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Suppression of TLRs signaling pathways by 1-[5-methoxy-2-(2-nitrovinyl)phenyl]pyrrolidine

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ABSTRACT

Toll-like receptors (TLRs) play significant roles in recognizing the pathogen-associated molecular patterns that induce innate immunity, and subsequently, acquired immunity. In general, 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, which lead to the activation of nuclear factor-kappa B (NF-κB) and interferon regulatory factor 3 (IRF3). 1-[5-methoxy-2-(2-nitrovinyl)phenyl]pyrrolidine (MNP) has been previously synthesized in our laboratory. To evaluate the therapeutic potential of MNP, its effect on signal transduction via the TLR signaling pathways was examined. MNP was shown to inhibit the activation of NF-κB and IRF3 induced by TLR agonists, as well as to inhibit the expression of cyclooxygenase-2, inducible nitric oxide synthase, and interferon inducible protein-10. MNP also inhibited the activation of NF-κB and IRF3 induced by the overexpression of downstream signaling components of the MyD88- or TRIF-dependent signaling pathways. These results suggest that MNP can modulate MyD88- and TRIF-dependent signaling pathways of TLRs, leading to decreased inflammatory gene expression.

1. Introduction

The innate immune response represents the first line of host defense against infectious bacteria and viruses by recognizing microbial pathogens and triggering defense responses [1]. The toll-like receptor (TLR) family consists of membrane-bound pattern recognition receptors (PRRs) that play critical roles in host defense against invading microbial pathogens since they recognize many pathogen-associated molecular patterns (PAMPs), including LPS (recognized by TLR4), peptidoglycan (TLR1 and TLR2), lipoprotein (TLR2 and TLR6), viral double-stranded RNA (TLR3), viral single-stranded RNA (TLR7 and TLR8), bacterial and viral cytosine phosphate guanine dinucleotide oligodeoxynucleotides (TLR9), and flagellin (TLR5) [2]. Signaling through TLRs can trigger two different signaling pathways, the first is a myeloid differentiation primary response protein 88 (MyD88)-dependent pathway that leads to the rapid activation of nuclear factor kappa B (NF-κB), and the second is a MyD88-independent pathway requiring the Toll/interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP)-inducing interferon β (TRIF) that leads to the activation of IFN regulatory factor 3 (IRF3), and delayed NF-κB activation. Both pathways can induce the production of proinflammatory cytokines, chemokines, and type I interferon [2].

All TLRs, with the exception of TLR3, signal inflammation via the conserved canonical MyD88-dependent signaling pathway. This pathway mediates the activation of TNF receptor-associated factor-6 and IKKβ complex, which results in the activation of the transcription factors NF-κB and AP-1 [2]. In addition to the proinflammatory signals, TLR3 and TLR4 use a TRIF-dependent signaling pathway. TRIF mediates the phosphorylation and activation of IRF3, and the subsequent expression of type I IFNs and IFN-inducible genes via TANK-binding kinase 1 (TBK1) and IKKε [3,4].

1-[5-methoxy-2-(2-nitrovinyl)phenyl]pyrrolidine (MNP) (Fig. 1A), which contains 5-methoxy-2-nitrovinyl phenyl and pyrrolidine moieties, has been synthesized in our laboratory for use as a fundamental building block for the preparation of biologically active compounds. Deregulation of the TLR-mediated signaling pathways can lead to the development of various chronic inflammatory diseases [5]. Therefore, understanding the manner in which anti-inflammatory factors modulate TLR-mediated signaling pathways and target gene expression may be a potential therapeutic strategy for the treatment of various chronic inflammatory diseases. Therefore, the aim of this study was to determine whether MNP can modulate TLR signaling pathways, which play important roles in innate immunity.

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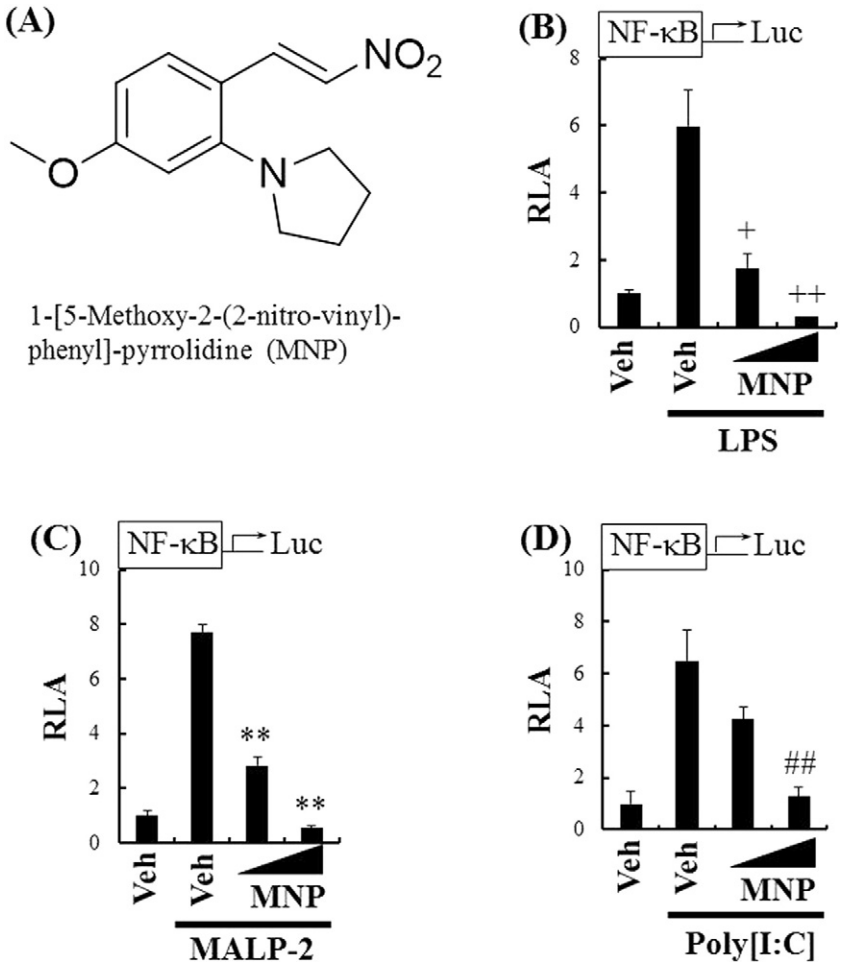

Fig. 1. MNP suppresses NF-κB activation induced by TLR agonists. (A) Chemical structure of 1-[5-Methoxy-2-(2-nitro-vinyl)-phenyl]-pyrrolidine (MNP). (B-D) RAW264.7 cells were transfected with NF-κB luciferase reporter plasmid, pre-treated with MNP (20, 50 μM) for 1 h, and then treated with LPS (10 ng/ml) (B), MALP-2 (10 ng/ml) (C), or Poly[I:C] (10 μg/ml) (D) for an additional 8 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described in Materials and methods. Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean ± SEM (n = 3). +, Significantly different from LPS alone, $p < 0.05$ (+), $p < 0.01$(++). *, Significantly different from MALP-2 alone, $p < 0.01$ (**). #, Significantly different from Poly[I:C] alone, $p < 0.01$ (##). Veh, vehicle; MNP, 1-[5-Methoxy-2-(2-nitro-vinyl)-phenyl]-pyrrolidine.

2. Materials and methods

2.1. Preparation of 1-[5-methoxy-2-(2-nitrovinyl)phenyl]pyrrolidine (MNP)

MNP was synthesized using a modification of an established procedure [6]. An oven-dried 100-ml flask containing a stirring bar was charged with 4-methoxy-2-(pyrrolidin-1-yl)benzaldehyde (1.026 g, 5 mmol), KF (0.291 g, 5 mmol), Me_2NH_2Cl (0.815 g, 10 mmol), nitromethane (12.1 ml, 225 mmol) and toluene (12.5 ml). The flask was equipped with a Dean-Stark apparatus and refluxed for 2 h. The solvent was 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 evaporated. The residue was then purified by flash chromatography (ethyl acetate/hexane = 1/20) to afford 1-[5-methoxy-2-(2-nitrovinyl)phenyl]pyrrolidine (MNP) (0.499 g, 40%) as a dark-red solid. Mp 175 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, J = 13.2 Hz, 1H), 7.41 (d, J = 13.2 Hz, 1H), 7.34 (d, J = 8.8 Hz, 1H), 6.42 (dd, J = 8.8 Hz, 2.4 Hz, 1H), 6.36 (d, J = 2.0 Hz, 1H), 3.84 (s, 3H), 3.41–3.34 (m, 4H), and 2.01–1.95 (m, 4H). ^{13}C NMR (100 MHz) δ 163.6, 153.2, 139.5, 132.4, 131.5, 111.8, 105.8, 100.8, 55.3, 52.8, and 25.7.

2.2. Reagents

Purified LPS was purchased from List Biological Laboratories (San Jose, CA, USA). Macrophage-activating lipopeptide of 2 kDa (MALP-2) was purchased from Alexis Biochemicals (Braunschweig, Germany). Polyinosinic-polycytidylic acid (poly[I:C]) was purchased from Amersham Biosciences (Piscataway, NJ, USA). All other reagents were purchased from Sigma-Aldrich, unless otherwise stated.

2.3. Cell culture

RAW264.7 cells (the murine monocyte-macrophage cell line, ATCC TIB-71) and 293 T cells (human embryonic kidney cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air.

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). COX-2, iNOS, and IP-10-luciferase reporter plasmids and p65 and constitutively active IRF3 (IRF3CA) expression plasmids were from Daniel Hwang (University of California, Davis, CA, USA). A 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,