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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※ 代謝產物之研究-HPLC 分析法
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計畫類別：個別型計畫

計畫編號：NSC91－2314－B－038－024－

執行期間：91 年 08 月 01 日 至 92 年 07 月 31 日

計畫主持人：張懷嘉

共同主持人：陳瑞明

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

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執行單位：台北醫學大學麻醉學科

中　華　民　國　92　年　09　月　20　日

行政院國家科學委員會專題研究計畫成果報告

大鼠肝臟微粒體細胞色素 P450s 對 2,6-雙異丙烷酚代謝產物之研究-HPLC 分析法

- STUDY OF METABOLITES OF 2,6-DIISOPROPYLPHENOL BY RAT HEPATIC CYTOCHROME P450S- AN HPLC ANALYSIS

計畫編號：NSC91－2314－B－038－024－
執行期限：91 年 08 月 01 日至 92 年 07 月 31 日
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Abstract

2,6-Diisopropylphenol, an intravenous anesthetic agent, has been worldwide used in a variety of surgical procedures for induction and maintenance of anesthesia. The beneficial characteristics of 2,6-diisopropylphenol include rapid onset, short duration of action and rapid elimination. One of the major advantages of this anesthetic over other injectable anesthetic agents is that relatively rapid and complete recovery occurs in most patients after repeated dosing or with relatively prolonged intravenous infusions. This property is attributable to rapid and extensive biotransformation of 2,6-diisopropylphenol to multiple metabolites, primarily by the liver.

In humans, 2,6-diisopropylphenol is eliminated from the body only after being metabolized. The major metabolite in human is the glucuronic acid conjugate of 2,6-diisopropylphenol. To date, UDP-glucuronosyltransferase 1A9 is the major enzyme that has been shown to mediate this reaction. Another metabolic pathway of 2,6-diisopropylphenol is via the ring hydroxylation, which accounts for approximately 40% of the dose. This oxidative metabolism of 2,6-diisopropylphenol is catalyzed by cytochrome P450 (CYP) to form 4-hydroxy-2,6-diisopropylphenol, which is then glucuronidated at either the C1- or C4-hydroxyl positions or is sulphated at the C4-hydroxyl position by a sulfotransferase.

The ring oxidation of 2,6-diisopropylphenol by CYPs is species-dependent. Guitton et al. (1998) reported that multiple human CYP isoforms might be involved in the liver metabolism of 2,6-diisopropylphenol. Kraus et al. (2000) showed that CYP2B11 was the major enzyme in dogs for metabolizing the ring hydroxylation of 2,6-diisopropylphenol. In human, CYP2B6 and to a lesser extent CYP2C9 were demonstrated to be involved in the oxidative metabolism of 2,6-diisopropylphenol. Rats are common and excellent animal models used in the pharmacological and toxicological studies of drugs. However, study about which CYP isoforms contribute to the 2,6-diisopropylphenol metabolism in rat liver is rare.

The present study is aimed to characterize the isoform(s) of CYPs metabolizing 2,6-diisopropylphenol in rat livers. After treating with CYP inducers, rat liver microsomes will be prepared, and CYP activities and proteins will also be determined. In vitro biotransformation of 2,6-diisopropylphenol with/without CYP-specific inhibitors or antibodies and HPLC analyses of the metabolites will be carried out to evaluate which CYP isoform in rat liver is involved in the 2,6-diisopropylphenol metabolism. Identification of the major CYP isoform responsible for 2,6-diisopropylphenol hydroxylation in rat livers may aid better understandings of 2,6-diisopropylphenol about the pharmacological and toxicological characteristics of the intravenous anesthetic.

Keywords: 2,6-Diisopropylphenol, Rat liver microsomes, Cytochrome P450s, Ring oxidation, Immune inhibition, Chemical inhibition, HPLC analysis

Introduction

2,6-Diisopropylphenol (I.C.I.35868), the active

ingredient of the intravenous anesthetic agent Diprivan, has been world-wide used in varieties of surgical procedures for induction and maintenance of anesthesia (Sebel and Lowdon, 1989).. The beneficial characteristics of 2,6-diisopropylphenol include rapid onset, short duration of action and rapid elimination. However, certain adverse effects such as cardiac depression or hypotension are observed in surgical patients during clinical applications of this intravenous anesthetic agent (Merin, 1990; Lippmann, 1991). Studies in human neutrophils and alveolar macrophages demonstrate that 2,6-diisopropylphenol can modulate intracellular calcium concentration, inhibit cellular chemotaxis, phagocytosis, reactive oxygen species production and also have potential effects on suppression of immune responses as well (Mikawa et al., 1998; Kotani et al., 1998). Being similar to phenol-containing α-tocopherol and butylated hydroxytoluene in structure, 2,6-diisopropylphenol has been reported to possess an antioxidant effect (Murphy et al., 1992). Previous studies have shown that 2,6-diisopropylphenol could protect varieties of tissues, cells or organelles from oxidative insults induced by different types of free radicals or reactive oxygen species (Aarts et al., 1995; Demiryürek et al., 1998). Our previous study has also shown that 2,6-disopropylphenol can protect macrophages from nitric oxide-induced apoptosis (Chang et al., 2002).

2,6-Diisopropylphenol is used for its short-lasting anaesthetic action, which results from rapid decay of its plasma concentrations (Simons et al., 1988; Vree et al., 1987). One of the major advantages of this drug over other injectable anesthetic agents is that relatively rapid and complete recovery occurs in most patients after repeated dosing or with relatively prolonged intravenous infusions.(Apfelbaum et al.,1993) This property is attributable, in part, to rapid and extensive biotransformation of 2,6-diisopropylphenol to multiple metabolites, primarily by the liver.

In humans, 2,6-diisopropylphenol is eliminated from the body only after being metabolized, because less than 0.3% of the dose is excreted from the body as the parent compound and most of the drug (73%) is eliminated as water-soluble conjugates via the kidneys on the first day (Simons et al., 1988; Simons et al., 1992; Sneyd et al., 1994). In human beings, the major metabolite is the glucuronic acid conjugate of 2,6-diisopropylphenol, which accounts for 53-73% of the total metabolites, depending principally on the administered dose of 2,6-diisopropylphenol (Simons et al., 1988; Sneyd et al., 1994). To date, UDP-glucuronosyltransferase 1A9 is the only enzyme that has been shown to mediate this reaction (Sutherland et al., 1993).

However, the oxidation of 2,6-diisopropylphenol via ring hydroxylation(Vree et al.,1999) accounts for approximately 40% of the dose (Simons et al.,1998), and this reaction is catalysed by cytochrome P450 (CYP) to form 4-hydroxypropofol, which is then glucuronidated at either the C1- or C4-hydroxyl positions or is sulphated at the C4-hydroxyl position by a sulfotransferase (Guitton et al.,1998). Although the glucuronide and sulfate conjugates of 2,6-diisopropylphenol appear to be pharmacodynamically inactive, 4-hydroxypropofol is reported to have approximately one third of the hypnotic activity of 2,6-diisopropylphenol (Simons et al., 1991).

The cytochrome P450-dependent (CYP) monooxygenase system consists of multiplicity of P450 hemoprotein, a flavoprotein NADPH-cytochrome P450 reductase, and phospholipids. The microsomal monooxygenases catalyze the oxidative, reductive, and peroxidative metabolism of a variety of xenobiotics including drugs, carcinogens, and environmental pollutants (Estabrook 1996). The P450 enzymes are markedly responsive to the stimulatory and inhibitory effects of many physiological and encironmental factors. Such responsiveness plays an important role in xenobiotic metabolism and toxicity (Nebert, 1994).

Using 11 different cDNA-expressed CYPs, it was recently shown that the hydroxylation of 2,6-diisopropylphenol can be mediated by multiple hepatic CYP isoenzymes (Simons et al., 1998). Based on the correlation of hepatic microsomal 2,6-diisopropylphenol hydroxylase activities to immunoquantified CYP2C9 levels and inhibition of this activity by a CYP2C9 antibody and sulphaphenazole, a purported selective CYP2C9 chemical inhibitor, it was concluded that CYP2C9 contributed by at least 50% to the oxidation of 2,6-diisopropylphenol. However, the correlation was not very strong ($r = 0.78$), inhibition by sulphaphenazole was relatively weak (< 39% inhibition), and immunoinhibition was only performed in livers containing a high amount of CYP2C9 protein.

The ring oxidation of 2,6-diisopropylphenol by CYPs is species-dependent. In 1998, Guitton et al. demostrated that multiple human CYP isoforms may be involved in the liver metabolism of 2,6-diisopropylphenol. Kraus et al. (2000) shows that the ring hydroxylation of 2,6-diisopropylphenol in dogs is primarily mediated by CYP2B11 and breed differences in 2,6-diisopropylphenol metabolism may result from differences in the liver content of this CYP. In humain, CYP2B6 and to a lesser extent CYP2C9 contribute to the oxidative metabolism of 2,6-diisopropylphenol (Court et al., 2001). However, study about which CYP isoform in the rat liver is involved in the ring oxidation of 2,6-diisopropylphenol is few.

2,6-Diisopropylphenol is an intravenous anesthetic used for induction and maintenance of anesthesia. This anesthetic is used for its short-lasting

anaesthetic action. The ring oxidation of 2,6-diisopropylphenol by hepatic CYPs is an important pathway for eliminating this anesthetic from the body. The CYP isoforms involved in the metabolism of 2,6-diisopropylphenol are species-dependent. Previous studies have shown that CYPs 2B6 and 2C11 are two major isoforms contributing to the ring oxidation of 2,6-diisopropylphenol in human and dog livers, respectively (Kraus et al., 2000; Court et al., 2001). However, the isoform of CYP for ring-oxidizing 2,6-diisopropylphenol has not been determined in rat livers. The rat is an excellent animal model for evaluating the pharmacologies and toxicologies of anesthetics. Therefore, the purpose of the present study is to characterize the isoform(s) of CYP metabolizing 2,6-diisopropylphenol. Identification of the major CYP isoform responsible for 2,6-diisopropylphenol hydroxylation in rats may aid better pharmacological and toxicological understandings of 2,6-diisopropylphenol in the clinical application.

Materials and Methods

Reagent and Solutions: All chemicals used were of analytical grade unless otherwise stated. Double-distilled water was used. Cyclohexane (spectroscopic grade), 2-propanol, trifluoroacetic acid and sodium dihydrogen orthophosphate were obtained from BDH (Poole, U.K.).. Acetonitrile (HPLC grade) was obtained from Fisons (Loughborough, U.K.). Tetramethylammonium hydroxide (TMAH) (25% in methanol) and thymol were obtained from Fluka (Buchs, Switzerland). Internal standard solution. A solution of thymol was prepared in methanol (1 mg/ml) and further diluted with methanol to an appropriate working concentration. Phosphate buffer solution (0.1 M). Sodium dihydrogen orthophosphate (13.6 g) was dissolved in 1 L of distilled water. Dilute TMAH solution. The HPLC mobile phase consisted of 600 ml of acetonitrile, 400 ml of distilled water and 1 ml of trifluoroacetic acid. The mobile phase was degassed by the passage of helium prior to use.

Preparation of Microsomal Fractions: Microsomes from rat livers were prepared by differential centrifugation as described previously (Kremers et al., 1981). Microsomal fractions were aliquoted in small volumes and stored at -80 °C in potassium phosphate buffer (200 mM, pH 7.4) containing lmM EDTA and glycerol (20%, v/v) before use. Microsomal proteins were assessed according to a modification of Lowry's method using bovine serum albumin as standard (Hartree, 1972).

CYP Contents and Enzyme assay: Total CYP content was measured using the method developed by Omura and Sato (Omura and Sato, 1964). Coumarin 7-hydroxylase and 7-pentoxyresorufin O-dealkylase at a substrate concentration of 100 μM were determined fluorometrically as described by Aitio and colleagues (1978). Instrumentation consisted of fluorescence spectrophotometerthermostatted (37℃) cuvette holder. Briefly, reactions were performed in a quartz cuvette containing NADPH cofactor mix and 300 ug microsomal protein to a final volume of 2 ml. Substrates (1 uM final concentration) were added in 10 ul DMSO. Resorufin formation was monitored with excitation and emission wavelengths set at 530 nm and 585 nm respectively. At the end of each incubation, 100 pmoles resorufin in 10 ul DMSO was added to calibrate the fluorescence output. Activities were calculated from the slope of the initial linear protein of the fluorescence intensity-time curve and expressed relative to microsomal protein content and incubation time (pmole/mg/min).

Immunobloting analysis: Hepatic microsomes was separated by sodium dodecylsulfate-polyacrylamide gel electrophosesis using precast 7.5% gels. Proteins were then electrophoretically transferred to nylon membranes. These blots were blocked with 5% powdered nonfat milk in TBS-Tween (0.15 M NaCl, 0.04 M Tris, pH 7.7, and 0.06% Tween 20) and then incubated in TBS-Tween/0.1% bovine serum albumin containing a 1:500 dilution of a polyclonal antirat CYP2B1/2 antibody. After washing, the blots were incubated in a 1:500 dilution of horse radish peroxidase conjugated secondary antibody, washed, and then chemiluminesence detection was performed with exposure to radiographic film. The film was then scanned, and the area and density of identified bands were quantitated.

In Vitro Biotransformation Assays: Typical in vitro incubations consisted of hepatic microsomes, drug substrate, and an nicotinamide adenine dinucleotide phosphate (NADPH) regeneration system in a final volume of 250 l of phosphate buffer. Incubation time (at 37°C) and microsomal protein content were minimized to ensure linearity of product formation with respect to these variables. Metabolite formation was quantified by high-performance liquid chromatography (HPLC). HPLC apparatus were obtained from Waters (Milford, MA) and consisted of a dual-head pump with autoinjector serially connected to a reverse-phase C-18 Bondapack column with either an ultraviolet absorbance or fluorescence detector depending on the assay method used.

Microsomal propofol hydroxylation activities were determined as described (Court et al.1999). Briefly, glass gas chromatography vials were prepared on ice containing 50 μl liver microsomes (100 μg/ml protein, final concentration) and 100 μl of an NADPH regeneration system(0.5mM NADP; 3.75 mM racemic isocitrate ;1 unit/ml isocitrate dehydrogenase; 6 mM $MgCl_2$). Reactions were started by addition of 100 μl propofol working solution

(0-100 μM) propofol for kinetic studies, 20 M propofol for inhibition studies, with 0.2% DMSO). All working solutions were made up in 50 mM phosphate buffer (pH7.5) and the total incubation volume was 250 μl. The tubes were immediately vortexed and placed in a 37℃ agitating water bath. After 10 min the reaction was stopped by addition of 250 μl acetonitrile containing the internal standard (25-50 ng thymol) to each tube, and immediately vortexed and placed on ice. This mixture was transferred to polypropylene tubes and spun at 2000 g in a microcentrifuge for 5 min at 4 ℃.. The supernatant was transferred to vials for immediate analyses or for storage at 4℃ until assay within 2 weeks.

HPLC Analysis: The high-performance liquid chromatograph used consisted of a solvent delivery system set to deliver a solvent flow of 1.5 ml/min, an automatic sample injector and an fluorescence detector. The excitation and emission wavelengths were 276 and 310 nm, respectively, and both monochromator slit widths were 10 nm. A C_{18} reversed-phase column was used at ambient temperature.

Microsomal propofol hydroxylation activities were determined as described (Court et al.1999). The dry residues of propofol and its metabolites from in vitro metabolism by rat liver microsomes was redissolved in HPLC mobile phase (200 μl) and an aliquot (100 μl) of the solute was submitted to HPLC analysis. The mobile phase consisted of 50% acetonitrite, 40% water and 10% methanol with a flow rate 2ml/mim. A standard curve was generated by assay of samples containing varying amounts of a fixed amount of internal standard. Metabolite concentrations were then calculated by linear regression of calibration curve data using measured metabolite-internal standard peak height ratios. Enzyme activity was calculated by dividing the amount of product formed by incubation time and microsomal protein content, and expressed as nmol/min/mg. Incubation time (10 min) and microsomal protein concentration (100 μg/ml) were minimized to ensure linearity of product formation with respect to these variables.

Immunoinhibition: An Immuno-inhibition study was performed using goat anti-rat CYP2B1 and pre-immune sera. Rat liver microsomes (20 ug protein) were pre-incubated with a mixture of inhibitory and control sera (total 400 ug serum protein) for 30 min at room temperature in a volume of 100 ul. The mixture of inhibitory and control serum was adjusted such that the ratio of inhibitory serum to microsomal protein was 0:1, 5:1, 10:1, 15:1, and 20:1. The reaction was started by adding 50 ul propofol (final concentration 20uM in 50 mM phosphate buffer and 0.2% DMSO) and 100 ul of the NADHP cofactor solution. This mixture was vortexed and incubated for 10 min at 37℃. The reaction was stopped by adding 250 ul acetonitrile / thymol solution, vortexed and placed on ice. The samples were extracted and analyzed by HPLC. The percent inhibition of activity at each ratio of inhibitory serum to microsomal protein was calculated as the percent decrease from control (i.e. no inhibitory sera added) activity.

Chemical Inhibition Study: Effective chemical inhibitors were used as probes of specific CYP isoforms involved in the metabolism of propofol. Inhibitors were introduced into the incubation medium simultaneously with the substrate except for troleandomycin and orphenadrine; these were preincubated with microsomes and the NADPHgenerating system at 37 ^{o}C for 20 min to produce sufficient reactive metabolites, which led to specific inhibition. The reaction was then continued with substrate for an additional 10 min. Inhibitors used as probes of specific CYP-mediated activities were diethyldithiocarbamate (100 μM; CYP2E1), sulphaphenazole (50 μM; CYP2C9), quinidine (10 μM; CYP2D6), midazolam (100 μM; CYP3A4), troleandomycine (100 μM; CYP3A4), 7-8 benzoflavone (10 μM; CYPlA1), coumarin (100 μM; CYP2A6) and orphenadrine (50 μM; CYP2B6), which are known to suppress more than 80% of the activities mediated by the CYP isoform given in parentheses (Newton DJ et al., 1995). Clotrimazole (50 μM) and n-octylamine (1 mM) were also used for their low specificity in inhibiting CYP-mediated activities.

Statistical Analysis: Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Student's *t* test. Otherwise, representative data are shown.

Results

Table 1 Body wights, liver weights and microsomal proten levels in untreated and phenobarbital-treated rats

	Control	Phenobarbital
BW(g)	313 ± 25	279 ± 5
LW(g)	13.0 ± 0.8	14.0 ± 0.9
LW/BW(%)	4.16 ± 0.14	5.37 ± 0.26*
	12.9 ± 4.3	17.6 ± 1.8*

Microsomal protein(mg/ml)	

Fig. 3 Effects of microsomal protein concentrations on the metabolism of PPF to 3-OH-PPF in phenobarbital-treated rats.

Table 2 Effects of phenobarbital on PRODactivities of rat hepatic microsomes

	Control	Phenobarbital
PROD activitiy	20 ± 16	1501 + 621

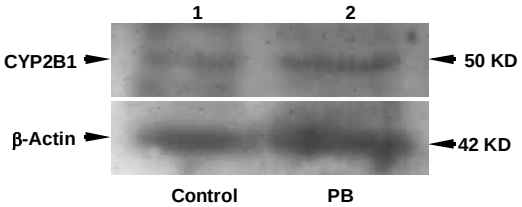

Fig. 4 HPLC profile of PPF metabolism by untreated rat liver microsomes.

Fig. 1. Effects of phenobarbital on CYP2B1 protein in rat liver microsomes.

Fig. 5 HPLC profile of PPF metabolism by phenolbarbital-treated rat liver microsomes.

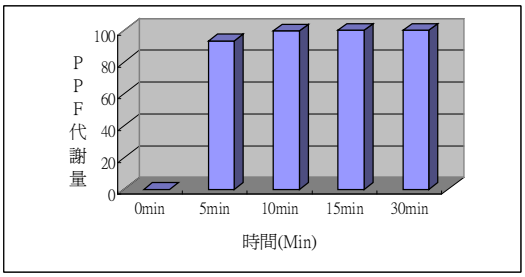

Fig. 2 Time-dependent effects of metabolism of propofol to 3-OH-PPF by phenobarbital-treated liver microsomes.

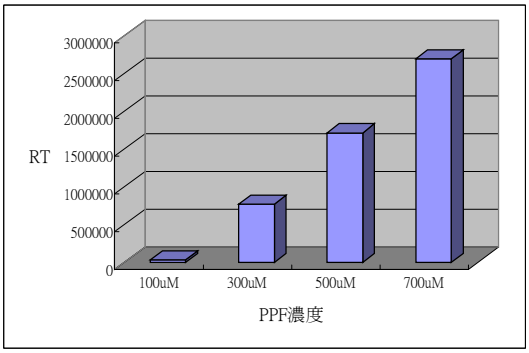

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Table 1. Cytotoxic effects of propofol on macrophages

Treatment	Cell viability (% of control)	LDH (U/L)	Cell cycle, % G1/S Phase	G2/M phase
1 hour				
PPF, 0 μM	100	65 ± 18	56 ± 3	28 ± 3
PPF, 3 μM	100 ± 11	70 ± 25	55 ± 3	28 ± 2
PPF, 30 μM	104 ± 22	58 ± 11	54 ± 3	30 ± 5
PPF, 300 μM	101 ± 15	80 ± 22	55 ± 3	28 ± 3
6 hours				
PPF, 0 μM	100	74 ± 16	55 ± 3	28 ± 3
PPF, 3 μM	96 ± 25	71 ± 15	55 ± 2	29 ± 3
PPF, 30 μM	108 ± 33	74 ± 20	56 ± 3	28 ± 3
PPF, 300 μM	72 ± 16*	113 ± 10*	54 ± 3	29 ± 3
24 hours				
PPF, 0 μM	100	78 ± 22	54 ± 2	29 ± 3
PPF, 3 μM	101 ± 11	77 ± 21	55 ± 4	29 ± 2
PPF, 30 μM	103 ± 13	82 ± 15	57 ± 5	29 ± 6
PPF, 300 μM	56 ± 18*	175 ± 18*	68 ± 6*	18 ± 6*

Macrophages were treated with 3, 30 and 300 μM propofol (PPF) for 1, 6 and 24 hours. Cell viability, lactate dehydrogenase (LDH) release and cell cycle were analyzed to determine the toxicity of PPF to macrophages. Each value was expressed as Mean ± SD for n = 9. *Values were considered to be statistically different from the respective control when the *P* value was less than 0.05.

Table 2. Concentration- and time-dependent effects of propofol on phagocytotic activities of macrophages

PPF, μM	Phagocytosis, cell number x 10^2 1 h	6 h	24 h
0	176 ± 43	185 ± 65	204 ± 54
3	194 ± 62	172 ± 58	212 ± 41
30	225 ± 81	86 ± 36*	73 ± 21*
300	174 ± 42	72 ± 22*	58 ± 21*

Macrophages were treated with 3, 30 and 300 μM propofol (PPF) for 1, 6 and 24 hours. Phagocytotic activities were determined by counting the fractions of macrophages that digested at least one fluorescent particle. Each value was expressed as expressed as Mean ± SD for n = 6. *Values were considered to be statistically different from the respective control when the *P* value was less than 0.05.

Table 3. Effects of propofol on cellular ATP levels

Time, h	ATP (pmole)
0	35 ± 11
1	20 ± 9*
6	24 ± 7*
24	26 ± 9*

Macrophages were exposed to 30 μM propofol (PPF) for 1, 6 and 24 hours. The amounts of cellular adenosine triphosphate (ATP) were detected by a bioluminescence assay. Each value was expressed as Mean ± SD for n = 6. *Values were considered to be statistically different from the respective control when the *P* value was less than 0.05.

Legends to figures

Fig. 1. Concentration- and time-dependent effects of propofol (PPF) on chemotaxic activities of macrophages. Macrophages were exposed to 3, 30 and 300 μM PPF for 1, 6 and 24 hours, respectively. Chemotaxic activities were assayed by the Transwell cell culture chamber inserts as described in Materials and Methods. Each value was expressed as Mean ± SEM for n > 6. *Values were considered to be statistically different from the respective control when the *P* value was less than 0.05.

Fig. 2. Concentration- and time-dependent effects of propofol (PPF) on oxidative ability of macrophages. Macrophages were exposed to 3, 30 and 300 μM PPF for 1, 6 and 24 hours. The levels of intracellular reactive oxygen species were determined by the flow cytometric method. Each value was expressed as Mean ± SEM for n > 6. *Values were considered to be statistically different from the respective control when the *P* value was less than 0.05.

Fig. 3. Effect of propofol (PPF) on IFN-γ mRNA in lipopolysaccharide (LPS)-activated macrophages. Messenger RNA from macrophages exposed to 1 ng/ml LPS, 30 μM PPF and a combination of PPF and LPS were prepared for RT-PCR analysis of IFN-γ (A, *top panel*) and β-actin (A, *bottom panel*). Intensities of DNA bands were quantified by a digital analysis system as described in Materials and Methods (B). Each value was expressed as Mean ± SEM for 3 determinations. *Values were considered to be statistically different from the respective control when the *P* value was less than 0.05.

Fig. 4. Concentration- and time-dependent effects of propofol (PPF) on the membrane potential of macrophage mitochondria. Macrophages were

exposed to 3, 30 and 300 μM PPF for 1, 6 and 24 hours. Mitochondrial membrane potential were determined by the flow cytometric method. Each value was expressed as Mean ± SEM for n > 6. *Values were considered to be statistically different from the respective control when the *P* value was less than 0.05.