International Immunopharmacology 24 (2015) 36–41

Contents lists available at ScienceDirect

International Immunopharmacology

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

Suppressive effects of 1-[4-fluoro-2-(2-nitrovinyl)phenyl]pyrrolidine on the Toll-like receptor signaling pathways

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ARTICLE INFO

Article history:
Received 18 September 2014
Received in revised form 31 October 2014
Accepted 31 October 2014
Available online 10 November 2014

Keywords:
Toll-like receptor
NF-κB
IRF3
Cyclooxygenase-2
Inducible nitric oxide synthase

ABSTRACT

When various pathogens invade a host, toll-like receptors (TLRs) play a significant role in recognizing the pathogen-associated molecular patterns carried by the pathogens to induce innate immune reaction, followed by acquired immunity reaction. TLRs have two downstream signaling pathways, the myeloid differential factor 88 (MyD88)-dependent and toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent pathways. To evaluate the therapeutic potential of 1-[4-fluoro-2-(2-nitrovinyl)phenyl]pyrrolidine (FPP), previously synthesized in our laboratory, its effect on signal transduction via the TLR signaling pathways was examined. FPP inhibited the activation of nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3) induced by TLR agonists, as well as inhibited the expression of cyclooxygenase-2, inducible nitric oxide synthase, and interferon inducible protein-10. FPP also inhibited the activation of NF-κB and IRF3 when induced by the overexpression of downstream signaling components of the TLRs. As a result, FPP has potential to become a new therapeutic drug for many inflammatory diseases.

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1. Introduction

Toll-like receptors (TLRs) were the first pattern recognition receptors (PRRs), and initiate a series of signaling processes that constitutes the first line of host defense for anti-microbial responses. [1]. TLRs are type 1 transmembrane glycoproteins that contain ectodomains, transmembrane domains, and intracellular TLR/IL-1 receptor (TIR) domains [2]. The ectodomains are the sensing domain, which is involved in the recognition of microbial components via the leucine rich repeats (LRRs). The recognition by the ectodomains of TLRs recruits intracellular adaptor proteins, such as myeloid differentiation primary-response protein 88 (MyD88) and Toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-β (TRIF), to initiate downstream signaling events [2].

MyD88 is the immediate downstream adaptor molecule recruited by activated TLRs, except for TLR3. It recruits the kinase interleukin-1 receptor-associated kinase (IRAK)-4, and induces the IRAK-4-mediated phosphorylation of IRAK-1. Phosphorylated IRAK-1 then associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), leading to the activation of NF-κB. TRIF is another adaptor molecule, responsible for the activation of the MyD88-independent signaling pathway to lead to the activation of various transcription factors including NF-κB and interferon (IFN) regulatory factor (IRF) 3/7 [3,4]. The activation of these transcription factors induces the expression of various oncogenes and pro-inflammatory mediators.

Inflammation is a part of the nonspecific immune response that can be initiated by invading microbial pathogens or tissue injuries. Dysregulated activation of the TLRs can lead to the development of many chronic inflammatory diseases. Accumulating evidence has pointed to a relationship between the TLRs and various inflammatory diseases including inflammatory bowel disease, psoriasis, diabetes mellitus, and rheumatoid arthritis [5]. Therefore, understanding how anti-inflammatory factors modulate TLR-mediated signaling pathways and target gene expression may provide new opportunities for the development of effective therapeutics for chronic inflammatory diseases.

1-[4-Fluoro-2-(2-nitrovinyl)phenyl]pyrrolidine (FPP), which contains 4-fluoro-3-nitrovinyl phenyl and pyrrolidine moieties, can be readily prepared from the corresponding benzaldehyde and nitromethane by condensation reaction for use as a synthetic building block in the synthesis of biologically active molecules. Appropriately, we sought to determine whether FPP can modulate TLR signaling pathways, which play an important role in innate immunity. FPP suppressed the activation of NF-κB and IRF3 and the expression of target genes induced by TLR agonists. These results raise the important possibility that TLR-mediated inflammatory

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http://dx.doi.org/10.1016/j.intimp.2014.10.033
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responses and the consequent risks for many chronic inflammatory diseases could be regulated by FPP.

2. Materials and methods

2.1. Preparation of 1-(4-fluoro-2-((E)-2-nitrovinyl)phenyl)pyrrolidine (FPP)

FPP was synthesized by the modification of an established procedure [6]. Briefly, an oven dried 100 ml flask containing a stirring bar was charged with 2-(pyrrolidin-1-yl)-5-fluorobenzaldehyde (0.966 g, 5.0 mmol), KF (0.019 g, 0.33 mmol), Me_2NH_2Cl (0.815 g, 10 mmol), nitromethane (11.2 ml, 203 mmol) and toluene (11 ml). The flask was equipped with a Dean-Stark apparatus and refluxed for 5 h. The solvent was then removed under reduced pressure and the residue was diluted with CH_2Cl_2 (30 ml), and washed with H_2O (20 ml). The organic layer was dried over anhydrous Na_2SO_4 and then evaporated. The residue was purified by flash chromatography (ethyl acetate/hexane = 1/20) to afford FPP (0.768 g, 65%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.34 (d, J = 13.4 Hz, 1H), 7.46 (d, J = 13.4 Hz, 1H), 7.09–7.05 (m, 2H), 6.92–6.89 (m, 1H), 3.28–3.24 (m, 4H), 1.99–1.96 (m, 4H); ^{13}C NMR (100 MHz) δ 156.5 (d, J = 239.3 Hz), 148.0, 137.8, 135.9, 120.6(d, J = 7.7 Hz), 119.5(d, J = 21.9 Hz),117.7 (d, J = 7.6), 114.8(d, J = 21.9 Hz), 53.2, 25.4.

2.2. Cell viability test

Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based colorimetric assay. Viability tests were performed by adding a small amount of the CellTiter 96 AQ_{ueous} One Solution Reagent (Promega, Madison, WI, USA) directly to culture wells, incubating for 4 h, and then recording the absorbance at 490 nm with a 96-well plate reader.

2.3. Cell culture

RAW 264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293T (human embryonic kidney cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Hyclone), 100 units/ml Penicillin, and 100 μg/ml Streptomycin (Thermo, UT). Cells were maintained at 37 °C in a 5% CO_2/air environment.

2.4. Plasmids

An NF-κB(2×)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). An IFNβ PRDIII-I-luciferase reporter plasmid and a wild-type TBK1 expression plasmid were kind gifts from Kate Fitzgerald (University of Massachusetts Medical School). Heat shock protein 70 (HSP70)-galactosidase reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). Wild-type MyD88 was provided by Jurg Tschopp (University of Lausanne, Switzerland). A TRIF expression plasmid was provided by Shizo Akira (Osaka University, Japan). Wild-type IKKβ was obtained from Michael Karin (University of California, San Diego, CA). All DNA constructs were prepared in large scale using the EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

2.5. Transfection and luciferase assay

The assays were performed as described previously [7]. RAW264.7 or 293T cells were transfected with a luciferase plasmid and various expression plasmids containing the signaling components using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. The HSP70-β-galactosidase plasmid was co-transfected as an internal control. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector. Luciferase and β-galactosidase enzyme activities were determined using the Luciferase Assay and β-galactosidase Enzyme Systems (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized to β-galactosidase activity to determine the relative luciferase activity.

2.6. Immunoblotting

Equal amounts of cell extracts were resolved by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. The membranes were blotted with the primary antibody and secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL). The reactive bands were visualized with the enhanced chemiluminescence system (Amersham).

2.7. Data analysis

Data were obtained from triplicate experiments. Values are expressed as the mean ± standard error of the mean (SEM). Differences in the data were evaluated using the Student's t test. p-Values of <0.05 were taken to indicate statistically significant differences.

3. Results and discussion

3.1. FPP suppresses the activation of NF-κB induced by TLR2 or TLR4 agonists

To evaluate the cytotoxic nature of FPP in RAW 264.7 cells, the cytotoxicity was determined using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based colorimetric assay. Treatment with 30 μM FPP reduced the population viability by 10%, but the population viability was decreased by 53% by 50 μM FPP (Fig. 1B). Therefore, all subsequent experiments utilized 20 or 30 μM FPP.

To identify whether FPP modulates the TLR-mediated signaling pathways, NF-κB activation induced by LPS (TLR4 agonist) or MALP-2 (TLR2 and TLR6 agonist) was used as the readout for the activation of TLRs. All TLR signaling pathways culminate in NF-κB activation, which induces the activation of target genes involved in innate and adaptive immune responses and inflammation [8]. Therefore, the inhibition of NF-κB activation is considered to be an important strategy for anti-inflammatory therapies. Appropriately, the effect of FPP on NF-κB activation was determined by the NF-κB luciferase reporter assay. FPP was observed to inhibit the activation of NF-κB induced by LPS (Fig. 1C) and MALP-2 (Fig. 1D).

3.2. FPP suppresses the expression of COX-2 and iNOS induced by TLR2 or TLR4 agonists

It was next investigated whether FPP could regulate the expression levels of iNOS and COX-2, which are two of the most important enzymes in certain inflammatory diseases. The next experiment assessed the capability of FPP to regulate the expression of COX-2 when induced by LPS or MALP-2. COX-2 is one of the target genes regulated through NF-κB activation in macrophages. FPP was found to suppress the expression of COX-2 induced by LPS and MALP-2 in RAW264.7 cells, as determined by the COX-2 luciferase reporter assay (Fig. 2A, B) and Western blotting (Fig. 2C, D).

It was next determined whether FPP could regulate the expression of iNOS, another target gene regulated through the activation of NF-κB. FPP suppressed both the LPS- and MALP-2-induced expressions of iNOS in RAW264.7 cells, as determined by the iNOS luciferase reporter assay (Fig. 3A, B) and Western blotting (Fig. 3C, D).

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