Molecular and Cellular Endocrinology xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Cordyceps sinensis polysaccharide CPS-2 protects human mesangial cells from PDGF-BB-induced proliferation through the PDGF/ERK and TGF-β1/Smad pathways

Ying Wang [a,b,1], Dan Liu [b,1], Huan Zhao [b], Huixing Jiang [c], Min Wang [b,*], Hongping Yin [a,b,*]

[a] State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, People's Republic of China
[b] School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009, Jiangsu, People's Republic of China
[c] First Clinical Medical College, Nanjing University of Traditional Chinese Medicine, Nanjing Traditional Chinese Medicine Hospital, Nanjing 210010, Jiangsu, People's Republic of China

ARTICLE INFO

Article history:
Received 26 June 2013
Received in revised form 29 October 2013
Accepted 25 November 2013
Available online xxxx

Keywords:
Cordyceps sinensis
Polysaccharide
Mesangial cells
TGF-β1
PDGF-BB
ERK

ABSTRACT

CPS-2, a *Cordyceps sinensis* polysaccharide, has been demonstrated to have significant therapeutic activity against chronic renal failure. However, little is known about the underlying molecular mechanism. In this study, we found that CPS-2 could inhibit PDGF-BB-induced human mesangial cells (HMCs) proliferation in a dose-dependent manner. In addition, CPS-2 notably suppressed the expression of α-SMA, PDGF receptor-beta (PDGFRβ), TGF-β1, and Smad 3 in PDGF-BB-treated HMCs. Furthermore, PDGF-BB-stimulated ERK activation was significantly inhibited by CPS-2, and this inhibitory effect was synergistically potentiated by U0126. CPS-2 could prevent the PDGFRβ promoter activity induced by PDGF-BB, and return expression of PDGFRβ, TGF-β1, and TGFβRI to normal levels while cells were under PDGFRβ and ERK silencing conditions and transfected with DN-ERK. Taken together, these findings demonstrated that CPS-2 reduces PDGF-BB-induced cell proliferation through the PDGF/ERK and TGF-β1/Smad pathways, and it may have bi-directional regulatory effects on the PDGF/ERK cellular signaling pathway.

1. Introduction

Chronic kidney disease (CKD) is a progressive loss in renal function over a period of months or years. The glomerulus, as the filtration unit of the kidney, consists of mesangial cells (MCs) embedded in an extracellular matrix (ECM). MCs play an important role in the extracellular formation of the glomeruli, from which it derives its structural and functional integrity (EI-Nahas, 2003; Xia et al., 2011). A number of growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), have been found to regulate the proliferation of MCs. These growth factors can also affect the production of the ECM and thus have an impact on glomeruli function.

MCs are known to express PDGF subunit B (PDGF-B) chain mRNA and secrete the gene product as a homodimer (PDGF-BB) *in vitro*. PDGF-BB has been demonstrated to be a potent mitogen and chemoattractant, and to be able to stimulate ECM production. Hayama et al. found that ECM production in human mesangial cells (HMCs) could be accelerated by PDGF or TGF (Hayama et al., 2000). Bonner revealed that PDGF ligands and PDGF receptors are expressed at low levels in the normal adult kidney; however, they have increased expression levels during renal development and during the progression of renal fibrogenesis, and exert action through ligand–receptor binding (Bonner, 2004). The binding of PDGF to PDGF receptor-β (PDGFRβ) leads to phosphorylation, followed by recruitment and activation of specific signaling molecules that may mediate proliferation and other downstream effects (Park et al., 2013). TGF-β1 is also implicated as a key mediator in fibrogenesis to stimulate collagen deposition. Elevated TGF-β1 was accompanied by excessive ECM accumulation (Okamoto et al., 2009; Willis and Borok, 2007). Furthermore, Li et al. found TGF-β1 promoted the expression and secretion of matrix metalloproteinase-9 (MMP-9), an enzyme that actively reorganizes the ECM (Li et al., 2008).

Cordyceps sinensis, as a traditional Chinese medicine (TCM), has been used for thousands of years to prevent and treat human diseases. Wang et al. found that *C. sinensis* water extract had significant activity in the treatment of hyperglycemia, respiratory disease, liver disease, renal dysfunction, and renal failure (Wang et al., 2009). A novel structurally determined polysaccharide CPS-2, which was obtained from cultured *C. sinensis*, showed a remarkably protective effect on chronic renal failure caused by fulgurizing kidney in rats (Wang et al., 2010). Nevertheless, little is known about the underlying molecular mechanism.

* Corresponding authors. Address: 24 Tong jia xiang, Nanjing 210009, Jiangsu, People's Republic of China. Tel.: +86 025 83271108; fax: +86 025 83271249 (H. Yin).
E-mail addresses: minwang@cpu.edu.cn (M. Wang), yinhongping63@hotmail.com (H. Yin).
[1] Both the authors worked equally to this work.

0303-7207/$ - see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.mce.2013.11.018

In order to clarify the mechanism of action of CPS-2 in generating protection against chronic renal failure, we hypothesized that CPS-2 relieves renal injury through the activation of the PDGFRβ/ERK and TGF-β1/Smad signaling pathways based on the fact that *C. sinensis extract* is shown to interact with ERK signaling pathway (Noh et al., 2010). In the present study, we have investigated the effects of CPS-2 in HMCs against PDGF-BB-induced cell proliferation and explored the interaction of CPS-2 with the PDGFRβ/ERK and TGF-β1/Smad signaling pathways. We found that CPS-2 protects HMCs from PDGF-BB-induced proliferation through both pathways, which may be the possible molecular mechanism of the renal protective effects of CPS-2.

2. Materials and methods

2.1. Reagents

Alpha-smooth muscle actin (α-SMA) monoclonal antibody was acquired from Epitomics Inc. (Burlingame, California, USA). The antibodies to MMP-2 and MMP-9 were from Sino Biological Inc. (Beijing, China). The antibodies to Smad 2/3, TGF-β1, β-actin, and ERK 1/2 and phospho-ERK1/2 were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies to PDGFRβ, TGF-β receptor I (TGFβRI), and TGF-β receptor II (TGFβRII) were from Bioworld Technology, Inc. (Minneapolis, Minnesota, USA). The reagents used for reverse transcription (RT)-PCR were purchased from Bio-Rad Corporation (Hercules, California, USA) and TRNzol Plus was purchased from Biouniquer Corporation (Jiangsu, China). PDGF-BB was obtained from Peprotech (Rocky Hill, New Jersey, USA). MEK1/2 inhibitor U0126 was obtained from Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Cell culture

The human mesangial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in an atmosphere containing 5% CO_2. Cells between passages 3 and 5 were used for experiments. After preincubation in serum-free DMEM for 12 h and 24 h with different concentrations of CPS-2 (12.5, 25, and 50 μg/ml), cells were treated with 20 ng/ml PDGF-BB for 24 h in DMEM containing 2% fetal calf serum.

2.3. Cell proliferation assay

MTT reduction assay was used as quantitative index of cell viability. After 24, 36, and 48 h incubation with different compounds as described above, 20 ml MTT (5 mg/ml, Invitrogen Corp., Carlsbad, CA, USA) was added and cells were cultured for an additional 4 h. Subsequently, cells were lysed using dimethylsulfoxide (150 μl/well). When the formazan crystals were completely dissolved, the optical density (OD) was measured at 490 nm by a Microplate Reader (BioTek Instruments, Winooski, VT, USA). Assays were performed in sextuplicate and repeated three times independently.

2.4. Real-time PCR

Total RNA was extracted from cells treated with CPS-2 (12.5, 25, and 50 μg/ml) for various lengths of time using TRNzol Plus reagent according to the manufacturer's instructions (Biouniquer). RNA concentrations were quantified by microplate reader (BioTek Instruments). Total cDNAs were synthesized using olig(dT)18 primer by BU-SuperScript RT KIT (Biouniquer). Real-time PCR (RT-PCR) was performed using an ABI 7700 Prism Sequence Detection System and TaqMan primer probes (Applied Biosystems, Foster City, CA, USA). The total reaction volume was 20 μl: 4 μl cDNA, 10 μl SYBR® Green Supermix iTaq™, 0.5 μl of each primer and 5 μl diethyl pyrocarbonate (DEPC)-treated water. Cycle parameters were as follows: activation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s and then annealing and extension at 60 °C for 30 s. Calculations of the expression levels were carried out using the absolute curve method and then normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. All experiments were conducted three times independently.

2.5. Western blot assay

We plated HMCs used for experiments into 6-well plates. The quiescent non-dividing cells obtained were treated with different stimulants as required by each experiment. Cells were harvested by centrifugation at 1500 rpm for 5 min, washed twice with PBS, suspended in RIPA lysis buffer for whole-cell lysates, and then incubated on ice for 30 min. The homogenates were centrifuged at 14,000 rpm for 15 min, and the supernatant was used for Western blot analysis. The protein concentration was determined using the Bradford method, as per the manufacturer's instructions (Beyotime Technology, Jiangsu, China). Supernatants were mixed with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (60 mM Tris–HCl, 2% SDS, 10% glyserol, 0.1% bromophenol blue, and 14.4 mM mercaptoethanol) and boiled for 5 min. Samples were subsequently loaded into each lane of a 10% SDS–polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked for 1 h in 5% (w/v) non-fat milk and then incubated with a primary antibody at room temperature for 1 h. Having been washed 3 times in TBST buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated rabbit IgG (diluted 1:5000) for 1 h at room temperature, and then washed 3 times. Finally, proteins were detected using an enhanced chemiluminescence system BeyoECL Plus (Beyotime). The immunoblotted bands were quantified by Gel-Pro Analyzer 4.0 software (Media Cybernetics, Rockville, MD, USA) and the protein of interest was normalized to β-actin. All experiments were performed three times independently.

2.6. Luciferase reporter vector containing PDGFRβ promoters

The luciferase reporter vector containing the PDGFRβ promoter was constructed by inserting a region of approximate 1.3 Kb containing the promoter into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). The promoter fragment was prepared by amplifying HMC genomic DNA using the specific primers 5′-CGGGGTACCGTTCATCTACACTGGAATAGG-3′ and 5′-CCGCTCGAGAGAACAGAGGGATGGAGGAA-3′, which were designed based on the GenBank sequence of *Homo sapiens* PDGFRβ NC_000005.9. The PDGFRβ gene promoter was inserted into the pGL3-Basic vector in front of the luciferase gene using Kpn I and Xho I. Then, the constructed PDGFRβ promoter-luciferase reporter vector was analyzed by restriction analysis and DNA sequencing from both ends which confirmed that >80% of the ~1.3 Kb promoter was present and revealed that within that sequence there was <0.3% single nucleotide variations from the published data in GenBank. To study promoter region-specific effects, progressive deletions of the PDGFRβ promoter were achieved by PCR. The PCR protocol employed for all above amplifications was 94 °C for 3 min followed by 35 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 2 min. The products were purified, digested and inserted into the reporter vector using the aforementioned restriction sites.

Please cite this article in press as: Wang, Y., et al. *Cordyceps sinensis* polysaccharide CPS-2 protects human mesangial cells from PDGF-BB-induced proliferation through the PDGF/ERK and TGF-β1/Smad pathways. Molecular and Cellular Endocrinology (2013), http://dx.doi.org/10.1016/j.mce.2013.11.018