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INHIBITION BY REACTIVE ALDEHYDES OF SUPEROXIDE ANION RADICAL PRODUCTION FROM STIMULATED POLYMORPHONUCLEAR LEUKOCYTES AND PULMONARY ALVEOLAR MACROPHAGES

EFFECTS ON CELLULAR SULFHYDRYL GROUPS AND NADPH OXIDASE ACTIVITY

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Abstract—Alpha,beta-unsaturated aldehydes such as acrolein (ACR) and crotonaldehyde (CRO) have been shown previously in our laboratory to inhibit the production of superoxide anion radical (O_2^-) by stimulated phagocytic cells *in vitro* in a dose-related manner. Based on the known reactivity of these compounds towards cellular sulfhydryls (SH), the present studies were aimed at investigating cellular SH status in relation to O_2^- production. Plasma membrane surface SH groups were measured using carboxypyridinedisulfide and monitoring the resultant formation of mixed disulfides through assay of thione released into the supernatant fraction. Intracellular non-protein sulfhydryls were measured using 5,5′-dithiobis-2-nitrobenzoic acid. In both human polymorphonuclear leukocytes (PMN) and rat pulmonary alveolar macrophages (PAM) there was a dose-related decrease in surface SH and soluble SH after ACR and CRO treatment. Propionaldehyde, a three-carbon saturated aldehyde, was without effect. The decrease in surface SH was greater than the decrease in soluble SH. In addition, in PMN and PAM preincubated with 5–40 μM ACR, there was a dose-related inhibition in the rate of O_2^- production with no effect on the lag time as measured by cytochrome *c* reduction. In stimulated PMN, there was a dose-related decrease in the rate after addition of 5–40 μM ACR. These data suggest that changes in SH status by reactive aldehydes can modulate the activity of the plasma membrane NADPH oxidase responsible for O_2^- production.

Central to the antimicrobial function of phagocytic cells is the ability to produce reactive oxygen species. These are generated during an oxygen burst which occurs as a result of a complex series of events initiated by interaction of the plasma membrane with soluble membrane-active agents or with insoluble particulates [1]. These initial events lead within a minute to the activation of a plasma membrane NADPH-dependent oxidase and include such phenomena as calcium release from the plasma membrane [2] followed by changes in membrane potential [3]. Elucidation of this pathway has, to a large extent, resulted from the observation that individuals with the rare inherited disorder chronic granulomatous disease (CGD), who have recurrent infections leading to death at an early age, are unable to produce a burst of oxygen consumption or the resulting superoxide anion radical and other reactive species that play a role in the bacteriocidal process [4].

In this laboratory we have been evaluating whether the mechanism by which exposure to a variety of air pollutants leads to a heightened susceptibility to pulmonary infection might be related to a CGD-like impairment in the bacteriocidal capabilities of phagocytic cells produced by these pollutants. In our previous studies we have shown that pulmonary alveolar macrophages obtained from rats exposed to ozone or nitrogen dioxide, both of which are known to potentiate pulmonary bacterial infections in laboratory animals, have a loss in their ability to produce superoxide anion radical upon stimulation [5]. More recently, we demonstrated that a series of reactive aldehydes are able to inhibit superoxide anion radical production in stimulated human neutrophils [6]. Such aldehydes are themselves components of indoor and outdoor air pollution mixtures and, at least in the case of acrolein, have been shown to potentiate bacterial infections in laboratory animals [7]. Furthermore, a variety of reactive aldehydes are formed as a result of lipid peroxidation [8], a known concomitant of both ozone [9] and nitrogen dioxide [10] exposure. Theoretically, a reaction of ozone with glycoproteins in mucus could also produce the type of short-chain carbonyl compounds able to inhibit superoxide anion radical production in stimulated human neutrophils.

In the present study we have extended our previous work in human neutrophils to evaluate whether rat pulmonary alveolar macrophages (PAM) are also susceptible to inhibition of superoxide anion radical production by reactive aldehydes. We then explored the possibility that such aldehydes produce their adverse effects on pulmonary alveolar macrophage

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and neutrophil membrane functions by interacting with membrane sulfhydryl groups. Previous studies have clearly demonstrated the ability of such aldehydes to alkylate protein sulfhydryl groups [11]. Furthermore, a potential role for sulfhydryls in the maintenance of normal phagocytic membrane interaction with components known to stimulate the oxidative burst has been suggested previously [12].

METHODS

Isolation of alveolar macrophages from rats. Female Sprague–Dawley rats (250–350 g) were anesthetized with 60 mg/kg sodium pentobarbital injected intraperitoneally. A tracheal cannula was inserted through an incision in the neck and 8 ml of phosphate-buffered saline (PBS), pH 7.4, was carefully instilled into the lung via a syringe attached to the cannula. This procedure generally was repeated four times. Six rats were used for each preparation. The lung lavage fluid was centrifuged at 400 *g* for 10 min. The cell pellets were resuspended in balanced salt solution (BSS: 128 mM NaCl, 12 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM glucose, 4 mM phosphate buffer, pH 7.4), placed on a Ficoll–Paque (Pharmacia) gradient, and centrifuged at 400 *g* for 45 min at 4°. Macrophages, which sediment at the interphase of the gradient, were removed, pooled, and washed once with BSS. The macrophage pellet was then resuspended in BSS, and the cells were counted on a hemocytometer and diluted in BSS to a final concentration of 1×10^7 cells/ml.

Lag time and rate of superoxide anion radical production in PAM. Lag time and rate of superoxide anion radical (O_2^-) production in alveolar macrophages were measured in a continuous system by monitoring the reduction of ferricytochrome *c* over time. Cells, 0.7×10^6/ml, and 0.57 mg/ml cytochrome *c* were equilibrated to 37° in a microcuvette followed by stimulation with the soluble oxygen burst initiator phorbol myristate acetate (PMA, 142 ng/ml). The reference cuvette contained all components of the sample cuvette as well as superoxide dismutase. Optical density at 550 nm was recorded continuously at a constant temperature of 37°. This technique allows for the determination of the lag time as well as the rate of superoxide anion radical production. Lag time was measured from the time of addition of PMA to the time at which extrapolation of the linear component of the rate intercepts the baseline, and the rate of O_2^- production was calculated from the linear increase in optical density at 550 nm as described by Newburger *et al.* [12].

Effects of aldehydes on the lag time and rate of superoxide production. Polymorphonuclear leukocytes or alveolar macrophages (0.7×10^6/ml) were preincubated with aldehydes (10^{-4}–10^{-6} M) for 10 min at 37° in a microcuvette. Ferricytochrome *c* was added, and the temperature was allowed to equilibrate to 37°, followed by stimulation with PMA. To observe the effects of aldehydes on the rate of O_2^- production, the cells were equilibrated to 37° in a microcuvette, and cytochrome *c* was added followed by stimulation with 142 ng/ml PMA. After 3–4 min, when linearity of O_2^- production had been established, aldehydes were added to the cuvette in various concentrations and the reaction was allowed to continue.

Inhibition of superoxide production in PAM by aldehydes. Superoxide anion radical production was measured as the superoxide dismutase inhibitable reduction of ferricytochrome *c* as described previously [6]. Alveolar macrophages (1.7×10^6/ml) were preincubated with the aldehydes (10^{-3}–10^{-7} M) for 30 min at 37°. PMA (71 ng/ml) was then added, and the cells were incubated for an additional 30 min for the measurement of O_2^-. As in our previous studies [6], removal of the supernatant fraction and thus any unreacted aldehyde, followed by resuspension of the cells and stimulation, did not affect the amount of O_2^- produced. From the observed inhibition of superoxide anion radical production, concentrations of aldehydes which caused a 50% inhibition in O_2^- production were calculated.

Determination of sulfhydryl groups on the surface of polymorphonuclear leukocytes and alveolar macrophages. Polymorphonuclear leukocytes (PMN) were isolated from the blood of healthy human volunteers as described previously [6]. PMN, 5×10^6 cells, and PAM, 2.5×10^6 cells, each in a volume of 3.0 ml, were treated at 25° with 10^{-3} M carboxypyridinedisulfide (CPDS, Sigma) in PBS, pH 7.2, for 10 and 20 min respectively. Reaction between cell surface sulfhydryl (SH) groups and CPDS results in the formation of mixed disulfides and the release of thione into the supernatant fraction [13, 14]. The samples were then centrifuged at 400 *g* for 5 min, and the absorbance of the supernatant fraction was measured spectrophotometrically at 344 nm using 10^{-3} CPDS in the reference cuvette. One mole of thione released corresponds to one mole of cell surface sulfhydryl groups reacted with CPDS. The number of SH groups per 10^6 cells was calculated using 1×10^4 M^{-1} as the extinction coefficient of the thione [14]. The effect of aldehydes on cell surface sulfhydryl groups was examined by preincubating cells with 10^{-3}–10^{-6} M aldehydes for 30 min at 37°. The cells were then centrifuged at 4° at 400 *g* for 5 min to remove any unbound aldehyde prior to incubation with CPDS.

Determination of soluble sulfhydryl groups in PMN and PAM. Soluble intracellular sulfhydryl groups were determined by the method of Jocelyn [15]. PMN, 5×10^6 cells in a final volume of 2.5 ml, or PAM, 2.5×10^6 cells in a final volume of 1.25 ml, was incubated with or without aldehyde for 30 min at 37° and centrifuged to remove unreacted aldehyde. The cells were then treated with CPDS and the supernatant fraction was examined as described above to determine cell surface sulfhydryl groups. After CPDS treatment, the cell pellets were washed once with 3.0 ml PBS and resuspended in 2.0 ml of deionized water for 20 min at room temperature [16], followed by the addition of 1.0 ml of 0.2 M phosphate buffer, pH 6.8. The samples were centrifuged at 800 *g* for 5 min at 4° to remove cell debris. An aliquot (2.8 ml) of the supernatant fraction was transferred to a cuvette, and 0.2 ml of 10 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Calbiochem) was added. The absorbance at 412 nm was recorded 2.0 min after the addition of DTNB. The concentration of SH

groups was calculated using the molar extinction coefficient at 412 nm of 13,600 [17]. In all experiments, cell viability after aldehyde incubations was measured by Trypan blue staining and was found not to be affected.

RESULTS

Production of O_2^- in rat alveolar macrophages stimulated by PMA was preceded by a lag time during which no superoxide was produced, followed by a linear phase of superoxide generation. Figure 1 shows that, as the concentration of PMA was increased, the lag time shortened and the rate of O_2^- production increased, up to a PMA concentration of 214 ng/ml. Figure 2A shows the effect of pretreatment with acrolein on the lag time of activation and the rate of superoxide production in human polymorphonuclear leukocytes. Concentrations of 5 and 20 μM acrolein affected the lag time very little; however, there was a dose-related decrease in the rate of superoxide production of 20 and 70% respectively. After incubation with 100 μM acrolein, there was no measurable superoxide production. A similar decrease in the rate of superoxide anion radical production, with no change in the lag time, was also observed in rat pulmonary alveolar macrophages incubated with acrolein (Fig. 2B).

To examine the effects only on the rate of O_2^- production, acrolein was added to cells 3 min after stimulation with PMA. Figure 3 shows the dose–response relationship between acrolein concentration and decrease in the rate of superoxide production. Immediately after addition of 100 μM acrolein, there was a marked decrease in rate, followed by a flattening of the curve, indicating that no further production of O_2^- was occurring. Acrolein concentrations of 20 and 1 μM decreased the rate of activity to a lesser extent in a concentration-dependent manner.

As shown in Table 1, inhibition of O_2^- production in macrophages by acrolein, *trans,trans*-muconaldehyde, *trans*-4-hydroxynonenal and crotonaldehyde was dose-related within a narrow, 10-fold range of concentrations. In PAM, the relative order of inhibition and the IC_{50} values of the reactive aldehydes were similar to those we have reported previously for PMN [6]. The order of inhibitory activity was acrolein > *trans,trans*-muconaldehyde > *trans*-4-hydroxynonenal > crotonaldehyde with the latter compound being about ten times less potent than acrolein. In contradistinction, propionaldehyde, a saturated three-carbon aldehyde, had no effect on O_2^- production in PAM following a 30-min incubation. Values for nmoles O_2^- produced in 15 min following propionaldehyde incubation were: control, 36.4; 1 μM, 33.5; 10 μM, 34.8; and 100 μM, 35.3 nmoles O_2^-/15 min.

To examine a possible target site for aldehydes in phagocytic cells which may be involved in inhibition of the membrane oxidase that reduces O_2 to O_2^-, cell sulfhydryl groups were studied. Table 2 shows the dose-dependent decrease in available membrane sulfhydryls in PMN and PAM treated with aldehydes. Incubation of PMN with 100 μM acrolein resulted in a 53% decrease in plasma membrane surface sulfhydryl groups, whereas the same concentration of crotonaldehyde resulted in a decrease of 28%. In PAM, 100 μM acrolein decreased plasma membrane sulfhydryls by 69%, and 100 μM crotonaldehyde by 37%.

Intracellular soluble protein sulfhydryl groups (Table 3) were also decreased in a concentration-related manner after cells were incubated with reactive aldehydes. Acrolein (100 μM) and crotonaldehyde (100 μM) decreased soluble sulfhydryls in

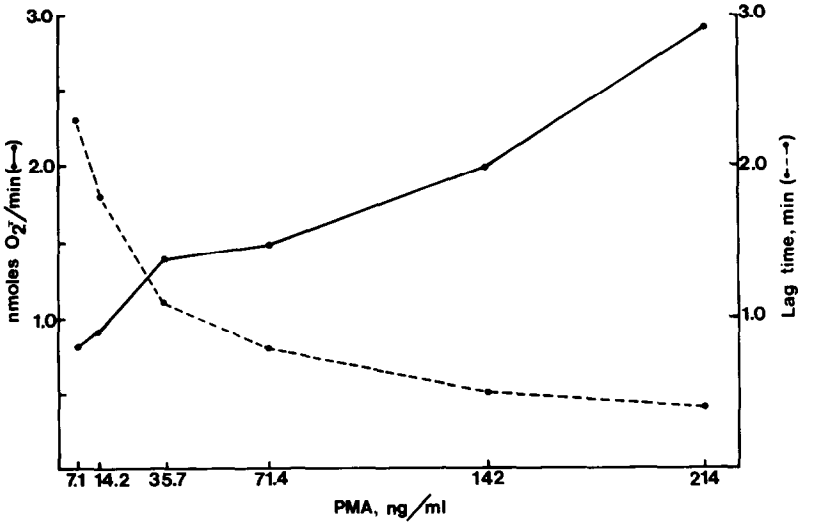

Fig. 1. Lag time and rate of O_2^- production in PAM stimulated with PMA. Cells and ferricytochrome *c* were equilibrated to 37° in a microcuvette, followed by stimulation with PMA. The increase in absorbance at 550 nm was recorded at a constant temperature of 37°. A reference cuvette contained all components of the sample cuvette in addition to superoxide dismutase. Lag time and rate were measured from the resultant curves and plotted as a function of PMA concentration.

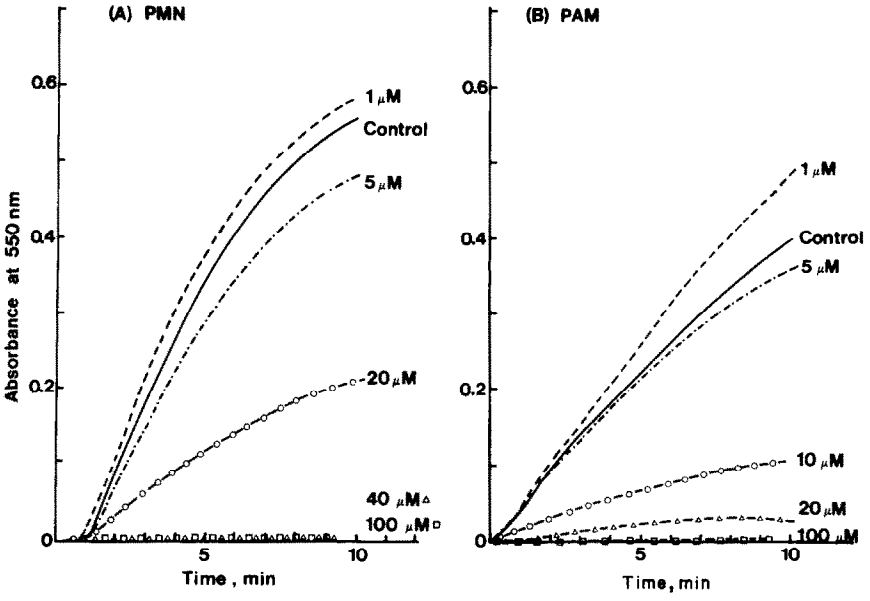

Fig. 2. Effect of acrolein on lag time and rate of O_2^- production in phagocytic cells. (A) PMN were preincubated with acrolein for 10 min at 37°, and O_2^- production was measured as described in Methods. (B) PAM, same conditions.

PMN by 41 and 9% respectively. Macrophages under the same conditions had a decrease in soluble sulfhydryl groups of 61 and 12% respectively.

DISCUSSION

In previous studies we found that reactive aldehydes which have an alpha,beta-unsaturated functional group inhibit the release of O_2^- in stimulated human neutrophils [6]. The present work indicates that a similar inhibition of O_2^- production was caused by reactive aldehydes in stimulated rat pulmonary alveolar macrophages with IC_{50} values in the range observed for PMN. In addition, the IC_{50} values reported are within the range of concentrations that might be encountered when aldehydes are inhaled. This is based on the report [7] that one 40-ml puff of cigarette smoke contains as high as 90 ppm acrolein.

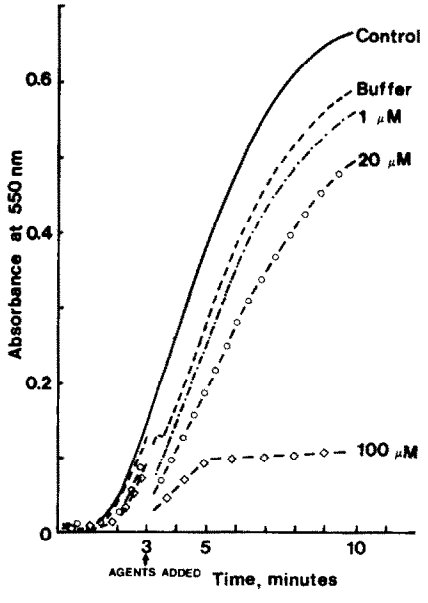

Fig. 3. Effect of acrolein on rate of O_2^- production in PMN. Acrolein was added to PMA-stimulated PMN after a linear rate had been established, and the reaction was allowed to continue.

Of the aldehydes examined, crotonaldehyde was the least effective in inhibiting O_2^- production *in vitro* in PMA-stimulated PAM and acrolein the most effective. The effectiveness of inhibition of O_2^- production correlated with the degree of electrophilicity at the beta carbon of the aldehyde, which is directly related to the alkylating potential of these compounds. The toxicity of alpha,beta-unsaturated aldehydes has also been directly related to the electrophilicity of the beta carbon [18]. Thus, the reactivity series observed in the present and previous studies [6] suggests that alkylating potential plays a role in the inhibition of O_2^- production by reactive aldehydes in human PMN and rat PAM.

It has been noted that alpha,beta-unsaturated aldehydes may mediate their effects in biological systems by alkylating cellular sulfhydryls [11]. Thus, the carcinostatic activity of compounds like *trans*-4-hydroxynonenal, acrolein and related aldehydes has been ascribed to their binding to essential cellular sulfhydryls, a process leading to inhibition of DNA, RNA and protein synthesis [11]. *In vitro*, reactive aldehydes form mono-adducts with cellular thiols such as glutathione and cysteine by addition across the reactive double bond of the aldehyde. Di-adducts containing two moles of thiol can also be formed through alkylation of the aldehydic functional group

Table 1. Inhibition of superoxide anion radical (O_2^-) production in rat alveolar macrophages by reactive aldehydes

Concentration (μM)	O_2^- production (% of control)* Acrolein	Muconaldehyde	4-Hydroxynonenal	Crotonaldehyde
1	96	100	99	ND†
10	95	113	109	ND
20	48	84	115	ND
50	14	20	86	ND
70	5	21	56	ND
100	2	23	28	100
200	ND	ND	ND	66
500	ND	ND	ND	2
700	ND	ND	ND	7
			IC_{50}‡ (μM)	
	24	31	77	240

Cells, 1.7×10^6/ml, were preincubated with aldehydes for 30 min at 37° followed by stimulation with 71 ng/ml PMA for 30 min at 37°. Each value is the mean of two to six experiments.

* The mean ± SD for O_2^- produced by control cells was 26.7 ± 5.2 nmoles/30 min.

† Not determined.

‡ Concentrations of reactive aldehydes at which 50% inhibition of superoxide anion radical production was observed. Values were calculated by linear regression.

Table 2. Decrease in plasma membrane surface sulfhydryl groups in phagocytic cells treated with alpha,beta-unsaturated aldehydes

Aldehyde	Concentration (μM)	% Decrease in surface SH*† PMN	PAM
Acrolein	1	27 (2)	9 (3)
	10	42 (3)	31 (3)
	100	53 (3)	69 (3)
	1000	54 (2)	
Crotonaldehyde	1	1 (2)	5 (3)
	10	−4 (3)	12 (3)
	100	28 (3)	37 (3)
	1000	46 (3)	
Propionaldehyde	1	−6 (3)	−7 (3)
	10	−7 (3)	−5 (3)
	100	−8 (3)	2 (4)

* Control values for surface SH ranged from 2.0 to 6.5 nmoles/10^6 cells in PMN and 3.6 to 9.9 nmoles/10^6 cells in PAM.

† The percent decrease is the average of two to four experiments; the number of experiments is in parentheses.

Table 3. Decrease in soluble intracellular sulfhydryl groups in phagocytic cells treated with alpha,beta-unsaturated aldehydes

Aldehyde	Concentration (μM)	% Decrease in soluble SH*† PMN	PAM
Acrolein	1	23 (2)	3 (2)
	10	28 (3)	31 (2)
	100	41 (3)	61 (2)
	1000	45 (2)	
Crotonaldehyde	1	−8 (2)	4 (3)
	10	1 (3)	8 (3)
	100	9 (3)	12 (3)
	1000	12 (3)	
Propionaldehyde	1	−5 (3)	5 (3)
	10	5 (3)	4 (3)
	100	−3 (3)	7 (3)

* Control values for soluble SH ranged from 2.0 to 6.2 nmoles/10^6 cells in PMN and 4.4 to 9.8 nmoles/10^6 cells in PAM.

† The percent decrease is the average of two to three experiments; the number of experiments is in parentheses.

as well as the alpha,beta double bond [19]. The present studies show that inhibition of O_2^- production in PAM and PMN was associated with decreases in intracellular soluble SH. Since the oxidase which metabolizes oxygen to O_2^- is located in the plasma membrane of PAM [20, 21] and PMN [22], it is conceivable that a membrane SH alkylation process by these reactive aldehydes is involved in the inhibition. Since these aldehydes are low molecular weight neutral molecules, it is also not surprising that they might cross the plasma membrane and alkylate intracellular soluble SH. Tsan [16] recently reported a decrease in non-protein cellular SH in neutrophils produced by chloromethyl ketones which correlated with their ability to inhibit O_2^- production in these cells. The present studies, however, demonstrate that there was a much steeper decrease in O_2^- production as a function of aldehyde concentration compared to the decrease in cell surface SH levels. This might reflect a subpopulation of sulfhydryls associated with the oxidase that is particularly sensitive to attack by reactive aldehydes. This subject, as well as the relation of intracellular soluble SH to the activity of the membrane oxidase, requires further study.

The membrane oxidase in PAM and PMN is a cryptic enzyme with a complex mechanism of activation. Once the oxygen burst initiator has come in contact with the phagocytic cell, a lag time is observed followed by a steady production of O_2^-. Newburger *et al.* [12] found that the lag time of the enzyme and its rate respond differently to different chemical and physical influences, suggesting that activation and activity are separable processes. Thus, pH changes were found to affect the rate but not the lag time, changes in temperature affected both, and treatment with *N*-ethylmaleimide (NEM), a known SH alkylating compound, was found to inhibit only the rate. Our results show that reactive aldehydes, similarly to NEM, decrease the activity but not the activation of the enzyme as seen by a decrease in the rate of O_2^- production but no significant change in the lag time. Our finding of a decrease in cell membrane SH levels in association with this effect of reactive aldehydes is also in keeping with the Newburger *et al.* observations of an effect of NEM, a known sulfhydryl alkylator.

Although the biochemical effects of ozone in the lung have been studied in some detail [23], the chemical fate of inhaled ozone is largely unknown. Since ozone is a potent oxidant which can produce reactive aldehydes in the lung, the present studies suggest that the functional impairment of PAM observed after ozone exposure could be derived from an interaction with reactive aldehydes formed in the lung as a result of ozone exposure.

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