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STUDIES ON THE *IN VITRO* INTERACTION OF MITOMYCIN C, NITROFURANTOIN AND PARAQUAT WITH PULMONARY MICROSOMES

STIMULATION OF REACTIVE OXYGEN-DEPENDENT LIPID PEROXIDATION*

MICHAEL A. TRUSH†, EDWARD G. MIMNAUGH, ERIKA GINSBURG and THEODORE E. GRAM
Drug Interactions Section, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, Bethesda, MD 20205, U.S.A.

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Abstract—*In vitro* experiments were performed to evaluate the capacity of the redox cycling compounds mitomycin C (MC), nitrofurantoin (NF) and paraquat (PQ) to stimulate pulmonary microsomal lipid peroxidation. It was observed that the interaction of MC, NF or PQ with rat or mouse lung microsomes in the presence of an NADPH-generating system and an O_2 atmosphere resulted in significant lipid peroxidation. All three compounds demonstrated similar concentration dependency, similar time courses and the ability to generate lipid peroxidation-associated chemiluminescence. The stimulation of lipid peroxidation by MC, NF or PQ was inhibited significantly by superoxide dismutase, glutathione, ascorbic acid, catalase and EDTA, agents which either scavenge reactive oxygen and/or prevent the generation of secondary reactive oxygen metabolites. In addition, the ability of MC or NF, but not PQ, to stimulate lipid peroxidation was reduced significantly following preincubation with microsomes and NADPH under N_2 (15–20 min) prior to incubation under O_2. During this period under N_2, MC and NF underwent reductive metabolism of their quinone and nitro moieties respectively. Thus, it appears that under aerobic conditions the pulmonary microsomal-mediated redox cycling of MC, NF and PQ is accompanied by the stimulation of reactive oxygen-dependent lipid peroxidation.

The interaction of mitomycin C, nitrofurantoin, or paraquat (Fig. 1) with microsomes, in the presence of NADPH, results in the formation of the reduced radical species of these chemicals [1–3]. Under aerobic conditions, these reduced radical species rapidly reoxidize, reforming the parent substrate and the reduced species of oxygen, namely superoxide [1, 4–6]. The redox cycling process is mediated by NADPH cytochrome P-450 reductase as indicated by studies using either the purified enzyme or antibodies raised against this enzyme [7–10]. Thus, in tissues capable of mediating this process, toxic reactions resulting from enhanced reactive oxygen generation become a distinct possibility. Moreover, the lung would be expected to be a likely target organ for such agents because of its complement of microsomal NADPH cytochrome P-450 reductase and its high oxygen tension, and indeed mitomycin C, nitrofurantoin and paraquat are known to induce life-threatening pulmonary toxicity in humans [11–13].

We have recently characterized *in vitro* conditions demonstrating that paraquat can stimulate reactive oxygen-dependent lipid peroxidation in pulmonary microsomes [14], a subject that has been in dispute in the literature [15, 16]. Further, this paraquat-induced lipid peroxidation was enhanced by O_2 or by using microsomes from α-tocopherol-deficient animals. The present investigation was undertaken to determine whether mitomycin C or nitrofurantoin, like paraquat, is also capable of stimulating *in vitro* reactive oxygen-dependent lipid peroxidation in pulmonary microsomes.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (weighing 200–250 g) were obtained from Taconic Farms, Germantown, NY, and male CDF_1 mice (weighing 20–25 g) were obtained from Charles River, Boston, MA. Animals were fed standard Purina rat chow and allowed water *ad lib*. They were killed by cervical dislocation, and the lungs from ten rats or twenty-five mice were combined to yield a pool of tissue. Similarly, the livers of six rats were pooled.

Microsomal preparation. Microsomal fractions were obtained from lungs or livers following homogenization in 2 vol. of 150 mM KCl–50 mM Tris–HCl buffer (pH 7.4), dilution of the homogenates to 25% (w/v), centrifugation of the homogenates at 9,000 *g* for 20 min, and finally centrifugation of the supernatant fractions at 100,000 *g* for 1 hr [17]. The resulting microsomal pellets were resuspended in KCl–Tris–HCl buffer and were centrifuged again at 100,000 *g* for 1 hr. Unless otherwise stated, the washed microsomes were resuspended in KCl–Tris–HCl buffer that had been bubbled with O_2.

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† Author to whom enquiries should be addressed: Dr. M. A. Trush, Building 37, Room 6D28, National Cancer Institute, NIH, Bethesda, MD 20205, U.S.A.

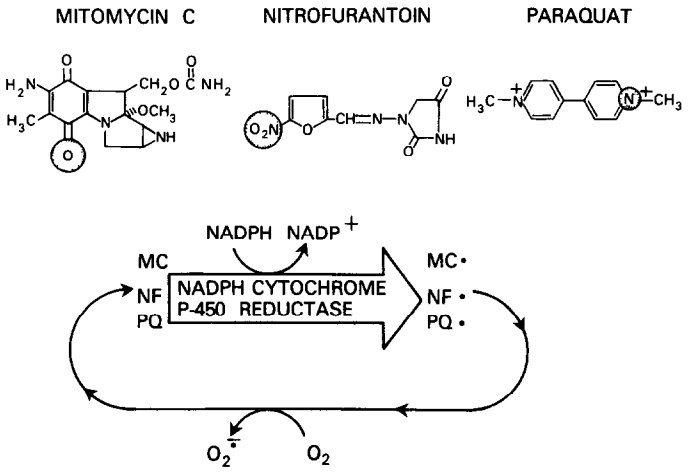

Fig. 1. Structures of mitomycin C, nitrofurantoin and paraquat with the electron accepting group of each outlined in the shaded region. Also illustrated are the processes involved in the redox cycling of these compounds which results in the generation of superoxide anion ($O_2^{\cdot}$).

Microsomal protein was determined by the method of Lowry *et al.* [18] and was diluted to a concentration of 3.5 mg protein/ml in O_2 saturated KCl–Tris–HCl buffer.

In vitro *lipid peroxidation*. Microsomes (final concentration 0.5 mg microsomal protein/ml) were incubated at 37° in the presence or absence of mitomycin C, nitrofurantoin or paraquat with an NADPH-generating system in open chloride-meter vials (1.8 cm × 4.0 cm) in a total volume of 1.75 ml. The NADPH-generating system consisted of NADP (1.9 mM), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (1.1 I.U./ml) and magnesium chloride (9 mM). Exogenous ferrous iron was not added to the mixtures. Unless indicated otherwise, incubations were conducted in a Dubnoff metabolic shaker fitted with a covered hood connected to an O_2 tank (flow rate 5.0 l/min). Reactions were stopped by the addition of 0.75 ml of a 2.0 M trichloroacetic acid (TCA)–1.7 N HCl solution, and the samples were centrifuged at 1000 *g*. Aliquots (0.5 ml) of the supernatant fractions were then reacted with 2.0 ml of 1% thiobarbituric acid and the chromophore was developed at 90° for 10 min. After the samples were cooled, the absorbance at 533 nm was determined, and the nmoles of malonaldehyde (MDA) formed was calculated based on an experimental extinction coefficient of 1.53×10^5 M^{-1} cm^{-1} at 533 nm. Where utilized, inhibitors were added directly to the incubation vials. Zero time blanks containing all components of the incubation mixture were used throughout.

Lipid peroxidation-associated chemiluminescence (CL). The generation of lipid peroxidation-associated CL was monitored in a Packard liquid scintillation spectrometer (model 3003) operated at ambient temperature and in the out-of-coincidence mode [19] with the following settings: gain 100%, window A-∞ with discriminators set at 0–1000 and input selector 1 + 2. The reaction mixture consisted of rat lung microsomes, the NADPH-generating system, and mitomycin C, nitrofurantoin or paraquat added to previously dark-adapted polyethylene scintillation vials. All additions, as well as the CL counting procedure itself, were performed in a darkened area. To begin the procedure, microsomes were incubated at 37° for 10 min in the presence or absence of the test chemicals; in order to maintain an aerobic environment in the reaction mixture during this time and during the course of the experiment each vial was capped with a rubber septum fitted with two 21 gauge needles, one of which was connected to an O_2 tank (flow rate 1.0 l/min) by means of tygon tubing. Following the 10-min preincubation period, each vial was transferred to the scintillation counter, background CL was determined, the NADPH-generating system was added, the rubber septums were repositioned, and the vials were returned to the 37° incubator. CL was then monitored at 10-min intervals at a counting time of 0.5 min. The results are expressed as counts $\times 10^{-4}$/0.5 min corrected for background activity.

Anaerobic metabolism of mitomycin C and nitrofurantoin. Evaluation of the NADPH-dependent anaerobic microsomal metabolism of mitomycin C and nitrofurantoin was assessed spectrophotometrically by substrate disappearance following incubation with rat lung or liver microsomes (0.5 mg protein/ml) and an NADPH-generating system at 37° under N_2 for various times. Disappearance of mitomycin C was monitored by the loss of the quinone moiety and that of nitrofurantoin by the loss of the nitro group. For mitomycin C, the reaction was terminated by adding 1 ml of 5% $ZnSO_4$ followed by 1 ml of saturated $Ba(OH)_2$. Following centrifugation of the samples at 1000 *g*, the absorbance of the supernatant fraction was determined at 363 nm as described by Kennedy *et al.* [20]. In order to compensate for the absorbance of NADPH at this wavelength, reactions containing microsomes and the NADPH-generating system were incubated for the same length of time as those containing mitomycin C; these values were then subtracted from the absorbance values obtained from those reactions

containing mitomycin C. Similarly, to minimize the contribution by NADPH at 363 nm, the composition of the NADPH-generating system was modified from that used in the *in vitro* lipid peroxidation assay as follows: NADP (0.72 mM), glucose-6-phosphate (5.0 mM), glucose-6-phosphate dehydrogenase (1.1 I.U./ml) and magnesium chloride (9.0 mM). In the experiments with nitrofurantoin, the reactions were stopped by adding 2 ml of 20% TCA, the samples were centrifuged at 1000 *g*, and the absorbance of the supernatant fractions was determined at 400 nm [9, 21]. In this case the composition of the NADPH-generating system was the same as that used in the *in vitro* lipid peroxidation experiments. For both chemicals, an incubated blank containing microsomes and the chemical was included; the reactions were conducted in duplicate, and all solutions were saturated with N_2 prior to incubation.

Chemicals. NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, catalase 10,000 units/mg protein), glutathione, ascorbic acid, superoxide dismutase (2900 units/mg protein) and paraquat (methyl viologen) were obtained from the Sigma Chemical Co., St. Louis, MO. Dimethylurea was obtained from the Aldrich Chemical Co., Milwaukee, WI. Mitomycin C (Bristol Labs) and nitrofurantoin (Norwich Pharmacal Co.) were obtained through the Division of Cancer Treatment, NCI. All other chemicals were of the highest purity available.

Statistics. Data were evaluated by Student's *t*-test, and results were considered significant if $P < 0.05$ [22].

RESULTS

Stimulation of pulmonary microsomal lipid peroxidation. Figs. 2–4 illustrate the enhanced lipid peroxidation that resulted when mitomycin C, nitrofurantoin or paraquat, in a wide range of concentrations, was incubated with rat and mouse lung microsomes under conditions in which these compounds may undergo redox cycling, i.e. an O_2 atmosphere and a steady state level of NADPH. In agreement with other reports [15, 16, 23], mouse lung microsomes were peroxidized to a greater extent than rat lung microsomes in both the absence and presence of these compounds. Similarly, the chemically enhanced lipid peroxidation was initiated in mouse lung microsomes at concentrations of these chemicals 4 to 10-fold lower than that required to initiate peroxidation in rat lung microsomes. Paraquat appeared to be more effective than the other two compounds in stimulating peroxidation with rat lung microsomes at the lower substrate concentrations, although the overall pattern of response was eventually quite similar for all three chemicals.

The data presented in Table 1 demonstrate that the stimulation of lipid peroxidation by all three compounds was dependent on aerobic conditions and that maximum stimulation occurred under O_2. This is not surprising since O_2 is an electron acceptor from the reduced radical species of these chemicals (Fig. 1). Deletion of the NADPH-generating system or utilization of boiled microsomes in the presence of reducing equivalents did not result in significant lipid peroxidation (data not shown).

Characterization of the time course of MDA generation revealed that this response was essentially linear for 2 hr with both rat and mouse lung microsomes (Fig. 5). Only the incubation of paraquat with mouse lung microsomes and NADPH approached the level of 100% peroxidation (80–90 nmoles/mg protein) cited by Kornbrust and Mavis [23], suggesting that significant peroxidizable substrate remained even after 2 hr of incubation, particularly in rat lung microsomes. With each chemical substrate there was a 30-min lag period with rat lung microsomes prior to the generation of significant amounts

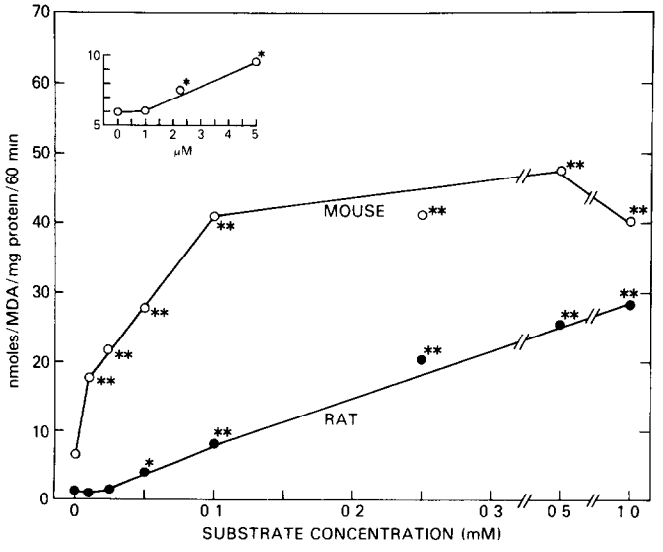

Fig. 2. Stimulation by mitomycin C of lipid peroxidation in rat and mouse lung microsomes during incubation under O_2 for 60 min in the presence of an NADPH-generating system. The inset illustrates the effect of 1.0, 2.5 or 5.0 μM mitomycin C with mouse lung microsomes. Each point is the mean of three to five determinations. Key: (*) $P < 0.05$, and (**) $P < 0.01$ from control.

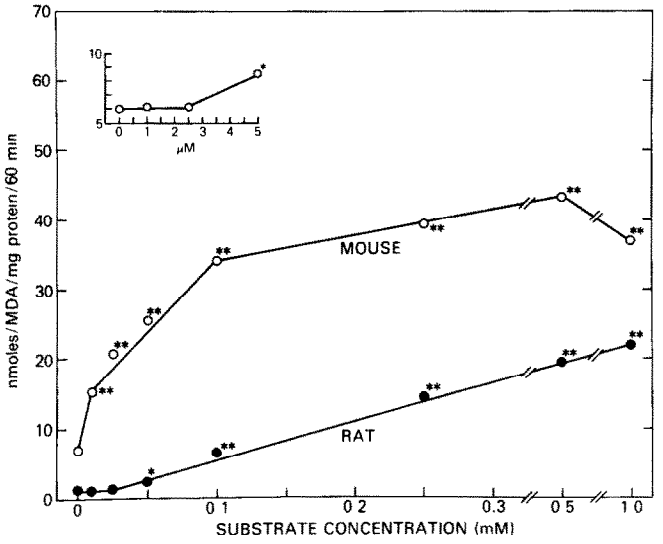

Fig. 3. Stimulation by nitrofurantoin of lipid peroxidation in rat and mouse lung microsomes during incubation under O_2 for 60 min in the presence of an NADPH-generating system. The inset illustrates the effect of 1.0, 2.5 or 5.0 μM nitrofurantoin with mouse lung microsomes. Each point is the mean of three to five determinations. Key: (*) $P < 0.05$, and (**) $P < 0.01$ from control.

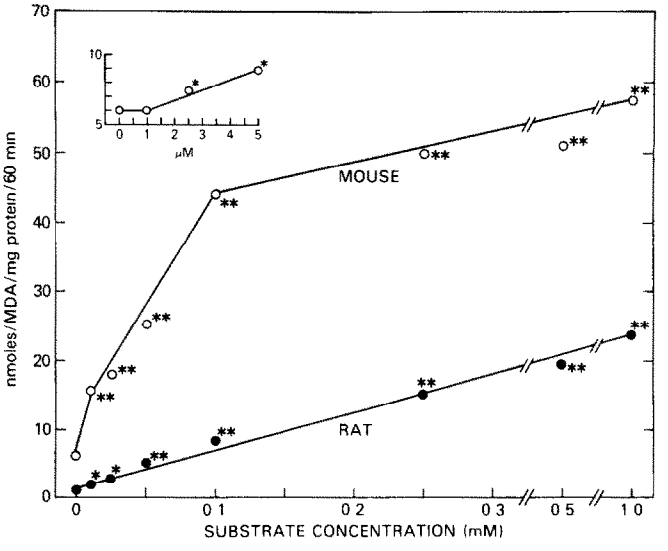

Fig. 4. Stimulation by paraquat of lipid peroxidation in rat and mouse lung microsomes during incubation under O_2 for 60 min in the presence of an NADPH-generating system. The inset illustrates the effect of 1.0, 2.5 or 5.0 μM paraquat with mouse lung microsomes. Each point is the mean of three to five determinations. Key: (*) $P < 0.05$, and (**) $P < 0.01$ from control.

Table 1. Effect of the atmosphere on the stimulation of lipid peroxidation in rat lung microsomes by mitomycin C, nitrofurantoin or paraquat

Addition	MDA [nmoles·(mg protein)$^{-1}$·60 min^{-1}]		
	Atmosphere		
	Air	Oxygen*	Nitrogen*
None	0.5 ± 0.2†	0.7 ± 0.3	0.5 ± 0.4
Mitomycin C (1 mM)	13.4 ± 0.9‡	26.4 ± 1.1‡§	1.4 ± 0.6§
Nitrofurantoin (1 mM)	12.0 ± 0.2‡	23.6 ± 1.1‡§	0.0 ± 0.0§
Paraquat (1 mM)	10.9 ± 1.0‡	26.5 ± 1.0‡§	0.6 ± 0.2§

* All solutions were bubbled with either O_2 or N_2 prior to incubation and the incubations were conducted in Dubnoff metabolic shakers with covered hoods (flow rate of O_2 or N_2 5 l/min). Rat lung microsomes (0.5 mg/ml) were incubated in the presence of an NADPH-generating system in the absence or presence of mitomycin C, nitrofurantoin or paraquat under the indicated atmosphere for 60 min. Lipid peroxidation was determined by the thiobarbituric acid method as described in Materials and Methods.

† Mean ± S.D., N = 3–5.

‡ $P < 0.01$, from no addition.

§ $P < 0.01$, from air.

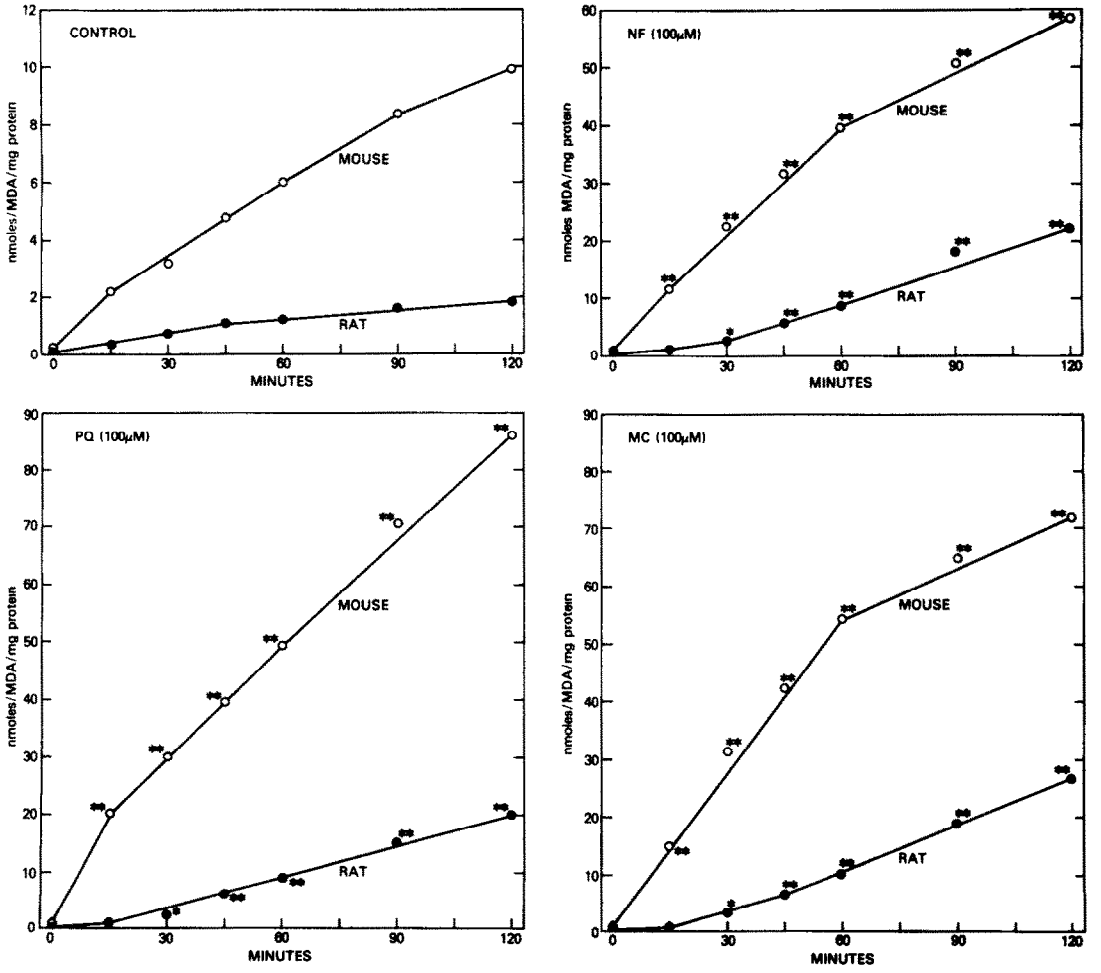

Fig. 5. Time course of lipid peroxidation during incubation under O_2 of rat and mouse lung microsomes with an NADPH-generating system in the absence or presence of mitomycin C, nitrofurantoin or paraquat. Note different ordinates in the presence of chemicals. Each point is the mean of four determinations. Key: (*) $P < 0.05$, and (**) $P < 0.01$ from control.

of MDA. We observed previously that with paraquat (10^{-4} M) and rat lung microsomes this lag period could be reduced if vitamin E-deficient microsomes were used or if the paraquat concentration was increased to 10^{-3} M with control microsomes [14]. However, even under these conditions the extent of peroxidation at 15 min was still not equivalent to that observed in the present investigation with 10^{-4} M paraquat and mouse lung microsomes, emphasizing that specific species differences, which control the rate of lipid peroxidation, exist at the microsomal level.

Lipid peroxidation-associated chemiluminescence. Howes and Steele [24] initally demonstrated that liver microsomal lipid peroxidation is accompanied by the generation of a product(s) in an electronically excited state which upon relaxation to ground state is accompanied by the emission of photons (chemiluminescence). The relationship between MDA generation and this chemiluminescence (CL) was further defined by Wright *et al.* [25]. In addition, Cadenas *et al.* [26] observed that paraquat, administered *in vivo* or *in vitro*, augmented hydroperoxide-induced CL in the isolated perfused rat lung. Figure 6 demonstrates that mitomycin C, nitrofurantoin and paraquat can elicit CL from pulmonary microsomes, while Fig. 7 depicts the concomitance in CL and MDA generation, suggesting

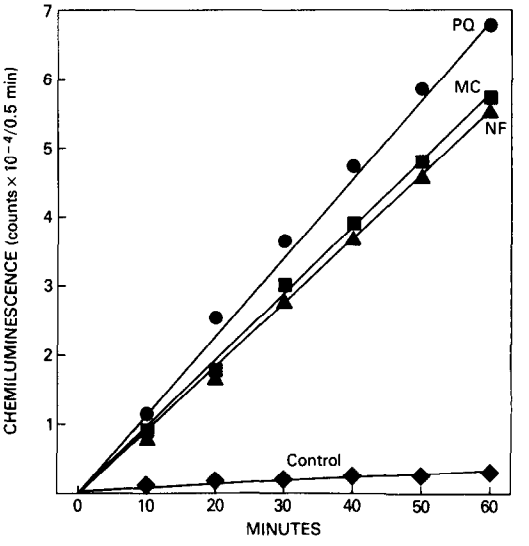

Fig. 6. Time course of generation of chemiluminescence resulting from the incubation of rat lung microsomes with an NADPH-generating system in the absence or presence (1 mM) of mitomycin C, nitrofurantoin or paraquat. Each point is the mean of four experiments.

Table 2. Effect of various agents on mitomycin C, nitrofurantoin or paraquat-stimulated lipid peroxidation in rat or mouse lung microsomes*

Agent	MDA [nmoles·(mg protein)$^{-1}$·60 min^{-1}]		
	MC (10^{-4} M)	NF (10^{-4} M)	PQ (10^{-4} M)
Rat lung microsomes			
None	8.4 ± 0.6	6.6 ± 1.7	10.2 ± 1.4
Superoxide dismutase (10^{-6} M)†	0.5 ± 0.2‡	0.6 ± 0.4‡	0.5 ± 0.2‡
Superoxide dismutase (10^{-7} M)	0.7 ± 0.2‡	0.9 ± 0.2‡	0.4 ± 0.3‡
Glutathione (10^{-3} M)	1.3 ± 0.3‡	1.6 ± 0.6‡	2.0 ± 0.6‡
Glutathione (10^{-4} M)	4.7 ± 1.4‡	5.4 ± 0.7	7.5 ± 0.8‡
Ascorbic acid (10^{-3} M)§	2.8 ± 0.4‡	2.5 ± 0.2‡	3.5 ± 1.0‡
Ascorbic acid (10^{-4} M)§	6.0 ± 2.1	5.2 ± 0.9	11.7 ± 2.5
EDTA (10^{-6} M)	1.9 ± 0.6‡	1.5 ± 0.5‡	1.7 ± 0.7‡
EDTA (10^{-7} M)	7.1 ± 1.2	6.3 ± 1.3	9.8 ± 1.8
Dimethylurea (10^{-2} M)	1.1 ± 0.4‡	2.7 ± 1.0‡	2.2 ± 0.9‡
Dimethylurea (10^{-3} M)	3.1 ± 1.0‡	5.0 ± 0.6	5.3 ± 0.4‡
Dimethylurea (10^{-4} M)	6.3 ± 1.2	5.8 ± 1.0	7.3 ± 0.9
Catalase (10^{-6} M)	1.0 ± 0.3‡	0.9 ± 0.2‡	0.8 ± 0.2‡
Mouse lung microsomes			
None	37.1 ± 2.6	36.8 ± 0.5	45.3 ± 5.6
Superoxide dismutase (10^{-6} M)	5.6 ± 0.4‡	5.5 ± 0.3‡	5.9 ± 0.6‡
Glutathione (10^{-3} M)	21.5 ± 1.7‡	19.1 ± 1.1‡	21.1 ± 1.7‡
Ascorbic acid (10^{-3} M)	26.8 ± 2.3‡	25.1 ± 1.8‡	42.1 ± 2.5
EDTA (10^{-6} M)	2.4 ± 0.2‡	2.5 ± 0.3‡	3.5 ± 0.3‡
Dimethylurea (10^{-2} M)	7.0 ± 0.9‡	9.3 ± 1.6‡	7.2 ± 1.3‡
Catalase (10^{-6} M)	10.1 ± 1.9‡	11.5 ± 0.5‡	12.6 ± 2.4‡

* Lung microsomes (0.5 mg/ml) and the NADPH-generating system were incubated in the presence of the various additions noted above for 60 min under O_2. Lipid peroxidation was determined as described in Materials and Methods. Each value is the mean ± S.D., N = 3–5.

† Boiled superoxide dismutase or an equivalent amount of bovine serum albumin had no significant inhibitory effect.

‡ $P < 0.05$.

§ Incubation of ascorbic acid with the NADPH-generating system did not stimulate lipid peroxidation.

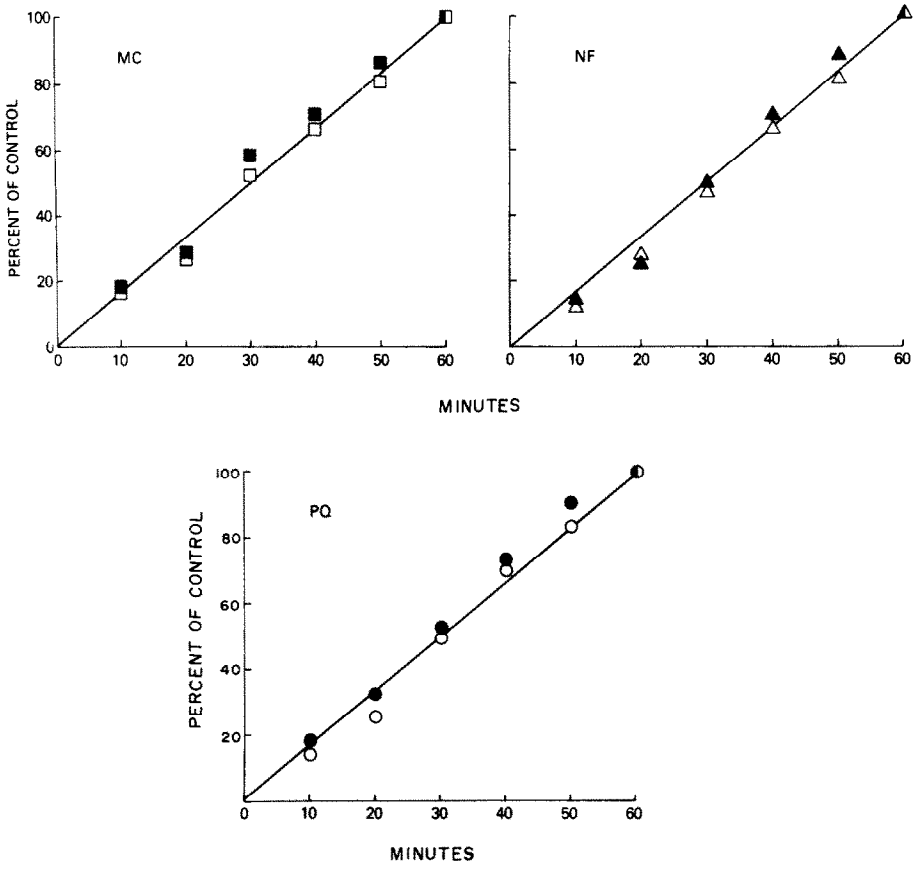

Fig. 7. Correlation between the rate of generation of chemiluminescence (closed symbols) and the rate of generation of lipid peroxidation (open symbols) resulting from the incubation of 1 mM mitomycin C, nitrofurantoin or paraquat with rat lung microsomes. Lipid peroxidation was determined in a group parallel to that used to generate the CL. The data presented are the percentage of control, at each time, of the value obtained at 60 min for each parameter. The 60-min control values (mean ± S.D.) for CL and lipid peroxidation are as follows: mitomycin C, 5.4 ± 0.5 counts × 10^{-4}/0.5 min and 23.1 ± 2.3 nmoles MDA·(mg protein)$^{-1}$·60 min^{-1}; nitrofurantoin, 5.3 ± 0.6 counts × 10^{-4}/0.5 min and 18.3 ± 3.6 nmoles MDA·(mg protein)$^{-1}$·60 min^{-1}; paraquat, 6.6 ± 0.5 counts × 10^{-4}/0.5 min and 20.5 ± 1.6 nmoles MDA·(mg protein)$^{-1}$·60 min^{-1}. Each point is the mean of three determinations and at each time point the values for both parameters were significantly different ($P < 0.05$) from microsomes incubated in the absence of these chemicals.

a relationship between lipid peroxidation and the origin of CL.

Involvement of reactive oxygen. Evidence for the involvement of reactive oxygen in the stimulation of lipid peroxidation by these chemicals was investigated by two approaches: (1) by incubating microsomes in the presence of scavengers of activated oxygen, and (2) by examining whether microsomal metabolism altered the molecular moiety of these chemicals responsible for the generation of reactive oxygen.

The results of the studies with the reactive oxygen scavengers are summarized in Table 2. In general, significant inhibition occurred in both rat and mouse lung microsomes, although in some instances the degree of inhibition was less with mouse lung microsomes. Superoxide dismutase (SOD) was an extremely effective inhibitor indicating that the superoxide anion, the initial reactive oxygen metabolite resulting from the reoxidation of these chemicals (Fig. 1), contributes significantly to this chemically mediated lipid peroxidation. Glutathione and ascorbic acid, normal cytosolic constituents of the lung, also inhibited the enhanced lipid peroxidation but not as effectively as SOD. In addition, catalase, the metal chelator EDTA, and the hydroxyl radical scavenger dimethylurea [27] were inhibitory, suggesting that secondary oxygen metabolites, possibly arising by non-enzymatic metal-mediated reactions, may also play a role in initiating pulmonary microsomal lipid peroxidation.

It has been demonstrated using electron spin resonance that, under anaerobic conditions, paraquat, nitrofurantoin and mitomycin C are converted by microsomes in the presence of NADPH to their reduced radical species [1, 2, 10]; however, only paraquat remains unchanged while mitomycin C and nitrofurantoin undergo secondary anaerobic metabolism. The anaerobic metabolism of mitomycin C is accompanied by reduction of the quinone moiety

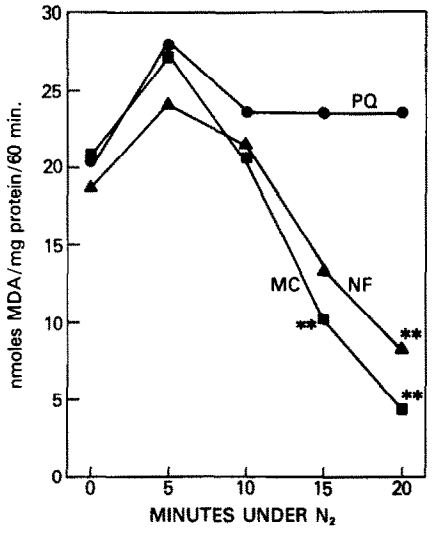

Fig. 8. Effect of anaerobic incubation on the stimulation of lipid peroxidation by mitomycin C, nitrofurantoin or paraquat. Rat lung microsomes were preincubated with 250 μM mitomycin C, nitrofurantoin or paraquat under N_2 (0–20 min) in the presence of the NADPH-generating system; the atmosphere was then changed to O_2 and the incubations were continued for an additional 60 min, after which the amount of MDA was then determined. Each point is the mean of four determinations. Key: (**) $P < 0.01$ from no incubation under N_2.

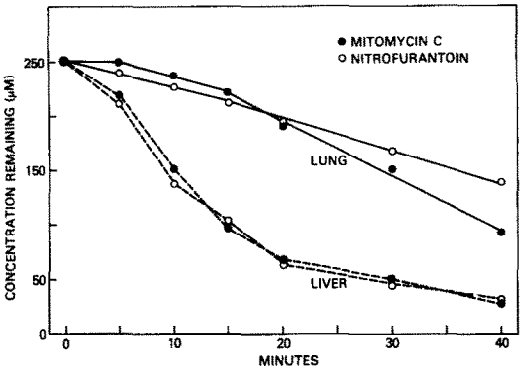

Fig. 9. Time course of the NADPH-dependent reductive metabolism of 250 μM mitomycin C or nitrofurantoin by rat lung or liver microsomes. The rates of metabolism by the lung or liver, respectively, were as follows: mitomycin C, 5.6 nmoles·(mg protein)$^{-1}$·min^{-1} or 13.3 nmoles·(mg protein)$^{-1}$·min^{-1}; nitrofurantoin, 3.6 nmoles·(mg protein)$^{-1}$·min^{-1} or 11.4 nmoles·(mg protein)$^{-1}$·min^{-1}. Each point is the mean of four determinations.

(Fig. 1) to the hydroquinone [20], while nitrofurantoin (Fig. 1) undergoes reduction of its nitro group possibly to the primary amine [9, 21]. When mitomycin C and nitrofurantoin were preincubated under N_2 with microsomes and NADPH for 15–20 min, subsequent incubation under O_2 resulted in a reduced capacity to stimulate lipid peroxidation (Fig. 8). On the other hand, the ability of paraquat to stimulate lipid peroxidation was not diminished following anaerobic incubation under the same conditions.

The data presented in Fig. 9 confirm that the anaerobic incubation of mitomycin C and nitrofurantoin with both rat lung and liver microsomes and an NADPH-generating system results in chemical alteration of both mitomycin C and nitrofurantoin. Like the aerobic stimulation of lipid peroxidation by these agents, the reductive metabolism was dependent on NADPH (data not shown). Similarly, incubation under O_2 for 60 min in the presence of NADPH and lung microsomes did not result in significant metabolism of either compound (data not shown).

The patterns of the responses in Figs. 8 and 9 are quite similar; however, the extent of peroxidation at 20 min (Fig. 8) was much less than would be expected from the amount (175–200 μM) of either mitomycin C or nitrofurantoin remaining with the lung microsomes at 20 min (Fig. 9), but it more closely agreed with the concentrations (100–150 μM) of these chemicals remaining at 40 min (Fig. 9). This discrepancy was surprising since there appeared to be a good correlation between chemical concentration and rate of lipid peroxidation (Figs. 2 and 3). An explanation may be that the anaerobic metabolism of these chemicals continued until the samples became completely reoxygenated following conversion from the N_2 to the O_2 atmosphere (Fig. 8). Thus, in this situation less of the parent chemical was available for redox cycling than was actually determined at the 20-min time point (Fig. 9).

DISCUSSION

Studies with experimental animal models have demonstrated that the pulmonary toxicity resulting from paraquat or nitrofurantoin administration was enhanced significantly by vitamin E deficiency and/or exposure to an oxygen-enriched atmosphere [28–30]. It has been noted also that oxygen exposure increases the pulmonary toxicity of mitomycin C in humans [31]. In addition, the pathophysiology of the pulmonary damage evoked by these agents exhibits similar patterns and endpoints: inflammation, hemorrhage, edema and interstitial fibrosis. Thus, these *in vivo* observations coupled with the inherent ability of these compounds to undergo aerobic futile redox cycling suggest that mitomycin C, nitrofurantoin and paraquat may induce lung injury through a common mechanism.

Bus *et al.* [10, 29, 32] presented data implicating microsomal lipid peroxidation as a biochemical mechanism for the pulmonary toxicity of paraquat; however, the inability of subsequent investigators to demonstrate *in vitro* stimulation by paraquat of rat lung microsomal lipid peroxidation has raised questions as to the validity of this hypothesis [15, 16]. In addition, even the involvement of reactive oxygen in paraquat-stimulated lipid peroxidation with mouse lung microsomes has been subjected to reconsideration [33]. However, data presented in the present manuscript demonstrate that not only paraquat but mitomycin C and nitrofurantoin stimulate reactive oxygen-dependent lipid peroxidation in both rat and mouse lung microsomes. The stimulation of *in vitro* peroxidation by these chemicals, like their *in vivo*

toxicity, exhibited common features: similar concentration dependency, similar time courses, enhancement by O_2, the ability to induce lipid peroxidation-associated chemiluminescence, and inhibition by a heterogeneous group of scavengers of reactive oxygen.

In addition to the innate ability of a chemical to generate reactive oxygen, microsomal factors probably determine to what extent this activity is expressed as lipid peroxidation. These factors include the microsomal NADPH cytochrome P-450 reductase activity, the content of peroxidizable polyunsaturated fatty acids and the content of microsomal vitamin E. With regard to these factors, mouse lung microsomes exhibit 2 to 3-fold greater activity of NADPH cytochrome P-450 reductase [17, 34], and have approximately 3-fold less vitamin E [23] and approximately an equivalent amount of peroxidizable substrate [23] as compared to rat lung microsomes. Thus, the higher rate of lipid peroxidation observed with mouse lung microsomes is probably reflective not only of a greater ability to enzymatically mediate the redox cycling of these compounds but also of an impaired capacity to protect against the effect of enhanced reactive oxygen generation. Since α-tocopherol protects against superoxide through conversion to the inactive tocopherol quinone [35], the progressive depletion of tocopherol by this process would be expected to facilitate the ability of reactive oxygen species to promote lipid peroxidation. The time for such a process to occur, viz. depletion of endogenous microsomal tocopherol, probably accounts for the lag period in initiation of lipid peroxidation in rat lung microsomes. Furthermore, the higher content of vitamin E in rat lung microsomes should make it easier for other scavengers or defenses of reactive oxygen to control lipid peroxidation *in vitro*, and presumably *in vivo*, which is in agreement with the data presented.

The significant inhibition of peroxidation by superoxide dismutase with both rat and mouse lung microsomes clearly indicates the involvement of reactive oxygen in this chemically stimulated lipid peroxidation and, in particular, a prominent role for superoxide. Whether superoxide initiates *in vitro* lipid peroxidation directly or indirectly through either a Fenton-type reaction or by maintaining arachidonate-associated iron in the more reactive ferrous form is not clear [36, 37]. The data suggest also that secondary oxygen metabolites are involved, possibly hydroxyl radicals, as demonstrated by the effect of catalase, dimethylurea and EDTA. Interestingly, ascorbic acid, which inhibited lipid peroxidation *in vitro*, has been shown to exert a protective effect against the *in vivo* toxicity of paraquat [38]. Similarly, it has been demonstrated that a nitrofurantoin preparation which contained vitamin C elicited less pulmonary toxicity than preparations lacking vitamin C [12]. An intriguing possibility for the biochemical mechanism of this protection is that ascorbic acid may react with the vitamin E radical to generate the active antioxidant form of vitamin E [39], thus allowing it to interact with reactive oxygen again.

Generation of the reactive oxygen to mediate pulmonary microsomal lipid peroxidation was shown to be dependent on the ability of these chemicals to undergo redox cycling as demonstrated by the experiments in which mitomycin C and nitrofurantoin were subjected to anaerobic metabolism to products no longer capable of undergoing redox cycling. Although the anaerobic metabolism of both mitomycin C and nitrofurantoin results in the formation of potentially cytotoxic products [9, 20], significant *in vivo* generation of such intermediates in the lung is probably unlikely considering the high O_2 tension of the lung. Further, Boyd *et al.* [9] have demonstrated that [^{14}C]nitrofurantoin did not preferentially bind in the lung as did another furan containing lung toxin, 4-ipomeanol. In addition, the enhancement of the *in vivo* toxicity of all three compounds by O_2 implies the involvement of an oxygen-dependent mechanism. Considering the present *in vitro* data, lipid peroxidation is worthy of further consideration as a toxigenic mechanism.

Although activated oxygen metabolites exhibit reactivity toward a variety of biomolecules [40, 41], microsomal membrane lipids, in particular polyunsaturated fatty acids of phospholipids, are regarded as prime target molecules because of their chemical lability and their proximity to the site of reactive oxygen generation. Moreover, in light of recent observations demonstrating that microsomal lipid peroxidation is accompanied by the formation of diffusible reactive products, lipid peroxidation should not be viewed as a process destructive only to the endoplasmic reticulum, or its consequences necessarily confined to the cell in which peroxidation occurred [42]. Depending on the reactivity of such products, they could serve to amplify even minute amounts of *in vivo* lipid peroxidation. Further, in addition to their ability to alter enzymatic activity associated with the endoplasmic reticulum, these products originating from microsomal peroxidation have the ability to elicit an inflammatory response [43], a process commonly associated with the pulmonary damage induced by these chemicals. Demonstration of the ability of mitomycin C, nitrofurantoin and paraquat to enhance the generation of such products by pulmonary microsomes could provide valuable insight into the role of lipid peroxidation in the *in vivo* toxicity of these chemicals.

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