行政院國家科學委員會補助專題研究計畫成果報告

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執行期間：91 年 08 月 01 日 至 92 年 07 月 31 日

計畫主持人：陳瑞明

共同主持人：陳大樑

本成果報告包括以下應繳交之附件：

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行政院國家科學委員會專題研究計畫成果報告

以體外與體內實驗模式探討 2,6-雙異丙烷酚之抗發炎作用

- IN VITRO AND IN VIVO STUDIES ON THE ANTI-INFLAMMATORY EFFECTS OF 2,6-DIISOPROPYLPHENOL

計畫編號：NSC91－2314－B－038－027－
執行期限：91 年 08 月 01 日至 92 年 07 月 31 日
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Abstract

Septicemia induced by gram-negative bacterial infection is a serious and life-threatening clinical syndrome occurred in intensive care unit patients. The prevalence of sepsis in hospitalized patients appears to significantly increase over the past decades, encountered by both surgeons and internists. Lipopolysaccharide, an endotoxin produced by gram-negative bacteria, is a main cause for endotoxic sepsis. Administration of rats with lipopolysaccharide can induce a sepsis-like syndrome. Immune cells in septic patients can be activated by lipopolysaccharide and increase the releases of pro-inflammatory cytokines and nitric oxide into blood and tissues. These pro-inflammatory cytokines and nitric oxide are important effectors to induce multiple-organs failure in septicemia. Decreasing the productions of inflammatory cytokines and nitric oxide induced by lipopolysaccharide will be an effective treatment for increasing the survival of septic patients.

2,6-Diisopropylphenol, a fat-soluble intravenous anesthetic agent, is widely used in surgical operations for induction and maintenance of anesthesia. Previous studies have shown that 2,6-diisopropylphenol can protect cells from HOCl, superoxide, hydrogen peroxide and hydroxyl radical induced insults. Our previous project (NSC89-2314-B-038-038) has shown that 2,6-diisopropylphenol can protect macrophages from nitric oxide-induced apoptosis. A manuscript from the results of this project has been accepted and will be published on the Canadian Journal of Anesthesia. Study from our another project (NSC90-2314-B-038-045) has further demonstrated that 2,6-diisopropylphenol inhibited lipopolysaccharide-induced inducible nitric oxide synthase mRNA and decreased the levels of nitric oxide. During septicemia-induced inflammation, nitric oxide, hydrogen peroxide and cytokines play important roles in the multiple organ failure syndromes. Study about the effects of 2,6-diisopropylphenol on lipopolysaccharide-induced cytokines is rare. Based on our previous studies of 2,6-diisopropylphenol, this project is designed to evaluate the anti-inflammatory effects of the intravenous anesthetic agent on in vitro macrophages and in vivo septic animals.

This project used macrophage-like Raw 264.7 cells as the experimental model to evaluate the effects of 2,6-diisopropylphenol on modulating pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in lipopolysaccharide-activated macrophages. This study is expected to know more about the interaction between drugs during the anesthesia of septic patients.

Keywords: septicemia, 2,6-diisopropylphenol, in vitro macrophages, lipopolysaccharide, nitric oxide, cytokines, anti-oxidation, anti-inflammation

Introduction

2,6-Diisopropylphenol is an intravenous anesthetic agent with high lipophilicity. The anesthetic agent is widely used in surgical operation for induction and maintenance of anesthesia because it posses rapid onset, short duration of action and rapid elimination (Bryson et al., 1995). 2,6-Diisopropylphenol and α-tocopherol are structurally similar; both contain a phenol group that donates a hydrogen atom to free radicals to terminate their activities (Aarts et al., 1995). Previous studies

have demonstrated that 2,6-diisopropylphenol is an antioxidant which scavenges lipidperoxyryl radicals, hydroxyl radicals, hydrogen peroxide and superoxide (Aarts et al., 1995; Demiryurek et al., 1998; Mikawa et al., 1998). The antioxidant effect of 2,6-Diisopropylphenol against lipid peroxidation is demonstrated in human plasma, rat liver mitochondria, microsomes and brain synaptosomes (Murphy et al., 1992; Eriksson et al., 1992; Aarts, et al., 1995). 2,6-Diisopropylphenol can protect thymocytes and erythrocytes from peroxinitrite- and 2,2'-azo-bis(2-amidinopropane) dihydrochloride-induced apoptosis (Salgo and Pryor, 1996; Murphy et al., 1996). In vitro cell studies have provided the similar evidences that 2,6-diisopropylphenol can protect various types of cells from oxidative stress-induced cell death (Salgo and Pryor, 1996; Navapurkar et al., 1998; Kelicen et al., 1997).. Our previous study has also shown that 2,6-diisopropylphenol can protect mouse macrophages from nitric oxide-induced apoptosis (Chang et al., 2002).

2,6-Diisopropylphenol can suppress immunological response. Mikawa et al (1998) rported that 2,6-diisopropylphenol inhibited the normal function of neutrophils, including chemotaxis, phagocytosis and production of reactive oxidants. 2,6-Diisopropylphenol in mixtures with lidocaine has antibacterial activity (Gajrai et al., 1998). In an animal study, 2,6-diisopropylphenol has been shown to attenuate cytokine response, base deficit, and activation of neutrophils to endotoximia (Taniguchi et al., 2000). However, another study using septic sheep as an experimental model reveals that 2,6-diisopropylphenol was a safe anesthetic agent when used in healthy sheep, but has potentially harmful side effects when given to septic sheep (Booke et al., 1996).

Because 2,6-diisopropylphenol has the potential antioxidant and immunosuppressing effects, the intravenous anesthetic agent might decrease the inflammatory responses for the intra- or post-operated patients. Immunosuppression might further decrease the inflammation-induced tissue or cell damage or increase the risk of bacteria infection. Therefore, evaluating the anti-inflammatory effects of 2,6-diisopropylphenol and its possible mechanism will be basically and clinically important. In comparison, study about the effects of 2,6-diisopropylphenol on macrophages is little although macrophages are critically involved in the pathogenesis of endotoxemia (Freudenberg et al., 1986).

Septicemia is a serous and life-threatening syndrome encountered by both surgeons and internists. A major complication in septic patients in intensive care units is impaired respiration, often leading to adult respiratory distress syndrome and onset of multiple organ failure (Polk and Shields, 1977; Kaplan et al., 1979). Sepsis causes the appearance in plasma of a series of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6, and in tissues of nitric oxide and other oxidants (Wichtermann et al., 1980; Ertel et al., 1991; Walley et al., 1996). This phenomenon has been termed the systemic inflammatory response syndrome. This condition appears to place organs (liver, lung and kidneys) at risk of injury and failure. It has been postulated that during septicemia the lung is especially susceptible to injury in the presence of a direct intrapulmonary insult, such as ischemia, blunt thoracic injury, intrapulmonary presence of bacteria, or ventilator-induced pulmonary injury, to name only a few examples (Moore and More, 1995).

Lipopolysaccharide (LPS) is a gram-negative bacterial outer membrane component. LPS is an important contributing factor to the pathogenesis of sepsis syndrome. Infection caused by LPS constitutes one of the major causes of the sepsis syndrome, characterized by hypotension, tachycardia, tachypnea, disseminated intravascullar coagulation, and multiple organ system failure. The mortality rate for septic patients continues to be unacceptably high, in spite of therapeutic intervention and rigorous supportive care (Bone, 1991).

Major tissue injury, especially when associated with infection, is commonly attended by widespread and uncontrolled activation of the mononuclear phagocyte cell population (Molloy et al., 1993). This process is responsible for the release of massively increased quantities of several inflammatory mediators that spill over into the general circulation to exert systemic effects, collectively constituting the 'sepsis syndrome'. Recent evidence suggests that the preinflammatory cytokines and TNF, IL-1, and IL-6 play a pivotal role in mediating this aberrant host response to major trauma and infection (Galanos and Freudenberg, 1993; Wolkow, 1998).

Septicemia induced by gram-negative bacterial infection is a serious and life-threatening clinical syndrome occurred in intensive care unit patients. LPS has been implicated as a main cause for endotoxic sepsis. LPS can activate the immune cells such as macrophages in septic patients and increase the releases of pro-inflammatory cytokines and nitric oxide into blood and tissues, in which may further induce multiple-organs failure (Polk and Shields, 1977; Kaplan et al., 1979). Inhibiting inflammatory cytokines and nitric oxide production could be beneficial to the survival of septic patients. 2,6-Diisopropylphenol is one of widely used intravenous anesthetic agents for induction and maintenance of anesthesia. Previous and our resent studies have shown that the intravenous anesthetic agent has antioxidant effects on varieties of cells or tissues against various types of oxidants, including nitric oxide and hydrogen peroxide (Aarts et al., 1995; Demiryurek et al., 1998; Mikawa et al., 1998; Chang et al., 2002). However, Study on the

anti-inflammatory effects of 2,6-diisopropylphenol on LPS-induced cytokines is rare.

Based on our previous studies of 2,6-diisopropylphenol, this project is designed to evaluate the anti-inflammatory effects of the intravenous anesthetic agent on in vitro macrophages and in vivo septic animals. This project is expected to know more about the drug-drug interaction occurred in the anesthesia of septic patients.

Materials & Methods

Cell Culture and Treatment

Mouse macrophage-like Raw 264.7 cells: The cell line is purchased from ATCC (USA). The cells are seeded in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum as well as 1000 U/ml of penicillin and 1000 μg/ml of streptomycin. Cells are maintained in an incubator with a condition of 37°C as well as 5% CO_2 and 95% air. The nutrient medium is renewed twice a week.

Macrophages are treated alone with different concentrations of propofol (pure compound, Zeneca, UK) dissolved in dimethylsulfoxide (DMSO) or LPS dissolved in PBS buffer, or the cells are co-treated with these two chemicals for various time intervals.

Determination of Cell Viability

Mitochondrial function of macrophage is assayed by the ability of viable cells to convert soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble dark blue formazan according to the method of Carmichael et al. (1987). In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well of a 96-well plate is measured photometrically. MTT is dissolved in PBS at a concentration of 5 mg/ml and sterilized by passage through a 0.22-μm filter. This stock solution is added (one part to 10 parts medium) to each well of a 96-well tissue culture plate, and the plate was incubated at37 °C for 4 hr. DMSO is added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, the plates are read on a microplate reader at a wavelength of 570 nm. A standard curve is set up using 200-50,000 cells/well, and the absorbency is directly proportional to the number of cells over this range.

ELISA analyses of inflammatory cytokines

The amount of cytokines (TNF-α, IL-1, IL-4, IL-6 and IL-10) in the culture supernatants or blood samples are determined with a commercially available ELISA kit (Endogen, Boston, MA). Briefly, 50 μl of the sample plus the biotinylated antibody reagent are added to each of the wells on the mivrotiter plates which were pre-coated with a capture antibody to cytokines (TNF- α, IL-1, IL-4, IL-6 and IL-10) and incubated at room temperature for two hours. After the preparation has been washing buffer, 100 μl of diluted streptavidin-horseradish peroxidase concentrate are added to each well and the preparations are incubated at room temperature for 30minutes. After the mixture is washed whit the washing buffer, 100 μ l of a premixed tetramethylbenzidine substrate solution are added to each well and plate was developed at room temperature in the dark for 30 minutes. The reaction is terminated by the addition of 100 μl of provided stop solution (0.18M H_2SO_4). The absorbency is measured at 450 nm with a microplate photometer. The cytokines (TNF- α, IL-1, IL-4, IL-6 and IL-10) concentration are determined using a standard curve.

Immunoblotting analyses of cytokines and iNOS proteins

The concentrations of proteins from macrophages or tissues are determined with the BCA method (Pierce, Rockford, U.S.A.). Protein (100 μg) is resolved on 12.5% polyacrylamide gels and blotted onto nitrocellulose sheets using the semidry blot system (TE 70; Hoefer Scientific Instruments, San Francisco, CA) at 2 mA/cm2 for 60 min in 25 mM Tris-HCl, pH 8.3; 192 mM glycine; and 20% methanol. The membrane is blocked overnight at room temperature with a blocking reagent (20 mM Tris, pH 7.4; 125 mM NaCl; 0.2% Tween 20; 4% nonfat dry milk; and 0.1% sodium azide). Then it is incubated for 1 h with the primary antibodies, washed three times, and then incubated with alkaline phosphatase-conjugated rabbit anti-mouse or goat anti-rabbit antibody in PBS and 0.5% Tween 20 for another 45 min with gentle shaking. After three final washes, the proteins are made visible by the Bio-Rad NBT-BCIP color development system.

RT-PCR analyses of inflammatory cytokines and iNOS

RNA is isolated from the guanidinium buffer by the well-established acid guanidinium–phenol–chloroform method. The amount of isolated RNA is measured by a spectrophotometer. By incubation at 40 °C for 60 min, cDNA is synthesized from 2.5 μg RNA with 20 μl total reaction mixture including tris-HCl buffer, 1 mm deoxyribonucleoside triphosphate, and 0.125 μm oligo dT primers, as well as 20 U RNase inhibitor and 0.25 U avian myeloblastosis virus reverse transcriptase. After 60 min incubation, the reverse transcriptase is inactivated by heating to 95°C for 2 min. The semiquantitative reverse transcription PCR mixture (50 μl) contains cDNA synthesized from 0.5 μg RNA 10 mm tris–HCl (pH 8.3), 50 mm KCl, 2.5 mm MgCl2, 0.2 mm dNTP, 0.2 μm 5' and 3' oligonucleotide primers, and 2.5 U Taq polymerase (Takara, Tokyo, Japan). The reaction mixture then is amplified in a DNA thermocycler (Perkin-Elmer, Irvine, CA). Each cycle consists of denaturation at 94°C for 1 min, annealing at 56 – 60 °C for 1 min, and extension at 72 °C for 1 min. The PCR products are separated by electrophoresis on a 1.8 % agarose gel containing 0.5 μg/ml ethidium bromide. PCR products are visualized on a transilluminator (model

FBTIV-816, Fisher Scientific, Pittsburgh, PA) at a 312-nm wavelength and photographed with Polaroid 667 film (Japan Polaroid, Tokyo, Japan). The band images are obtained by scanning the photograph with a ScanJet 3P (Hewlett-Packard, Cupertino, CA). The total intensity (average intensity × total pixels) of each band is measured with Mocha software (Jandel Scientific Software, San Rafael, CA). To evaluate the relative amount of cytokine mRNA in each sample, the cytokine (or iNOS) : β-actin ratio of the intensity of ethidium bromide luminescence for each PCR product is calculated.

Statistical Analysis

The statistical significance of the difference between control and treated groups was evaluated by Student's *t*-test. A P value < 0.05 was considered as statistically significant.

Results

Table 1. Effects of propofol and lipopolysaccharide on macrophage viability

	Cell Viability, values at O.D. 550 nm		
	1h	6h	24h
Control	0.965 ± 0.157	0.917 ± 0.148	1.124 ± 0.274
LPS	0.859 ± 0.187	0.932 ± 0.135	1.107 ± 0.314
PPF	0.947 ± 0.213	0.924 ± 0.098	1.081 ± 0.195
PPF + LPS	0.859 ± 0.210	0.897 ± 0.212	1.036 ± 0.261

Macrophages were treated with 50 μM propofol (PPF), 1 ng/ml lipopolysaccharide (LPS) and a combination of PPF and LPS for 1, 6 and 24 hours. Cell viability was determined by a colorimetric MTT method as described in Materials and Methods. Each value is expressed as Mean ± SEM for n = 12.

Table 2. Suppressive effects of propofol on lipopolysaccharide-induced macrophage TNF-α protein

	TNF-α, pg/ml		
	1h	6h	24h
Control	46 ± 13	28 ± 11	27 ± 10
LPS	156 ± 44*	61 ± 12*	58 ± 16*
PPF	49 ± 13	23 ± 9	28 ± 8
PPF + LPS	62 ± 19†	49 ± 14†	31 ± 9†

Macrophages were treated with 50 μM propofol (PPF), 1 ng/ml lipopolysaccharide (LPS) and a combination of PPF and LPS for 1, 6 and 24 hours. The levels of TNF-α protein released from macrophages to culture medium were quantified by an ELISA kit as described in Materials and Methods. Each value is expressed as Mean ± SEM for n = 9. *Values are significantly different from the respective control, P < 0.05. †Values are considered to have statistical difference between a combination of PPF and LPS- and LPS-treated groups, P < 0.05.

Table 3. Suppressive effects of propofol on lipopolysaccharide-induced macrophage IL-1β protein

	IL-1β, pg/ml		
	1h	6h	24h
Control	78 ± 18	45 ± 16	37 ± 14
LPS	321 ± 85*	412 ± 98*	134 ± 33*
PPF	69 ± 19	39 ± 12	24 ± 6
PPF + LPS	158 ± 33†	85 ± 21*†	65 ± 29†

Macrophages were treated with 50 μM propofol (PPF), 1 ng/ml lipopolysaccharide (LPS) and a combination of PPF and LPS for 1, 6 and 24 hours. The levels of IL-1β protein released from macrophages to culture medium were quantified by an ELISA kit as described in Materials and Methods. Each value is expressed as Mean ± SEM for n = 9. *Values are significantly different from the respective control, P < 0.05. †Values are considered to have statistical difference between a combination of PPF and LPS- and LPS-treated groups, P < 0.05.

Table 4. Suppressive effects of propofol on lipopolysaccharide-induced macrophage IL-6 protein

	IL-6, pg/ml		
	1h	6h	24h
Control	5.6 ± 1.8	4.1 ± 1.4	2.8 ± 1.0
LPS	16.8 ± 4.7*	35.1 ± 7.9*	6.7 ± 2.3*
PPF	6.8 ± 2.1	3.7 ± 1.0	1.9 ± 0.8
PPF + LPS	7.1 ± 1.7†	5.4 ± 2.3†	2.4 ± 0.7†

Macrophages were treated with 50 μM propofol (PPF), 1 ng/ml lipopolysaccharide (LPS) and a combination of PPF and LPS for 1, 6 and 24 hours. The levels of IL-6 protein released from macrophages to culture medium were quantified by an ELISA kit as described in Materials and Methods. Each value is expressed as Mean ± SEM for n = 9. *Values are significantly different from the respective control, P < 0.05. †Values are considered to have statistical difference between a combination of PPF and LPS- and LPS-treated groups, P < 0.05.

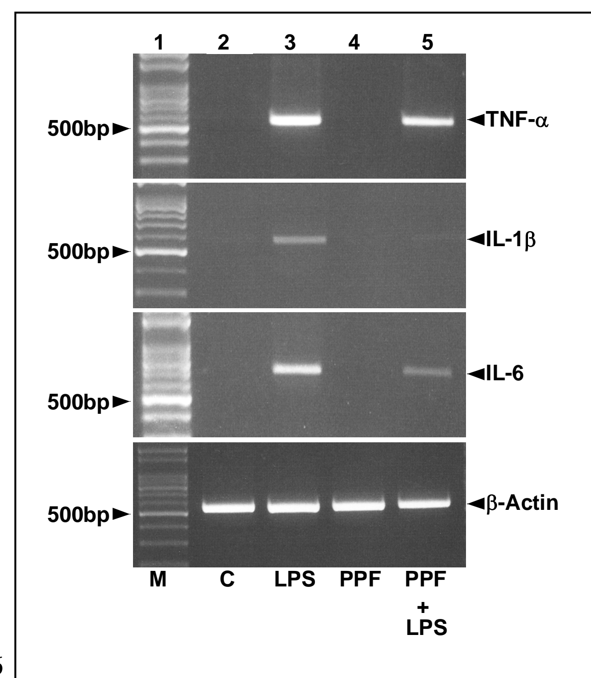

Fig. 1. Effects of propofol (PPF) on LPS-induced inflammatory cytokine mRNA.. Macrophages were exposed to LPS, PPF and a combination of PPF and LPS for 1 hour. RT-PCR analyses of TNF-α, IL-1β and IL-6 were carried out. The levels of β-actin mRNA were used as an internal control.

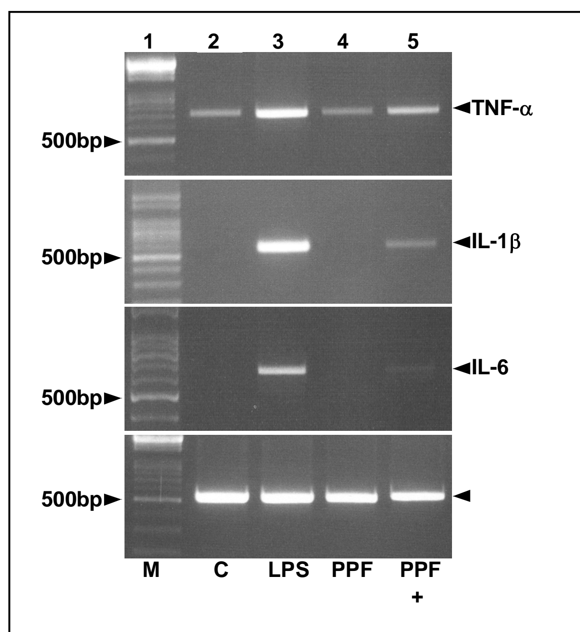

Fig. 2. Effects of propofol (PPF) on LPS-induced inflammatory cytokine mRNA. Macrophages were exposed to LPS, PPF and a combination of PPF and LPS for 6 hours. RT-PCR analyses of TNF-α, IL-1β and IL-6 were carried out. The levels of β-actin mRNA were used as an internal control.

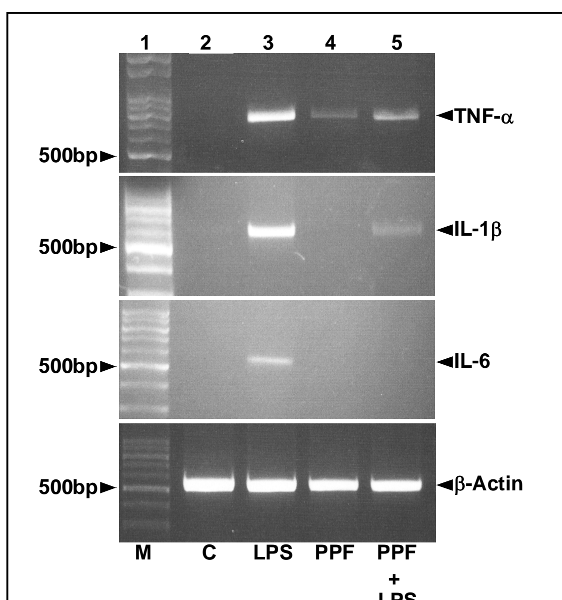

Fig. 3. Effects of propofol (PPF) on LPS-induced inflammatory cytokine mRNA. Macrophages were exposed to LPS, PPF and a combination of PPF and LPS for 24 hours. RT-PCR analyses of TNF-α, IL-1β and IL-6 were carried out. The levels of β-actin mRNA were used as an internal control.

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