Prevention of Liver Fibrosis by the Purinoceptor Antagonist Pyridoxal-Phosphate-6-Azophenyl-2',4'-Disulfonate (PPADS)

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Abstract. Background: Hepatic stellate cells (HSC) are important mediators of liver fibrosis. HSC express purinergic receptors for extracellular ATP that induce fibrogenesis. Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) is a highly bioavailable purinoceptor inhibitor. We sought to determine whether PPADS could prevent experimental liver fibrosis in rats. Materials and Methods: The effect of PPADS as an inhibitor of HSC purinoceptors was compared to the effect of suramin using confocal video microscopy. Rats were treated with CCl₄, dimethylnitrosamine, or common bile duct ligation in the presence or absence of PPADS. Fibrosis in liver sections was assessed using Trichrome and Sirius red stains. In HSC isolated from experimental animals, proliferation was determined by bromodeoxyuridine uptake, apoptosis was determined using Annexin V flow cytometry, and transcription of α(1)-procollagen and fibronectin were determined using quantitative RT-PCR. Results: Both PPADS and suramin inhibited HSC purinoceptor activation, but PPADS had a more durable effect. PPADS completely blocked the development of cirrhosis due to CCl₄ or dimethylnitrosamine but not due to bile duct ligation. PPADS inhibited HSC proliferation, but had no effect on HSC apoptosis. PPADS inhibited transcription of α(1)-procollagen and fibronectin by HSC. Conclusion: Blockade of purinergic receptors is a novel approach to prevention of non-biliary liver fibrosis. The primary action of PPADS is to inhibit HSC proliferation and fibrogenesis. Future design of purinergic receptor inhibitors may be an effective pharmacologic treatment to prevent liver fibrosis.

Abbreviations: PPADS: Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate; DMN: dimethylnitrosamine; RT-PCR: reverse-transcriptase polymerase chain reaction.

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(22). Since P2Y receptors induce fibrogenesis by HSC, and PPADS injection can block organ P2Y receptors, we hypothesized that PPADS may be an effective pharmacologic treatment to prevent liver fibrosis. We tested this hypothesis by evaluating the effect of PPADS at preventing liver fibrosis induced by CCl₄. Here we demonstrate that PPADS markedly inhibited CCl₄-induced liver fibrosis. We further demonstrate that the effect of PPADS is due to blockade of basement membrane component synthesis by HSC and blockade of HSC proliferation.

Materials and Methods

Materials. Suramin, PPADS and dimethylnitrosamine (DMN) were purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals were of the highest quality available.

Experimental animals. Adult male Sprague-Dawley rats (180-250 g) were used for all experiments. Treatment of animals was within the prescribed guidelines of the Yale University Institutional Animal Care and Use Committee.

Isolation and culture of HSC from normal rats. HSC were isolated from normal adult male rats by in situ Pronase/collagenase perfusion followed by Nycodenz density gradient centrifugation, as described previously (20). HSC were plated on glass coverslips and used for microscopic studies 7 days after isolation, at which time the cells are known to be typical of activated HSC (23).

Confocal video microscopic determination of cytosolic Ca²⁺ signals. ATP-induced Ca²⁺ signals in HSC were monitored using confocal video microscopy as described previously (20). Briefly, HSC were loaded with the Ca²⁺-sensitive fluorophore fluo-4/AM (Molecular Probes) and mounted on a specially designed stage for use on a confocal microscope. HSC were also loaded with either suramin or PPADS (50 µM each) for 15 min prior to stimulation. HSC were then perfused with ATP (100 µM), and serial changes in fluo-4 fluorescence were determined using a Zeiss LSM 510 confocal imaging system. Fluorescence was excited using a Kr/Ar laser at 488 nm; emitted fluorescence >515 nm was collected. In separate experiments, HSC were loaded with either suramin or PPADS as described above, then washed x 2 and placed in HEPES buffer alone for 10 min. ATP-induced changes in fluo-4 fluorescence were then observed as described above.

Experimental liver fibrosis models and PPADS protocol. For CCl₄ treatment, rats (180-250 g) were provided drinking water containing 1% Phenobarbital starting two weeks prior to and during the entirety of CCl₄ treatment, in order to up-regulate cytochrome P₄₅₀ enzyme levels (24). Rats were injected subcutaneously with CCl₄ (0.1 ml, CCl₄ diluted 1:6 in olive oil) three times per week for 6-8 weeks.

For control experiments, rats were injected in identical fashion with olive oil alone (CCl₄-free vehicle). For dimethylnitrosamine (DMN) treatment, rats were injected intraperitoneally with 100 µl DMN (13.6 g/L) diluted 1:100 with 0.15 mol/L sterile saline per 100 g body weight (25). The injections were given three times per week for 10 weeks.

Common bile duct ligation (BDL) was performed as described previously (26). Briefly, rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The abdomen was washed with isopropl alcohol, and midline laparotomy was performed. The common bile duct was exposed using blunt dissection and ligated with dual sutures. Animals were used 2 weeks after BDL.

Assessment of liver histology. Rat livers were fixed in 4% paraformaldehyde in PBS, sectioned in 10-µm thick slices, and stained with either Masson Trichrome or Sirius red stains (27). Trichrome sections were observed using light microscopy, and Sirius red sections were observed using polarized light microscopy.

Isolation of HSC from rats with animal models of cirrhosis. HSC were isolated from rats treated with CCl₄ or DMN ± PPADS by a modification of the Nycodenz gradient method. After in situ Pronase/collagenase perfusion, cells underwent Nycodenz gradient centrifugation. However, in this case, the upper layers were carefully removed until a cell-dense pellet was obtained. Cells from the pellet were then plated for subsequent functional or molecular studies. These cells were shown to be typical of activated hepatic stellate cells by typical light microscopic morphology and homogeneous expression of α-smooth muscle actin in a stress-fiber distribution.

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Assessment of HSC proliferation. Cell proliferation was measured by BrdU ELISA as described previously (26) (Cell Proliferation ELISA, BrdU Colorimetric, Roche Diagnostics GmbH, Nonnenwald, Germany). HSC were loaded with BrdU for six hours then fixed and denatured, and anti-BrdU antibody was added. Colorimetric substrate was added, and BrdU incorporation was quantitated using a multi-plate reader.

Assessment of HSC apoptosis. HSC apoptosis was determined using Annexin V-PE staining according to manufacturer instructions (BD Biosciences, San Jose, CA, USA). Briefly, HSC were isolated, plated on 96-well plates, washed, and stained. Positive cell fractions were identified by fluorescence-activated cell sorting (FACS).

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Alterations in expression of α(1)-procollagen (procollagen-1) or fibronectin mRNA in HSC were determined using real-time RT-PCR. Total RNA was isolated using standard methods and subjected to real-time PCR using the ABI-PRISM 7700 (Applied Biosystems, Foster City, CA, USA). Detection of procollagen-1 or fibronectin was accomplished by labeling with 6-FAM normalized to a VIC-labeled GAPDH probe. PCR was performed using the following cycling parameters: reverse transcription at 48°C x 30 min; activation of AmpliTaq polymerase (Applied Biosystems) at 95°C x 10 min; PCR cycling 40 cycles of 95°C x 15 sec (denaturation) and 60°C x 1 min (annealing/extension).

Statistical analysis. Data are expressed as mean±standard deviation where appropriate. Comparisons between individual groups were made with two-tailed t-tests.

Results

Blockade of HSC P2Y receptor activation by PPADS. The effect of PPADS on ATP-induced Ca²⁺ signals in activated rat HSC (primary HSC 7 from rat liver days after isolation) was
compared to the effect of suramin, which has previously been shown to inhibit ATP-induced Ca²⁺ signals in these cells (20). Both PPADS and suramin completely inhibited ATP-induced Ca²⁺ signals in activated HSC (n≥5 per experiment; not shown). However, PPADS had a more durable effect on inhibition of ATP-induced Ca²⁺ signals. Activated HSC were treated with suramin or PPADS had an approximate two-fold increase in fluo-4 fluorescence after treatment with ATP. Similar changes were noted when cells were treated with suramin, washed, then treated with ATP (‡p=NS vs. ATP alone). However, cells treated with PPADS then washed had no increase in fluo-4 fluorescence after ATP treatment (*p=0.002 vs. control; p=0.02 vs. suramin).

**Effect of PPADS on normal liver.** To determine whether PPADS treatment had any adverse effect on normal rats, animals were treated with either PPADS or vehicle alone (n=5 rats/group). PPADS had no effect on normal liver histology. Furthermore, no differences in animal behavior, feeding, or weight were noted. Thus, PPADS alone had no discernable effect on normal rat liver or other rat function.

**Effect of PPADS on CCl₄-induced liver fibrosis.** The effect of PPADS on CCl₄- induced liver fibrosis was determined by liver histology. Liver sections were obtained from rats treated with CCl₄ ± PPADS and stained with either Trichrome or Sirius red stains. As seen in Figure 2, PPADS blocked the development of cirrhosis induced by CCl₄ completely.

**Effect of PPADS on serum markers of liver inflammation and dysfunction.** The effect of PPADS, CCl₄, and CCl₄ + PPADS was determined on the following serum markers: albumin, bilirubin, ALT, AST and GGT. No differences were noted between CCl₄-treated rats and CCl₄ + PPADS-treated rats for any of the serum markers tested (Table I). These findings suggest that PPADS has no effect on liver inflammation or function.

**Effect of PPADS on HSC proliferation and apoptosis.** The effect of PPADS administration on HSC proliferation was assessed by BrdU uptake. HSC were isolated from control, CCl₄-treated, and CCl₄ + PPADS-treated rats. CCl₄ induced a modest increase in HSC proliferation that was completely inhibited by PPADS administration (Figure 3). The effect of PPADS administration on HSC apoptosis was determined by Annexin V FACS. HSC were isolated from control, CCl₄-treated, and CCl₄ + PPADS-treated rats. Annexin V staining was present in 14.9% of HSC from control rats, 50.2% of HSC from CCl₄-treated rats, and 50.9% of HSC from CCl₄ + PPADS treated rats. Taken together, these findings demonstrate that PPADS inhibits the increase in HSC proliferation induced by CCl₄ treatment but has no effect on HSC apoptosis.

**Effect of PPADS on transcription of α(1)-procollagen and fibronectin.** The effect of PPADS administration on transcription of α(1)-procollagen (procollagen-1) and fibronectin was determined by real-time quantitative RT-PCR. HSC were isolated as described above, RNA was isolated, and quantitative RT-PCR was performed. As seen in Figure 4, CCl₄ treatment induced a threefold increase in procollagen-1 transcription and twofold increase in

### Table I. Changes in serum markers in experimental models.

<table>
<thead>
<tr>
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<th>Control</th>
<th>PPADS</th>
<th>CCl₄</th>
<th>CCl₄ + PPADS</th>
</tr>
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<tbody>
<tr>
<td>Albumin</td>
<td>3±0.1</td>
<td>3.2±0.0</td>
<td>2.9±0.1</td>
<td>2.8±0.06</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.05±0.03</td>
<td>0.12±0.01</td>
<td>0.32±0.04*</td>
<td>0.27±0.02**</td>
</tr>
<tr>
<td>ALT</td>
<td>84.5±12.5</td>
<td>81.3±24.4</td>
<td>118±40</td>
<td>128±20</td>
</tr>
<tr>
<td>AST</td>
<td>155±45</td>
<td>122±29</td>
<td>299±87*</td>
<td>296±51**</td>
</tr>
<tr>
<td>GGT</td>
<td>3±0.0</td>
<td>3±0.0</td>
<td>34.7±13.4*</td>
<td>27.7±15.5**</td>
</tr>
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*p<0.05 vs. control; **p=NS vs. CCl₄; p<0.05 vs. PPADS.
Figure 2. PPADS blocks liver fibrosis induced by CCl$_4$ administration. Rats were treated with either CCl$_4$ alone or CCl$_4$ + PPADS for 6-8 weeks as described in the Materials and Methods section. Animals were sacrificed, and livers were sectioned and stained with Trichrome stain or Sirius red stain. A) Trichrome stained micrographs. As seen in the representative images, CCl$_4$-treated animals developed bridging fibrosis and nodule formation; however, neither bridging fibrosis nor nodules were noted in CCl$_4$ + PPADS-treated animals. B) Sirius red stained micrographs. The extent of collagen deposition can be better quantitated in the Sirius red stained micrographs. Bridging fibrosis and nodule formation are clear in the representative image from the CCl$_4$-treated animals; however, these features are absent in the representative micrograph from CCl$_4$ + PPADS-treated animals. Images shown are representative of >10 animals per group.
fibronectin transcription, which was completely inhibited by PPADS administration. These findings demonstrate that PPADS inhibits the increase in procollagen-1 and fibronectin transcription induced by CCl4 treatment.

**Effect of PPADS on DMN-induced and BDL-induced liver fibrosis.** To ensure that the effects of PPADS on development of liver fibrosis were not an artifact of the CCl4 model of cirrhosis, rats were treated with either DMN ± PPADS or BDL ± PPADS. As in CCl4 cirrhosis, DMN cirrhosis was completely inhibited by PPADS administration (Figure 5A). However, BDL cirrhosis was not at all inhibited by PPADS administration (Figure 5B). These findings provide further evidence that PPADS inhibits development of non-biliary but not biliary liver fibrosis in the models examined.

**Discussion**

These studies have demonstrated that the purinergic receptor antagonist PPADS prevents development of experimental liver fibrosis and have provided at least two potential mechanisms of action for PPADS: blockade of transcription of basement membrane proteins and inhibition of HSC proliferation. Previously, we reported that purinergic signals activated transcription of basement membrane proteins in HSC from normal livers (20). However, this is the first evidence that extracellular nucleotides may regulate proliferation of HSC. Both of these findings themselves prompt two important questions: (i) What is the source of extracellular nucleotides in the liver acting on HSC? (ii) How does activation of HSC P2Y receptors induce protein transcription and proliferation? The first question as to the source of extracellular nucleotides in the liver has several possible answers. Since ATP is present in cells in millimolar concentrations, yet P2Y receptors are activated by high nanomolar to low micromolar concentrations of ATP and other nucleotides, injury of hepatocytes and other liver cells is a plausible source of ATP. An alternative potential mechanism for release of nucleotides such as ATP in the liver is regulated release. Regulated release may take place via stored nucleotide granules or vesicles; this mechanism is important in control of platelet aggregation (28) and neurotransmission (29). Regulated release of ATP may also take place through secretory channels. Evidence suggests that ATP-binding cassette (ABC) proteins such as CFTR and the hepatocyte canalicular transporters regulate
Figure 5
Figure 5. PPADS blocks cirrhosis due to dimethylnitrosamine (DMN) administration, but not due to common bile duct ligation (BDL). A) Effect of PPADS on DMN cirrhosis. Rats were treated with DMN ± PPADS as described above, and livers were harvested for Trichrome and Sirius red staining. DMN-treated animals developed fibrosis bridging from central-to-central area and nodule formation. However, neither feature was noted in rats treated with DMN + PPADS. B) Effect of PPADS on BDL cirrhosis. Rats underwent BDL as described above, and livers were harvested for Trichrome and Sirius red staining. BDL rats developed fibrosis bridging from portal-to-portal area and nodule formation. Animals that underwent BDL and were treated with PPADS also developed these features to the same extent. Micrographs are representative of five animals per group.
channel-mediated secretion of ATP (30, 31). In fact, these mechanisms are not mutually exclusive, as platelet nucleotide-rich dense granules also express high amount of the ABC protein MRP4 (32), suggesting that this protein may contribute to ATP release. In any case, the relative contributions of these potential mechanisms in liver fibrosis certainly deserve further investigation.

The second question as to the signal transduction mechanisms regulating purinergic activation of transcription and proliferation in HSC also has several possible answers. The best-known second messenger for P2Y receptors is cytosolic Ca$^{2+}$, and nuclear Ca$^{2+}$ signals may be important regulators of transcription and proliferation (33, 34). However, several other second messengers have been identified as important downstream effectors of purinoceptors, especially with regards to proliferation. These include extracellular signal-related kinases 1/2, p38 mitogen activated kinase, and c-Jun N-terminal kinase, cAMP-dependent protein kinase, and protein kinase C (18, 19, 35, 36). Future work from our laboratory and others will hopefully clarify which, if any, of these mechanisms is important in the transduction from purinergic signal to HSC fibrogenesis and proliferation. This, in turn, may hopefully facilitate anti-fibrogenic drug design.

A final question raised by the findings of this manuscript is why PPADS blocked non-biliary but not biliary fibrosis. One potential explanation is that biliary fibrosis is mediated by distinct cells (portal fibroblasts/myofibroblasts) from HSC and that purinergic signals are not activators of fibrogenesis in portal fibroblasts. The expanding attention to the importance of portal fibroblasts in biliary fibrosis has led to the understanding that portal fibroblasts are distinct from HSC (7, 37-40) and that portal fibroblasts are of particular importance in biliary fibrosis (5, 41-43). This is consistent with unpublished data from our laboratory, which demonstrates that HSC and portal fibroblasts express distinct P2Y receptors. The current work suggests that the P2Y receptors expressed by portal fibroblasts do not link to fibrogenesis, suggesting that PPADS may only function to prevent non-biliary liver fibrosis.

In summary, we believe we have presented evidence of a novel agent for the prevention of liver fibrosis and have provided several potential mechanisms of this effect. It is our hope that this and related findings will lead eventually to an effective pharmacologic approach to the prevention of liver fibrosis in chronic liver disease, which is desperately needed.

References

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