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Novel anti-inflammatory function of NSC95397 by the suppression of multiple kinases

Yanyan Yang [a,1], Woo Seok Yang [a,1], Tao Yu [a,1], Young-Su Yi [a], Jae Gwang Park [a], Deok Jeong [a], Ji Hye Kim [a], Jeong Su Oh [a], Keejung Yoon [a], Jong-Hoon Kim [b,*], Jae Youl Cho [a,*]

[a] Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] Department of Veterinary Physiology, College of Veterinary Medicine, Biosafety Research Institute, Chonbuk National University, Jeonju 561-756, Republic of Korea

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ABSTRACT

NSC95397 (2,3-bis-[(2-hydroxyethyl)thio]-1,4-naphthoquinone) is a CDC25 inhibitor with anti-cancer properties. Since the anti-inflammatory activity of this compound has not yet been explored, the aim of this study was to examine whether this compound is able to modulate the inflammatory process. Toll like receptor (TLR)-mediated inflammatory responses were induced by lipopolysaccharide (LPS), a TLR4 ligand, and pam3CSK, a TLR2 ligand, in peritoneal macrophages and RAW264.7. The molecular mechanism of NSC95397's anti-inflammatory activity was studied using immunoblotting analysis, nuclear fractionation, immunoprecipitation, overexpression strategies, luciferase reporter gene assays, and kinase assays. NSC95397 dose-dependently suppressed the production of nitric oxide (NO), tumor necrosis factor (TNF)-α, and prostaglandin (PG)E_2, and diminished the mRNA expression of inflammatory genes such as inducible NO synthase (iNOS), cyclooxygenase (COX)-2, interferon (IFN)-β, and TNF-α in peritoneal macrophages and RAW264.7 cells that were stimulated by LPS and pam3CSK. This compound also clearly blocked the activation of NF-κB (p65), AP-1 (c-Fos/c-Jun), and IRF-3 in LPS-treated RAW264.7 cells and TRIF- and MyD88-overexpressing HEK293 cells. In addition, biochemical and molecular approaches revealed that this compound targeted AKT, IKKα/β, MKK7, and TBK1. Therefore, these results suggest that the anti-inflammatory function of NSC95397 can be attributed to its inhibition of multiple targets such as AKT, IKKα/β, MKK7, and TBK1.

1. Introduction

When viruses, bacteria, or fungi enter the human body, innate immunity, the first line of defense against infections, is activated within minutes or hours [1]. This line of defense includes phagocytic uptake, antigenic peptide presentation, and the direct killing of infected pathogens by the activation of immune cells such as macrophages, neutrophils, and dendritic cells [2]. Of these different components of innate immunity, macrophages are considered to be central inflammatory cells since they have a powerful machinery to attack external pathogens and are widely distributed and maintained in the human body [3]. While neutrophils immediately respond and then disappear after the inflammatory response, macrophages are more long-lived [4].

The activation of macrophages by the release of interferon (IFN)-γ from Th1 cells involves many different cellular and molecular events that manage the local and systemic inflammatory response [4]. These cells produce a great amount of inflammatory mediators such as prostaglandin (PG)E_2 and thromboxane, which play critical roles in regulating vasoconstriction, platelet aggregation, neutrophil chemotaxis, and smooth muscle contraction; these cells also produce various cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6, which are able to stimulate fever, the secretion of corticosteroids, and the induction of leukocytosis [5].

Abbreviations: PG, prostaglandin; NO, nitric oxide; COX, cyclooxygenase; iNOS, inducible NO synthase; TNF-α, tumor necrosis factor α; ERK, extracellular signal-related kinase; TLR, Toll-like receptors; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor-κB; AP-1, activator protein-1; Na CMC, sodium carboxyl methylcellulose; JAK2, Janus kinase 2; JNK, c-Jun N-terminal kinase; EIA, enzyme immunoassay; ELISA, enzyme linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (a tetrazole); IRF-3, interferon regulatory factor-3; DTT, dithiothreitol; PI3K, phosphoinositide 3-kinase; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-polymerase chain reaction; and STAT-1, signal transducer and activator of transcription 1.

* Corresponding authors.
E-mail addresses: jhkim1@chonbuk.ac.kr (J.-H. Kim), jaecho@skku.edu, jaecho67@gmail.com (J.Y. Cho).
[1] These authors contributed equally to this work.

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Past studies have shown how the molecular events in activated macrophages are carried out under infectious conditions. The activation of toll-like receptors (TLR) is regarded as a major event in the body's response to bacterial, fungal, and viral infections [6]. The activation of TLRs requires cellular adaptor molecules including myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) to connect external stimuli to the intracellular signaling machinery composed of protein tyrosine kinases [*e.g.*, Syk, janus kinase (JAK), and Src], serine/threonine kinases [*e.g.*, AKT, phosphatidylinositide 3-kinases (PI3K), IκB kinase (IKK), TANK-binding kinase (TBK)1, IKKε, and phosphoinositide-dependent kinase-1 (PDK1)], and mitogen-activated protein kinases [extracellular signal-regulated kinase (ERK), p38, and c-Jun-N-terminal kinase (JNK)] [7–9]. Eventually, the signaling cascade is linked to the transcriptional activation of inflammatory genes including iNOS, COX-2, and various cytokines by inducing the translocation of transcription factors such as nuclear factor (NF)-κB, activator protein (AP)-1, interferon regulatory factor (IRF)-3, and signal transducers and activators of transcription (STAT)-1, and increasing their binding to the promoter sites of the target genes [10,11].

Even though macrophage-mediated inflammatory responses are critical for the protection of the host, higher and more sustained levels of inflammation are now widely accepted as one of the major contributors to a variety of serious diseases such as cancer, atherosclerosis, diabetes, and Alzheimer's disease [12]. This is because our defense systems are attacking our body's own cells, tissues, and organs by releasing toxic radicals and necrotic cytokines resulting in the loss of function [13]. Indeed, inflammation was called the "secret killer" in a 2004 edition of *Time* magazine. A great amount of evidence has indicated that anti-inflammatory strategies could help our body prevent a variety of chronic diseases. Therefore, the development of safe and strong anti-inflammatory drugs could be essential to preventing serious diseases.

NSC95397 (2,3-bis-[(2-hydroxyethyl)thio]-1,4-naphthoquinone) is an inhibitor of CDC25 [14], which is an essential molecule regulating the cell cycle [15]. Due to the importance of CDC25 in cell proliferation, this compound and its derivatives were initially designed as anti-cancer drugs [16,17]. In fact, several researchers have reported the effectiveness of this compound in inducing cytotoxicity and proliferation blockades in various tumor cells including neuroendocrine tumor cells and prostate cancer cells [18,19].

Although the evidence is still emerging, a previous study reporting that CDC25 stimulates NF-κB activation *via* the serine 32-phosphorylation of IκBα [20] has encouraged us to test whether CDC25 inhibitors are able to suppress various inflammatory responses. Indeed, it was identified that NSC95397 was able to inhibit the secretion of NO at the initial screening test (data not shown). In addition, some quinone-type compounds have also been shown to have anti-inflammatory properties [21,22]. Based on these points, we aimed to carefully evaluate in this study the anti-inflammatory activities of NSC95397 in macrophage-mediated inflammatory responses induced by the stimulation of TLR2, a receptor that recognizes G(+) bacteria-derived components such as peptidoglycan, and TLR4, a receptor that responds to G(−) bacteria-derived components such as lipopolysaccharide (LPS). Furthermore, the molecular targets of this compound were also identified using various molecular and biochemical approaches.

2. Materials and methods

2.1. Materials

NSC95397 [NSC, (2,3-bis[(2-hydroxyethyl)thio]-1,4-naphthoquinone, purity > 97%], an inhibitor of CDC25 (Fig. 1A), BAY 11-7082 [BAY, (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile, purity > 98%] and SP600125 [1,9-pyrazoloanthrone, anthrapyrazolone, purity > 98%] was purchased from Calbiochem (La Jolla, CA). BX795 (purity > 98%) was obtained from InvivoGen (San Diego, CA). Polyethylenimine (PEI), the tetrazole 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA), sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), pam3CSK, and lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). The enzyme immune assay (EIA) and enzyme immunosorbent assay (ELISA) kits that were used to determine PGE_2 levels, and the protein A-coupled Sepharose beads were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Fetal bovine serum (FBS), penicillin, streptomycin, TRIzol reagent, and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD). All other chemicals used in this study were of analytical grade and obtained from Sigma Chemical Co. Phospho-specific and total antibodies against p65, c-Fos, c-Jun, IRF3, TBK1, inhibitor of κB (IκB), IKKα/β, IKKβ, AKT, p85 (Y458/Y199), Syk, Src (Y416), IKKε, tyrosine, ERK, JNK, p38, mitogen-activated protein kinase (MKK)3/6, MKK4, MKK7, HA, lamin A/C, and β-actin were obtained from Cell Signaling (Beverly, MA). β-Tubulin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. DNA constructs

Luciferase constructs containing binding sites for IRF-3, NF-κB, and AP-1 were gifts from Prof. Hae Young Chung (Pusan National University, Pusan, Korea) and Addgene (Cambridge, MA). FLAG-MyD88, CFP-TRIF, HA-AKT, FLAG-IKKβ, FLAG-MKK7, and FLAG-JNK1 were purchased from Addgene (Cambridge, MA). FLAG-TBK1 was a gift from Prof. Joo Young Lee (Catholic University, Bucheon, Korea).

2.3. Preparation of peritoneal macrophages

Six-week-old male C57BL/6 mice were purchased from B&K (Fremont, CA). The mice had access to food pellets (Samyang, Daejeon, Korea) and water *ad libitum* and were housed under a 12 h light/12 h dark cycle. All studies were performed in accordance with the guidelines that were established by the Sungkyunkwan University Institutional Animal Care and Use Committee. Peritoneal exudates were obtained from C57BL/6 male mice (7–8 weeks old and weighing 17–21 g) by lavage 4 days after intraperitoneal injection of 1 ml sterile 4% thioglycollate broth (Difco Laboratories, Detroit, MI), as previously reported [23]. After the exudates were washed with RPMI1640 medium containing 2% FBS, peritoneal macrophages (1×10^6 cells/ml) were plated in 100 mm tissue culture dishes for 4 h at 37 °C in a 5% CO_2 humidified atmosphere.

2.4. Cell culture and drug preparation

RAW264.7 cells, a murine macrophage cell line, as well as peritoneal macrophages and HEK293 cells were maintained in RPMI1640 media supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS. The cells were grown at 37 °C and 5% CO_2 in humidified air. The stock solution (10 mM) of NSC95397 was prepared using DMSO.

2.5. Determination of NO, PGE_2, and TNF-α production

After the pre-incubation of the RAW264.7 cells or peritoneal macrophages (1×10^6 cells/ml) for 18 h, the cells were treated with NSC95397 (0 to 40 μM) for 30 min and then further incubated