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Original article

Punicalagin, a PTP1B inhibitor, induces M2c phenotype polarization via up-regulation of HO-1 in murine macrophages

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ABSTRACT

Current data have shown that punicalagin (PUN), an ellagitannin isolated from pomegranate, possesses anti-inflammatory and anti-oxidant properties; however, its direct targets have not yet been reported. This is the first report that PTP1B serves as a direct target of PUN, with IC_{50} value of 1.04 μM. Results from NPOI further showed that the K_{on} and K_{off} of PUN-PTP1B complex were 3.38e2 M^{-1} s^{-1} and 4.13e-3 s^{-1}, respectively. The active site Arg24 of PTP1B was identified as a key binding site of PUN by computation simulation and point mutation. Moreover, inhibition of PTP1B by PUN promoted an M2c-like macrophage polarization and enhanced anti-inflammatory cytokines expression, including IL-10 and M-CSF. Based on gene expression profile, we elucidated that PUN treatment significantly up-regulated 275 genes and down-regulated 1059 genes. M1-like macrophage marker genes, such as *Tlr4, Irf1/2, Hmgb1, and Stat1* were down-regulated, while M2 marker genes, including *Tmem171, Gpr35*, *Csf1*, *Il1rn*, *Cebpb, Fos, Vegfa*, *Slc11a1*, and *Bhlhe40* were up-regulated in PUN-treated macrophages. *Hmox-1*, a gene encoding HO-1 protein, was preferentially expressed with 16-fold change. Inhibition of HO-1 obviously restored PUN-induced M2 polarization and IL-10 secretion. In addition, phosphorylation of both Akt and STAT3 contributed to PUN-induced HO-1 expression. This study provided new insights into the mechanisms of PUN-mediated anti-inflammatory and anti-oxidant activities and provided new therapeutic strategies for inflammatory diseases.

1. Introduction

Macrophages are critical sentinels in both innate and adaptive immunity that undergo a continuum of functional activation states under certain homeostatic and pathological conditions [1]. Based on cues in their local microenvironment, activated macrophages possess anti-microbial, phagocytic, and pro-inflammatory properties, but might also contribute to tissue remodeling, T cell suppression, and resolution of inflammation [2]. Activated macrophages can be classified into M1 macrophages (classically activated) by lipopolysaccharides (LPS) or interferon (IFN)-γ and M2 macrophages (alternatively activated) by interleukin (IL)-4 or IL-10. Moreover, M2 macrophages can be further divided into M2a, M2b, and M2c subtypes with specific secretory cytokines, surface markers and biological functions. M2a macrophages, activated by IL-4 and IL-13, highly express IL-10, IL-1ra, MHC II, CD206 and contribute to repair and regeneration; M2b macrophages, stimulated by immune complexes and toll-like receptor agonists, are characterized by IL-10, TNF-α, IL-1β, IL-6, MHC II and CD86 and contribute to immune-regulation; M2c macrophages, sensitized by IL-10, show high expression of IL-10, TGF-β, CD206 and SPHK1 and facilitate resolution of inflammation and T cell suppression [3,4]. Since different phenotypes perform various functions, modulating their functions may provide a promising therapy in diseases that these macrophages participate in.

Protein tyrosine phosphatase-1B (PTP1B), also known as protein tyrosine phosphatase non-receptor type 1(PTPN1), is a non-receptor tyrosine phosphatase that is an attractive drug target for treatment of over-nutrition and obesity-induced insulin resistance [5,6]. There is increasing experimental evidence showing that PTP1B also plays a pivotal role in modulating the response of macrophages to pro-inflammatory and anti-inflammatory stimulation. In PTP1B knockdown and knockout macrophages, LPS challenge exhibits increased signal transducers and activators of transcription 3 (STAT3) phosphorylation and IL-10 mRNA expression, while IL-10 stimulation contributes to

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STAT3 phosphorylation and anti-inflammatory gene up-regulation as compared to wild-type macrophages [7,8]. Moreover, PTP1B over-expression is reported to dephosphorylate STAT6, which suppresses anti-inflammatory cytokine IL-4 production, and has important implications in inflammatory diseases [9]. Given that IL-10/STAT3 and IL-4/STAT6 are two crucial signaling pathways in M2c and M2a phenotypes polarization, respectively, inhibition of PTP1B may lead to M2 macrophage polarization.

Punicalagin (PUN) is the most abundant ellagitannin in pomegranate, a well-documented traditional Chinese medicine used for inflammatory diseases [10,11]. Our previous research showed that PUN possesses considerable anti-inflammatory and anti-oxidant properties [12,13]. Besides, accumulating evidence indicates that PUN exhibits a variety of capacities including anti-apoptosis, anti-glycation, and anti-aging activities [14–17]. However, despite the increasing interests on the biological and pharmacological activities of PUN, there are still no documents reporting the direct target of punicalagin, which may help us to better our understanding of pharmacological actions of PUN.

In this study, we reported for the first time that PUN is a promising inhibitor of PTP1B and promotes M2c phenotype polarization via up-regulation of heme oxygenase (HO)-1, a well-documented enzyme possessing anti-oxidant and anti-inflammatory properties, in macrophages. Both Akt and STAT3 participate in PUN-induced HO-1 up-regulation. Our findings provided novel important evidence to better understand the activities of PUN and the underlying mechanisms of PUN-induced macrophage polarization in inflammatory diseases.

2. Materials and methods

2.1. Reagents

Punicalagin [> 98% high-performance liquid chromatography (HPLC) purity] was purchased from Tauto Biotech (Shanghai, China). LPS (*Escherichia coli* O55:B5), WP1022, LY294002, zinc protoporphyrin-IX (Znpp-IX), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and Ethanolamine-HCl were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), antibiotic-antimycotic and TRIzol® reagent were purchased from Gibco (Grand Island, NY, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). The ELISA kits for GM-CSF, M-CSF, IL-1β, IL-6, IL-4, IL-10, and TNF-α were purchased from Cusabio (Wuhan, China). The antibodies for flow cytometry, APC-CD68, FITC-F4/80, PE-CD206, APC-CD86, and FITC-MHCII were purchased from Becton Dickinson (Oxford, UK). Antibodies for Histone, GAPDH, PTP1B, HO-1, pSTAT1, pSTAT3, pSTAT6, Akt, iNOS and Arg-1 were purchased from Cell Signaling Technology (Danvers, MA, USA). The goat anti-mouse antibody was purchased from Li-cdr Odyssye® (Lincoln, NE, USA). SKi Sensor FC-Carboxyl chip (Cat no. SKI-Sensor-FC-C) was purchased from Silicon Kinetics (San Diego, CA, USA).

2.2. Cell culture

RAW264.7 cells were bought from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM medium supplemented with 10% FBS and antibiotics (100 U/ml streptomycin and 100 U/ml penicillin) at 37 °C in a humidified incubator with 5% CO_2. BALB/c mouse peritoneal macrophages were obtained as described previously. Briefly, 4% thioglycolate broth was intraperitoneally injected 3 days before extraction. The mice were sacrificed and peritoneal cells were isolated after intraperitoneal injection of cold PBS. Cells were then seeded onto dishes (2×10^6 cells/dish) for incubation and experiments.

2.3. Pharmacophore mapping prediction of potential targets

Pharmmapper server is a web server using pharmacophore mapping approach to predict potential drug targets (http://59.78.96.61/pharmmapper). Briefly, the Mol2 format file of PUN (PubChem CID: 44584733) was referred to the Pharmmapper server. During the course of operation, the maximum conformations were configured to 300, and the number of reserved matched targets was 300. The submission ID was recorded and could be used to review the pharmacophore results.

2.4. PTP1B activity detection

PTP1B activity was assayed on cells collected with 100 μl pNPP buffer (25 mM Hepes, pH 7.2, 50 mM NaCl, 5 mM dithiothreitol, 2.5 mM EDTA). The colorimetric pNPP hydrolysis assay based on the ability of phosphatases to catalyze the hydrolysis of pNPP to pnitrophenol, a chromogenic product was used. The intensity of the color reaction was measured at 410 nm on a Bio-Rad microplate spectrophotometer. Results were normalized by protein concentration. The IC_{50} of PUN on PTP1B was calculated by SPSS Probit regression.

2.5. PUN/PTP1B binding coefficient assessment via Nanopore Optical Interferometry

To further investigate the PUN-PTP1B complex, we used Nanopore Optical Interferometry (NOPI) to calculate the binding coefficient. Briefly, SKi FC-Carboxyl chip was activated with the mixture of 0.4 M EDC and 0.1 M NHS, followed by PTP-1B immobilization on surface. 1 M ethanolamine-HCl (pH 8.5) was injected to block the remaining activated NHS groups. PBS (pH 7.4) was used as a running buffer. 10 mM Glycine-HCl, pH 1.5 was used as a regeneration buffer. The experiments were conducted at 25 °C. Serial dilutions of PUN (0, 3, 6, 12, 24, 48, 96, 192 μM) were injected to estimate the binding with PTP1B protein.

2.6. Molecular docking by AutoDock

To further characterize the interaction between PUN and PTP1B protein, we employed Autogrid and Autodock to compute the binding affinity as well as binding sites. Briefly, the Mol2 file of PUN was drawn and converted by Chemdraw and then saved as final coordinate pdb file. The 3D crystal structure of PTP1B (PDB ID: 10NY) was obtained from the Protein Data Bank (PDB) at http://www.rcsb.org. The grid procedure was operated by AutoGrid in a grid box of 60*60*60 Å with a 1.0 Å to set aside the flexible rotations of PUN and to enfold all active sites. Afterwards, the simulation was performed for 30 times by employing Autodock 4.0 software and the data was exported.

2.7. PTP1B mutation and purification

To understand the binding mechanisms of PUN-PTP1B complex, three key residents of PTP1B were mutated. Briefly, protein site-directed mutagenesis (C215S, R221A, and R24A) was performed by employing pET-28a (Kan+) vector at *Nco*I–*Xho*I. Then recombinant wild type and mutant PTP1B proteins were over expressed using *E.coli* Rosatta expression system at 37 °C/ 220 rpm condition for 10 h and purified to homogeneity. Protein expression was identified by Western blot and concentration was calculated by BCA detection.

2.8. Overexpression/knockdown

2.8.1. Ptpn1

We used lentiviral transfection to construct *ptpn1* overexpression (*ptpn1* OE) RAW264.7 cells. Briefly, pGMLV-PA6 system was employed to amplify *ptpn1* genes including 6217M_PTPN1-F (*Eco*RI), 6217M_PTPN1-R (*Bam*HI) and vector. Then CMV-M_PTPN1-PGK-puro

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