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(*E*)-3-(3,4-dihydroxy-2-methoxyphenyl)-1-(2,4-dihydroxyphenyl) prop-2-en-1-one, a novel licochalcone B derivative compound, suppresses lipopolysaccharide-stimulated inflammatory reactions in RAW264.7 cells and endotoxin shock in mice

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ABSTRACT

Activated macrophages mediate inflammation, as they release nitric oxide and pro-inflammatory cytokines in various inflammatory diseases. Suppressing macrophage activation may alleviate inflammatory processes. Here, we report that (*E*)-3-(3,4-dihydroxy-2-methoxyphenyl)-1-(2,4-dihydroxyphenyl)prop-2-en-1-one (DDP), a novel licochalcone B derivative compound, inhibits inflammatory reactions in macrophages and protects mice from endotoxin shock. *In vitro* experiments showed that DDP suppressed the generation of nitric oxide and pro-inflammatory cytokines by suppressing the activation of nuclear factor-κB and activator protein-1 and simultaneously inhibited its upstream inflammatory signaling cascades in lipopolysaccharide in RAW264.7 cells. In an animal model, DDP protected BALB/c mice from lipopolysaccharide-induced endotoxin shock, possibly through inhibition of the production of inflammatory cytokines. DDP inhibited the production of inflammatory mediators and may be a potential target for treatment of various inflammatory diseases.

1. Introduction

Inflammation is a process by which the body's immune cells and chemicals protect from infection with foreign substances, such as bacteria and viruses. Inflammation is characterized by increased blood flow to tissues causing increased temperature, redness, swelling, and pain [1]. Inflammation can be a valuable phenomenon triggered by noxious stimuli and conditions, such as infection and tissue injury [2]. However, this typical view of inflammation as a reaction to injury or infection might need to be expanded to account for inflammatory processes induced by other types of adverse conditions [1]. Human diseases that are associated with these conditions, including atherosclerosis, asthma, type 2 diabetes, and neurodegenerative disease, are all characterized by chronic low-grade inflammation.

During the inflammation process, among immune cells macrophages play a central role in mediating many different immunopathological phenomena including the overproduction of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α), and other inflammatory mediators, such as nitric oxide (NO) and prostaglandins [1–3]. Since a number of diseases are linked to the inflammatory response [3], substances including synthetic and natural chemicals that can regulate inflammatory reaction have been studied.

Since the nuclear factor-kappa B (NF-κB) pathway plays a key role in controlling inflammation [4,5], it is a critical target for the anti-inflammatory activity of various medicines and phytochemicals [6–8]. NF-κB is an essential transcription factor that regulates gene expression for various cytokines, chemokines, growth factors, and cell-adhesion molecules [4,5]. In unstimulated cells, NF-κB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein called inhibitor-κB (Iκ-B). Following activation by several inflammatory stimuli, such as lipopolysaccharide (LPS), cytokines, oxidants, and viruses, the NF-κB heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target genes, including genes encoding

Abbreviations: AP-1, activator protein-1; Iκ-B, inhibitor-κB; iNOS, inducible NO synthase; NO, nitric oxide; NF-κB, nuclear factor-kappa B; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor.

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for pro-inflammatory cytokines, adhesion molecules, chemokines, and inducible enzymes, such as inducible NO synthase (iNOS) [4,5].

In addition to NF-κB, mitogen-activated protein kinases (MAPKs) including the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38, are essential to regulate the production of inflammatory mediators [9]. The MAPKs comprise a family of protein-serine/threonine kinases that link extracellular signals to the machinery that controls a variety of fundamental cellular outputs, such as cell proliferation, survival, death, and differentiation [10]. Upon activation of the MAPKs, transcription factors present in the cytoplasm or nucleus are phosphorylated and activated, leading to expression of target genes resulting in a biological response [9,10]. In conjunction with the activation of NF-κB and MAPK activation, expression of multiple genes is induced that together regulate the inflammatory response. Because NF-κB and MAPK play such a pivotal role in the amplifying loop of the inflammatory response, they have become logical targets for new anti-inflammatory treatments [11–13].

In our on-going project directed toward the discovery of novel anti-inflammatory agents based on a naturally occurring licochalcone, important active constituents of licorice, we have synthesized several licochalcone derivatives. (*E*)-3-(3,4-dihydroxy-2-methoxyphenyl)-1-(2,4-dihydroxyphenyl)prop-2-en-1-one (DDP) markedly suppresses LPS-induced NO in RAW 264.7 macrophages [14]. DDP is a novel licochalcone B derivative which has 3,4-dihydroxy-2-methoxy substituent and a resorcinol type ring A. Although licochalcone B has several pharmacological attributes including anti-cancer [15], anti-oxidant, and anti-inflammatory [16,17] activity, no report on DPP's anti-inflammatory activities or the molecular mechanisms involved has been published.

Therefore, as a part of our ongoing screening program to evaluate the anti-inflammatory potentials of compounds, we investigated the molecular mechanisms underlying the anti-inflammatory properties of DDP in activated macrophages and a murine model of sepsis.

2. Methods

2.1. Reagents

DDP was synthesized as previously described [14]. LPS derived from *Escherichia coli* (0111:B4), dimethylsulfoxide (DMSO), and G418 were obtained from Sigma–Aldrich (St Louis, MO, USA). Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/EDTA were purchased from Hyclone (Logan, UT, USA). Cell Titer 96 Aqueous One Solution was purchased from Promega (Madison, WI, USA). Protein detection BCA kit was obtained from Thermo Scientific Pierce (Rockford, IL, USA). The antibodies used in this study were anti-inhibitor of NF-κB (Iκ-Bα) mouse monoclonal, anti-NF-κB p65 rabbit polyclonal, anti-phospho-JNK (pT183, pY185) rabbit polyclonal, anti-JNK (rabbit polyclonal, anti-phospho-pERK1/2 (pT202, pY204) rabbit polyclonal, anti-ERK1/2 rabbit polyclonal, anti-phospho-p38 (pT180, pY182) rabbit polyclonal, anti-p38 rabbit polyclonal, anti-c-Fos rabbit monoclonal, anti-C-Jun rabbit monoclonal (Cell Signaling Technology, Danvers, MA, USA), and anti-poly (ADP-ribose) polymerase (PARP) rabbit monoclonal and anti-β-actin mouse monoclonal (Sigma–Aldrich).

2.2. Cells

RAW264.7 murine macrophages obtained from the Korean Cell Bank (Seoul, Korea) were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37° C in 5% CO_2. RAW NF-kB/SEAP reporter cell line was obtained from IMGENEX (San Diego, CA, USA) and cultured in DMEM containing 10% FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418.

2.3. Cell proliferation and lactate dehydrogenase toxicity assay

The effect of DDP on cell proliferation and cytotoxicity was tested using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) and a CytoTox 96® Non-Radioactive Cytotoxicity assay kits, respectively. RAW264.7 cells were plated at a density of 2×10^4 cells in a 96-well flat-bottom plate, and DDP was added to each plate at the indicated concentrations. After a 24 h incubation period, cell proliferation and cytotoxicity were measured according to the manufacturer's instructions. Unless otherwise mentioned, control treatment refers to the cells treated with an equal amount of DMSO; the maximum amount of DMSO was maintained at <0.1% v/v.

2.4. Measurement of nitrite and cytokines

The amount of nitrite and cytokines (IL-1β, IL-6, TNF-α) released by the mouse macrophages was determined by measuring the amount of each substance in the RAW264.7 cell culture supernatant. RAW264.7 cells were plated at a density of 5×10^5 cells in a 24-well cell culture plate with 1000 μl of culture medium and incubated for 12 h. The cells were then treated with various doses of DDP in 100 ng/ml LPS and incubated for another 24 h. The amount of nitrite and cytokines was measured using the Griess reagent system (Promega) and an ELISA kit (eBiosciences, San Diego, CA, USA) according to the manufacturer's instructions.

2.5. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

RAW264.7 cells were plated at 1×10^6 cells/ml in 6-well plates, incubated overnight, and treated with different concentrations of DDP for 1 h, followed by treatment with 100 ng/ml of LPS and incubation for an additional 24 h. Total RNA from RAW264.7 cells was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions.

Total RNA (2 μg) was reverse-transcribed to cDNA by using the SuperScript® III First-Strand Synthesis System (Invitrogen) in a total volume of 20 μl. Real-time PCR assay was carried out with LightCycler Nano (Roche Diagnostics, Germany) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). PCR primers used in this study were: murine iNOS: F 5′-CCT CCT CCA CCC TAC CAA G3′, R 5′-CAC CCA AAG TGC TTC AGT CA-3′; murine IL-1β: F 5′-TTC TCC ACA GCC ACA ATG AG-3′, R 5′-ACG GAC CCC AAA AGA TGA AG-3; murine IL-6: F 5′-CAT CCA GTT GCC TTC TTG GGA-3′, R 5′-CCA GTT TGG TAG CAT CCA TC-3′; murine TNF-α: F-5′-TGT CTC AGC CTC TTC TCA TT-3′, R 5′-AGA TGA TCT GAG TGT GAG GG-3′; murine GAPDH: F 5′-TCT TGC TCA GTG TCC TTG C-3′, R 5′-CTT TGT CAA GCT CAT TTC CTG G-3′. Transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene were quantified as endogenous RNA of reference to normalize each sample. Relative quantities were estimated by the delta-delta-Ct method. The results were normalized as relative expression in which the average value of the iNOS, IL-1β, IL-6, and TNF-α mRNA was divided by the average value of GAPDH mRNA.

2.6. NF-κB reporter assay

RAW NF-κB/SEAP reporter cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and treated with various concentrations of DPP and stimulated with 500 ng/ml LPS for