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Propofol reduces nitric oxide biosynthesis in lipopolysaccharide-activated macrophages by downregulating the expression of inducible nitric oxide synthase

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Abstract Nitric oxide is an active oxidant that contributes to the physiology and pathophysiology of macrophages. Propofol has been widely used in intravenous anesthesia. It possess antioxidant and immunomodulating effects. This study aimed to evaluate the effects of propofol on nitric oxide production in lipopolysaccharide-activated macrophages. Exposure of macrophages to propofol (25, 50 and 75 μM), to lipopolysaccharide (0.5, 1, 1.5 and 2 ng/ml) or to a combination of propofol and lipopolysaccharide did not affect cell viability. However, propofol at 100 μM led to significant cell death ($P < 0.05$). The levels of nitrite, an oxidative product of nitric oxide, were increased in lipopolysaccharide-treated macrophages in a concentration-dependent manner ($P < 0.01$), while propofol could concentration-dependently decrease the lipopolysaccharide-enhanced nitrite levels ($P < 0.01$). Immunoblotting analysis revealed that lipopolysaccharide increased the protein level of inducible nitric oxide synthase (iNOS). The co-treatment of propofol and lipopolysaccharide significantly reduced this lipopolysaccharide-induced iNOS protein ($357 \pm 49 \times 10^3$ versus $92 \pm 6 \times 10^3$ arbitrary units, $P < 0.01$). Analysis by reverse transcriptase-polymerase chain reaction showed that lipopolysaccharide induced mRNA of iNOS, but that the inductive effect was inhibited by propofol ($95 \pm 7 \times 10^2$ versus $30 \pm 4 \times 10^2$ arbitrary units, $P < 0.01$). This study has demonstrated that propofol, at therapeutic concentrations, could suppress nitric oxide biosynthesis by inhibiting iNOS expression in lipopolysaccharide-activated macrophages. The mechanism of suppression was at a pre-translational level.

Keywords Propofol · Macrophages · Lipopoly saccharide · Inducible nitric oxide synthase · Nitric oxide

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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) (Moncada et al. 1991). The diatomic free radical is an important regulator of vasoconstriction, neuronal transmission, immune response, and cell apoptosis (Moncada et al. 1991; Horibe et al. 2000; Chen et al. 2002). NO can be either the mediator of non-specific cellular immunity or the cause of autoimmune injury during inflammation (Nathan 1992). Lipopolysaccharide (LPS), a gram-negative bacterial outer membrane component, has been identified as one of critical factors involved in the pathogenesis of sepsis (Raetz et al. 1991). In response to stimuli, LPS can bind to membrane-localized Toll-like receptors, lead to the induction of specific signal transduction pathways, and release large amounts of NO into the general circulation to exhibit systemic effects (West et al. 1994; Schuster and Nelson 2000). In the pathophysiology of septic shock, the excessive production of NO following iNOS induction has been proposed as a major factor involved in the tissue damage (Lynn and Cohen 1995). The increases of NO in macrophages could be modulated by a variety of drugs, including anesthetic agents (Shimaoka et al. 1996; Chiou et al. 2001).

As a safe and effective intravenous anesthetic agent, propofol (PPF, 2,6-diisopropylphenol) is widely used for

induction and maintenance of anesthesia in surgical procedures, or for sedation in the intensive care unit (Sebel and Lowden 1989; Young et al. 2000). In studies on macrophages and neutrophils, PPF has been reported to impair cell functions and may contribute to the suppression of host immunity (Mikawa et al. 1998; Kotani et al. 1998). Being similar in structure to phenol-containing α-tocopherol and butylated hydroxytoluene, PPF has the potential for antioxidation by directly scavenging hydroxyl chloride, superoxide, hydrogen peroxide and hydroxyl radical (Murphy et al. 1992; Demiryurek et al. 1998). In addition to these oxidants, our previous study has further shown that PPF can protect macrophages from NO-induced cell death (Chang et al. 2002).

The effects of PPF on NO biosynthesis are different among various cell types. In cultured porcine aortic endothelial cells and rat ventricular myocytes, PPF has been shown to enhance NO production and cause the vasodilatation or negative chronotropy (Petros et al. 1993; Yamamoto et al. 1999). A study on canine pulmonary arterial rings revealed that PPF selectively attenuated acetylcholine-induced relaxation by inhibiting NO synthesis (Beutler and Poltorak 2001). PPF is often used for sedation of patients suffering from critical illness such as sepsis (Young et al. 2000). NO is an important effector in LPS-involved septic pathophysiology (Raetz et al. 1991; West et al. 1994). However, the role of PPF in regulating NO biosynthesis in LPS-activated macrophages is still unknown. This study aimed to evaluate whether PPF could modulate NO synthesis in LPS-activated macrophages, and to study the possible mechanism of any such modulation.

Materials and methods

Cell culture and drug treatment

A murine macrophage cell line, RAW 264.7, purchased from American Type Tissue Collection (Rockville, MD, USA) was used in this study as an experimental model. 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 μg/ml) in 75-cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2. The cells were grown to confluence prior to PPF administration. PPF, a pure compound sponsored by Zeneca Ltd (Macclesfield, UK), was freshly prepared for each independent experiment by dissolution in dimethyl sulfoxide (DMSO). The DMSO concentration in the medium was less than 0.1 % to avoid its toxicity to macrophages. LPS was dissolved in phosphate-buffered saline (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 (Liu et al. 2001). Ten-thousand macrophages were seeded in 96-well tissue culture clusters for overnight culture. After preincubation with PPF, LPS and a combination of PPF and LPS, cells were cultured with fresh medium containing 0.5 mg/ml MTT for another 3 h. 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 (Moncada et al. 1991). 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 Corp., Madison, 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, Wals/Salzburg, Austria). Preliminary studies revealed that exposure of macrophages to 1 ng/ml LPS for 6, 12, 16 and 24 h led to 0.5-, 5-, 10- and 18-fold increases of cellular nitrite. Thus, after treatment for 24 h, LPS resulted in a maximum increase in cellular nitrite. The time interval of 24 h was chosen for the subsequent experiments.

Immunoblotting analysis

The NO production in macrophages in response to LPS stimulation is due to de novo syntheses of iNOS protein (West et al. 1994). Thus, an immunoblotting analysis was carried out to determine whether PPF could modulate iNOS protein. After pretreatment with the drugs for 24 h, macrophages were washed with the phosphate-buffered saline, and the cell lysates were collected after dissolving the cells in 50 μl of ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.2, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM ethylenediaminetetraacetic accid). To avoid the degradation of cytosolic proteins by proteinases, a mixture of 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 5 μg/ml leupeptin was added to 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% nonfat milk at 37°C for 1 h. Immunodetection of cellular iNOS protein was carried out using a mouse monoclonal antibody against mouse iNOS protein (Transduction Laboratories, Lexington, KY, USA). Cellular β-actin was immunodetected by a mouse monoclonal antibody against mouse β-actin (Sigma, St. Louis, MO, USA) as an internal standard. Intensities of the immunoreactive bands were determined using the digital imaging system UVIDOCMW version 99.03 (Uvtec Ltd, Cambridge, UK).

Analysis of reverse transcriptase-polymerase chain reaction

Messenger RNA from macrophages exposed to PPF, or LPS or a combination of PPF and LPS was prepared for analyses of reverse transcriptase-polymerase chain reaction (RT-PCR) of iNOS and of β-actin according to the instruction of the ExpressDirect mRNA Capture and RT System for RT-PCR kit (Pierce). Oligonucleotides for PCR analyses of mouse iNOS and β-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, respectively, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-CGACTCCTTTTCCGCTTCCTGAG-3′. The oligonucleotide sequences of sense and antisense primers for β-actin mRNA analysis were 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′, respectively. The PCR reaction was carried out using 35 cycles including 94°C for 45 s, 60°C for 45 s and 72°C for

Table 1 Effects of propofol (PPF),lipopolysaccharide (LPS) and a combination of PPF and LPS on macrophage viability. Macrophages were exposed to PPF, LPS or a combination of PPF and LPS for 1, 6 and 24 h. Cell viability was determined by the MTT assay as described in Materials and methods section. Each value represents mean ± SEM for $n = 12$

Treatment		Cell viability (% of control)		
Agent	Concentration	1 h	6 h	24 h
Control		100	100	100
PPF	25 μM	100 ± 5	99 ± 8	103 ± 6
	50 μM	102 ± 6	104 ± 6	97 ± 8
	75 μM	99 ± 4	97 ± 5	93 ± 7
	100 μM	97 ± 7	84 ± 5*	68 ± 8*
LPS	0.5 ng/ml	101 ± 7	101 ± 6	104 ± 7
	1.0 ng/ml	95 ± 8	98 ± 7	105 ± 6
	1.5 ng/ml	104 ± 5	103 ± 6	99 ± 5
	2.0 ng/ml	103 ± 4	99 ± 5	101 ± 7
PPF + LPS	25 μM + 1.0 ng/ml	96 ± 5	95 ± 8	91 ± 9
	50 μM + 1.0 ng/ml	93 ± 8	102 ± 6	99 ± 3
	75 μM + 1.0 ng/ml	101 ± 5	98 ± 5	102 ± 4
	100 μM + 1.0 ng/ml	95 ± 8	79 ± 4*	65 ± 8*
	50 μM + 0.5 ng/ml	94 ± 9	94 ± 9	96 ± 7
	50 μM + 1.0 ng/ ml	100 ± 7	101 ± 7	104 ± 8
	50 μM + 1.5 ng/ml	97 ± 6	100 ± 5	95 ± 9
	50 μM + 2.0 ng/ml	97 ± 8	95 ± 6	98 ± 3

*$P < 0.05$, values are considered to be statistically different from the respective control

2 min, and the products were loaded and separated in a 1.8% agarose gel containing 0.1 μg/ml ethidium bromide. The amounts of β-actin mRNA in macrophages were detected as an internal standard. The intensities of DNA bands in the agarose gel were quantified with the aid of UVIDOCMW version 99.03 digital imaging system as described above.

Statistical analysis

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 range test was less than 0.05.

Results

The exposure of macrophages to 25, 50 and 75 μM PPF for 1, 6 and 24 h did not affect the cell viability (Table 1). However, treatment with 100 μM PPF for 6 and 24 h 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 with 100 μM PPF and 1 ng/ml LPS for 6 and 24 h led to significant cell death of 21 and 35%, respectively. Treatment with 50 μM PPF and various concentrations of LPS was not cytotoxic to macrophages (Table 1).

LPS at 0.5, 1, 1.5 and 2 ng/ml resulted in significant 6-, 14-, 18- and 20-fold increases of nitrite in the culture medium of macropahges, respectively (Fig. 1). Exposure of macrophages to 50 μM PPF blocked LPS-enhanced nitrite levels by 40, 53, 56, and 50%, respectively. PPF did not influence the amounts of nitrite released from macrophages (Fig. 2). Following treatment with 25, 50, 75 and 100 μM PPF, the LPS-enhanced nitrite levels were significantly decreased by 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 induce the expression of iNOS protein (lane *3*). Exposure of macrophages to PPF suppressed LPS-induced iNOS protein (lane *4*). The amounts of β-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 by 74% the LPS-enhanced iNOS protein (Table 2).

After pretreatment with the drugs, mRNA from macrophages was prepared for RT-PCR analyses of iNOS and β-actin (Fig. 4). The molecular size of RT-PCR products for iNOS and β-actin mRNA was 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

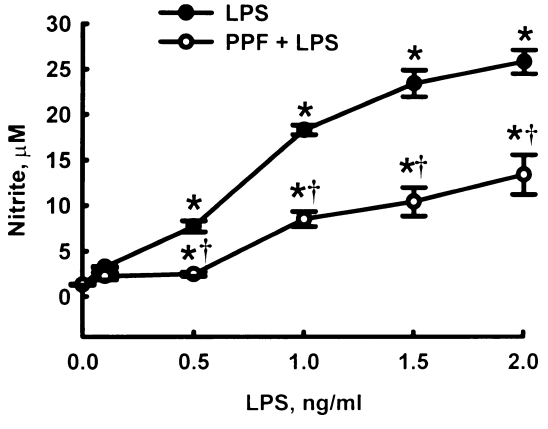

Fig. 1 Concentration-dependent effects of lipopolysaccharide (LPS) on nitrite production. Macrophages were exposed to 0.5, 1, 1.5 and 2.0 ng/ml LPS and a combination of 50 μM propofol (PPF) and LPS at various concentrations for 24 h. The amounts of nitrite in the culture medium were determined by the Griess reaction method. Data are expressed as means ± SEM for $n = 12$. *$P < 0.05$, values significantly different from the respective control. †$P < 0.05$, values significantly different from LPS-treated groups

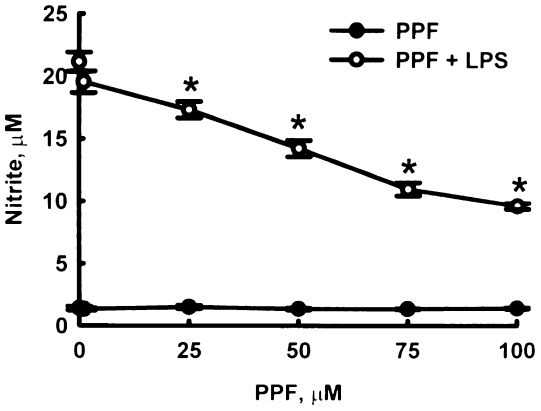

Fig. 2 Concentration-dependent inhibitory effects of propofol (PPF) on lipopolysaccharide (LPS)-enhanced nitrite production. Macrophages were exposed to 25, 50, 75 and 100 μM PPF and a combination of 1 ng/ml LPS and PPF at various concentrations for 24 h. The amounts of nitrite in the culture medium were determined by the Griess reaction method. Data are expressed as means ± SEM for $n = 12$. $^{\dagger}P < 0.05$, values for the combination of PPF and LPS significantly different from those with LPS alone

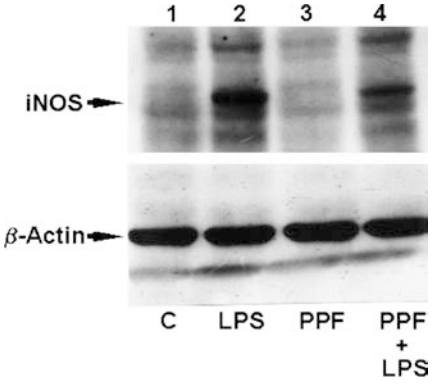

Fig. 3 Immunoblotting analysis of inducible nitric oxide synthase (iNOS) from untreated (*C*) macrophages, and those treated with lipopolysaccharide (*LPS*), propofol (*PPF*) or a combination of PPF and LPS. Macrophages were exposed to 1 ng/ml LPS, 50 μM PPF and a combination of PPF and LPS. Cytosolic proteins were prepared and subjected to protein blot analysis in which mouse monoclonal antibody was used for probe for iNOS protein. The expression of β-actin protein was regarded as an internal standard. The molecular sizes of iNOS and β-actin are 130 and 42 kD, respectively

mRNA was apparently induced (lane *3*). PPF did not enhance the expression of iNOS mRNA (lane *4*). Co-treatment with PPF and LPS inhibited the endotoxin-induced iNOS mRNA (lane *5*). The amounts of β-actin mRNA were detected and quantified as an internal standard (Fig. 4, *bottom panel*). Quantification of RT-PCR products revealed that PPF significantly inhibited by 68% the LPS-induced iNOS mRNA (Table 2).

Discussion

Our present study demonstrates that PPF could modulate the levels of NO production, measured as nitrite, in

Table 2 Effects of propofol (PPF) on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) protein and mRNA in macrophages. Cytosolic proteins and cellular mRNA from macrophages exposed to LPS, PPF or a combination of PPF and LPS were isolated for immunoblotting and RT-PCR analyses, respectively. Intensities of the protein and mRNA bands (in arbitrary units) were obtained from densitometric analyses of the protein blot and RT-PCR using a digital imaging system. Each value represents mean ± SEM for $n > 3$ (*n.d.* not detectable)

Treatment	iNOS, (arbitrary units)	
	Protein	mRNA
Control	n.d.	n.d.
LPS	357086 ± 48612	9478 ± 674
PPF	n.d.	n.d.
PPF + LPS	91587 ± 6475*	3042 ± 414*

*$P < 0.05$, values are considered to be statistically different from the LPS-treated group

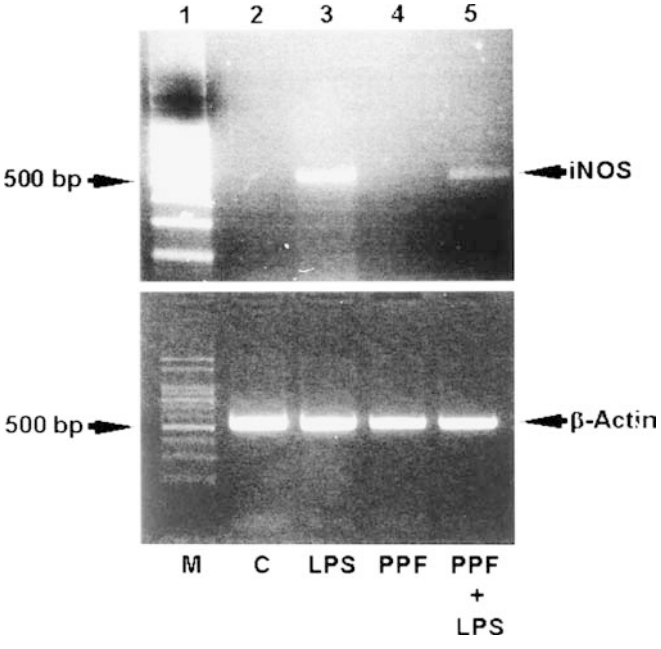

Fig. 4 RT-PCR analysis of inducible nitric oxide synthase (iNOS) from untreated (*C*) macrophages, and those treated with lipopolysaccharide (*LPS*), propofol (*PPF*), or a combination of PPF and LPS. Macrophages were exposed to 1 ng/ml LPS, 50 μM PPF and a combination of PPF and LPS. Cellular mRNA was prepared for RT-PCR analysis of iNOS. The expression of β-actin mRNA was regarded as an internal standard

LPS-activated macrophages. This study showed that a therapeutic concentration of PPF, 50 μM, caused significant decreases 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-enhancement of cellular NO in macrophages. 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 responsive to LPS stimulation (Figs. 3 and 4). This result is similar to previous studies, according to

which the calcium-independent iNOS protein is involved in the NO production in LPS-activated macrophages (Raetz et al. 1991; Nathan 1992; West et al. 1994). PPF, at a clinically relevant concentration, 50 μM, significantly decreased LPS-enhanced cellular NO production (Figs. 1 and 2). Simultaneously, PPF inhibited LPS-induced protein and mRNA of iNOS (Figs. 3 and 4; Table 2). Thus, PPF could inhibit the LPS-related induction of iNOS and hence suppress the amounts of NO in macrophages. From the present data, we suggest that the mechanism by which PPF is involved in NO suppression is at the pretranslational level.

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 (Kirkland et al. 1993; Schuster and Nelson 2000). The binding efficiency of LPS to CD-14 and Toll-like receptors plays a critical role in determining the inductive strength with regard to iNOS (Kirkland et al. 1993). Because PPF is highly lipophilic, it may accumulate in cellular membrane (Sebel and Lowdon 1989). The accumulation of PPF might disturb the membrane integrity, affect the conformation of CD-14 and Toll-like receptors, decrease the binding efficiency of LPS to these membrane proteins, and finally inhibit iNOS expression. However, other mechanisms are also possibly involved in the PPF-induced 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 binding between PPF and LPS may interfere with LPS and the membrane protein interaction, and decrease iNOS induction.

This study provides another affecting mechanism about the antioxidant and immunosupressive characteristics of PPF. Structurally, PPF is similar to α-tocopherol and butylated hydroxytoluene, and has been indicated as having antioxidant potential (Demiryurek et al. 1998; Cudic and Ducrocq 2000). Previous studies revealed that PPF could directly scavenge hydroxyl chloride, superoxide, hydrogen peroxide and hydroxy radical, and protect varieties of tissues or cells from injuries caused by these oxidants (Murphy et al. 1992; Kokita and Hara 1996; Demiryurek et al. 1998). Our present study further showed that, in LPS-activated macrophages, PPF could decrease cellular oxidative stress via the suppression of NO biosynthesis. NO, just like hydrogen peroxide, is one of important effectors produced by macrophages to decompose ingested microorganisms and tumor cells (Nathan 1992; Albina et al. 1993). The effects of PPF on descending NO biosynthesis may further explain the immunosuppressive characteristics of this intravenous anesthetic agent. NO per se can increase cellular stress and contributes to the pathophysiology of sepsis (Le et al. 1995; Lynn and Cohen 1995). Therefore, the PPF-caused NO suppression in LPS-activated macrophages may be helpful to decrease the oxidative damage of tissues and cells during sepsis.

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

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