Prolyl oligopeptidase attenuates hepatic stellate cell activation through induction of Smad7 and PPAR-γ

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Abstract. Prolyl oligopeptidase (POP) is a serine endopeptidase widely distributed in vivo with high activity in the liver. However, its biological functions in the liver have remained largely elusive. A previous study by our group has shown that POP produced N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) and thereby exerted an anti-fibrogenic effect on hepatic stellate cells (HSCs) in vitro. It was therefore hypothesized that POP may affect the activation state of HSCs and has an important role in liver fibrosis. The HSC-T6 immortalized rat liver stellate cell line was treated with the POP inhibitor S17092 or transfected with recombinant lentivirus to overexpress POP. Cell proliferation and apoptosis were determined using a Cell Counting Kit-8 and flow cytometry, respectively. The activation status of HSCs was determined by examination of the expression of α-smooth muscle actin (α-SMA), collagen I, monocyte chemoattractant protein-1 (MCP-1), transforming growth factor (TGF)-β-Smad signaling and peroxisome proliferator activated receptor-γ (PPAR-γ). Inhibition by S17092 decreased, whereas lentiviral expression increased the activity of POP and cell proliferation, while neither of the treatments affected cell apoptosis. Of note, S17092 significantly increased, whereas POP overexpression decreased the expression of α-SMA and MCP-1 without affecting the expression of collagen I and TGF-β1. Furthermore, S17092 caused a reduction, whereas POP overexpression caused an upregulation of Smad7 protein and PPAR-γ, but not phosphorylated-Smad2/3 expression. In conclusion, POP attenuated the activation of HSCs through inhibition of TGF-β signaling and induction of PPAR-γ, which may have therapeutic potential in liver fibrosis.

Introduction

Prolyl oligopeptidase (POP; enzyme ID, EC 3.4.21.26; molecular weight, 80 kDa), also known as prolyl endopeptidase (Prep), is a serine endopeptidase that hydrolyzes proline-containing peptides shorter than 30 amino acids, specifically at the carboxyl terminal of internal proline residues (1). POP was first discovered in the human uterus in 1971 (2) and later it was detected in a wide range of species and in most tissues of mammals, with the highest enzyme activity generally detected in the brain (2). Several bioactive neuropeptides, such as neurotensin, bradykinin, arginine-vasopressin (AVP), thyrotropin-releasing hormone (TRH), and substance P (SP), are known to be POP substrates in vitro. However, the functions of POP in peripheral tissues have largely remained unknown (1-4). In the liver, the protein density of POP is low, while its activity is surprisingly high (5,6). It was moderately present in the cytoplasm and nuclei of hepatocytes as well as in the nuclei of Kupffer and hepatic endothelial cells (6), but its biological functions in the liver are not well studied.

Chronic liver diseases caused by various etiologies lead to liver fibrosis, which is characterized by excessive deposition of extracellular matrix (ECM) in the liver (7). The activation of HSCs has been reported to have critical roles in the development of hepatic fibrogenesis (8). It is known that the activated HSCs are the major α-smooth muscle actin (α-SMA)-producing cells and the principle source of deposited ECM, including collagen I, collagen III and proteoglycans, in liver fibrosis (9-13).

The activation of HSCs is mainly mediated by damaged hepatocyte-derived growth factors, such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), endothelin (ET), fibroblast growth factor (FGF) and connective tissue growth factor (CTGF) (14). Among these...
growth factors, TGF-β1 is recognized as a major profibrogenic cytokine by promoting and maintaining HSC activation, proliferation, as well as collagen production and deposition through the TGF-β1/Smad pathway (15-18).

Peroxisome proliferator activated receptor-γ (PPAR-γ) was initially identified as a key regulator of adipogenesis (19), while increasing evidence has confirmed that PPAR-γ is a key factor in HSC activation and phenotypic alteration, maintaining HSCs in a quiescent phase, and suppressing the production of type I collagen, α-SMA and TGF-β1. Thus, PPAR-γ has an important role in reducing and preventing liver fibrosis (20,21). PPAR-γ can disrupt the TGF-β signaling pathway and Smad-dependent promoter activity, directly antagonizes the activation and/or function of Smad3 in fibroblasts without affecting the protein expression of stimulatory Smad3. However, PPAR-γ can increase the expression of inhibitory Smad7 (22-24). PPAR-γ can also restore the ability of HSCs to accumulate retinyl palmitate, a feature of quiescent HSCs (25-27). Together with a decrease in α-SMA expression, re-expression of PPAR-γ is thought to be an indicator of attenuated HSC activation and even transition to the quiescent state.

A previous study by our group showed that N-acetyl-serine l-aspartyl-lysyl-proline (AcSDKP), a tetrapeptide hydrolyzed from thymosin-β4 by POP (28), exerts an anti-fibrogenic effect on HSCs in vitro (29,30). Other studies also showed that thymosin-β4 has a protective effect against carbon tetrachloride-induced acute hepatotoxicity and inhibits HSC activation (31,32). However, whether POP can directly affect the activation of HSCs has remained elusive. POP was found to participate in liver inflammation (33) and regulate hepatocyte proliferation and differentiation (34,35). It was therefore hypothesized that POP may have an important role in regulating the functions of HSCs and inhibiting liver fibrosis.

To test this hypothesis, a pharmacological inhibitor of POP activity, S17092, was employed (36), as well as a lentiviral overexpression method to induce the protein expression of POP in the HSC-T6 immortalized rat liver stellate cell line. The results demonstrated that POP can attenuate HSC activation and may protect against liver fibrosis.

Materials and methods

Cell culture. HSC-T6, which is an immortalized rat liver stellate cell line that has a stable phenotype and biochemical characteristics compared to primary stellate cells (13,37), was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). HSC-T6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified incubator at 37°C with 5% CO₂.

Infection with recombinant lentivirus. A recombinant lentivirus overexpressing POP was constructed by Genechem (Shanghai, China) according to the mRNA sequence of the rat Prep gene in GenBank (NM_031324). Cytomegalovirus-driven green fluorescent protein (GFP) reporter gene, which was included in the recombinant lentivirus, was used for detecting the transduction efficiency. HSC-T6 cells were infected with empty lentivirus (mock) or POP-expressing lentivirus at a multiplicity of infection of 10 in 1 ml of an enhanced infection solution (Eni.S) containing 5 µg/ml polybrene (an infection enhancer; Genechem) for 12 h.

Cell proliferation assay. The proliferation rate of HSC-T6 cells was detected using a Cell Counting Kit-8 (CCK-8; Dojindo, Shanghai, China) according to the manufacturer's instructions. In brief, HSC-T6 cells were seeded on 96-well plates at a density of 1x10⁴ cells per well. After culturing for 24 h, cells were treated with increasing concentrations of S17092 (Sigma; Merck Millipore, Darmstadt, Germany) (0, 5, 10, 25, 50 or 100 µg/ml) or vehicle (dimethyl sulfoxide; Sigma, Merck Millipore) for 24 or 48 h. For viral infection, HSC-T6 cells were seeded at a density of 2x10⁵ cells per well, infected with mock-transfected or POP-expressing lentivirus for 12 h, and the transfection mixture was subsequently replaced with normal culture medium and cells were cultured for an additional 24 or 48 h. CCK-8 stain was added and the absorbance (optical density) at 450 nm was detected using a microplate reader (uQuant; Biotek, Winooski, VT, USA).

Cell apoptosis detection. Cell apoptosis was determined using an Annexin V-phycocerythrin (PE)/7-aminooctanoylmethionin D (AAD) Apoptosis Detection kit (cat. no. 559763; Becton-Dickinson, San Jose, CA, USA) according to the manufacturer's instructions. Cells (~2x10⁴) seeded on 3.5-cm dishes were treated with S17092 (0, 5 or 10 µg/ml) for 24 h. Cells (~5x10⁴) seeded on 3.5-cm dishes were infected with mock-transfected or POP-expressing lentivirus for 12 h, and the transfection mixture was replaced with normal culture medium and cells were cultured for an additional 24 h. Cells were subsequently harvested and stained with Annexin V-PE and 7-AAD at room temperature for 15 min. Apoptotic cells were detected using a FACSscan flow cytometer and analyzed by CellQuest software (version 5.1; Becton-Dickinson).

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from treated cells using TRIzol (Takara Bio Inc., Dalian, China) and was reverse-transcribed into complementary DNA using primer-script RT master mix (cat. no. RR036A; Takara Bio Inc.) under the following conditions: 37°C for 15 min, 85°C for 5 sec and 4°C 1 h. qPCR was performed in a 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR Premix Ex Taq (Tli RaHa; Plus cat. no. RR420A; Takara Bio, Inc.) under the following conditions: 95°C for 30 sec, 95°C for 5 sec and 60°C for 34 sec (40 cycles), 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. Primers of target genes were synthesized by Sangon Biotech (Shanghai, China) and their sequences are listed in Table I. Primer specificity was confirmed by a dissociation curve using 7500 system SDS software (Applied Biosystems). GAPDH was used as the internal control (38).

Western blot analysis. Treated cells were lysed in ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor phenylmethylsulfonyl fluoride (PMSF; Beyotime Institute of Biotechnology, Shanghai, China). Total protein was measured using the bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). Mouse
anti-α-SMA (cat. no. A5228; 1:1,000), rabbit anti-TGF-β1 (cat. no. SAB4502954; 1:1,000) and rabbit anti-POP (cat. no. SAB2104515; 1:500), rabbit anti-PPAR-γ (cat. no. AB61087; 1:1,000) were obtained from Sangon Biotech (Shanghai, China), rabbit anti-phosphorylated (p)-Smad 2/3 (cat. no. AP0326; 1:1,000) was purchased from Bioworld Technology (St. Louis Park, MN, USA), and mouse anti-beta tubulin (cat. no. AT819; 1:1,000) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). A secondary horseradish peroxidase-conjugated goat anti-mouse antibody (cat. no. A9919; 1:60,000) and goat anti-rabbit antibody (cat. no. A9169; 1:50,000) were purchased from Sigma (Merck Millipore). Western blot analysis was performed as previously described (29). Immune complexes were detected using immobilon western chemiluminescent horseradish peroxidase substrate (Millipore Corp., Billerica, MA, USA). Bands were quantified using Image Lab version 2.0.1 (Bio-Rad Laboratories, Hercules, CA, USA). Tubulin was used as a loading control.

**POP activity assay.** Intracellular AcSDKP was measured using the AcSDKP enzyme immunoassay kit (Bertin Pharma, Montigny-le-Bretonneux, France) modified for the cells (29,39). In brief, cells were lysed with RIPA buffer containing 10 µmol/l captopril and 1 mmol/l PMSF. The cell number was determined using a Fuchs-Rosenthal counting chamber. Lysates were centrifuged at 14,000 x g for 10 min and supernatants were extracted with methanol. Samples and standards were then processed according to the manufacturer's instructions.

**Statistical analysis.** Results were expressed as the mean ± standard error of the mean. At least three independent experiments were performed for each assay. Data were analyzed by Student's t-test or one way analysis of variance followed by the Mann-Whitney U test using SAS software (Release 8.02 TS Level 02 M0; SAS Institute, Cary, NC, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Pharmacological inhibition of POP activity and lentiviral induction of POP protein expression in HSC-T6 cells.** As a potent inhibitor of POP, S17092 has been considered as a promising therapeutic compound for memory impairment (40). In the present study, the effects of S17092 on HSC-T6 cells were investigated. S17092 treatment did not affect the protein levels of POP (Fig. 1A); however, it dose-dependently decreased the activity of POP as indicated by the intracellular levels of AcSDKP (Fig. 1B). To induce the activity of POP, HSC-T6 cells were transfected with a lentiviral overexpression vector. The protein levels of POP in HSC-T6 cells transduced with POP overexpression vector were ~2.5-fold increased compared with those in mock- and non-transfected control HSC-T6 cells (Fig. 1C). Consistently, the intracellular levels of AcSDKP were ~1.5-fold increased, indicating that POP activity was increased (Fig. 1D). Of note, after transduction with POP-expressing lentivirus, the mRNA levels of POP were ~8-fold increased compared to those in mock-transfected and non-transfected control HSC-T6 cells (Fig. 1E). These results showed that POP activity in HSC-T6 cells can be pharmacologically inhibited or induced using a lentivirus.

**POPs activity is essential for the proliferation of HSC-T6 cells but does not affect apoptosis.** To investigate the effect of POP on the growth of HSC-T6 cells, cell proliferation and apoptosis were tested under pharmacological inhibition and lentiviral induction conditions. S17092 inhibited the proliferation of HSC-T6 cells in a dose-dependent manner (Fig. 2A), whereas POP overexpression promoted cell proliferation by 80% compared to mock- and non-transfected control HSC-T6 cells after 48 h of incubation (P<0.05) (Fig. 2B). However, S17092 or lentiviral induction of POP did not

<table>
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<th>Gene</th>
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<tr>
<td>TGF-β1</td>
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<td>Forward: 5'-ATTCCCTGGCGTACCTTGGG-3' Reversible: 5'-AGGCCCTTATCCGCTCCT-3'</td>
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<td>POP</td>
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<td>Colla1</td>
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<td>GAPDH</td>
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<td>Forward: 5'-GGGCAGGAGCAAGATCAT-3' Reversible: 5'-CCAGTGAGTCCCGTTCAC-3'</td>
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POP, prolyl oligopeptidase; TGF, transforming growth factor; SMA, smooth muscle actin; MCP, monocyte chemoattractant protein; Colla1, collagen type I α 1 chain.
affect the apoptotic rate of HSC-T6 cells (Fig. 2C and D). These results suggested that POP increases the proliferation of HSCs in vitro.

**POP inhibits the expression of α-SMA and MCP-1 without affecting collagen I and TGF-β1.** To assess the potential influence of POP on liver fibrosis, several markers for the activation of HSCs were tested after pharmacological inhibition or lentiviral induction of POP. Compared with the vehicle, S17092 dose-dependently increased the mRNA expression of α-SMA and MCP-1 in HSC-T6 cells at the concentration of 5 and 10 µg/ml without affecting collagen I and TGF-β1 mRNA expression (Fig. 3A). By contrast, POP overexpression in HSC-T6 resulted in a significant decrease in the expression of α-SMA and MCP-1 mRNA but not of collagen I and TGF-β1 mRNA when compared to mock- and non-transfected control HSC-T6 cells (Fig. 3B). Western blot analysis further confirmed that the protein levels of α-SMA were dose-dependently increased by S17092 and significantly decreased after lentiviral induction (Fig. 3C). However, the protein levels of TGF-β1 were not affected by either S17092 or lentiviral vector. These results indicated that POP reduces the expression of pro-fibrotic genes.
**POP increases Smad7 and PPAR-γ without affecting p-Smad 2/3.** Smad proteins are intracellular mediators of the signal transduction of TGF-β (41). The protein levels of p-Smad2/3 were not affected by either S17092 treatment or POP overexpression (Fig. 4A). However, the Smad7 protein was significantly downregulated in S17092-treated cells (Fig. 4B). By contrast, Smad7 was significantly upregulated in lentivirus-induced cells compared to mock- and non-transfected control HSC-T6 cells (Fig. 4B). Of note, PPAR-γ was also markedly decreased following S17092 treatment and increased in following lentiviral induction (Fig. 4C). These results showed that POP affects the Smad7 and PPAR-γ signaling pathways.

**Discussion**

The present study showed that POP attenuated the activation of HSC-T6 cells, as indicated by the inhibited expression of MCP-1 and α-SMA. This activated phenotype may be caused by increased Smad7 and PPAR-γ levels.

Liver fibrosis is associated with complex molecular and cellular mechanisms, while HSCs are considered to have a pivotal role in this process, mainly participating in intrahepatic inflammation and excessive deposition of ECM in the liver (15,42). The results of the present study showed that POP decreased α-SMA, which is a marker for HSC activation. Furthermore, in HSC-T6 cells, POP also inhibited MCP-1, which is a crucial pro-inflammatory and pro-fibrotic cytokine mainly produced by activated HSCs in the liver (43). These results suggested that POP attenuates HSC activation and thus their pro-fibrotic features.

TGF-β signaling is critical in promoting liver fibrosis (15,16). While, according to the results of the present study, inhibition by S17092 and POP overexpression did not affect TGF-β1 expression and p-Smad2/3 levels in HSC-T6 cells, POP participated in the regulation of Smad7 levels. Following POP inhibition, Smad7 was decreased, while it was increased after POP overexpression. These data suggested an inhibitory effect of POP on TGF-β1 signaling. Smad7 is an inhibitory peptide, which blocks TGF-β1 signaling by physical interaction with the activated TGF-β receptor 1 and prevention of the docking and phosphorylation of Smad2/3, thus inhibiting its pro-fibrogenic and pro-inflammatory activities and HSC activation (44,45).

Apart from de novo expression of α-SMA, reduced PPAR-γ expression is also a marker for HSC activation (20,21). The results of the present study showed that POP inhibitor significantly decreased, while lentivirus-mediated overexpression of POP increased the expression of PPAR-γ. Together with decreased α-SMA levels in HSCs, this indicated attenuated HSC activation.

The decrease of intracellular Ac-SDKP after POP inhibition by S17092 as well as the increase of intracellular Ac-SDKP after vector-mediated overexpression of POP demonstrated that POP activity was successfully manipulated in HSC-T6 cells. It may be speculated that the effects of POP on HSCs may be mediated via Ac-SDKP, since a previous study has demonstrated the anti-inflammatory and anti-fibrotic effects of Ac-SDKP on HSCs (29). However, there are differences between the effects of POP and Ac-SDKP on HSCs. In the present study, POP inhibited TGF-β1 signaling via increasing Smad7 without affecting TGF-β1 and p-Smad2/3. This effect is different from that of Ac-SDKP, which decreases the expression of TGF-β1 and p-Smad2/3 without affecting Smad7 (29). Furthermore, Ac-SDKP inhibits HSC proliferation (29),

![Figure 4. Effects of POP on Smad proteins and PPAR-γ signaling in HSC-T6 cells. Western blot analysis of (A) p-Smad 2/3, (B) Smad7 and (C) PPAR-γ in HSC-T6 cells treated with S17092 (0, 5 or 10 µg/ml) for 24 h or transduced with mock-transfected or POP-expressing lentivirus for 3 days. *P<0.05, **P<0.01. NS, not significant; POP, prolyl oligopeptidase; PPAR, peroxisome proliferator activated receptor; p-Smad, phosphorylated Smad; mock, empty lentivirus.](image-url)
which is in contrast to the stimulatory effect of POP on the proliferation of HSCs, as shown by decreased cell proliferation after POP inhibition and increased cell proliferation after POP overexpression in the present study. Indeed, this difference has also been shown in other cell types, such as cancer cells and nervous tissue (46-48). In fact, POP was found to be important in promoting hepatocyte proliferation and liver regeneration (34,49,50). The effect may be mediated by POP present in cell nuclei, and to not be associated with its catalytic activity but with protein-protein interactions to regulate gene expression and protein secretion (35,51-53). It has been reported that POP binds to growth-associated protein 43 to control growth cone and synaptic function (46). Of note, HSC activation is generally characterized by increased expression of α-SMA and proliferation (54). However, after activation, α-SMA expression in HSCs in the S-phase of the cell cycle is low or not present (55). Recent studies showed that POP inhibitors impeded cell growth via reducing the expression of retinoblastoma protein (pRb) and Ki-67 and increasing the expression of p53, p27kip1 and pRb2/p130 in cancer cells (47,48). Collectively, the effects of POP on HSCs may not or not exclusively be due to increases in Ac-SDKP. However, it is difficult to separate the effects of POP and Ac-SDKP on HSCs since the mechanisms of the effect of Ac-SDKP on HSCs are currently elusive and no inhibitor or reliable neutralizing antibody is available to investigate these.

In conclusion, the present study showed that POP attenuated HSC activation and decreased its pro-inflammatory and pro-fibrotic features, strongly suggesting a protective effect of POP against liver fibrosis. Further studies are required to investigate specific mechanisms of POP in regulating HSC proliferation and activation and its possible anti-fibrotic effect in vivo.

Acknowledgements

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