynucleotidyl transferase enzyme was omitted (data not shown). Liver sections from fibrotic rats showed positive TUNEL staining cells with immunoreactivity localized to the margin of the fibrous septa and parenchyma (Fig. 4). However, the number of positive cells for TUNEL staining significantly decreased in hepatic sections of animals treated with AM1241 and F13A compared with the vehicle group (6 ± 1 versus 3 ± 0 positive cells/field, p < 0.001 and 5 ± 0 versus 3 ± 1 positive cells/field, p < 0.001, respectively). Finally, we measured the amount of active caspase-3 in livers of control, vehicle-treated, AM1241-treated, or F13A-treated animals. As shown in Fig. 4, the amount of activated caspase-3 was significantly higher in fibrotic rats than in controls. Of interest,
both CB2 receptor stimulation and APJ blockade significantly reduced activated caspase-3 expression in the hepatic tissue of fibrotic rats. These findings indicate that chronic in vivo CB2 stimulation or APJ blockade prevents proangiogenic and apoptotic phenomena in the liver of CCl4-induced fibrotic rats.

TABLE 2

<table>
<thead>
<tr>
<th>Genes (fold change)</th>
<th>Control (n = 10)</th>
<th>CCl4-Treated Rats</th>
<th>CCl4 + AM1241 (n = 10)</th>
<th>CCl4 + F13A (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFRβ</td>
<td>1.02 ± 0.11</td>
<td>3.74 ± 0.81†</td>
<td>1.22 ± 0.27**</td>
<td>4.87 ± 0.92†††</td>
</tr>
<tr>
<td>TGFβRI</td>
<td>1.08 ± 0.21</td>
<td>1.46 ± 0.40</td>
<td>0.64 ± 0.06</td>
<td>2.05 ± 0.15†</td>
</tr>
<tr>
<td>Col1α2</td>
<td>1.04 ± 0.12</td>
<td>9.40 ± 1.31††</td>
<td>7.42 ± 1.61</td>
<td>9.80 ± 1.95†††</td>
</tr>
<tr>
<td>α-SMA</td>
<td>1.10 ± 0.02</td>
<td>9.19 ± 1.70††</td>
<td>3.57 ± 0.89**</td>
<td>6.39 ± 0.48†</td>
</tr>
<tr>
<td>MMP2</td>
<td>0.97 ± 0.08</td>
<td>8.13 ± 1.39††</td>
<td>4.35 ± 0.62</td>
<td>9.49 ± 1.23††</td>
</tr>
<tr>
<td>MMP9</td>
<td>1.04 ± 0.07</td>
<td>20.05 ± 4.12†††</td>
<td>23.5 ± 5.42</td>
<td>14.46 ± 3.88††</td>
</tr>
<tr>
<td>TIMP1</td>
<td>1.05 ± 0.18</td>
<td>12.42 ± 1.90†††</td>
<td>6.28 ± 1.10**</td>
<td>8.97 ± 1.39†††</td>
</tr>
<tr>
<td>TIMP2</td>
<td>1.02 ± 0.08</td>
<td>3.11 ± 0.35†††</td>
<td>1.89 ± 0.15**</td>
<td>3.56 ± 0.53†</td>
</tr>
</tbody>
</table>

* p < 0.05, compared with vehicle-treated rats (one-way ANOVA with the Newman-Keuls post hoc test and the Kruskal-Wallis test with the Dunn post hoc test when appropriate).
** p < 0.01.
*** p < 0.001.
† p < 0.05, compared with control rats.
‡‡ p < 0.01.
††† p < 0.05.

Fig. 4. Effect of CB2 receptor activation and APJ blockade on apoptosis. A, representative TUNEL assay in hepatic tissue of control rats and fibrotic rats receiving vehicle (CCl4) or treated with the CB2 receptor agonist (CCl4 + AM1241) or the APJ antagonist (CCl4 + F13A). The number of positive cells was determined by counting the number of positively stained cells in eight independent fields per animal (original magnification, 200×). B, bars at the bottom show the quantitative measurement of TUNEL-positive cells in all animals. C, Western blot for activated caspase-3 on liver tissue of control rats (CT), fibrotic rats receiving vehicle (CCl4), and fibrotic rats treated with either AM1241 (CCl4 + AM1241, 1 mg/kg per day b.wt.) or F13A (CCl4 + F13A, 75 μg/kg per day b.wt.) for 5 weeks. Eighty micrograms of protein was loaded per lane. D, bars at the bottom show the densitometric analysis of all the samples normalized to β-actin. Results are given as means ± S.E. a, p < 0.05; b, p < 0.01; c, p < 0.001 versus control; ††, p < 0.01; †††, p < 0.001 versus CCl4.
Effect of CB2 Receptor Activation and APJ Blockade on mRNA of Hepatic Profibrogenic Genes and Protein Expression of PDGFRβ and TIMP1. For further insight into the effect of AM1241 and F13A in fibrotic rats, we measured hepatic mRNA expression of a panel of selected genes involved in cytokine signaling (TGFβ1 and PDGFRβ), collagen synthesis (Col1α2), stellate cell activation (α-SMA), and extracellular matrix (ECM) turnover (MMP2, MMP9, TIMP1, and TIMP2) (Table 2). After 13 weeks of CCl4 treatment, all the genes analyzed were up-regulated in fibrotic rats compared with control animals. TGFβ1 was the least, albeit significantly, activated transcript (approximately a 2-fold increase), whereas the most intensely up-regulated messengers were those related to ECM turnover, such as MMP9 and TIMP1. The antifibrogenic properties displayed by AM1241 and F13A were paralleled by a decrease in mRNA expression in these genes. In fact, both the CB2 agonist and the APJ antagonist inhibited PDGFRβ expression and significantly reduced the degree of activation of HSCs as shown by the decrease in the mRNA expression of α-SMA. However, the most interesting finding was that both treatments significantly altered the expression balance of the transcripts involved in ECM turnover, thus favoring ECM degradation. In fact, although neither AM1241 nor F13A treatment modified MMP9 expression, both compounds induced a significant reduction in TIMP abundance, which, in turn, resulted in a marked increase in the MMP/TIMP gene expression ratio (Table 3).

Because inhibition of mRNA expression of PDGFRβ and TIMP1 appeared to be major contributory factors to the antifibrotic properties of both AM1241 and F13A treatments, we next assessed whether CB2 receptor stimulation or APJ blockade was also associated with lower hepatic abundance of PDGFRβ and TIMP1 proteins. As shown in Fig. 5, PDGFRβ and TIMP1 expression were significantly reduced in fibrotic rats treated with AM1241 or F13A compared with fibrotic animals treated with vehicle.

### Table 3

<table>
<thead>
<tr>
<th>Gene Ratio</th>
<th>CC14-Treated Rats</th>
<th>CB2 Stimulation</th>
<th>APJ Blockade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>AM1241</td>
<td>Vehicle</td>
</tr>
<tr>
<td>MMP2/TIMP1</td>
<td>0.91 ± 0.24</td>
<td>0.68 ± 0.21</td>
<td>1.43 ± 0.27</td>
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<tr>
<td>MMP2/TIMP2</td>
<td>2.86 ± 0.48</td>
<td>2.18 ± 0.27</td>
<td>2.80 ± 0.26</td>
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<tr>
<td>MMP9/TIMP1</td>
<td>1.79 ± 0.40</td>
<td>4.56 ± 0.96</td>
<td>2.13 ± 0.54</td>
</tr>
<tr>
<td>MMP9/TIMP2</td>
<td>6.75 ± 1.36</td>
<td>15.7 ± 3.47</td>
<td>4.39 ± 1.04</td>
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</table>

* p < 0.05; unpaired Student’s t test.

### Discussion

The results of this investigation indicate that chronic administration of either AM1241 or F13A reduces hepatic collagen deposition in rats under a non-discontinued fibrosis induction program. These findings indicate that long-term CB2 receptor stimulation or signaling disruption of the hepatic apelin system prevents fibrosis progression in CC14-treated rats.
treated rats. Our results also indicate that the molecular mechanisms ultimately underlying these phenomena are coincident despite the marked dissimilarities between the CB2 and APJ signaling pathways, thus opening new avenues for preventing fibrosis progression in liver diseases.

In fact, the chronic administration of either AM1241 or F13A to rats under a CCl4-induced fibrosis/cirrhosis protocol resulted in significantly decreased hepatic collagen deposition, which was associated with a significant amelioration in systemic and portal hemodynamics, reduced angiogenesis, inflammatory infiltrate, and apoptosis compared with that in rats under the same fibrosis induction protocol but treated with vehicle. Moreover, animals receiving the CB2 agonist also showed signs of attenuated liver inflammation as indicated by decreased serum AST and ALT enzymes. All these changes were framed by reduced expression of messengers related to PDGF signaling, HSC activation, and ECM turnover.

Our group and others have previously described the anti-fibrogenic properties of CB2 receptor stimulation in experimental models of advanced liver disease (Julien et al., 2005; Liu et al., 2008; Muñoz-Luque et al., 2008). Whereas the experimental design of these studies focused on fibrosis regression, here we assessed whether CB2 agonism is able to prevent fibrosis progression even under conditions of maintaining the hepatic injury. We administered the CB2 receptor agonist AM1241 to rats under a fibrosis induction protocol. AM1241 is among the most selective receptor agonists currently available. For CB2 and CB1 receptors, the binding affinity ($K_i$) is 3.4 and 239.4 nM, respectively, and previous experiments have provided pharmacological and biochemical evidence that AM1241 selectively activates the CB2 receptor in vivo in mice, rats, and human cell lines (Malan et al., 2001; Ibrahim et al., 2003; Yao et al., 2006). The absence of central effects induced by CB2 agonism has been the major rationale to propose this mechanism as an anti-fibrogenic therapy. However, previous studies showed that pharmacological activation of the CB2 receptor signaling pathway may also induce inflammation in adipose tissue but not in the liver (Deveaux et al., 2009). In agreement with these findings, treatment with the CB2 agonist, in addition to stopping fibrosis progression and angiogenesis, was also associated with decreased serum levels of AST and ALT and reduced inflammatory infiltrate. An interesting finding of this study was that in contrast to what we had previously found in cirrhotic rats (Muñoz-Luque et al., 2008), administration of AM1241 inhibited apoptosis in fibrotic animals. Although we do not have any experimental data to explain this phenomenon, we believe that it is probably related to the different degrees of active fibrogenesis between the two groups of CCl4-treated animals. In fact, in the former study, the rats had fully established cirrhosis and the active fibrogenesis was much lower than that in the animals of the current investigation that were within the initial phases of fibrosis development (Gressner et al., 2007; Iredale, 2007). There are a number of potential mechanisms mediating the effects of CB2 receptor stimulation on hepatic fibrosis. They are probably related to the strong abundance of these receptors in nonparenchymal and biliary cells located within and at the edges of fibrotic septa that directly mediate growth arrest and antifibrotic and proapoptotic actions in hepatic cells (Julien et al., 2005; Liu et al., 2008). Whatever the case, however, our results indicate that selective pharmacological activation of the CB2 receptor is effective in preventing fibrosis progression in experimental liver disease.

There is much experimental evidence indicating that the hepatic apelin system is an important mediator of the initiation and maintenance of the inflammatory and fibrogenic processes occurring in the cirrhotic liver (Principe et al., 2008; Melgar-Lesmes et al., 2010, 2011). In fact, AP is selectively expressed in HSCs of humans and rats with cirrhosis and markedly stimulates PDGFRβ, collagen type 1 (Col1), and cell viability in LX-2 cells, a human cell line of activated stellate cells. In contrast, APJ blockade significantly regressed hepatic fibrosis and angiogenesis in cirrhotic animals and prevented the induction of PDGFRβ and Col1 expression induced by profibrogenic agents in LX-2 cells. In the current investigation, we chemically disrupted APJ signaling using F13A. This is an analog of apelin-13 in which the phenylalanine at the C terminus of the peptide is substituted by an alanine residue that behaves as an AP-specific antagonist (Melgar-Lesmes et al., 2010). Interaction of this competitive antagonist with APJ fully abolishes the biological activity of AP (Lee et al., 2005).

Acquisition of a proliferative, proinflammatory, and contractile phenotype by quiescent stellate cells is the most characteristic response of activated HSCs to chronic liver injury. Our experiments indicate that the APJ antagonist exerts its antifibrogenic effect by acting on different steps of this process. Chronic administration of F13A strongly reduced α-SMA, a well accepted marker of hepatic myofibroblasts, suggesting that APJ antagonism in vivo represses the activation of HSCs in CCl4-treated rats.

The current investigation indicates that in addition to favoring cell viability, CB2 stimulation or APJ blockade also interferes with the production of profibrogenic mediators produced during chronic liver injury and the concomitant tissue repair. In this regard, the inhibitory effect on PDGF signaling shared by both AM1241 and F13A is noteworthy, considering that PDGF is the most potent proproliferative cytokine for HSCs (Pinzani et al., 1989; Friedman, 2008).

Pharmacological stimulation of the CB2 receptor or inhibition of AP activity also appears to affect the synthesis of molecules implicated in ECM remodeling. The net deposition of scar tissue depends on the balance between synthesis and degradation. The latter reflects the relative activity of MMPs and their inhibitor TIMPs, which are mainly produced by HSCs and other inflammatory cells (Iredale, 2007; Friedman, 2008). In experimental and human cirrhosis, fibrosis appears to be the result not only of excessive ECM synthesis but also of reduced degradation, which is caused by the up-regulation of TIMPs, inactivating the concurrently secreted MMPs (Iredale, 2007). According to these mechanisms, the untreated fibrotic rats in our experiments presented a marked induction of Col1α2 gene expression as well as a significant up-regulation of the MMPs. This result can be explained as a compensatory mechanism designed to eliminate the excess of scar tissue. However, the concomitant TIMP induction overwhelmed MMP activity, thereby leading to a net ECM deposition in the liver. ECM remodeling is indeed regulated by the balance between MMPs and TIMPs rather than by their absolute levels (Iredale, 2007). Therefore, several investigations have proposed that the inhibitory activity of TIMPs is the leading regulator of the remodeling process (Arthur,
therefore, point to PDGF signaling and TIMP1 activity as TIMP1 messenger and protein abundance. These results, expression and alter MMP/TIMP balance by decreasing noids and AP antifibrogenic effects, both inhibit PDGFR differences in the signaling pathways driving endocannabi-
through common mechanisms. In fact, despite the marked
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References

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