Protease-activated receptor 1 knockout reduces experimentally induced liver fibrosis

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1 Institut National de la Santé et de la Recherche Médicale (INSERM), U889, Bordeaux; 2 IFR 66, Université Victor Segalen Bordeaux 2; 3 Centre Hospitalier Universitaire (CHU) de Bordeaux, Hôpital Pellegrin, Department of Pathology; 4 Université Victor Segalen Bordeaux 2, Animalerie spécialisée; 5 Centre National de la Recherche Scientifique (CNRS), UMR5084 and Université Victor Segalen Bordeaux 2, Bordeaux, France

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Rullier A, Gillibert-Duplantier J, Costet P, Cubel G, Haurie V, Petibois C, Taras D, Dugot-Senant N, Deleris G, Bioulac-Sage P, Rosenbaum J. Protease-activated receptor 1 knockout reduces experimentally induced liver fibrosis. Am J Physiol Gastrointest Liver Physiol 294: G226–G235, 2008. First published October 25, 2007; doi:10.1152/ajpgi.00444.2007.—Thrombin inhibition protects against liver fibrosis. However, it is not known whether the thrombin profibrogenic effect is due to effects on blood coagulation or to signaling via protease-activated receptors (PARs). We took advantage of the lack of blood coagulation defects in PAR-1-knockout mice. Acute carbon tetrachloride (CCl4) toxicity was similar in wild-type (WT), PAR-1−/− mice, respectively, 36% and 56% decrease in PAR-1−/− and PAR-1+/− mice, respectively (P < 0.001). Similar results were obtained for area of activated fibrogenic cells (64% and 79% decrease in PAR-1−/− and PAR-1+/− mice, respectively, P < 0.001). These findings were corroborated by measurements of type I collagen, matrix metalloproteinase-2, and PDGF-β receptor mRNA levels. There was also a significant decrease in T lymphocyte infiltration in PAR-1-deficient mice. Altogether, these results suggest that thrombin profibrogenic effects are independent of effects on blood coagulation and are instead due to direct effects on fibrogenic cells and possibly on T lymphocytes.

LIVER FIBROSIS is the major complication of most chronic liver diseases, leading eventually to cirrhosis and hepatocellular carcinoma. Fibrosis deposition results from the activation of fibrogenic cells, the major cells being hepatic stellate cells and portal fibroblasts (22). Activated fibrogenic cells have a myofibroblastic phenotype characterized notably by the expression of α-smooth muscle actin (ASMA) and are responsible for a high-level synthesis of extracellular matrix components, particularly collagen I, and of extracellular matrix remodeling enzymes such as matrix metalloprotease (MMP)-2 (2). Thrombin is a multifunctional serine protease that plays a central role in hemostasis by converting soluble fibrinogen into an insoluble fibrin clot and by promoting platelet aggregation (11). Thrombin has also a cytokine-like activity exerted via specific cell surface receptors called protease-activated receptors (PARs). PARs belong to the seven transmembrane domain G protein-coupled receptor superfamily. There are altogether four PARs, three of them, PAR-1, PAR-3, and PAR-4, being thrombin receptors. All PARs are activated by cleavage of the amino-terminal sequence of the extracellular exodomain, resulting in a new amino-terminal sequence that acts as a tethered ligand and initiates signal transduction (11). PAR-1 is expressed by several human cellular types such as platelets, endothelial cells, fibroblasts, smooth muscle cells, and T lymphocytes (12, 14, 34). Besides its procoagulant effects, many studies have shown the implication of thrombin and PAR-1, its main receptor, on inflammation, fibrogenesis, and extracellular matrix remodeling in several organs and particularly in kidney and liver. Thus we (34) and others (27) have shown that PAR-1 was overexpressed in human liver fibrosis. In vitro, thrombin via PAR-1 stimulates rat fibroblasts (16, 26) and human (24) hepatic stellate cell proliferation; it regulates their migration (18) and their capacity to synthesize extracellular matrix components (17) and protease inhibitors (30). Finally, we recently demonstrated (15) that a thrombin antagonist was protective against carbon tetrachloride (CCL4)-induced fibrosis.

The profibrotic effects of thrombin could result from one of two pathways. Activation of hemostasis would result in the occurrence of fibrin deposition and microthrombi in the liver parenchyma (31), leading to hypoxia and subsequent fibrogenesis (10, 37). However, PAR-1 signaling is known to result in the increased expression of a series of extracellular matrix molecules (7, 17) and profibrogenic mediators such as connective tissue growth factor (CTGF) (8) or monocyte chemotactic protein (MCP)-1 (26) and could thus account for the profibrogenic effect of thrombin. Fiorucci et al. (16) showed that a PAR-1 antagonist reduced liver fibrosis induced by bile duct ligation. These results are in favor of a significant role of PAR-1 signaling in liver fibrogenesis. Indeed, since rodent platelets do not express PAR-1 but instead use PAR-3 together with PAR-4, PAR-1 antagonists do not alter blood coagulation (9) and their protective effects are thus the consequence of the blockade of PAR-1 function in other cells such as fibro/myofibroblasts. However, the use of pharmacological antagonists may lead to improper results because of incomplete
effects on one hand or nonspecific effects on the other (13). Thus in this study we used mice in which PAR-1 expression was abolished via homologous recombination to clarify the implication of PAR-1 in liver fibrogenesis.

MATERIALS AND METHODS

Animals

PAR-1-deficient mice were established by Connolly et al. (9) and were studied in the C57BL/6 background. We used only male animals, aged 10–12 wk and weighing 31.0 ± 2.5 g at the onset of the study. The mice were allowed food and water ad libitum and were housed at a constant temperature with a 12:12-h light-dark cycle during the study. Genotyping was performed with a tail blood sample spotted onto Flinders Technology Associates (FTA) filter paper (Whatman, Banbury, UK) that was processed according to instructions from the manufacturer. Primers PAR-1s (5'-GGA GAA AAA TGA AAG CGT CCT GC) and PAR-1as (5'-AGC CTG GCA TCA GGT GTT CTA SACC TTA TGT AC) were used to amplify a 660-bp product from the wild-type allele, whereas primer PAR-1as ne (5'-TGA GAC GTG CTA CTT CCA TTT GTC AC) in combination with PAR-1s was used to amplify a 400-bp product from the targeted allele. Fragments were amplified with Hot start polymerase (Qiagen, Venlo, The Netherlands) with a PCR buffer containing 1.5 mM MgCl₂ in a final volume of 50 μl under the following conditions: 94°C for 1 min, 61.9°C for 1 min, 72°C for 1 min for 35 cycles. PCR products were visualized by electrophoresis on 1.5% agarose gels containing ethidium bromide. Knockout, heterozygous, and paired littermate wild-type animals were used. This study was performed in accordance with the European Community Standards on the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the University of Bordeaux 2.

Carbon Tetrachloride-Induced Liver Lesions

CCl₄ acute liver toxicity. To assess the necrotic and inflammatory changes caused by acute exposure to CCl₄ (Sigma, St Quenlin-Fallavier, France), a single intraperitoneal injection of 300 μl/kg (in olive oil) was administered to PAR-1⁺/⁺, PAR-1⁻/⁻, and PAR-1⁻/⁻ mice (5 mice/group). Mice without any treatment were used as controls. Blood and liver samples were obtained after 48 h. Liver tissue was snap-frozen or fixed in 10% neutral formalin. Sera were collected for biochemical analysis.

CCl₄-induced liver fibrosis. Liver fibrosis was induced by intraperitoneal injection of 300 μl/kg body wt CCl₄ mixed with olive oil three times weekly for 6 wk. Four groups of mice were studied: three groups received CCl₄ (PAR-1⁺/⁺ mice, PAR-1⁻/⁻ mice, and PAR-1⁻/⁻ mice; 15 animals/group), whereas one control group of PAR-1⁺/⁺ mice received only the CCl₄ solvent (n = 6). Mice were killed after 6 wk of treatment, 2 days after the last injection. Blood and sera were collected. Liver samples were harvested, weighed, and rapidly sliced: sections from several lobes were fixed in 10% neutral formalin for histological examination; other parts were snap-frozen in liquid nitrogen for RNA extraction or Western blot. Spleen weight was also measured.

Measure of CCl₄ Acute Liver Toxicity

Acute liver toxicity was assessed by three different methods in a blinded fashion. First, we tested the levels of serum aminotransferases on an automated analyzer in the Biochemistry Department of Pellegrin Hospital in Bordeaux. Second, we analyzed the extent of liver peroxidation induced by CCl₄ in microsomal fractions of mouse liver by Fourier-transformed infrared (FT-IR) spectroscopy as previously described (29). Ward’s algorithm was used on OPUS 4.0 software (Bruker, Ettlingen, Germany) for spectra classification on the spectral intervals that are the most representative of the (C-O) sugar absorptions (1,130–930 cm⁻¹).

Third, the area of centrolobular necrosis was measured with a histomorphometric method based on hematoxylin-eosin and safran-stained slides. Slides were examined with a Zeiss Axiosplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany). Images were acquired with an AxioCam camera (Carl Zeiss Vision, Hallbergmoos, Germany) by means of the Axiovision image processing and analysis system (Carl Zeiss Vision), and quantitative data were obtained with a computerized image analysis (KS300, Carl Zeiss Vision). Sampling corresponded to three sections randomly taken from left, median, and right major lobes. Analysis was performed on an average of 10 fields per sample taken randomly with the ×10 objective. Necrosis was expressed as a percentage of necrotic area on the total surface of liver analyzed.

Fibrosis Assessment

Four-micrometer-thick sections from formalin-fixed paraffin embedded liver tissue were prepared and stained with picrosirius red [saturated picric acid in distilled water containing 0.1% (wt/vol) picrosirius red F3B (BDH Chemicals, Poole, UK)] to allow visualization of liver fibrosis. Sections were mounted with Eukitt (O Klinder, Freiburg, Germany) before examination. The analysis was performed on the whole surface of the sample, including three sections randomly taken from left, median, and right major lobes with the ×4 objective of a Coolscope microscope (Nikon, Champigny sur Marne, France) and Lucia version 5.0 software (Nikon) in a blinded fashion. Large centrolobular veins (diameter >150 μm) and large portal tracts were excluded. Fibrosis deposition was expressed as a percentage of picrosirius red-stained area on the total area of the sample.

Table 1. Primer sequences used for real-time quantitative RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLP0</td>
<td>F: 5'-GCC GAC GTC GAA GTC CAA CT-3'</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCA TCA GCA CCA GAC CCT TC-3'</td>
<td></td>
</tr>
<tr>
<td>Collagen α₁(I)</td>
<td>F: 5'-ATG TGC AGT TTT GTG GAC CT-3'</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAG CTG ACT TCA GGA AGT TC-3'</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>F: 5'-CTG GTC TGG TTT CTT CTA ATG CA-3'</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGG TAT CCA TCT CCA TGG TCC-3'</td>
<td></td>
</tr>
<tr>
<td>PDGF-β receptor</td>
<td>F: 5'-TTC CAG GAG TAC TAC CAG CTT-3'</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGG GGG GCT GAT GAC TAG G-3'</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>F: 5'-TTC TGG GGC TGC TGG TCA C-3'</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>R: 3'-ACA CCT GCT GCT GCT GAT C-3'</td>
<td></td>
</tr>
<tr>
<td>CTFG</td>
<td>F: 5'-CCC TAG CTG CTT ACC GAC T-3'</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAT TCC ACA GGT CTT AGA ACA GC-3'</td>
<td></td>
</tr>
<tr>
<td>PAR-1</td>
<td>F: 5'-AGC CCT CCT CTG AAC GTG CT-3'</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCG GAG AAG TAG TAG CTG ATC T-3'</td>
<td></td>
</tr>
</tbody>
</table>

RLP0, human acidic ribosomal phosphoprotein P0; PAR-1, protease-activated receptor-1; MMP-2, matrix metalloprotease-2; PDGF-β, platelet-derived growth factor-β; MCP-1, monocyte chemotactic protein-1; CTFG, connective tissue growth factor; F, forward; R, reverse.
Table 2. \( \text{CCL}_4 \) acute liver toxicity: aminotransferase levels and centrolobular necrosis in the three treated groups

<table>
<thead>
<tr>
<th></th>
<th>PAR-1(^{-/-})</th>
<th>PAR-1(^{-/-})</th>
<th>PAR-1(^{-/-})</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASAT, IU/l</td>
<td>1,002 (981-1,023)</td>
<td>2,217 (1,176-3,258)</td>
<td>1,423 (963-2,976)</td>
<td>ns</td>
</tr>
<tr>
<td>ALAT, IU/l</td>
<td>2,379 (1,443-3,315)</td>
<td>4,394 (2,047-6,741)</td>
<td>3,916 (3,333-6,564)</td>
<td>ns</td>
</tr>
<tr>
<td>Centrolobular necrosis, % of section area</td>
<td>24.25 (22.5-32)</td>
<td>32 (22.8-35)</td>
<td>29.5 (27-29.7)</td>
<td>ns</td>
</tr>
</tbody>
</table>

CCL\(_4\), carbon tetrachloride; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; ns, not significant.

Immunolabeling for ASMA was performed on serial sections of formalin-fixed paraffin-embedded liver tissue with a monoclonal antibody (Dako, Glostrup, Denmark; clone 1A4) diluted to 1/400, after heat-induced antigen retrieval (citrate buffer pH 6, 10 min). The signal was amplified by EnVision dextran polymer (Dako) and revealed with liquid diaminobenzidine substrate (Dako). Results were analyzed with the same histomorphometric method and the \( \times 20 \) objective.

**Real-Time Quantitative RT-PCR**

Total RNA was extracted from liver samples with Nucleospin RNA II (Macherey Nagel, Düren, Germany). The quality of the total RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under UV light. RNA was reverse transcribed with Superscript II (Promega, Charbonnières-les-Bains, France). Nucleotide sequences of primers for collagen \( \alpha_1 \) (I), MMP-2, platelet-derived growth factor (PDGF)-\( \beta \) receptor, MCP-1, CTGF, and PAR-1 target genes, and RLP0 (encoding the human acidic ribosomal phosphoprotein P0, used as a control) are shown in Table 1. No-template and no-reverse transcriptase PCR were also performed as controls.

All PCR reactions were performed with a Stratagene X4000 thermocycler (Stratagene, Amsterdam, The Netherlands) and the SYBR Green PCR Core reagents kit (Bio-Rad, Marnes-la-Coquette, France). Five microliters of diluted complementary DNA samples (produced from 3 ng total RNA) was added to 20 \( \mu \)l of the PCR master mix. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 45 cycles at 95°C for 15 s and 65°C for 1 min. The experiments were performed with duplicates for each data point. Each sample was normalized on the basis of its expression of the RLP0 gene as previously described (4). All amplified sequences were also verified by sequencing in a ABI prism 3130 xl Genetic Analyser (Hitachi High Technologies, Tokyo, Japan). To compare quantitative values from one sample to another, the relative expression of each gene was also normalized to the sample displaying the smallest amount of RNA, called the calibrator. Each sample-normalized value was divided by the calibrator-normalized value to give the final relative expression level.

**Hypoxia Assessment**

To detect liver hypoxic areas, intraperitoneal injection of 60 mg/kg pimonidazole (Hypoxyprobe-1 kit, Chemicon International, Temecula, CA) was performed 1 h before death. Pimonidazole is a stable and aqueous soluble molecule that specifically forms adducts in hypoxic cells. Such adducts are stable with time and can be detected by a specific monoclonal antibody called Hypoxyprobe 1-Mab-1 on formalin-fixed paraffin-embedded tissues. The signal was detected according to the manufacturer’s procedure. Briefly, after inhibition of endogenous peroxidase, sections were incubated with 0.01% Pronase 40 min at 40°C and then with the Dako blocking system for 5 min at room temperature before incubation with Hypoxyprobe 1-Mab-1 (1/50) for 45 min at room temperature. After washing, sections were incubated with biotin-SP-conjugated F(ab’\(^{2}\)) fragment of a rabbit anti-mouse IgG. The signal was detected after application of peroxidase-conjugated streptavidin followed by liquid diaminobenzidine substrate. Positive hepatocytes were counted with a Coolscope microscope (Nikon) in 12 nonoverlapping fields at the \( \times 20 \) objective.

**Assessment of T Lymphocyte Infiltration**

To study T lymphocyte infiltration, we used an immunohistochemical method with a polyclonal CD3 antibody (Dako) diluted to 1/100 after heat-induced antigen retrieval (citrate buffer pH 6, 10 min). The
staining was amplified as described above. Centrolobular CD3-positive lymphocytes were counted in 12 fields at the ×40 objective with the Coolscope microscope.

**MMP-2 Immunoblots**

Sixteen-micrometer cryostat sections were solubilized in 25 μl of 10% glycerol-1% SDS at room temperature for 15 min. Protein concentration was estimated from A280 readings. Samples normalized for protein concentration were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The blots were incubated with a MMP-2 antibody from Santa Cruz Biotechnology (sc-13594; Santa Cruz, CA), and the signals were detected with enhanced chemiluminescence.

**Statistical Analysis**

All data are expressed as medians (range). A Kruskal-Wallis test was performed to compare groups. Results were considered to be significant when \( P < 0.05 \).

**RESULTS**

**Genotyping**

About only 7% of mice born from crossing between PAR-1/−/− males. This sub-Mendelian ratio is in accordance with previous publications (9) and is due to a roughly 50% embryonic lethality of PAR-1/−/− mice resulting

<table>
<thead>
<tr>
<th>Test</th>
<th>Controls</th>
<th>PAR-1+/+</th>
<th>PAR-1+/−</th>
<th>PAR-1−/−</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>34.10 (32.2-37.7)</td>
<td>33.5 (27.4-36.3)</td>
<td>33.1 (28.9-39.4)</td>
<td>32.45 (27.6-37.6)</td>
<td>ns</td>
</tr>
<tr>
<td>Spleen wt, g</td>
<td>0.089 (0.07-0.13)</td>
<td>0.115 (0.07-0.16)</td>
<td>0.103 (0.07-0.13)</td>
<td>0.108 (0.07-0.16)</td>
<td>ns</td>
</tr>
<tr>
<td>Liver wt, g</td>
<td>1.1 (0.8-1.4)</td>
<td>1.4 (1-1.6)</td>
<td>1.4 (1-1.8)</td>
<td>1.4 (0.6-1.8)</td>
<td>0.05</td>
</tr>
<tr>
<td>Liver wt/body wt</td>
<td>0.03 (0.02-0.03)</td>
<td>0.04 (0.02-0.05)</td>
<td>0.04 (0.02-0.04)</td>
<td>0.04 (0.02-0.05)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ASAT, IU/l</td>
<td>194.5 (151-223)</td>
<td>279 (183-435)</td>
<td>290 (186-789)</td>
<td>285.5 (125-487)</td>
<td>ns</td>
</tr>
<tr>
<td>ALAT, IU/l</td>
<td>29.5 (21-39)</td>
<td>82 (59-123)</td>
<td>85 (50-151)</td>
<td>87.5 (56-143)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Fig. 2. Effect of PAR-1 deficiency on fibrosis deposition.** Fibrosis was stained with picrosirius red, and the staining was quantified by histomorphometry. A: control. B–D: mice treated for 6 wk with CCl\(_4\) [PAR-1+/+ (B), PAR-1+/− (C), PAR-1−/− (D)] (picrosirius red; ×10). E: box and whiskers plot of % of section area occupied by fibrosis. Top and bottom of each box represent 75th and 25th percentiles, respectively. Whiskers represent the range, and the line in the box represents the median value of the distribution. WT, wild type; HT, heterozygous; KO, PAR-1 gene-knockout mice. Kruskal-Wallis test showed a significant difference between groups (\( P < 0.001 \); \( n = 15 \) each for PAR-1+/+, PAR-1+/−, and PAR-1−/− animals and 6 for controls).
from hemorrhage and cardiovascular failure at midgestation because of abnormal endothelial function (19).

**CCL4 Acute Liver Toxicity**

Since CCL4-induced fibrosis is dependent on the ability of CCL4 to induce liver necrosis and inflammation, we took great care to ensure that all three groups of mice had a similar response to an acute CCL4 challenge. No mice died during this experiment. Serum aminotransferase levels were very high in animals receiving CCL4 (Table 2). There was, however, no significant difference between PAR-1\textsuperscript{+/+}, PAR-1\textsuperscript{+/−}, and PAR-1\textsuperscript{−/−} mice. Similarly, PAR-1\textsuperscript{+/−} and PAR-1\textsuperscript{−/−} mice did not differ from PAR-1\textsuperscript{+/+} mice regarding the extent of centrolobular necrosis (Table 2). Finally, we used FT-IR spectroscopy to provide a global analysis of liver peroxidation level induced by CCL4 in microsomal fractions of mouse liver. With Ward’s algorithm for spectra classification (classification is represented in dendrogram), this allowed a clear discrimination between controls and CCL4-treated animals, although the three treated groups clustered together (Fig. 1). We also measured liver infiltration by CD3-positive lymphocytes. Median value was 49.0 (range 32–95) in control untreated mice and 52.5 (47–73), 38.0 (36–59), and 45.0 (39–48) for PAR-1\textsuperscript{+/+}, PAR-1\textsuperscript{+/−}, and PAR-1\textsuperscript{−/−} mice treated with CCL4, respectively. These values did not differ significantly ($P = 0.07$ by ANOVA).

**CCL4-Induced Liver Fibrosis**

**Vital parameters and liver function tests.** During CCL4 treatment, three PAR-1\textsuperscript{+/+} mice and one PAR-1\textsuperscript{−/−} mouse died; the difference in mortality was not statistically significant between the three groups. After 6 wk of CCL4 treatment, mouse body weight and spleen weight were not different between the four groups (controls and PAR-1\textsuperscript{+/+}, PAR-1\textsuperscript{+/−}, and PAR-1\textsuperscript{−/−} treated mice), whereas liver weight and consequently liver weight-to-body weight ratio were significantly higher in all CCL4-treated groups compared with controls (Table 3); however, these three groups were not different from each other. Treatment with CCL4 also resulted in increased values of serum transaminases, with no difference between the three groups (Table 3).

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**Fig. 3.** Effect of PAR-1 deficiency on α-smooth muscle actin (ASMA) expression. Fibrogenic cell activation was assessed by staining for ASMA. The area stained positively was quantified by histomorphometry. A: control. B–D: mice treated for 6 wk with CCL4 [PAR-1\textsuperscript{+/+} (B), PAR-1\textsuperscript{+/−} (C), PAR-1\textsuperscript{−/−} (D)] (immunostaining ASMA; $\times$ 20). E: box and whiskers plot of % of section area occupied by ASMA-positive cells. Top and bottom of each box represent 75th and 25th percentiles, respectively. Whiskers represent the range, and the line in the box represents the median value of the distribution. Kruskal-Wallis test showed a significant difference between groups ($P < 0.001$; $n = 15$ each for PAR-1\textsuperscript{+/+}, PAR-1\textsuperscript{+/−}, and PAR-1\textsuperscript{−/−} animals and 6 for controls).
PAR-1 expression. PAR-1 expression was measured with real-time PCR in the livers of PAR-1+/+ control animals or those that received CCl4 for 6 wk. The median PAR-1/RLP0 value in controls was 6,802 (range 2,481–12,916), whereas it was 13,372 in CCl4-treated animals (range 9,455–22,256). The difference was highly significant (P = 0.008), in accordance with previous data (16).

Fibrosis measurement. CCl4 treatment induced fibrosis deposition predominantly located in centrilobular areas. The area of fibrosis increased by ~10-fold from 0.25 (0.13–0.35)% of field area in the control group that received only olive oil to 2.31 (1.79–3.20)% in CCl4-treated PAR-1+/+ mice. The area of fibrosis was decreased by 56% in PAR-1−/− mice (1.16%, 0.88–1.44) and by 36% (1.58%, 1.16–2.80) in PAR-1+/+ mice compared with PAR-1+/+ mice (P < 0.001) (Fig. 2).

ASMA staining was restricted to vessel walls in the control group, whereas in the CCl4-treated groups ASMA-positive cells were observed around the centrilobular areas and infiltrated the lobule. The area of ASMA-positive cells increased by 10-fold from 0.25 (0.21–0.37)% in the control group to 2.69 (1.58–3.76)% in CCl4-treated PAR-1+/+ mice. The area of ASMA-positive cells was decreased by 79% in PAR-1−/− mice (0.76%, 0.50–1.73) and by 64% (1.14%, 0.97–1.62) in PAR-1+/− mice compared with PAR-1+/+ mice (P < 0.001) (Fig. 3).

Expression of fibrosis-related molecules. We evaluated the mRNA levels of collagen α1(I), a major component of liver fibrosis, PDGF-β receptor, a marker of fibrogenic cell activation, and MMP-2, implicated in extracellular matrix remodeling, using real-time quantitative RT-PCR. The median collagen α1(I)-to-/RLP0 ratio was 2.25 (1–14) in the control group, whereas it reached 59.5 (18.80–233.20) in PAR-1+/+ mice treated for 6 wk with CCl4. As shown in Fig. 4A, PAR-1+/+ mice had a significantly lower expression of collagen α1(I) transcripts, whereas PAR-1+/+ mice showed intermediate levels (P = 0.008). The median PDGF-B receptor-to-RLP0 ratio was also increased from 1.50 (1–10.70) in the control group to 10.50 (2.80–38.90) in PAR-1+/+ mice treated for 6 wk with CCl4. There was a trend toward a decrease in PAR-1−/− mice and PAR-1−/− mice, although the difference did not reach statistical significance (P = 0.063) (Fig. 4B). The median MMP-2-to-RLP0 ratio was 1.60 (1–18.70) in the control group, whereas it was 64.45 (17–179) in PAR-1+/+ mice treated for 6 wk with CCl4. As for collagen α1(I), PAR-1−/− mice had a significantly lower expression of MMP-2 transcripts, whereas PAR-1+/− mice showed intermediate levels (P = 0.004) (Fig. 4C). MMP-2 expression was also analyzed in a subset of samples by Western blot. As shown in Fig. 4D, MMP-2 expression was, as expected, increased in CCl4-treated PAR-1+/+ mice compared with controls; the increase was abolished in PAR-1−/− mice.

Finally, we also assessed the expression of two cytokine genes known to be PAR-1 targets and relevant in the context of liver fibrosis, CTGF (8) and MCP-1 (26). The expression of both genes was increased in CCl4-treated PAR-1+/+ mice compared with controls, although modestly. In both cases, there was a decrease in PAR-1−/− mice, but it failed to reach statistical significance (Table 4).

Hypoxia. Figure 5 illustrates hypoxia staining in the different groups of animals. As previously described (10), the staining was localized preferentially in the cytoplasm of cen-

![Figure 4](http://ajpgi.physiology.org/)

Fig. 4. Effect of PAR-1 deficiency on the expression of specific transcripts. After 6 wk of CCl4, total RNA was extracted from livers and analyzed by real-time RT-PCR with primers specific for collagen α1(I) (A), platelet-derived growth factor-β receptor (PDGFRβ; B), or matrix metalloproteinase (MMP)-2 (C). Results were normalized to the expression of RLP0 transcripts. Box and whiskers plots of transcript levels are shown. Top and bottom of each box represent 75th and 25th percentiles, respectively. Whiskers represent the range, and the line in the box represents the median value of the distribution. P values are shown (n = 15 each for PAR-1+/+, PAR-1−/−, and PAR-1−/− animals and 6 for controls). D: Western blot for MMP-2 expression in liver extracts of untreated animals (controls) or in WT or PAR-1−/− animals that received CCl4.
Table 4. Expression of CTGF and MCP-1 transcripts

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>PAR-1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>PAR-1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>PAR-1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGF</td>
<td>6.6 (1.0-8.9)</td>
<td>16.7 (5.2-37.0)</td>
<td>15.1 (3.0-67.2)</td>
<td>11.3 (3.1-32.2)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>3.2 (1.0-7.3)</td>
<td>7.2 (2.8-17.8)</td>
<td>5.1 (1.2-27.1)</td>
<td>4.9 (1.7-11.4)</td>
</tr>
</tbody>
</table>

Values are medians (range) of gene expression results normalized to RLP0; n = 12 animals each for PAR-1<sup>+/+</sup>, PAR-1<sup>−/−</sup>, and PAR-1<sup>−/−</sup> and 5 for controls. Differences did not reach statistical significance.

trolobular hepatocytes. Quantitative analysis of all samples demonstrated that the median number of hypoxic hepatocytes was different among the four groups (Fig. 5; P < 0.001). The median number of hypoxic hepatocytes was 117.0 (86.0–186.0) in the control group, whereas it increased to 179.0 (67.0–231.0) in PAR-1<sup>+/+</sup> mice treated for 6 wk with CCl<sub>4</sub> (P = 0.049). In PAR-1<sup>−/−</sup> mice, it was down to control level (97.5, 62.0–145.0) (P = 0.001). However, the difference was not statistically significant between PAR-1<sup>−/−</sup> mice (162.0, 100.0–201.0) and PAR-1<sup>+/−</sup> mice.

**T lymphocyte infiltration.** Figure 6 illustrates CD3 staining in the different groups of animals. Quantitative analysis of all samples demonstrated that the median number of CD3-positive lymphocytes was different among the four groups (Fig. 6; P < 0.001). The median number of CD3-positive lymphocytes was 49.0 (32.0–95.0) in the control group, whereas it reached 178.5 (146.0–210.0) in PAR-1<sup>+/+</sup> mice treated for 6 wk with CCl<sub>4</sub>. The number of these cells decreased by 70% (87.5, 66.0–125.0) in PAR-1<sup>−/−</sup> mice and by 35% (130.0, 98.0–179.0) in PAR-1<sup>−/−</sup> mice.

**DISCUSSION**

In this work, we studied the impact of PAR-1 deficiency on mouse liver fibrogenesis in the CCl<sub>4</sub> model. We observed that complete PAR-1 deficiency led to a 56% decrease in area of fibrosis and almost 80% reduction in area of ASMA-positive cells. This was paralleled by decreased levels of collagen I, MMP-2, PDGF-<sub>B</sub> receptor, CTGF, and MCP-1 transcripts. These data indicate that PAR-1 deficiency afforded a significant protection against liver fibrosis. Animals with a single deleted allele had an intermediate protection. In addition, we found that PAR-1<sup>−/−</sup> mice had a significant reduction in the number of hypoxic hepatocytes. It is likely that this reduced hypoxia is the consequence of decreased fibrogenesis, which will improve oxygen supply (21). In turn, since hypoxia is a contributing factor to fibrogenesis (1, 10), its decrease will contribute to the reduction in liver fibrosis.
CCl₄ toxicity results from liver microsomal metabolism of CCl₄ leading to the generation of the toxic trichloromethyl radical that induces hepatocyte necrosis and apoptosis (32). This results in local inflammation that subsequently triggers fibrogenic cell activation. Since CCl₄ metabolism can be altered by a variety of environmental or genetic mechanisms, we took great care to ensure that CCl₄ toxicity was not altered in the different mouse genotypes under study. We found that PAR-1/H11001/H11001, PAR-1/H11001/H11002, and PAR-1/H11002/H11002 mice behaved similarly in response to CCl₄ regarding serum aminotransferases and the extent of liver necrosis as quantified by histomorphometry. There was also no difference in the number of T lymphocytes infiltrating the parenchyma after an acute lesion, although this infiltration was low in every group at the time point studied. We also used the recently developed method of FT-IR spectroscopy, which allows a global assessment of many biochemical parameters in the liver (29), and again found no difference among the three experimental groups. Thus it is likely that the differences we found on fibrosis and ASMA scores truly reflect the role of PAR-1 on fibrogenesis, although our data do not allow us to formally exclude the possibility of a defect in tissue repair mechanisms in PAR-1-deficient mice.

Our results confirm and extend the observations of Fiorucci et al. (16) These authors found that a synthetic PAR-1 antagonist protected against liver fibrosis in the bile duct ligation model in rats (16). Our own study adds several new pieces of information. First, it strongly suggests that PAR-1 may be a generic mediator of fibrogenesis since it is involved in liver fibrosis resulting from very different mechanisms and involving distinct populations of fibrogenic cells. Indeed, CCl₄-induced fibrosis is a highly inflammatory disease in which the main cellular actor is the hepatic stellate cell; on the other hand, bile duct-induced fibrosis is only very slightly inflammatory, and the main cell type responsible for fibrosis deposition is the portal fibroblast (3, 36). Second, the use of knockout animals in our study allows us to unambiguously ascribe a profibrogenic role to PAR-1, whereas studies with pharmacological antagonists may lead to improper results because of insufficient specificity of the molecules (13).

As pointed out above, an unresolved question is whether the reported liver profibrogenic effects of thrombin are due to its procoagulant effect or to its documented profibrogenic signaling via PARs (17, 24, 27). Our study allows us to answer this question. Indeed, mouse platelets do not express PAR-1, but instead use PAR-3 and PAR-4 to respond to thrombin. Thus PAR-1 depletion does not affect thrombin effect on platelets and altogether has no effect on blood coagulation (9). We can thus conclude that the profibrogenic effect of thrombin is
largely independent of blood coagulation and instead relies on its ability to activate PAR-1. This is also supported by data from Fiorucci et al. (16).

In addition to the direct effects of thrombin on fibrogenic cells, our results point to the possible role of T lymphocytes. T lymphocytes are now acknowledged as key contributors to liver fibrosis (35). We found that PAR-1-deficient animals had a significantly reduced infiltration of the liver with T lymphocytes. Since the expression of PAR-1 on T lymphocytes is well documented (25, 34) and since thrombin can elicit activation signals in T cells (20, 25), we suggest that PAR-1 deficiency may reduce T lymphocyte accumulation in the liver, which may participate in the decreased fibrogenic process. Since PAR-1 can also be activated by other ligands, such as activated protein C (APC) (33) or MMP-1 (5), it could be argued that the effects of PAR-1 deletion may not reflect only a defect in thrombin signaling. However, APC generation is thrombin dependent, and given that thrombin affinity for PAR-1 is much higher than that of APC the available concentrations of APC may be insufficient to activate PAR-1 (23). Regarding MMP-1, it is expressed at low levels in the liver and is not upregulated in the course of fibrosis. On the other hand, there is evidence that thrombin is generated in fibrotic liver, as evidenced by the presence of fibrin deposition in experimental injury (31) or human disease (28). Thus, although the role of alternative PAR-1 ligands cannot be completely excluded, most evidence points to thrombin as being the active PAR-1 ligand in this setting. In conclusion, we have shown that inactivation of PAR-1 induces a significant protection against liver fibrosis. These observations confirm and extend previous data obtained in the bile duct ligation model with a PAR-1 antagonist (16). Thus, altogether, these data suggest that PAR-1 antagonists may be useful in the clinical management of liver fibrosis. This is especially appealing, since contrary to direct thrombin inhibitors, PAR-1 antagonists do not provoke a bleeding risk, and there is moreover great hope that orally active molecules will be available (reviewed in Ref. 6). Finally, the fact that deletion of a single PAR-1 allele was enough to offer a very significant protection suggests that complete blockade of PAR-1 function may not be required for a therapeutic effect.

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REFERENCES


