行政院國家科學委員會專題研究計畫成果報告

2,6-雙異丙烷酚於發生菌血症時所產生的抗氧化與抗發炎作用之研究: (I)調控一氧化氮與細胞素生成之體外研究

Study of Antioxidative and Anti-inflammatory Effects of 2,6-Diisopropylphenol on Septicemia: (I) An in Vitro Study on Regulations of Nitric Oxide and Cytokines

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Abstract

Background. This study is aimed to evaluate the effects of propofol on nitric oxide production in lipopolysaccharide-activated macrophages.

Methods. Mouse macrophages (cell line Raw 264.7) were cultured and incubated with propofol, lipopolysaccharide and a combination of propofol and lipopolysaccharide. Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetr azolium bromide assay. The levels of nitrite, an oxidative product of nitric oxide, were quantified by the Griess reaction method. Analyses of immunoblot and reverse transcriptase-polymerase chain reaction were carried out to determine the protein and mRNA levels of inducible nitric oxide synthase (iNOS), respectively.

Results. Exposure of macrophages to propofol (25, 50 and 75 M), lipopolysaccharide (0.5, 1, 1.5 and 2 ng/ml) or a combination of propofol and lipopolysaccharide did not affect cell viability. However, propofol at 100 M significantly led to cell death ($P < 0.05$). The levels of nitrite were increased within lipopolysaccharide-treated macrophages in a concentration-dependent manner ($P < 0.01$). Propofol could concentration-dependently decrease the lipopolysaccharide-enhanced nitrite levels ($P < 0.01$). Exposure of macrophages to lipopolysaccharide increased the protein level of iNOS. Co-treatment of propofol and lipopolysaccharide significantly reduced this lipopolysaccharide-induced iNOS protein ($357 \pm 49 \times 10^3$ vs $92 \pm 6 \times 10^3$ arbitrary units, $P < 0.01$). Lipopolysaccharide induced iNOS mRNA, but the inductive effect was inhibited by propofol ($95 \pm 7 \times 10^2$ vs $30 \pm 4 \times 10^2$ arbitrary units, $P < 0.01$).

Conclusions. This study demonstrated that propofol, at therapeutic concentrations, could suppress NO biosynthesis through inhibiting iNOS expression in lipopolysaccharide-activated macrophages and the mechanism of suppression is at a pretranslational level.

Keywords: propofol; macrophages; lipopolysaccharide; inducible nitric oxide synthase; nitric oxide

Introduction

Nitric oxide (NO) is a gaseous free radical synthesized from L-arginine by calcium-dependent constitutive NO synthase or calcium-independent inducible NO synthase (iNOS).[1] The diatomic free radical is an important regulator of vaso-regulation,

neuronal transmission, immune response, and cell apoptosis.[1] [2] NO can be either the mediator of non-specific cellular immunity or the cause of autoimmune injury during inflammation.[3] Lipopolysaccharide (LPS), a gram-negative bacterial outer membrane component, has been identified as one of critical factors involved in the pathogenesis of sepsis.[4] LPS can bind to membrane-localized Toll-like receptors, lead to the induction of specific signal transduction pathways, and produce large amounts of NO into the general circulation to exhibiting systemic effects.[5] [6] In the pathophysiology of septic shock, the excessive production of NO following iNOS induction has been proposed to be a major factor involved in the tissue damage.[7] The increases of NO in macrophages could be modulated by a variety of drugs, including anesthetic agents.[8] [9]

As a safe and effective intravenous anaesthetic agent, propofol (PPF; 2,6-diisopropylphenol) is widely used for induction and maintenance of anaesthesia in surgical procedures or for sedation in the intensive care unit.[10] [11] In the studies of macrophages and neutrophils, PPF has been reported to impair cell functions and may cause immunosuppression.[12] [13] Being similar to phenol-containing -tocopherol and butylated hydroxytoluene in structure, PPF has the potential of antioxidation by directly scavenging hydroxyl chloride, superoxide, hydrogen peroxide and hydroxyl radical.[14] [15] In addition to these oxidants, our previous study has further shown that PPF can protect macrophages from NO-induced cell death.[16]

The effects of PPF on NO biosynthesis are different in various cell types. In cultured porcine aortic endothelial cells and rat ventricular myocytes, PPF has been shown to enhance NO production.[17] [18] A study on canine pulmonary arterial rings revealed that PPF selectively attenuated acetylcholine-induced relaxation by inhibiting NO synthesis.[19] PPF is an effective sedative for critically ill patients.[11] NO is an important molecule in LPS-involved septic pathophysiology.[4] [5] However, the role of PPF in regulating NO biosynthesis in LPS-activated macrophages is still unknown. This study is aimed to evaluate whether PPF could modulate NO synthesis in LPS-activated macrophages and its possible mechanism.

Materials and Methods

Cell culture and drug treatment

A murine macrophage cell line, Raw 264.7, was purchased from American Type Tissue Collection (Rockville, MD, USA). Macrophages 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 g/ml) in 75-cm^2 flasks at 37 ^oC in a humidified atmosphere of 5 % CO_2. The cells were grown to a confluence prior to PPF administration. PPF, a pure compound sponsored by Zeneca Limited (Macclesfield, Cheshire, UK), was freshly prepared by dissolving it in dimethyl sulfoxide (DMSO) for each independent experiment. DMSO in the medium was less than 0.1 % to avoid its toxicity to macrophages. LPS was dissolved in the phosphate-buffered saline buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4).

Determination of cell viability

To determine the appropriate concentrations of PPF and LPS that were not cytotoxic to macrophages, cell viability was assayed by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described previously.[2] Ten thousand macrophages were seeded in 96-well tissue culture clusters for overnight. After preincubation with PPF, LPS and a combination of PPF and LPS, cells were cultured with a new medium containing 0.5 mg/ml MTT for another three hours. The blue formazan product in cells was dissolved in DMSO and measured spectrophotometrically at a wavelength of 550 nm.

Quantification of nitrite

Nitric oxide has such a short half-life that it is easily oxidized to nitrite and nitrate.[1] In

order to evaluate whether PPF could modulate cellular NO, the amounts of nitrite were determined according to the technical bulletin of Promega's Griess Reagent System (Promega Corporation, Madeson, WI, USA). After exposure to the drugs, the culture medium of macrophages was centrifuged, and the supernatant was collected. Following a reaction of the supernatant with sulfaniamide and *N*-1-napthylethylenediamine, a colorimetric azo compound was formed and quantified by an Anthos 2010 microplate photometer (Anthos Labtec Instruments GmbH, Lagerhausstrasse, Wals/Salzburg, Aus). Preliminary studies revealed that exposure of macrophages to 1 ng/ml LPS for 6, 12, 16 and 24 hours led to 50 %, 5-, 10- and 18-folds increases of cellular nitrite. Thus, after treatment for 24 hours, LPS resulted in a maximum increase in cellular nitrite. The time interval of 24 hours was chose for the following experiments.

Immunoblotting analysis

The NO production in macrophages responsible to LPS stimulation is due to the de novo syntheses of iNOS protein.[5] Thus, an immunoblotting analysis was carried out to determine if PPF could modulate iNOS at its protein level. After pretreatment with the drugs, macrophages were washed with the phosphate-buffered saline, and the cell lysates were collected after dissolving the cells in an ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.2, 0.1 % SDS, 1 % Triton X-100, 1 % sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA). To avoid the degradation of cytosolic proteins by proteinases, a mixture of 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate and 5 µg/ml leupeptin was added in the RIPA buffer. Protein concentrations were quantified by a bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Cytosolic proteins (100 µg) were resolved on 12 % polyacrylamide gels and electrophoretically blotted onto nitrocellulose membranes. The membranes were blocked with 5 % non-fat milk at 37 ^{o}C for 1 hour. Immunodetection of cellular iNOS protein was carried out using a mouse monoclonal antibody against mouse iNOS protein (Transduction Laboratories, Lexington, KY, USA). The cellular -actin was immunodetected by a mouse monoclonal antibody against mouse –actin (Sigma, Saint Louis, MI, USA) as an internal standard. Intensities of the immunoreactive bands were determined using an UVIDOCMW Version 99.03 digital imaging system (Uvtec Limited, Cambridge, England, UK).

Analysis of reverse transcriptase-polymerase chain reaction

Messenger RNA from macrophages exposed to PPF, LPS and a combination of PPF and LPS was prepared for analyses of reverse transcriptase-polymerase chain reaction (RT-PCR) of iNOS and -actin mRNA according to the instruction of the ExpressDirect^{TM} mRNA Capture and RT System for RT-PCR kit (Pierce, Rockford, IL, USA). Oligonucleotides for PCR analyses of mouse iNOS and -actin were designed and synthesized by the Clontech Laboratories, Inc. (Palo Alto, CA, USA). The oligonucleotide sequences of upstream and downstream primers for iNOS mRNA analysis were 5'-CCCTTCCGAAGTTTCTGGCAGCAGC -3' and 5'-CGACTCCTTTTCCGCTTCCTGAG-3'. The oligonucleotide sequences of sense and antisense primers for -actin mRNA analysis were 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3'. The PCR reaction was carried out using 35 cycles including 94 ^{o}C for 45 sec, 60 ^{o}C for 45 sec and 72 ^{o}C for 2 min, and the products were loaded and separated in a 1.8 % agarose gel containing 0.1 g/ml ethedium bromide. The amounts of -actin mRNA in macrophages were detected as an internal standard. Intensities of DNA bands in the agarose gel were quantified with the aid of a UVIDOCMW Version 99.03 digital imaging system as described above.

The statistical significance of the

difference between control and PPF-treated groups was evaluated by the Student's *t*-test. A *P* value less than 0.05 was considered as statistically significant. The statistical difference between groups was considered significant when the *P* value of the Duncan's multiple rang test was less than 0.05.

Results

Exposure of macrophages to 25, 50 and 75 M PPF for 1, 6 and 24 hours did not affect cell viability (Table 1). However, treatment with 100 M PPF for 6 and 24 hours caused 16 and 32 % cell death, respectively. LPS at 0.5, 1, 1.5 and 2 ng/ml was not cytotoxic to macrophages (Table 1). Co-treatment of 100 M PPF and 1 ng/ml LPS for 6 and 24 hours significantly led to 21 and 35 % cell death. Treatment with 50 M PPF and various concentrations of LPS was not cytotoxic to macrophages (Table 1).

LPS at 0.5, 1, 1.5 and 2 ng/ml significantly caused 3-, 6-, 14-, 18- and 20-fold increases of nitrite in the culture medium of macropahges, respectively (Fig. 1). Exposure of macrophages to 50 M PPF block LPS-enhanced nitrite levels at 30, 40, 53, 56, 50 %, respectively. PPF did not influence the amounts of nitrite released from macrophages (Fig. 2). Following treatment with 25, 50, 75 and 100 M PPF, the LPS-enhanced nitrite levels were significantly decreased by the anesthetic agent at 18, 33, 52 and 55 %, respectively.

In untreated macrophages, iNOS protein was not detectable (Fig. 3, *top panel*, lane 1). Following treatment with LPS, the levels of iNOS protein were significantly increased (lane 2). PPF *per se* did not affect the expression of iNOS protein (lane 3). Exposure of macrophages to PPF suppressed LPS-induced iNOS protein (lane 4). The amounts of -actin protein in macrophages were immunodetected and used as an internal standard (Fig. 3, *bottom panel*). Quantification of immunorelated protein bands revealed that PPF significantly decreased 74 % LPS-enhanced iNOS protein (Table 3).

After pretreatment with the drugs, mRNA from macrophages was prepared for RT-PCR analyses of iNOS and -actin (Fig. 4). The molecular weights of RT-PCR products for iNOS and -actin mRNA were 497 and 540 base pairs, respectively. In untreated macrophages, iNOS mRNA was not detectable (Fig. 4, *top panel*, lane 2). Following treatment with LPS, iNOS mRNA was apparently induced (lane 3). PPF did not affect the expression of iNOS mRNA (lane 4). Co-treatment with PPF and LPS inhibited the endotoxin-induced iNOS mRNA (lane 5). The amounts of -actin mRNA was detected and quantified as an internal standard (Fig. 4, *bottom panel*). Quantification of RT-PCR products revealed that PPF significantly inhibited 68 % LPS-induced iNOS mRNA (Table 3).

Discussion

Our present study demonstrates that PPF could modulate the levels of NO production in LPS-activated macrophages. In the cell culture system, LPS significantly increased the amounts of nitrite in the culture medium of macrophages (Figs 1 and 2). NO is easily oxidized to nitrite and nitrate.[1] To determine the levels of cellular NO, the amounts of nitrite are sensitively detected by the Griess reaction method. The increase of nitrite in the culture medium of macrophages exposed to LPS may correspond to the enhancement of cellular NO induced by the endotoxin. This study showed that a therapeutic concentration of PPF, 50 M, caused a significant decrease in the amounts of nitrite in LPS-stimulated macrophages (Figs 1 and 2). The suppressive effect of PPF on cellular nitrite response means that this anesthetic was able to inhibit LPS-enhanced cellular NO in macrophages.

The possible mechanism of PPF's suppression to cellular NO production might be through its inhibition of iNOS. In parallel to the increase of cellular NO, the present study showed that the induction of iNOS at the protein and mRNA levels in macrophages was responsible to LPS stimulation (Figs 3 and 4). This result is similar to previous studies that the calcium-independent iNOS protein is involved in the NO production in LPS-activated macrophages.[3-5] Therefore, the LPS-caused increase of cellular NO in

macrophages is due to the de novo synthesis of mRNA and protein of iNOS induced by the endotoxin. PPF, at a clinically relevant concentration, 50 M, significantly decreased LPS-enhanced cellular NO production (Figs 1 and 2). Simultaneously, PPF inhibited LPS-induced the protein and mRNA of iNOS (Figs 3 and 4; Table 2). Thus, PPF could inhibit LPS-induced iNOS and then suppress the amounts of NO in macrophages. From the present data, we suggest that the mechanism of PPF-involved NO suppression is at the pretranslational level.

The signaling pathway of PPF-inhibited iNOS in LPS-activated macrophages is still unknown. CD-14 and Toll-like receptors are two membrane proteins that contribute to the regulation of NO synthesis in macrophages in responses to LPS stimulation.[6 20] The bonding efficacy of LPS to CD-14 and Toll-like receptors plays a critical role in determining the inductive strength of iNOS.[20] Because PPF is highly lipophilic, it may accumulate in cellular membrane.[10] The accumulation of PPF might disturb the membrane integrity, affect the conformation of CD-14 and Toll-like receptors, decrease the bonding efficiency of LPS to these membrane proteins, and finally inhibit iNOS expression. However, other mechanisms are possibly involved in the PPF-caused suppression of NO biosynthesis in LPS-activated macrophages. For example, our unpublished data reveal that PPF can bind to LPS and form a complex with a new **florescence spectrum. The bonding between PPF and LPS may interfere with LPS and the membrane protein interaction and decrease iNOS induction.**

Structurally, PPF is similar to -tocopherol and butylated hydroxytoluene and has been implicated to have the antioxidant potential.[15] Previous studies revealed that PPF could directly scavenge hydroxyl chloride, superoxide, hydrogen peroxide and hydroxy radical and protect varieties of tissues or cells from these oxidants-caused injuries.[14 15 21] A result reported by Cudic and Ducrocq (2000) shows the reaction of PPF with NO-derived peroxynitrite to yield 4-nitrosodioxypropofol and finally 4-nitrosopropofol.[22] However, our previous study revealed a different result that NO released from sodium nitroprusside, a NO donor, induced macrophage apoptosis, but the death effect can be blocked by PPF not through the direct scavenging of NO.[16] Our present study further showed that, in LPS-activated macrophages, PPF could decrease cellular oxidative stress via the suppression of NO biosynthesis.

According to the analyses of chemotaxis and phagocytosis, PPF has been thought to have immunosuppressive effects on macrophages and neutrophils.[12 13] Meanwhile, NO, just like hydrogen peroxide, is one of important effectors produced by macrophages to decompose ingested microorganisms and tumor cells.[3] Hydrogen peroxide has been shown to be directly scavenged by PPF in neutrophils.[12] During inflammation, large amounts of NO are produced following iNOS induction in macrophages.[5 7] The present study showed that PPF could decrease the production of cellular NO. Therefore, the effects of PPF on descending NO biosynthesis may further explain the immunosuppressive characteristics of this intravenous anesthetic agent.

In response to LPS stimulation, PPF might decrease the oxidative stress to cells or tissues through the suppression of NO synthesis in macrophages. The activation of macrophage-dependent proinflammatory cascade plays an important role in the development of increased susceptibility to subsequent sepsis for patients in the intensive care unit.[23] NO is one of major effectors released from activated macrophages.[3 5] The massive production of NO mediates macrophages and other cells undergoing apoptosis, which is closely related to many clinical situations, such as type-I diabetes mellitus or certain neurological disorders like Alzheimer's disease.[24 25] For critically ill patients, PPF is an effective sedative and anesthetic agent.[10 11] This study demonstrated that PPF significantly decreased the levels of cellular NO production in LPS-activated macrophages. NO *per se* can increase cellular stress and contributes to the pathophysiology

of sepsis.[7] From the present data, we suggest that the PPF-caused NO suppression in LPS-activated macrophages may be helpful to decrease the oxidative damage to tissues and cells during sepsis.

In conclusion, the present study demonstrates that PPF has the ability to decrease NO biosynthesis through the inhibition of iNOS at the levels of protein and mRNA in LPS-stimulated macrophages and its mechanism of suppression involves a pretranslational event. This protective effect might benefit the critically ill patients in clinical situation such as sepsis.

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