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Propofol inhibits lipoteichoic acid-induced *iNOS* gene expression in macrophages possibly through downregulation of toll-like receptor 2-mediated activation of Raf-MEK1/2-ERK1/2-IKK-NFκB

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ABSTRACT

Our previous study showed that propofol suppressed Gram-negative bacterial LPS-induced NO biosynthesis. Lipoteichoic acid (LTA), an outer membrane component of Gram-positive bacteria, can induce septic shock. This study was further aimed to evaluate the effects of propofol on LTA-induced *iNOS* gene expression in macrophages and its possible molecular mechanisms. Exposure of macrophages to LTA increased production of nitrite and intracellular reactive oxygen species, but propofol reduced such enhancements in concentration- and time-dependent manners. Treatment of macrophages with LTA-induced iNOS mRNA and protein productions. Meanwhile, propofol at a clinically relevant concentration of 50 μM significantly inhibited LTA-caused augmentations of iNOS mRNA and protein syntheses. In parallel, exposure to LTA increased translocation of nuclear factor-kappa B (NFκB) from the cytoplasm to nuclei. Propofol at 50 μM decreased such translocation. Analyses by an electrophoretic mobility shift and reporter gene further showed that propofol could alleviate LTA-induced transactivation of NFκB. Sequentially, propofol decreased phosphorylation of IKK, ERK1/2, MEK1/2, and Raf in LTA-stimulated macrophages. Application of toll-like receptor 2 (TLR2) small interference (si)RNA decreased the translation of this receptor and Raf phosphorylation in LTA-stimulated macrophages. Co-treatment with propofol and TLR2 siRNA synergistically ameliorated LTA-induced iNOS mRNA expression and nitrite production. Thus, this study shows that propofol can downregulate NO biosynthesis via inhibiting *iNOS* gene expression. The suppressive mechanism occurs possibly through reduction of TLR2-mediated sequential activation of Raf-MEK1/2-ERK1/2-IKK-NFκB.

1. Introduction

Sepsis, induced by an inadequate inflammatory and immunological host response to Gram-positive or -negative bacteria, is the major cause of mortality in intensive care medicine [1,2]. Lipoteichoic acid (LTA), a Gram-positive bacterial outer membrane component, has been shown to be one of the critical factors participating in the pathogenesis of sepsis [3]. Macrophages play a key role in cellular host defense against infection and tissue injury [4,5]. In response to stimuli, LTA can stimulate macrophages to produce massive amounts of inflammatory factors into the general circulation which exhibit systemic effects [6,7]. NO, one such inflammatory factor, is either a mediator of non-specific cellular immunity or the cause of autoimmune injury during inflammation [8,9]. In the pathophysiology of septic shock, excessive production of NO following induction of *iNOS* gene has been proposed as being a major factor involved in tissue damage [10]. Increases in NO in macrophages can be modulated by a variety of drugs, including anesthetic agents [11,12].

As a safe and effective intravenous anesthetic agent, propofol (2,6-diisopropylphenol) is widely used for inducing and maintaining anesthesia in surgical procedures or for sedation in intensive care units [13,14]. Propofol has the advantages of rapid onset, a short duration of action, and rapid elimination [15]. In studies of macrophages and neutrophils, propofol has been reported to impair cell functions and may contribute to the suppression of host immunity [12,16,17]. Being similar to phenol-containing α-tocopherol and butylated hydroxytoluene in structure, propofol has an antioxidative potential by downregulating the amounts of hydrogen peroxide, superoxide, and the hydroxyl radical [18]. In addition to those oxidants, our previous study further showed that propofol can protect macrophages from NO-induced cell death

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[19]. In response to exposure to LPS, a Gram-negative bacterial endotoxin, propofol decreases NO biosynthesis in macrophages via inhibition of iNOS messenger (m)RNA and protein productions [12].

During inflammation, LTA induces certain gene expressions, including the *iNOS* gene, in macrophages via toll-like receptor (TLR)-dependent mechanisms [20]. TLRs, which are type I transmembrane proteins with extracellular domains comprised largely of leucine-rich repeats and intracellular signaling domains, have at least 12 members found in mammalian cells [21]. TLR2 has been shown to be a major receptor in macrophages responsible for LTA stimulation [22,23]. After binding to endotoxins, the alteration in TLR2's conformation induces cascade activation of intracellular protein kinases [24,25]. Recent studies showed that the Raf protein can mediate the transduction of TLR signaling to diverse biological functions such as cell growth, survival, and differentiation [24,26]. After being phosphorylated by the Raf protein, activated mitogen-activated protein kinase kinase (MEK) 1/2 kinases sequentially trigger extracellular signal-regulated kinases (ERKs) 1/2 [27,28]. Activation of inhibitor kappa B kinase (IKK) by ERK1/2 then stimulates the translocation and transactivation of transcription factor nuclear factor-kappa B (NFκB), which induces the expressions of certain inflammatory genes [29]. NFκB-DNA-binding elements are found in the promoter region of *iNOS* genes [30]. Gram-negative and positive bacteria can stimulate macrophages through activating different TLR members [22,23,31]. In LPS-activated macrophages, we showed that propofol can reduce NO biosynthesis [5,32]. However, the effect of propofol on LTA-induced NO production is still unknown. Thus, this study was further aimed to evaluate the effects of propofol on regulation of LTA-induced NO biosynthesis and *iNOS* gene expression in macrophages and its possible signal-transducing mechanisms.

2. Materials and methods

2.1. Cell culture and drug treatment

Macrophage-like Raw 264.7 cells, purchased from American Type Culture Collection (Rockville, MD, USA), were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum, L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml) in 75-cm^2 flasks at 37 °C in a humidified atmosphere of 5% CO_2.

Propofol purchased from Aldrich (Milwaukee, WI, USA) was protected from light and freshly prepared by dissolving it in dimethyl sulfoxide (DMSO) for each independent experiment. The concentration of DMSO in the medium was kept to <0.1% to avoid toxicity of this solvent to macrophages. According to the clinical application, propofol at ≤50 μM is within a range of clinical plasma concentrations [33]. In this study, therapeutic concentrations of propofol were used to evaluate its effects on macrophages. Control macrophages were treated with DMSO only. LTA (Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4).

2.2. Assay of cell viability

Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously [34]. Briefly, macrophages (1×10^4 cells per well) were seeded in 96-well tissue culture plates overnight. After drug treatment, macrophages were cultured in new medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for a further 3 h. The blue formazan products in macrophages were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm.

2.3. Quantification of nitrite

Levels of cellular NO were quantified by measuring nitrite production in the culture medium of macrophages as described previously [35]. Briefly, after drug treatment, amounts of nitrite, an oxidative product of NO, in the culture medium of macrophages were detected following a technical bulletin of Promega's Griess Reagent System (Promega, Madison, WI, USA).

2.4. Quantification of intracellular reactive oxygen species (ROS)

Amounts of intracellular ROS were quantified by flow cytometry according to a previously described method [36]. Briefly, 1×10^5 macrophages were cultured in 12-well tissue culture clusters overnight, and then co-treated with propofol and 2,7-dichlorofluorescin diacetate, an ROS-sensitive dye. After drug treatment, macrophages were harvested and suspended in PBS buffer. The relative fluorescent intensities in cells were quantified by a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA).

2.5. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assay

Messenger (m)RNAs from macrophages exposed to LTA, propofol, or a combination of propofol and LTA were prepared for qRT-PCR analyses of iNOS and β-actin mRNA. Oligonucleotides for PCR analyses of iNOS and β-actin were designed and synthesized by Clontech Laboratories (Palo Alto, CA, USA). The oligonucleotide sequences of the upstream and downstream primers for these mRNA analyses were, respectively 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-CGACTCCTTTTCCGCTTCCTGAG-3′ for iNOS [12], and 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′ for β-actin [37]. A quantitative PCR analysis was carried out using iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). The relative quantification of our data was performed using the standard curve method according to the manufacturer recommendation. The relative expression level of the target gene was computed with respect to β-actin to normalize for variation in the quality of RNA and the amount of input cDNA. For each experimental sample, the amount of the target gene was determined from a standard curve. The cycle threshold obtained for each amount of pooled cDNA was allowed to draw a linear standard curve of amplification for each target gene. The cycle threshold of any given sample was reported to this standard curve both for the β-actin RNA and each mRNA. The amount of target gene was divided by the β-actin RNA amount to obtain a normalized target value.

2.6. Extraction of nuclear proteins and immunodetection

The amounts of nuclear transcription factors were quantified following a previously described method [38]. Briefly, after drug treatment, nuclear extracts of macrophages were prepared. Protein concentrations were quantified by a bicinchonic acid protein assay kit (Pierce, Rockford, IL, USA). Nuclear proteins (50 μg/well) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. After blocking, nuclear NFκB was immunodetected using a rabbit polyclonal antibody against mouse NFκB (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Total NFκB was immunodetected as the internal standard. Intensities of the immunoreactive bands were determined using a digital imaging system (Uvtec, Cambridge, UK).