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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※ **麻醉藥物 Propofol 調控內毒素脂多醣誘導巨噬細胞** ※
※ **氧化壓力之研究** ※
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行政院國家科學委員會專題研究計畫成果報告
麻醉藥物 Propofol 調控內毒素脂多醣誘導巨噬細胞氧化壓力之研究
Study of Modulation of Anesthetic Propofol on Oxidative Stress Induced by Endotoxin Lipopolysaccharide in Macrophage

計畫編號：NSC 89－2314－B－038－038－
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一、中文摘要

評估 propofol 保護巨噬細胞免於一氧化氮(nitric oxide: NO)傷害的作用機制。實驗結果顯示由 NO 所造成巨噬細胞的死亡和凋零作用，可以被 propofol 所拮抗。此一結果說明 propofol 對臨床病人的免疫功能可能具有一定程度的保護作用。

關鍵詞：Propofol、一氧化氮、細胞凋零

Abstract

To evaluate the potential effects of the clinically relevant concentrations of propofol on cell viability and nitric oxide-induced macrophage apoptosis. The amounts of nitric oxide in mouse macrophage-like Raw 264.7 cells were time-dependently increased when incubated with sodium nitroprusside. Parallel to the increase of nitric oxide, sodium nitroprusside simultaneously caused the cell death of macrophage in a concentration- and time-dependent manner. A therapeutic concentration of propofol (30 μM) exhibited no cytotoxicity to macrophages. After incubated with sodium nitroprusside for 1 and 6 hours, propofol could completely or partially block nitric-induced cell death, respectively. Administration of sodium nitroprusside to macrophages resulted in the time-dependent increase of apoptotic cells. Similar to the results of the cell viability analysis, propofol was able to protect macrophages from nitric oxide-induced apoptosis in 1 and 6 hour-treated groups but not in the 24 hour-treated group. Propofol *per se* did not alter the amount of basal and sodium nitroprusside-provided nitric oxide in macrophage. Propofol, at therapeutic concentrations, can protect macrophages from nitric oxide-induced cell apoptosis as well as cell death and might play a significant role in cellular functions of clinical patients.

Keywords: Propofol, Nitric oxide, Apoptosis.

二、Introduction

PPF (2, 6-diisopropylphnyl), an intravenous anesthetic agent, is widely used in varieties of surgical operations for induction and maintenance of anesthesia.[1] The beneficial effects of PPF in clinical applications are rapid onset, short time of action and rapid elimination. However, certain side effects of cardiac depression or hypotension is possibly observed in surgical patients applied by the intravenous anesthetic agent.[1-3] Studies of human neutrophil and alveolar macrophages reveal that PPF can inhibit cell chemotaxis, phagocytosis, reactive oxygen species (ROS) production and cell numbers, as well as modulate intracellular calcium concentration.[4-5] PPF could have potential effects on suppression of immune responses.

Structurally, PPF is similar to phenol-containing -tocopherol and butylated hydroxytoluene, and has been reported to possess an antioxidant characteristic from the examination of electron spin resonance spectroscopy.[6] Previous studies have shown that PPF could protect varieties of tissues, cells or organelles from different types of free radicals or ROS induced oxidative insults.[7-11] ROS, comprised of superoxide, hydroxyl radical, hydrogen peroxide, NO, and other oxidative derivatives, are constitutively produced as by-products of aerobic metabolisms in mammalian cells or are induced by pathophysiological conditions or xenobiotics.[12] Transformed and nontransformed cells can induce intracellular ROS through autocrine and paracrine mechanisms, and the induced ROS would interfere with endogenous survival factors leading to cell apoptosis.[12-13] In a chemiluminescence study of stimulated human leukocytes or cell-free systems, PPF has been verified to have the ability to scavenge hydroxyl radical, hydrogen peroxide and superoxide.[10]

NO, produced from L-arginine by nitric oxide synthase (NOS), is a gaseous radical with varieties of biological activities.[12,14] NO is one of the important ROS in mammalian cells.[12] Immunologically, the role of NO seems like a double-edged sword. NO is regarded as an important mediator of non-specific cell-mediated immunity because macrophages can release the radical to kill a range of infected pathogens.[15] However, NO induced by

proinflammatory cytokines or endotoxin lipopolysaccharide is cytotoxic to cells.[15-17] On the other hand, the massive induction of NO can cause macrophages themselves and neighboring cells undergoing apoptosis, which is related to severe medical symptoms, including sepsis, type-I diabetes mellitus or certain neurological disorders like Alzheimer's disease.[15-21] To reduce NO-induced oxidative stress will be helpful for the recovery of patients suffering from surgical operation. Study about the interaction of PPF and NO-induced oxidative stress is little.

SNP is a clinical medicine used to release NO for vasodilation. The NO donor is decomposed to NO under the presence of biological tissues, reducing agents or visible light.[22] The use of SNP has its biochemical advantage because the donor permits the investigation of NO's role in signaling transduction pathways without interfering with NOS-involved second messenger systems. The present study is designed to investigate the protective role of the relevantly clinical concentration of PPF in NO-induced cell insults using SNP as the NO donor and mouse macrophage RAW 264.7 cell line as the biological model from the aspects of cell viability, apoptotic analysis and NO quantification.

三、Materials and Methods

This study was designed to investigate the effects of PPF on NO-induced cell insults. To achieve this specific aim, mouse macrophage Raw 264.7 cell line, which was purchased from American Type Tissues Collection (Rockville, MD, USA), was used to be our bio-model. The immune cells were maintained 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 -cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2. Macrophages were grown to a confluence prior to PPF treatment.

PPF, a kind gift from Zeneca Limited (Macclesfield, Cheshire, UK), was stored under nitrogen and protected from light, heat and moisture. This intravenous anesthetic agent was freshly diluted with dimethyl sulfoxide (DMSO) for each independent experiment. To rule out the effect of DMSO on cytotoxicity, the solvent concentration in the medium was less than 0.1%. SNP was dissolved in PBS buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) to a 200 mM of stock concentration, then stored at –20°C, protected from light until it was used in related experiments.

The clinical plasma levels of PPF are between 10 and 50 [23] Preliminary analysis of macrophage viability, influenced by 3, 30 and 300 M PPF, revealed that higher than the therapeutic concentration of PPF was cytotoxic to macrophages, but less than or equal to the therapeutic concentration did not cause cell death. Therefore, PPF at 30 was chosen to be the administered concentration in the present study.

Following treatment of macrophages with SNP, PPF, and a combination of PPF and SNP for 1, 6 and 24 hours, cell viability was analyzed using a variation of a colorimetric 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) assay, which was based on the ability of live cells to reduce MTT to a blue formazan product.[24] Ten thousand macrophages were seeded in 96-well tissue culture plates for overnight. After treatment with the drugs, cells were renewed with a medium containing 0.5mg/ml MTT and then cultured for another three hours. The blue formazan product in cells was dissolved by DMSO and measured spectrophotometrically at a wavelength of 550 nm.

Apoptotic analysis was carried out by detecting the sub-G1 DNA content which resulted from cleavage of DNA during apoptosis to determine if PPF could block NO-induced cell programmed death according to the method of Nicoletti et al. (1991).[25] After treatment with the drugs, macrophages were harvested and fixed in 80% ethanol. In the process of centrifugation and washing, the fixed cells were stained with propidium iodide. The fluorescence intensities of individual nuclei was analyzed using a FACScan flow cytometer on the basis of a 560 nm dichroic mirror and a 600 nm band pass filter for collecting the red fluorescence due to the propidium iodide staining of DNA.

SNP was decomposed to NO under the presence of visible light, simple reducing agents or biological tissues.[21] To evaluate if PPF could directly scavenge NO, the amounts of NO in the culture medium of macrophages treated with SNP, PPF and a combination of PPF and SNP were determined. After treatment with the drugs, the culture medium was collected and centrifuged, and then the supernatants were stored at -70°C. NO has a short half-life that is easily oxidated to nitrite and nitrate. The amount of NO was assayed by reacting nitrite in a culture medium with sulfaniamide and *N*-1-napthylethylenediamine to form a colormetric azo compound according to the technical bulletin of Promega's Griess Reagent System (Promega Corporation, Madeson, WI, USA).

Statistical analysis was carried out between control and drug-treated groups by the Students t-test, and it was considered as a statistical difference when the *P* value was less than 0.05. The values between PPF and a combination of PPF and SNP-treated groups were considered as a statistical difference when the *P* value of the Duncan Multiple Rang test was less than 0.05.

四、Results

SNP was a NO donor that spontaneously released NO under visible light. Treatment of macrophages with SNP conentration- and time-dependently increased NO in macrophages (data not shown). In the 1 hour-treated group, SNP at 1.5 and 2.0 mM lead to 10 and 15% of macrophage death, respectively (Fig. 1). In the 6 hour-treated group, 0.5,

1.0, 1.5 and 2.0 mM of SNP suppressed 10, 17, 22 and 35% of cell viability, respectively. After treatment with 0.5, 1.0, 1.5 and 2.0 mM of SNP for 24 hour, macrophage viability was inhibited by 48, 75, 96 and 96%, respectively.

Administration of macrophages with 2 mM SNP for 1, 6 and 24 hours resulted in 15, 31 and 96% of cell death, respectively (Fig. 2). Under the present treated conditions, the relevantly clinical concentration of PPF was not cytotoxic to macrophages. In 1 hour-treated group, PPF completely blocked NO-mediated macrophage death. In the 6 hour-treated group, the intravenous anesthetic agent partially but significantly protected macrophage from NO-caused cell death. In the 24 hour-treated group, PPF did not inhibit macrophage death caused by SNP.

Apoptotic analysis revealed that treatment of macrophages with SNP for 1, 6 and 24 hours significantly augmented 18, 35 and 53% of cells processing apoptosis, respectively (Fig. 3). The therapeutic concentration of PPF did not affect macrophage apoptosis. In the 1 hour-treated group, PPF completely protected macrophages from NO-induced apoptosis. In the 6 hour-treated group, the anesthetic agent partially but significantly blocked NO-mediated macrophage apoptosis. In the 24 hour-treated group, PPF did not protect macrophages from NO-induced cell apoptosis.

Administration of macrophages with 2 mM SNP for 1, 6 and 24 hours resulted in about 5-, 6- and 7-fold increase in NO (Table 1). PPF at a therapeutic concentration did not affect the amount of NO in macrophages under the present treated conditions. In a combination of PPF and SNP, the intravenous anesthetic agent did not change the amounts of NO, which is released by SNP.

五、Discussion

Macrophages play a crucial role in bacterial defense for pre-, intra- or post-operated patients suffering from microorganism invasion. Macrophages can produce ROS to attack and decompose the invaded microorganisms and tumor cells.[15] However, over-production of ROS simultaneously harms macrophages themselves and the surrounding cells.[18] NO, one of the important ROS, can either be the effector of non-specific cell mediated immunity or be the cause of the pathogenesis of autotissue injury during inflammation.[26] NO is enormously produced by inducible NOS in endotoxin lipopolysaccharide- or cytokines-activated macrophages, and high concentrations of the free radical can drive macrophages to undertake a apoptotic pathway which uld lead to other more severe syndromes such as septic shock, neurotoxicity, autoimmune diseases and chronic inflammation.[15,17,19,20,22] Surgical operation is a typical treatment for intensive care unit patients suffering from sepsis. PPF is one of the widely used intravenou anesthetic agents, and structurally, the anesthetic has a potent antioxidant effect on hydrogen peroxide, hydroxyl radical and superoxide induced tissue or cell injury.[6-11] From the viewpoint of basic research and clinical application, to evaluate the effects of PPF on NO-induced macrophage insults is important.

NO released by SNP is cytotoxic to macrophages. The basal level of NO in the untreated macrophages is detectable (data not shown). SNP is an NO donor that can be enzyme or non-enzyme metabolically decomposed to NO.[20] After treatment with the NO donor, the amount of nitrite, an oxidative product of NO, in the culture medium is significantly augmented in a time dependent manner (Table 1). This means that the oxidative stress in macrophages is excited after SNP administration. Treatment with SNP causes concentration- and time-dependent decreases of macrophage viability (Fig. 1). The present results are similar to previous studies, which report that NO at high concentrations is cytotoxic to macrophages.[21,29,30] Macrophage NO in inoculated mice seven days after injection of *Salmonella typhimurium* mediates immunosuppression in the infectious inflammation.[27] Lipopolysaccharide is an endotoxin derived from invaded bacteria and is one of the important causes for bacterial induced immunosuppression. Our unpublished data reveals that administration of mouse macrophage Raw 264.7 cells with lipopolysaccharide resulted in about a seven fold increase in NO, similar to five to seven fold growth of NO magnified by SNP (Table 1). Thus, over-production of NO derived from SNP treatment, like the lipopolysaccharide stimulation, would result in the increase of intracellular oxidative stress leading to macrophage death.

Macrophage death induced by NO is basically through the apoptotic pathway. Apoptosis, or programmed cell death, is an energy-dependent model of cell death with certain characteristics, including shrinkage morphology, chromatin condensation and DNA cleavage.[28,29] Cells undergoing apoptosis are preceded by chromatin cleavage, and the reduced DNA content of apoptotic nuclei causes a unequivocal hypodiploid DNA peak in flow cytometry analysis.[25] Administration with SNP leads to the macrophage shrinkage (data not shown), and the increase of apoptotic cells, which corresponds to the appearance of the hypodiploid DNA peak (Fig. 3). Several lines of evidence from the morphological observation and flow cytometry analysis reveal that NO released by SNP causes macrophage apoptosis. Nevertheless, Dypbukt et al. (1994) reports that high concentrations of NO donors cause LDH release and necrotic cell death.[30] SNP at high concentrations did not rule out the possibility that the NO donor could partially induce macrophage necrosis.

A therapeutic concentration of PPF could block NO-induced cell death. During anesthesia, the dose is an important factor to determine whether the administered anesthetic agent is therapeutic or toxic to patients. The concentration of PPF used in this study is 30 M, which is in the range of clinical plasma

levels of PPF.[23] The relevantly clinical concentration of PPF is not cytotoxic to macrophages (Fig. 2). In preliminary studdies, 300
time-dependent increase of macrophage death (data not shown). In the treatment of a combination of PPF and SNP, the anesthetic agent sgnificantly protects macrophages from NO-induced cell apoptosis (Figs. 2 and 3). PPF has antioxidant characteristics to protect from lipid peroxidation in isolated liver mitochondria,[7] hydrogen peroxide-induced derangements in the isolated rat heart,[31] lipid hydroperoxides-caused haemoglobin oxidation,[32] 2, 2'-azobis (2-amidinopropane) dihydrochloride-, a free radical generator, induced death of isolated rat hepatocytes,[11] and non-receptor-mediated oxidative glutamate toxicity of neuronal cells.[33] This is the first time to verify that PPF is able to protect from NO-induced cell death.

The protective effect of PPF on NO-induced macrophage death is time-dependently descended. In the 1 hour-treated group, PPF completely protected NO-induced cell death, but the effect decreased and even no effect in 6 hour- and 24 hour-treated groups, respectively (Figs. 2 and 3). PPF has a clinical advantage of rapid onset and elimination.[1] The anesthetic agent is metabolized through cytochrome P450-dependent monooxygenases-involved mono-oxygenation to 2, 6-diisopropyl-1, 4-quinol or UDP glucuronosyltransfease-participated glucuronidation to glucuronide complex.[34] Cytochrome P450 isoenzymes are detectable in the untreated and inducer-treated murine macrophage cell line, RAW264.7.[35] In untreated macrophages and benzene-treated macrophages, UDP glucuronosyltransfease activity is detectable and able to be elevated.[36] Therefore, the lessened contents of PPF in macrophages owing to its metabolism by the related enzymes may explain the descent protection effect of the intravenous anesthetic agent on NO-induced cell death in 6 hour- and 24 hour-treated macrophages. Another possible reason for explaining the time-dependent decrease of the protective effect of PPF is that, due to the light and oxygen sensitivity of the anesthetic agent, PPF may be time-dependently decomposed after exposure to the visible light and aerobic conditions.

PPF does not affect NO production in control and SNP-treated macrophages, and, consequently, the protective mechanism of the intravenous anesthetic agent from NO-induced insults is not through direct scavenging of NO. Petros et al. (1993) reported that PPF was able to stimulate NO production in cultured porcine aortic endothelial cells from the determination of increased cyclic GMP formation.[37] The present study directly assays the amount of nitrite to represent the corresponded NO production in macrophages. The present data reveals that PPF does not influence the NO production in untreated and SNP-treated cells (Table 1). PPF, structurally similar to a free radical scavenger butylated hydroxytoluene and -tocopherol, has more efficacies than the known antioxidant in scavenging riboflavin radicals and in blocking malondialdehyde formation derived from lipid hydroperoxides of arachidonic acid.[38] In a human leukocyte chemiluminescence study, Demiryurek et al. (1998) reported that PPF possesses a direct scavenging effect on hydroxyl chloride, superoxide, hydrogen peroxide and hydroxyl radical.[10] The data presented here have revealed that PPF has not such effects on direct scavenging of NO. Therefore, the protective mechanism of PPF against NO-mediated damage may be through other signal transduction pathways.

There are multiple mechanisms of NO-induced cell apoptosis. The massive production of NO from the NO donors or inducible NO synthase mediates macrophage apoptosis or necrosis.[12,28-30] Toxicity of NO may result from the interaction with thiol- or iron-sulfur-containing proteins leading to protein dysfunction, or direct damage to DNA by NO itself or its oxidation product, peroxynitrite.[18] Hortelano et al. (1999) reports that the NO-stimulated release of cytochrome *c* from mitochondria precedes the change of mitochondrial transmembrane potential and causes macrophage apoptosis.[39] PPF has a high lipophilicity so that it might accumulate in the mitochondrial membrane and block NO-induced cytochome *c* release and membrane potential change. Other NO-signailing apoptotic or anti-apoptotic proteins, including p53, bcl-2/bax, and cyclooxygenase-2, probably interact with PPF and lessen the NO-induced toxicity.[18,28,29]

In conclusion, this study has demonstrated that a therapeutic concentration of PPF could protect mouse macrophage RAW264.7 cells from NO-induced cell insults from the aspects of analyses of cell viability and apoptosis. By directly detecting NO production, the present study has also revealed that the protective mechanism of PPF might not be through the direct scavenging of NO. It will be an interesting subject for future studies to investigate the possible protection mechanism of PPF from NO-induced macrophage insults.

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