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Effects of Nitrofurantoin on Lipid Peroxidation and Reactive Oxygen Radical Generation in Porcine Lung Microsome [1]

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ABSTRACT

In vitro effects of nitrofurantoin, an antimicrobial agent for acute and chronic urinary tract infection, on the lung microsomal lipid peroxidation and the generation of reactive oxygen radicals were investigated to elucidate the biochemical mechanisms of its in vivo pulmonary toxicity.

The interaction of nitrofurantoin with porcine lung microsome resulted in significant lipid peroxidation. In addition, nitrofurantoin stimulated the generation of reactive oxygen radicals, $O_2^{\cdot-}$, H_2O_2 as well as a highly reactive secondary oxygen species, OH·. The stimulation of lipid peroxidation was inhibited not only by superoxide dismutase and catalase, but also by hydroxyl radical scavengers, mannitol and thiourea. Neither singlet oxygen ($^1O_2$) was detected during the incubation of microsome with nitrofurantoin, nor lipid peroxidation was inhibited by singlet oxygen scavengers. When incubated anaerobically under the nitrogen atmosphere, the ability of nitrofurantoin to stimulatle lipid peroxidation was abolished.

It appears that NADPH-dependent metaboliam of nitrofurantoin in pulmonary microsome under aerobic condition is accompanied by the stimulation of lipid peroxidation through the mediation of reactive oxygen radicals, particularly hydroxyl radical. It is strongly suggested from these results that the stimulation of pulmonary microsomal lipid peroxidation by the reactive oxygen radical may be a in vivo mechanism of pulmonary toxicity caused by nitrofurantoin.

Key Words: nitrofurantoin, lipid peroxidation, oxygen radical, pulmonary microsome
Abbreviations: CAT; catalase, MDA; malondialdehyde, NBT; nitroblue tetrazolium, NF; nitrofurantoin, SOD; superoxide dismutase

INTRODUCTION

Nitrofurantoin (NF), a derivative of 5-nitrofurfural (N-[5-nitro-2-furfurylidine]-1-amino-hydantoin), is a urinary antiseptic used in acute and chronic urinary tract infection and has been known to have pulmonary toxicity with clinical symptoms of fever, dyspnea, dry cough and pulmonary infiltration and fibrosis (Holmberg *et al.*, 1980). The pulmonary toxicity caused by NF was considered initially

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a hypersensitivity reaction, but it was not proven in immunological studies. On the hand, a number of investigators reported the toxicity as a direct one, because it was produced in dose-dependent manner, more markedly in animals with Vit. E deficiency or in animals exposed to high oxygen tension environment (Boyd *et al.*, 1979; Smith and Boyd, 1983).

Based on in vitro studies using lung microsome, Mason and Holtzman (1975), Sasame and Boyd (1979) and Trush *et al.*, (1982) reported that NF increased the generation of reactive oxygen radicals with concomitant increase in lipid peroxidation. They suggested that reactive oxygen radicals may cause the pulmonary toxicity by enhancing the lipid peroxidation of microsomal membranes. And they also reported that the mechanism of pulmonary toxicity by NF is similar to that by paraquat, which is well known for its pulmonary toxicity. In contrast, several investigators insisted no causative relationships between pulmonary toxicities by NF or paraquat and reactive oxygen radicals, accompanied by increased microsomal lipid peroxidation (Ilett *et al.*, 1974; Kornbrust and Mavis, 1980; Shu *et al.*, 1979; Talcott *et al.*, 1979).

As far as the mechanism by which NF produces pulmonary toxicity is concerned, it is presently controversial. Moreover, even with the theories which insist the involvement of reactive oxygen radical, particular oxygen radical species which plays a crucial role in the toxicity has not been elucidated yet.

The present study was undertaken to examine the mechanism by which NF causes pulmonary toxicity. The effects of NF on reactive oxygen radical generation and lipid peroxidation were studied in in vitro lung microsomal preparation.

MATERIALS AND METHODS

Preparation of lung microsome

Lung microsome was prepared from fresh porcine lung obtained from the slaughter house by the method of Trush *et at.* (1982). About 100g of lung parenchyme, cleaned of pleura and bronchial trees, was cut into small pieces and homogenized in 4 volumes of cold homogenizing solution (150mM KCl, 50mM Tris-HCl, pH 7.4) with a polytron tissue disintegrator (PCU-1, Brinkman Inc.) for 20 sec (four times 5 sec with 15 sec intervals) at rheostat 7. The homogenate was centrifuged at 3,000g for 20min. The supernatant was passed through four layers of cheese cloth and the filtrate was centrifuged for 20 min at 12,000g to remove tissue debris and mitochondria. The supernatant was centrifuged at 100,000g for one hour and the resultant pellet was suspended in 40-50ml of cold homogenizing solution. The suspended pellet was recentrifuged at 100,000g for one hour and the final microsomal pellet was obtained. The extracted microsome was resuspended in homogenizing solution using glass-teflon homogenizer to make protein concentration of 10-15mg/ml and stored at −20 °C. All the procedures were performed at 0-4 °C. Protein concentration was determined by the method of Lowry *et al.* (1951).

Lipid peroxidation

Microsomal lipid peroxidation was estimated from measuring malondialdehyde (MDA) concentration by thiobarbituric acid method (Bidlack and Tappel, 1973). Lung microsome (0.5mg/ml) contained in reaction mixture consisting of 150mM KCl, 50mM Tris-HCl (pH 7.4), 0.5mM NADPH and NF or oxygen radical scavengers as indicated in Figures and Tables was incubated at 37 °C in a Dubnoff shaking water bath. The reaction was started with addition of NADPH and maintained aerobic condition by continuous flow (5L/min) of pure oxygen through the incubator fitted with a covered hood connected to an O_2 tank. In case of anaerobic reaction, nitrogen gas was continuously flowed through the incubator. A 1ml aliquot of reaction mixture was transferred to a tube containing same volume of cold 30% trichloroacetic acid to stop the reaction. The mixture was then centrifuged at 3,000g for 10 min. The supernatant (1.5ml) was mixed with an equal volume of 0.67% thiobarbituric acid and the chromophore was developed in a boiling water bath for 15 min. After cooling to room temperature, the

absorbance was measured at 532nm with spectrophotometer (Hitachi-Perkin Elmer,139 UV-VIS). The concentration of MDA was expressed as nmol/mg protein using the molar extinction coefficient of 1.52 x 10^5 M^{-1} cm^{-1} (Placer *et al.,* 1966).

Measurement of superoxide anion ($O_2^{-\cdot}$)

The generation of superoxide anion was estimated by measuring adrenochrome formed from oxidation of epinephrine by $O_2^{-\cdot}$ (Misra and Fridovich, 1972). An O_2-saturated 3 ml reaction mixture yielding the final concentrations of 1.5mg/ml microsome, 150mM KCl, 50mM Tris-HCl (pH 7.4),200μM epinephrine and NF or superoxide dismutase (SOD) as indicated in Figures was incubated in a spectrophotometer cuvette at 37 °C. The reaction was started with addition of NADPH. The oxidation of epinephrine to adrenochrome was followed by a dual-wavelength spectrophotometer (Aminco-Chance DW-2, American Instrument Co.) equipped with a thermostatted cell compartment. The changes in absorbance were recorded with a wavelength pair of 480nm-550nm.

Measurement of hydrogen peroxide (H_2O_2)

Hydrogen peroxide was estimated from measuring the formation of Fe $(SCN)_3$ from ferrous ammonium sulfate and potassium thiocyanate upon oxidation of Fe^{2+} to Fe^{3+} by H_2O_2 (Hildebrandt and Roots, 1975). Microsome (1.5mg/ml) contained in a reaction mixture of 150mM KCl, 50mM Tris-HCl (pH 7.4), 0.5mM NADPH and NF or catalase was incubated for 10 min at 37 °C in a Dubnoff shaking water bath, in which aerobic condition was maintained by O_2 flow (5L/min). In case of the reaction without exogenous catalase, 0.2mM sodium azide was used to inhibit the endogenous microsomal catalase. After starting the reaction with addition of NADPH, a 1ml aliquot was mixed with 1ml of cold 5% TCA to stop the reaction at constant time interval. The mixture was centrifuged for 10 min at 20,000g and the supernatant (1.5ml) was transferred to a tube containing 0.3ml of 10mM ferrous ammonium sulfate and 0.5ml of 2.5M potassium thiocyanate. The extinction of the $Fe(SCN)_3$ was measured at 480nm in a spectrophotometer and the concentration of H_2O_2 was estimated from the standard curve prepared by known concentrations of exogenous H_2O_2 in 50mM Tris-HCl (pH 7.4) buffer solution.

Measurement of hydroxyl radical (OH·)

The generation of OH· was estimated from determination of ethylene formed by interaction of methional and OH· (Beauchamp and Fridovich, 1970). Reaction mixture consisted of 1.5mg/ml microsome, 150mM KCl, 50mM Tris-HCl (pH 7.4), 1mM methional, 0.5mM NADPH and NF or reactive oxygen radical scavengers was incubated at 37 °C in a shaking water bath. The reaction mixture (1ml) was contained in 12.5ml glass vial filled with O_2 and sealed with gas-tight rubber cap. At 10 min after incubation, 0.5ml aliquot of gas phase above the reaction mixture was sampled with gas-tight syringe and injected into gas chromatograph (GCV, PYE-Unicam) to analyze ethylene. Gas chromatograph was equipped with a 1/8 inch x 3 meter stainless steel column of Chromosorb 102 and a flame ionization detector. The temperatures of column, detector and injector were 70 °C, 200 °C and 80 °C, respectively. The gas flow was 25ml/min for carrier (N_2), 35ml/min for hydrogen and 350ml/min for air. Amount of ethylene production was caluculated from integrated areas of chromatogram prepared by known amount of pure ethylene gas.

Measurement of singlet oxygen ($^1O_2$)

$^1O_2$ was analyzed from measuring o-dibenzoylbenzene, which is produced from interaction of 1,3-diphenylisobenzofuran with $^1O_2$, by thin layer chromatography (Pederson and Aust, 1973). The reaction mixture was consisted of 2mg/ml microsome, 150mM KCl, 50mM Tris-HCl (pH 7.4), 0.5mM NADPH, 5.4mM 1,3-diphenylisobenzofuran and 0.5mM NF, and was incubated for 10 min at 37 °C in

a Dubnoff shaking incubator under aerobic condition. A 0.5ml aliquot was transferred to a tube containing 0.5 ml cold 5% TCA and 0.6ml chloroform. The mixture was shaken vigorously and centrifuged for 20 min at 2,000g. A 0.3ml aliquot taken from chloroform layer was placed under a stream of nitrogen to remove the solvent. After resolubilizing the residue in 0.1ml chloroform, 15μl aliquot was applied to the silica gel G thin layer plate and developed by heptane-dioxane (3:1). 1,3-diphenylisobenzofuran spot was observed by its flurorescence under UV-lamp and o-dibenzoylbenzene spot was confirmed by spray with 0.3% 2.4-dinitrophenylhydrazine.

Miscellaneous

NF was purchased from Dai Woong Pharmaceutical Co. (Seoul, Korea) and used in 5mM solution prepared with addition of small amount of 0.2N NaOH. Thiobarbituric acid, ß-nicotinamide adenine dinucleotide phosphate, reduced form (ß-NADPH), catalase, superoxide dismutase, epinephrine, nitroblue tetrazolium, ß-methylthiopropionaldehyde (methional) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1,4-diazabicyclo-(2,2,2,) -octane, 1,3-diphenylisobenzofuran, o-dibenzoylbenzene were purchased from Aldrich Chemical Co. (Milwaukee, WI. U.S.A.). Mannitol, thiourea, histidine were from Kanto Chemical Co. (Tokyo, Japan) and ferrous ammonium sulfate, potassium thiocyanate were from Junsei Chemical Co. (Tokyo, Japan).

RESULTS

Effect of nitrofurantoin on lipid peroxidation

NADPH-dependent lipid peroxidation in porcine lung microsome was markedly enhanced by NF in

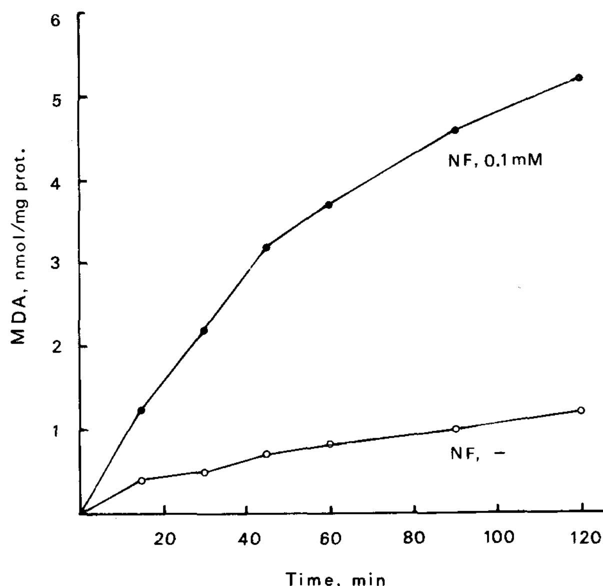

Fig. 1. Effect of nitrofurantoin on NADPH-dependent lipid peroxidation of porcine lung microsome. Porcine lung microsome, 0.5 mg/ml, was incubated in the reaction mixture containing 150 mM KCl, 50 mM Tris-HCl (pH 7.4), and 0.5 mM NADPH in the absence (o) or presence (•) of 0.1 mM nitrofurantoin (NF). The incubation was conducted at 37 °C in a Dubnoff metabolic shaking incubator fitted with a covered hood connected to an O_2 tank (flow rate:5 L/min). At the time interval indicated, 1 ml aliquot of reaction mixture was removed to test tube containing equal volume of 30% cold TCA, and the mixture was centrifuged. Malondialdehyde (MDA) content in the supernatant fraction was determined by the method of Bidlack and Tappel (1973) using thiobarbituric acid.

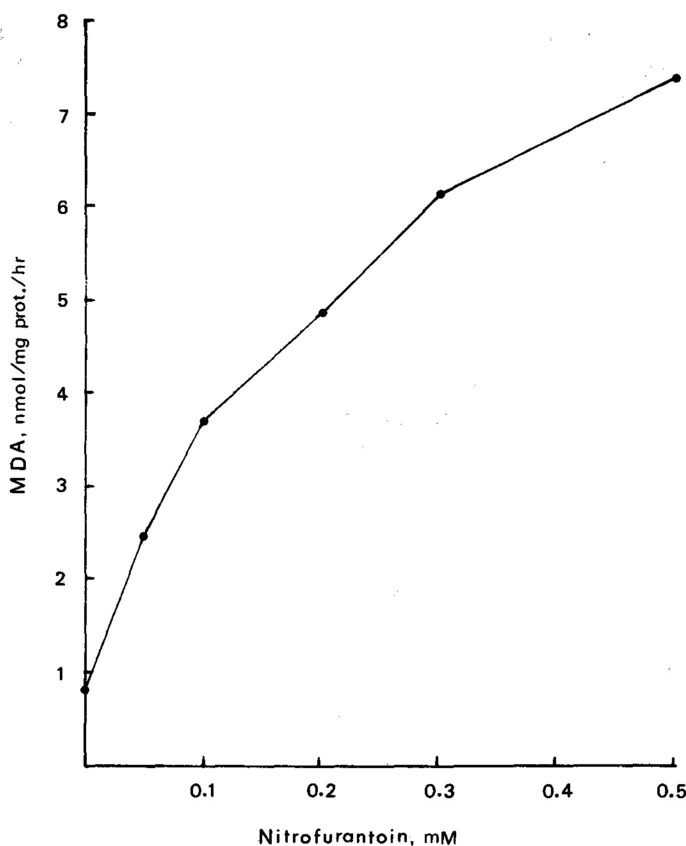

Fig. 2. Dose-response relation of nitrofurautoin on the NADPH-dependent lipid peroxidation of porcine lung microsome. Microsome (0.5 mg/ml) was incubated for 60 min with varying concentrations of nitrofurantoin under the same experimental conditions as in Fig. 1.

aerobic condition. In control experiment, content of a lipid peroxidation product, MDA was not so increased throughout the incubation period. However, the MDA production was markeldy enhanced with the addition of NF (Fig. 1). The extent of the increase was dependent on the dose of NF added to the reaction mixture (Fig. 2). At 60 min after incubation, the production of MDA was 0.81nmol/mg prot./hr in control experiment. Additions of 0.1mM and 0.5mM NF caused about 5-fold and 9-fold increase in the production of MDA, respectively. The half maximal stimulatory concentration of NF ($[NF]_{50}$) was 0.13mM (Fig. 3). In anaerobic condition maintained by replacing O_2 with N_2, the MDA production was never stimulated by NF. Under the atomosphere of air, the production was decreased to 80% of that in aerobic condition (Table). Deletion of NADPH or boiled microsome did not result in significant lipid peroxidation (data not shown). These results indicate that NF stimulates the peroxidation of lung microsomal lipid and that the stimulation is dependent on aerobic condition. This is noteworthy, since O_2 accepts an electron from the reduced radical species of NF formed during the metabolism (Mason and Holtzman, 1975), and since in vivo pulmonary toxicity induced by NF is more marked in rats exposed to atmosphere of high O_2 tension (Boyd *et al.*, 1979).

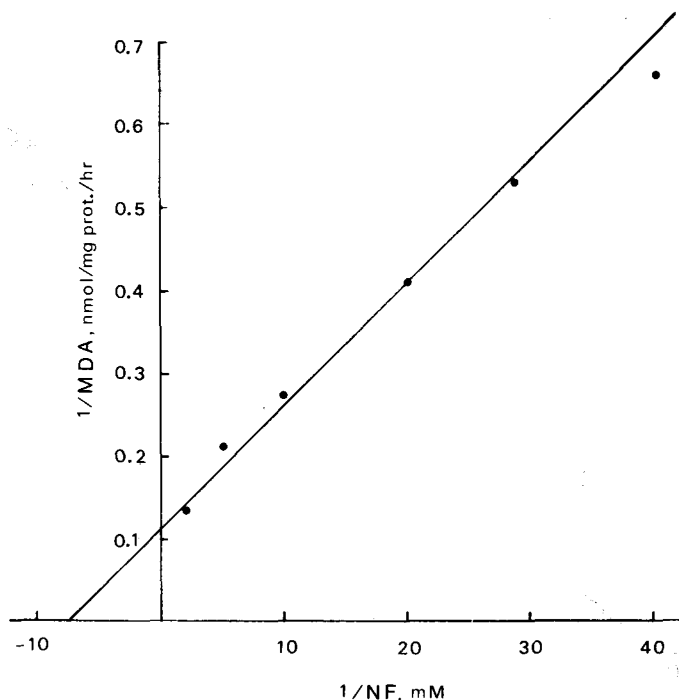

Fig. 3. Double reciprocal plots of malondialdehyde production and nitrofurantoin concentration. Double recirocal plots were made to estimate the half-maximal stimulatory concentration of nitrofurantoin for the stimulation of microsomal lipid peroxidation.

Table 1. Effect of oxygen on the stimulation of lipid peroxidation by nitrofurantoin in porcine lung microsome

Atmosphere	MDA produced	
	nmoles/mg protein/h	% control
Oxygen	5.15±0.24	100
Air	4.16±0.36	81.1
Nitrogen	0.00	0

Effects of reactive oxygen radical scavengers on NF-induced lipid peroxidation in lung microsome

The scavengers of reactive oxygen radicals were used for assessing involvement of oxygen radicals in NF-induced lipid peroxidation of lung microsome (Table 2). A selective scavenger of $O_2^-\cdot$, superoxide dismutase (SOD), appeared to be most effective among the scavengers studied, and it reduced the enhanced MDA formation to 19-57% of the control value at selected concentrations. Catalase, a scavenger of H_2O_2, prevented MDA production by NF partially. Additions of SOD (10^{-8} M) combined with catalase (10^{-7} M) were more effective than that of each agent alone in the same concentration. OH· radical scavengers, mannitol (Tauber and Babior, 1977) and thiourea (Simon *et al.*, 1981), also markeldy reduced MDA production in dose-dependent manner, and thiourea was slightly more effective than mannitol. Meanwhile, the scavengers of $^1O_2$, histidine and 1,4-diazabicyclo- (2,2,2,) -octane (Klebanoff, 1980) never prevented the production of MDA in any concentrations screened.

Table 2. Effects of various oxygen radical scavengers on NF-stimulated lipid peroxidation of porcine lung microsome

Additions		MDA produced	
		nmoles/mg protein/h	% control
None		5.63	100
SOD[a]	1.5×10^{-8}M	3.18	56.5
	1.5×10^{-7}M	1.04	18.5
Catalase	10^{-7}M	3.50	62.2
SOD + Catalase	10^{-8}M 10^{-7}M	2.03	36.1
Histidine	2mM	5.31	94.3
	5mM	5.78	102.7
DABCO[b]	1mM	5.51	97.9
	10mM	5.53	98.2
Mannitol	1mM	3.56	63.2
	10mM	1.84	32.7
Thiourea	1mM	2.58	45.8
	10mM	1.19	21.1

a: Superoxide dismutase
b: 1,4-diazabicyclo-(2,2,2)-octane

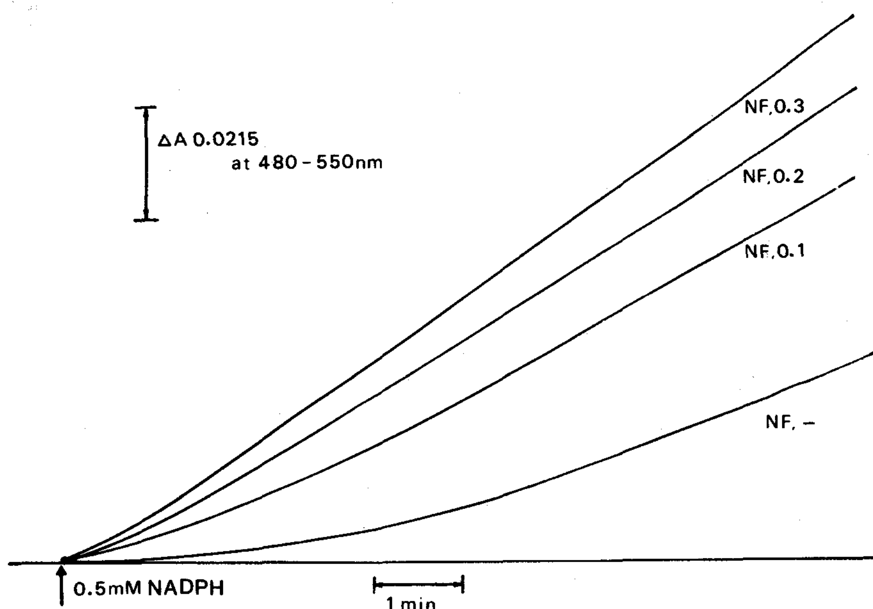

Fig. 4. Effect of nitrofurantoin on the generation of superoxide anion in procine lung microsome. O_2-saturated 3 ml reaction mixture containing 0.5 mg/ml microsome, 150 mM KCl, 50 mM Tris-HCl (pH 7.4), 200μM epinephrine, 0.5mM NADPH and 0.1—0.3mM nitrofurantoin was incubated in a spectrophotometer cuvette at 37 °C. The reaction was started by addition of NADPH. The oxidation of epinephrine to adrenochrome, an index of superoxide generation, was followed with an Aminco-Chance DW-2 dual-wavelength spectrophotometer. The changes in absorbance were recorded at 480—550nm for more than 10 min.

Effect of NF on the formation of reactive oxygen radical in lung microsome

Superoxide anion: Epinephrine is oxidized to adrenochrome in $O_2^{\cdot-}$-generating xanthine-xanthine oxidase system and this oxidation is inhibited by superoxide dismutase(McCord and Fridovich, 1969; Misra and Fridovich, 1972). In the present study, lung microsome incubated in the presence of NADPH and epinephrine under O_2 produced adrenochrome, and this production was stimulated by NF dose-dependently. At 10 min after incubation, 0.1, 0.2 and 0.3mM NF caused 2, 2.5 and 3-fold increase in the production of adrenochrome, respectively (Fig. 4). The increase in adrenochrome production was markedly prevented by SOD in concentration dependent fashion (Fig. 5).

These results indicate that the production of $O_2^{\cdot-}$ in lung microsome under the present experimental condition is stimulated by NF.

Hydrogen peroxide: Porcine lung microsome incubated with NADPH under O_2 atmosphere generated H_2O_2, and this generation was markedly enhanced by NF. In control experiment without NF, the amount of H_2O_2 produced was 8nmol/mg prot. Additions of NF (0.1-0.5mM) caused 13 to 23-fold increase in the formation of H_2O_2 (Fig. 6). The production of H_2O_2 was completely abolished by catalase (10^{-6} M) or nitroblue tetrazolium (5×10^{-4} M). The complete inhibition by nitroblue tetrazolium indicates that H_2O_2 is produced from dismutation reaction of $O_2^{\cdot-}$ (Klebanoff, 1980).

Hydroxyl radical: Beauchamp and Fridovich (1970) observed the ethylene production from methional incubated with xanthine and xanthine oxidase, and the production was inhibited either by SOD and catalase or by hydroxyl radical scavengers. From this, they proposed that OH· formed as a secondary consequence of the interaction of $O_2^{\cdot-}$ with H_2O_2 (Haber and Weiss, 1934) reacted with methional to produce ethylene.

As described above in the method, the production of ethylene analyzed by gas chromatograph was

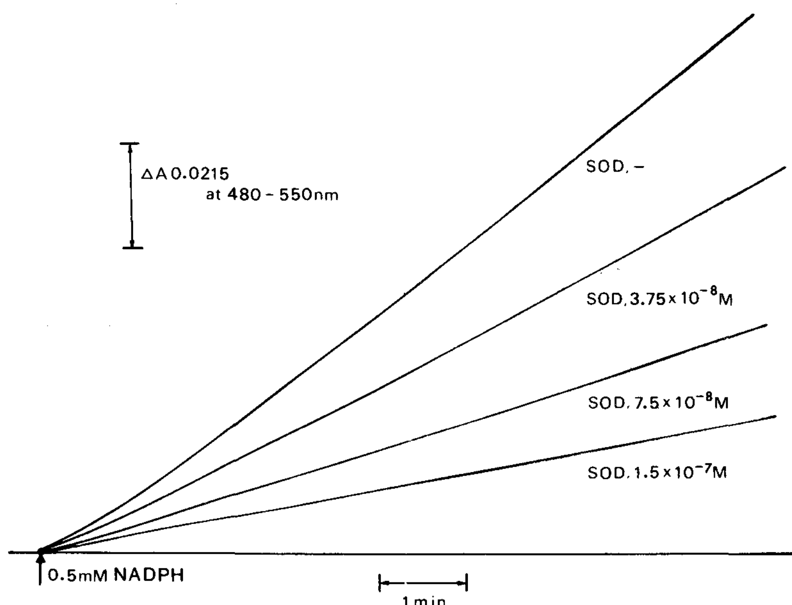

Fig. 5. Effect of superoxide dismutase on NF-stimulated generation of superoxide anion in procine lung microsome. The reaction was done with 0.3 mM nitrofurantoin in the presence of superoxide dismutase (SOD) under the same experimental conditions as in Fig. 4.

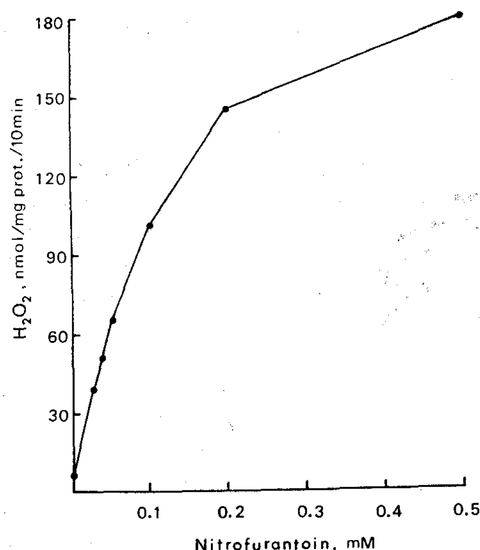

Fig. 6. Stimulation of hydrogen peroxide generation by nitrofurantoin in porcine lung microsome. Porcine lung microsome, 1.5 mg/ml, was incubated aerobically at 37$_7$C in the reaction mixture of 3 ml containing 150mM KCl, 50mM Tris-HCl (pH 7.4), 0.2mM sodium azide, 0.5mM NADPH and varying concentration of nitrofurantoin. At 10 min of incubation, a 1 ml aliquot was transferred to a test tube containing 1 ml of ice-cold 5% TCA and centrifuged at 20,000xg for 10min. The supernatant (1.5ml) was mixed with 0.3ml of 10mM ferrous ammonium sulfate and 0.5ml of 2.5M potassium thiocyanate. The optical density of the mixture was measured at 480nm.

Table 3. Effect of nitrofurantoin on hydroxyl radical production by porcine lung microsome

Concentration of nitrofurantoin	Ethylene formed in 10 min	
	nmoles/mg protein	stimulation fold
0	7	—
0.1mM	75.7	10.8
0.3mM	101.0	14.4
0.5mM	125.9	18.0

Table 4. Effect of various oxygen radical scavengers on nitrofurantoin-stimulated hydroxyl radical production by porcine lung microsome[a]

Additions		Ethylene formed in 10 min	
		nmoles/mg protein	% inhibition
None		75.7	—
Catalase	10^{-6}M	23.2	69.4
SOD	1.5×10^{-7}M	18.5	75.6
Catalase + SOD	10^{-6}M 1.5×10^{-7}M	14.3	81.6
Mannitol	10mM	68.5	9.5
	20mM	61.0	19.5
Thiourea	10mM	57.9	23.6
	20mM	51.1	32.6

a: Microsome was incubated with 0.1mM nitrofurantoin.

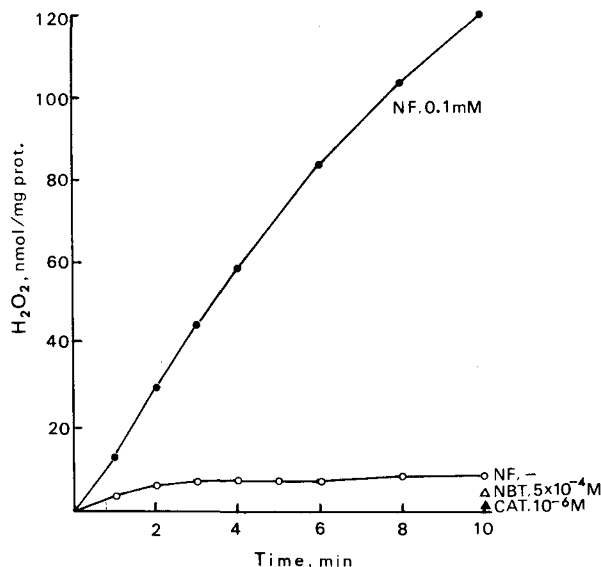

Fig. 7. Effect of catalase and nitroblue tetrazolium on the production of hydrogen peroxide in procine lung microsome. The microsome (1.5 mg/ml) was incubated with 0.1 mM nitrofurantoin in the presence of catalase or nitroblue tetrazolium (NBT). When catalase was added to the reaction mixture sodium szide was omitted. Other conditions were the same as in Fig. 6, O; no nitrofurantoin, ●; 0.1 mM nitrofurantoin only, Δ; 0.1 mM nitrofurantoin + $5x10^{-4}$M nitroblue tetrazolium and ▲; 0.1 mM nitrofurantoin + 10^{-6}M catalase.

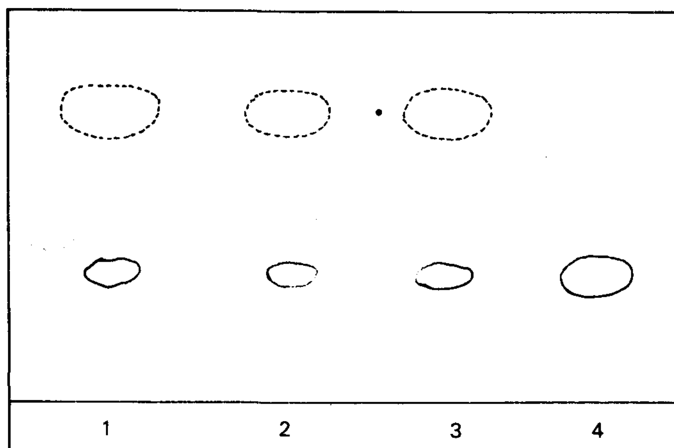

Fig. 8. Thin layer chromatogram of 1,3-diphenylisobenzofuran incubated with porcine lung microsome in the presence of nitrofurantoin. Singiet oxygen was analyzed by thin layer layer chromatographic method using 1, 3-diphenylisobenzofuran (Pederson and Aust, 1973). The reaction mixture containing 2 mg/ml microsome, 150 mM KCl, 50mM Tris-HCl (pH 7.4). 0.5mM nitrofurantoin were incubated aerobically for 10min at 37°C. For the chromatographic analysis, 0.5 ml of reaction mixture was removed and extracted with 0.6 ml of chloroform. Each extract, 15 μl, was applied to the Silica gel G thin layer plate and developed by heptane-dioxane (3:1). The spots were observed by the fluorescence(dotted circles) under UV lamp or after being sprayed with 0.5% 2,4-dinitrophenylhydrazine in 2N HCl (solid circles). 1; incubation mixture without nitrofurantoin, 2; incubation mixture with 0.5 mM nitrofurantoin, 3; 200 nmoles of 1,3-dephenylisobenzofuran only and 4; 200 nmoles of o-dibenzoylbenzen only.

7nmol/mg prot. in control experiment without NF. Additions of NF (0.1-0.5 mM) in the incubation mixture enhanced the production of ethylene by 11 to 18-fold (Table 3). The increased ethylene production was inhibited by SOD and catalase as well as by OH· scavengers, mannitol and thiourea (Table 4).

Singlet oxygen: The production of MDA stimulated by NF was never affected by $^1O_2$ scavengers, histidine and 1,4-diazabicyclo-(2,2,2)-octane (Table 2). This result indicated no involvement of $^1O_2$ in the NF-induced lipid peroxidation of lung microsome. It was confirmed by estimating the formation of $^1O_2$. The generation of $^1O_2$ was analyzed by determining o-dibenzoylbenzene, which is produced from interaction of 1,3-diphenylisobenzofuran with $^1O_2$, by thin layer chromatograph. As shown in Fig.8, the chromatogram demonstrated no differences in o-dibenzoylbenzene spots both in control and NF-treated microsome. It was concluded from this result that $^1O_2$ was never produced in porcine lung microsome incubated in the present experimental condition.

DISCUSSION

A molecular oxygen is reduced ultimately to water with acceptance of four electrons. Partial reduction of O_2, however, can occur in sequential univalent process with the formation of a number of reactive oxygen specieces, $O_2^{\overline{\cdot}}$, H_2O_2 and OH· . When oxygen accepts a single electron, it is converted to superoxide anion, and this dismutates spontaneously or enzymatically by SOD to produce H_2O_2. H_2O_2 can also be generated directly from oxygen by divalent reduction (Klebanoff, 1980). The interaction of $O_2^{\overline{\cdot}}$ and H_2O_2 results in the formation of secondary oxygen radicals, OH· (Haber and Weiss, 1934) and possibly singlet oxygen, $^1O_2$ (Kellogg and Fridovich, 1975). These oxygen radicals are highly reactive and can alter most types of cellular macromolecules. Based on in vitro experiments, these radicals have been known to oxidize polyunsaturated lipids and proteins containing oxidizable amino acids, and to damage nucleic acids (Fridovich, 1978; Klebanoff, 1980; McCord and Fridovich, 1978; Oh *et al.*, 1982).

Reactive oxygen radicals can be produced during normal electron transport in mitochondria (Boveris, 1977; Nohl and Hegner, 1978). But these normally produced radicals do not exert any significant harmful effect, because these are detoxified by endogenous oxygen radical quenchers, SOD, catalase and glutathione peroxidase. However, in certain pathologic conditions such as ischemic tissue injury or inflammatory process, the productions of reactive oxygen radicals are increased and contribute to development of the pathologic processes (Demopoulos *et al.*, 1980; Weissmann, 1979). Reactive oxygen radicals are also known to be involved in the toxicities produced by many kinds of exogenous chemicals, including adriamycin, bleomycin and a herbicide, paraquat (Frank, 1983; Smith and Boyd, 1983; Trush *et al.*, 1982).

Pulmonary toxicity is a serious side effect occurring in the course of treatment with NF (Holmberg *et al.*, 1982). In animal experiment the NF-induced pulmonary toxicity is developed dose-dependently, and more markedly in animals deficient with Vit. E or exposed to high O_2 environment (Boyed *et al.*, 1979). In in vitro study using pulmonary microsome, Mason and Holtzman (1975) observed that the metabolism of NF is similar to that of paraquat, which is well known for its pulmonary toxicity (Baldwin *et al.*, 1975; Bus *et al.*, 1974; Montgomery, 1976; Trush *et al.*, 1981, 1982). They reported that nitroaromatic compound incubated with pulmonary microsome in the presence of NADPH is reduced to nitroaromatic anion radical with acceptance of a single electron, and this radical again transfers an electron to O_2 to produce $O_2^{\overline{\cdot}}$. This was supported by other investigators (Sasame and Boyd, 1979; Trush *et al*, 1982) who observed the increased production of $O_2^{\overline{\cdot}}$ and H_2O_2 during NADPH-dependent oxidation-reduction of NF in pulmonary microsome of rat or mouse. In addition, they also observed the enhanced lipid peroxidation of microsome, accompanying the increased oxygen radical formation. In contrast, a number of investigators insisted no causative relationship between the pulmonary toxicity by NF or paraquat and the increased production of reactive oxygen radicals (Talcott

et al., 1979; Ilett *et al.*, 1974; Kornbrust and Mavis, 1980; Shu *et al.*, 1979). They reported no enhanced lipid peroxidation, and even with increased oxygen radical production, no concomitant lipid peroxidation by the drug.

In the present study, porcine lung microsome incubated with NADPH under O_2 produced $O_2^{-}\cdot$ and H_2O_2, and the productions were increased dose-dependently by NF. The lipid peroxidation of the microsome was also markedly enhanced by the drug. In agreement with Trush *et al.* (1982), the increased lipid peroxidation was prevented by SOD and catalase. In anaerobic conditions, however, lipid peroxidation was neither observed nor increased by NF. This results is in accordance with the observations of Mason and Holtzman (1975) and Sasme and Boyd (1979), who reported that NF, incubated with pulmonary microsome in anaerobic condition, is remained in radical state without transferring an electron to O_2, and does not increased the production of $O_2^{-}\cdot$ and H_2O_2. And in conditions maintained with atmospheric air, the degree of lipid peroxidation was much lower than that in aerobic condition made with pure O_2.

In addition to $O_2^{-}\cdot$ and H_2O_2, the production of OH· , analyzed by ethylene formation from methional, was also increased by NF in pulmonary microsome under aerobic condition. The increased production of OH· and accompanied peroxidation of microsomal lipid was prevented not only by OH· scavengers, but also by SOD and catalase. These results are in contrast to a study of Trush *et al.* (1982) who described the major role of $O_2^{-}\cdot$ in microsomal lipid peroxidation caused by NF. Based on in vitro studies using liver microsome, it was reported that NADPH-dependent lipid peroxidation is induced principally by OH· (Fong *et al.*, 1973; Lai *et al.*, 1977, 1978, 1979), not by $O_2^{-}\cdot$ or H_2O_2 alone (Svingen *et al.*, 1979). Consistent with these, the present results suggest that OH· , which may be generated by interaction of $O_2^{-}\cdot$ and H_2O_2, plays a principal role in the increase in lipid peroxidation of pulmonary microsome produced by NF.

Meanwhile, one potential problem for interpreting the present result is that the extent of the reduction of OH· formation by scavengers is much less than that of the prevention of lipid peroxidation by these agents. Such discrepancy, however, can be explained by a number of reports which showed only partial reduction of OH· formation by various OH· scavengers (Klebanoff and Rosen, 1978; Tauber and Babior, 1977; Weiss *et al.*, 1978). Lipid peroxidation initiated by a certain mechanism may be propagated further by following chain reactions (Aust and Svingen, 1982). Considering these concepts, if microsomal lipid peroxidation is initiated by highly reactive OH· , initial reduction of OH· formation even in small degree, will prevent further propagatory peroxidation reaction in large extent.

Microsome is considered to be easily attacked by reactive oxygen radicals, since microsomal membrane contains NADPH-dependent enzymes which mediate oxygen radical production as well as phospholipid consisting of readily peroxidizable unsaturated fatty acid (Aust and Svingen, 1982; Bus *et al.*, 1974; Trush *et al.*, 1982). Furthermore, toxic metabolites, which can be produced during microsomal lipid peroxidation process, can extend additional harmful effect to various organ tissues in wide range (Benedetti *et al.*, 1971 (1), (2)).

From the present result, it is strongly suggested that the stimulation of reactive oxygen radical, particulary OH· resulting in lipid peroxidation in pulmonary microsome may be a mechanism of in vivo pulmonary toxicity caused by NF.

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=국문초록=

Nitrofurantion이 폐장 미크로솜 지질과산화와 반응성 산소 라디칼 생성에 미치는 영향.

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항균제 nitrofurantion에 의한 폐독작용의 생화학적 기전을 규명하기 위한 연구 일환으로 *in vitro*에서 폐장 mircrosome 지질의 과산화 및 반응성 산소 radical (O_2^-·, H_2O_2, OH·, $^1O_2$)의 생성에 대한 nitrofurantion의 영향과 양자 간의 상호 관련성을 검토하였다.

Nitrofurantion은 호기성 반응 조건에서 돼지 폐장 mircrosome의 NADPH 의존성 지질 과산화를 용량 의존적으로 증가시킬 뿐 아니라 $O_2^- \cdot$, H_2O_2 및 두 radical의 상호 작용으로 2차적으로 형성되는 OH·의 생성 또한 촉진하였으며 $^1O_2$생성은 관찰되지 않았다. 이와 같은 폐장 microsome 지질 과산화 증가는 SOD 및 catalase에 의하여 억제될 뿐만 아니라 OH· 제거 물질인 mannitol, thiourea에 의하여도 현저히 억제되었으며, $^1O_2$제거 물질에 의하여는 영향을 받지 않았던 한편 염기성 반응 조건에서는 nitrofurantoin에 의한 지질 과산화가 관찰되지 않았다.

이상의 결과로 미루어 보아 nitrofurantoin은 폐장 microsome의 NADPH 의존적인 반응성산소 radical ($O_2^- \cdot$, H_2O_2 및 OH·)의 생성을 증가시키며 이들 중 특히 OH·에 의한 microsome막 지질 과산화를 촉진하는 것으로 결론지었고, 이와 같은 *in vitro* 현상은 nitrofurantion의 *in vivo* 폐독작용의 기전을 설명하는 일부가 될 것으로 사료하였다.