European Journal of Pharmacology 789 (2016) 195–201

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Cardiovascular pharmacology

Ameliorating effects of 1,8-cineole on LPS-induced human umbilical vein endothelial cell injury by suppressing NF-κB signaling *in vitro*

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ARTICLE INFO

Article history:
Received 10 April 2016
Received in revised form
20 June 2016
Accepted 21 July 2016
Available online 22 July 2016

Keywords:
1,8-Cineole
Human umbilical vein endothelial cell (HUVEC)
Lipopolysaccharide (LPS)
Nitric oxide (NO)
Nuclear transcription factor-kappa B (NF-κB)

ABSTRACT

1,8-Cineole (also known as eucalyptol) is a monoterpene that occurs naturally in many aromatic plants, 1,8-cineole has been reported to ameliorate dysfunction of endothelial cells. However, the mechanism of action of 1,8-cineole is incompletely understood. We investigated the protective effect of 1,8-cineole on lipopolysaccharide (LPS)-induced human umbilical vein endothelial cell (HUVEC) injury and the underlying mechanisms. HUVECs were preincubated with 1,8-cineole for 1.5 h, then exposed to LPS for 12 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase leakage assays showed 1,8-cineole reduced LPS-induced HUVEC injury significantly. Results from enzyme linked immunosorbent assays revealed that 1,8-cineole suppressed LPS-induced secretion of interleukin-6 and interleukin-8, and recovered nitric oxide to normal levels. 1,8-Cineole decreased phosphorylation of nuclear factor-kappa B (NF-κB) p65 and expression of inducible nitric oxide synthase, and simultaneously improved protein levels of endothelial nitric oxide synthase. Immunofluorescence confirmed 1,8-cineole moderates nuclear translocation of NF-κB. These results suggest that 1,8-cineole ameliorates HUVEC dysfunction significantly, and that this effect at least involves NF-κB suppression.

1. Introduction

Atherosclerosis is a crucial pathological factor in the development of cardiovascular diseases, and is the leading cause of death worldwide (Lee et al., 2015; Kondkar and Abu-Amero, 2015; Morris-Rosenfeld et al., 2014). Endothelial cells are crucial for maintenance of the physiological functions of the cardiovascular system. Endothelial dysfunction (especially inflammation) has been implicated in the initiation and propagation of atherosclerotic processes (Castellon and Bogdanova, 2016; Steyers and Miller, 2014; Mudau et al., 2012; Shen et al., 2012). Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and elicits inflammatory responses in immune and non-immune cells, including endothelial cells (Meng et al., 2010). Exposure of endothelial cells to LPS plays an important part in inflammation *via* production of pro-inflammatory cytokines/chemokines, cell adhesion molecules, and nitric oxide (NO) (Kim et al., 2006). Accumulating evidence has confirmed that nuclear transcription factor-kappa B (NF-κB) plays a central part in inflammation and apoptosis through diverse signaling cascades (Koshimizu et al., 2013). NF-κB activation mediates expression of numerous cytokines, which leads to further activation of NF-κB, thereby amplifying and perpetuating the inflammatory response (Ding et al., 2009). NO is produced by nitric oxide synthase (NOS). Endothelial nitric oxide synthase (eNOS)-derived NO is a key signaling molecule in vascular homeostasis and an important regulator of vascular tone and arterial pressure (Rogers et al., 2013). External stress stimuli such as hyperglycemia, LPS, and cytokines (e.g., tumor necrosis factor-α) have been shown to activate NF-κB, which reduces eNOS activity and up-regulates inducible nitric oxide synthase (iNOS) expression (Tsutsuki et al., 2012), finally leading to excessive production of NO and increasing cell damage (Rippe et al., 2012).

Several medicinal herbs have been shown to inhibit specific cellular and humoral immune responses (Chen et al., 2005; Xia et al., 2007; Singh et al., 2008; Shen et al., 2012). 1, 8-Cineole (also known as eucalyptol) is a monoterpene found naturally in many aromatic plants of the *Eucalyptus, Croton, Hyptis, Pectis, Rosamarinus, and Salvia* genera (Rocha et al., 2015). 1,8-Cineole has been suggested to have biological and pharmacological activities, including insecticidal (Tak and Isman, 2015a, 2015b), antimicrobial (Kifer et al., 2016), hepatoprotective (Murata et al., 2015), gastroprotective (Rocha et al., 2015), anti-anxiety (Kim et al., 2014) and

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http://dx.doi.org/10.1016/j.ejphar.2016.07.039
0014-2999/

anti-inflammatory (Kim et al., 2015; Juergens, 2014; Khan et al., 2013) activities. Recently, our research team identified and reported 1,8-cineole to be one of the major bioactive ingredients of the essential oil from Fructus Alpiniae Zerumbet (Zhang et al., 2014), which shows potent protection against injury to vascular endothelial cells (Chen et al., 2014a, 2014b; Tao et al., 2013; Shen et al., 2012). However, the mechanism of action of 1,8-cineole in amelioration of injury to endothelial cells is incompletely understood.

The present study demonstrated that 1,8-cineole suppresses the NF-κB pathway and, in turn, inhibits expression of the inflammatory phenotype of human umbilical vein endothelial cells (HUVECs) induced by LPS.

2. Materials and methods

2.1. Materials

1,8-Cineole was purchased from Aladdin (Shanghai, China). 1,8-cineole was dissolved in dimethyl sulfoxide (DMSO) as a stock solution, stored at –20 °C, and diluted with endothelial cell culture medium before each experiment. LPS (from *Escherichia coli* 0111: B4) was obtained from Sigma-Aldrich (Saint Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Solarbio (Beijing, China). Lysis buffer, NF-κB Activation-Nuclear Translocation Assay kit, NF-κB inhibitor (pyrrolidine dithiocarbamate (PDTC)) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). iNOS antibody (Immunoway Biotechnology, Staffordshire, UK), eNOS antibody (GeneTex, Irvine, CA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Bioworld Technology, Nanjing, China) antibody, and phospho-NF-κB-p65 (Cell Signaling Technology, Danvers, MA, USA) were also obtained. In all experiments, the final concentration of DMSO in the solution was $\leq 0.1\%$.

2.2. Cell culture

Primary HUVECs and the culture medium were purchased from ScienCell Research Laboratories (San Diego, CA, USA). The culture medium comprised basal medium, 5% fetal bovine serum, 1% endothelial cell growth supplement, and 1% penicillin/streptomycin solution. Cells were seeded in poly-L-lysine-coated cell culture flasks (25 cm^2; NEST, Shanghai, China), and maintained in an incubator with 95% humidity and 5% CO_2 at 37 °C. HUVECs were subcultured after trypsinization (0.25% trypsin, 0.5 mM EDTA) when they had grown to $\approx$ 90% confluence. Passages 3–6 were used. In all experiments, the culture medium was replaced with serum-free medium for 12 h before treatment with various concentrations of 1,8-cineole with or without LPS (2.5 μg/ml) as indicated for 12 h, and the vehicle control contained serum-free medium only.

2.3. Assay to measure cell viability

Cells were seeded at 1×10^4 cells per well into 96-well plates. After overnight growth, cells were incubated with 1,8-cineole (2.27, 0.57, 0.14 μM) for 1.5 h, and then exposed to LPS (2.5 μg/ml) for 12 h. At the end of treatment, 20 μl of 5 mg/ml MTT was added to the medium and incubated for 4 h at 37 °C. The supernatant was removed and 150 μl DMSO used to dissolve the precipitate. Absorbance of mixtures was determined at 490 nm using a microplate reader (ELx800; General Electric, Fairfield, CT, USA).

2.4. Lactate dehydrogenase (LDH) leakage from cells

LDH activity (a biomarker of cell injury) in a medium is related to the permeability of cell membranes. We detected LDH activity in culture supernatants using a commercial LDH Activity Assay kit (Bioworld Technology) in accordance with manufacturer instructions.

2.5. Measurement of nitric oxide (NO) production

The cultured HUVECs were seeded in 96-well plates and pre-incubated with 1,8-cineole for 1.5 h, then exposed to 2.5 μg/ml LPS for 12 h at 37 °C. Total NO production was estimated by spectrophotometric measurement of nitrite and nitrate concentrations in the cell culture supernatant fluid using Griess reagent with the Total Nitric Oxide Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Optical density at 540 nm was measured using a microplate reader (ELx800; General Electric). Concentrations were calculated by comparing absorptions with a standard curve.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cultured HUVECs were seeded in 96-well plates and pre-incubated with 1,8-cineole for 1.5 h, supernatants were collected after 12 h of stimulation with 2.5 μg/ml LPS at 37 °C, and stored at −20 °C until assay. Protein levels of interleukin (IL)-6 and IL-8 were determined by an ELISA kit according to manufacturer's instructions (QIYI Biological Technology, Shanghai, China). Absorbance was detected with a microplate reader (ELx800; General Electric) at 450 nm.

2.7. NF-κB localization by immunofluorescence

Detection of NF-κB nuclear translocation was carried out following the instructions of the commercial kit. Briefly, HUVECs on six-well plates were washed with phosphate-buffered saline (PBS) once, fixed with 4% paraformaldehyde, washed thrice with PBS, and blocked with 5% bovine serum albumin (BSA) for 1.5 h at room temperature. Then, cells were incubated overnight with primary antibody against NF-κB p65 at 4 °C, washed thrice in PBS, and incubated with Cy3-labeled secondary antibody for 1.5 h at room temperature. Finally, cells were stained with 4′,6-diamidino-2-phenylindole for 5 min, then observed using a fluorescence microscope (Eclipse TS100; Nikon, Tokyo, Japan). Because of the interference of red fluorescence in cytoplasm it is difficult to quantify red fluorescence in nuclei; thus, translocation of the NF-κB p65 subunit is estimated by observation, as discussed by other authors (Hu et al., 2012; Chen et al., 2014a, 2014b).

2.8. Protein extraction and western blotting

Total proteins were extracted from cultured HUVECs with lysis buffer containing 1 mM phenylmethanesulfonyl fluoride. Lysates were clarified by centrifugation at 12,000 × *g* for 20 min 4 °C and the concentration of protein in the supernatant measured using a BCA Assay kit with a microplate reader (ELx800, General Electric) at 570 nm. Subsequently, equal amounts (30–50 μg) of protein were separated by 6–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 2% BSA with TBST buffer for 1 h, then incubated overnight at 4 °C with rabbit anti-human p65 (1:1000 dilution), rabbit anti-human eNOS (1:1000), rabbit anti-human iNOS (1:600) polyclonal antibodies or rabbit anti-human GAPDH (1:10,000) monoclonal antibody. After washing, membranes were probed further with

Please cite this article as: Linghu, K., et al., Ameliorating effects of 1,8-cineole on LPS-induced human umbilical vein endothelial cell injury by suppressing NF-κB signaling *in vitro*. Eur J Pharmacol (2016), http://dx.doi.org/10.1016/j.ejphar.2016.07.039