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Pretreatment with EDU Decreases Rat Lung Cellular Responses to Ozone

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Pretreatment with EDU Decreases Rat Lung Cellular Responses to Ozone. BASSETT, D. J. P.,
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AND KERR, J. S. (1989). Toxicol. Appl. Pharmacol. 100, 32-40. The phenylurea compound
EDU (N-[2-(2-oxo-1-imidazolindinyl)ethyl]-N'-phenylurea) has been shown to protect plants
from the damaging effects of ozone exposure. Models of rat lung injury, based on acute exposure
to 2 ppm ozone for 3 hr and on exposure to 0.85 ppm ozone for 2 days, were used to determine
whether EDU pretreatment of rats protected lungs from oxidant injury. Rats were pretreated
with 100 mg/kg body wt EDU by ip administration for 2 days prior to and on the days of ozone
exposure. No adverse toxicological effects of EDU pretreatment were observed. Lung superoxide
dismutase (SOD) and catalase (CAT) activities were significantly enhanced from 636 to 882 U/
lung and from 599 to 856 U/lung, respectively. One day following acute exposure (2 ppm for 3
hr), an ozone-induced increase of polymorphonuclear leukocytes (PMNs) from 0.01 to 1.18
million cells/lung was decreased to 0.68 million by EDU pretreatment. No alteration occurred
in the degree of lung permeability indicated by increased lavage fluid albumin. EDU pretreat-
ment also significantly decreased ozone-induced increases in PMN recovery after 2 days expo-
sure to 0.85 ppm ozone from 5.54 to 2.12 million cells/lung. However, in this second case,
EDU pretreatment reduced the observed ozone damage, indicated by a decrease in lavage fluid
albumin and by a decrease in themacrophage and lymphocyte infiltration associated with this
length of ozone exposure. The observation that EDU treated cultured pulmonary arterial endo-
thelial cells increased SOD and CAT activities identified a potential lung site of EDU interaction.
These data demonstrated that although EDU pretreatment appears not to prevent initial ozone
damage, it does reduce the infiltration of PMNs and might therefore prevent amplification of
the injury associated with this cell type. © 1989 Academic Press, Inc.
by bronchoalveolar lavage analysis for inflammatory cells and for albumin, previously used to indicate changes in lung permeability (Bassett et al., 1988a,b; Guth et al., 1986; Roth, 1981).

Two separate models of ozone-induced rat lung injury were employed. The first examined the effects of EDU pretreatment on the initial infiltration of polymorphonuclear leukocytes (PMNs) that occurs during the first 24 hr following a single acute exposure to 2 ppm ozone for 3 hr (Bassett et al., 1988a). This increase in lavageable PMNs has previously been shown to be proportional to the length of ozone exposure and is associated with increased levels of lavage fluid albumin that are maximal at this 24-hr time point (Bassett et al., 1988a). The second model involved studying the effects of EDU pretreatment on lung inflammatory responses over a longer time period of ozone exposure when both macrophages and PMNs are still infiltrating into the lung (Bassett et al., 1988b) and bronchiolar and alveolar epithelia are undergoing proliferative repair (Evans et al., 1976a,b). Rats were exposed continuously for 2 days to 0.85 ppm ozone, by which time maximal increases in lung permeability and in the numbers of lavageable inflammatory cells have occurred (Bassett et al., 1988b).

It was expected that if EDU pretreatment had provided protection to the lung against the ozone-induced damage associated with these two exposure protocols, the albumin and the numbers of PMNs recovered in bronchoalveolar lavage fluid would be diminished. This study demonstrated that increased lung levels of SOD and CAT resulting from EDU pretreatment could be correlated in both models of ozone exposure with a decrease in the numbers of PMNs recovered by bronchoalveolar lavage.

METHODS

**Animals.** Male Sprague-Dawley virus-free rats (Hilltop, Scottsdale, PA) weighing 200–250 g were kept in high efficiency particulate-filtered (HEPA) air on a normal diet (RMH-1000, Agway, Syracuse, NY) for 5 to 10 days before exposure. Blood serum samples were taken routinely from at least two rats 2 weeks following the end of each experiment to ensure that the animals had been maintained free from Sendai virus, Kilham rat virus, rodent corona viruses, and mycoplasmal infections (Microbiological Associates, Rockville, MD). Rats were exposed to either air or ozone in separate 700-liter laminar-flow inhalation chambers (Baker, Sanborn, ME) that were equipped with a charcoal- and HEPA-filtered air supply. The animals remained in the chambers in separate stainless-steel wire cages with free access to food and water during and following exposures. The air flow to the chambers was adjusted to give at least 20 changes per hour, a relative humidity of 50–65%, and a temperature of 20–25°C. Rats were injected twice a day with EDU or carrier (control) (100 mg/kg body wt, ip) for 2 days prior to air and ozone exposures, twice on the day of 3 hr exposures to 2 ppm ozone, and once on each of the 2 days of continuous exposure to 0.85 ppm ozone. EDU injections were prepared by first dissolving 250 mg in 0.5 ml di-methyl sulfoxide (DMSO) with the subsequent addition of 2 ml methyl cellulose (0.25%).

**Ozone generation and analysis.** Ozone-containing air was introduced into the chamber at a rate of 2 liters/min from an ultraviolet light ozone generator (Orec Corp., Phoenix, AZ). Chamber ozone concentrations were monitored at different positions within the chamber at regular intervals by an ozone uv photometer (Model 1003AH, Dasibi Inc., Glendale, CA), which was routinely calibrated against a known ozone source (Model 1008 PC, Dasibi Inc.). Two different ozone exposure protocols were employed. In the first series of experiments, rats were exposed to either air or ozone for 3 hr with an additional 30 min allowed for chamber equilibration. Ozone concentrations were calculated from the averages obtained over each 30-min period, giving mean chamber concentrations of 2.07 ± 0.02 and 2.06 ± 0.05 ppm ozone (±SEM, n = 7) for two separate exposures. In the second series of exposures when rats were exposed continuously for 48 hr, ozone concentrations were averaged every 2.5 hr, giving a mean ozone concentration of 0.85 ± 0.02 ppm (±SEM, n = 20) with values that ranged from 0.78 to 0.94 ppm. Chamber analyses for nitrogen dioxide, using methods based on the Griess–Saltzmann reaction (Saltzman et al., 1984), demonstrated levels that were not significantly different than ambient concentrations of less than 0.015 ppm.

**Bronchoalveolar lavage.** Tracheas of anesthetized rats (sodium pentobarbital, 50 mg/kg body wt, ip) were cannulated. The chest cavity was opened and exsanguinated via abdominal arteries. The lungs were then gently lavaged with a single 7.5 ml vol of warm (37°C) phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS). The volume of lavage fluid recovered was recorded and was found not to be affected by the ozone exposures used in this study, giving a mean value of 6.3 ± 0.1 ml (±SEM
Cells and debris were separated by centrifugation and the resulting supernatant was lyophilized and then analyzed for albumin content by standard assay procedure based on its color reaction with bromocresol green (Sigma Chemical Co., St. Louis, MO) that is read at 630 nm (Rodkey, 1965). The recovery of cells was continued by additional lavages of the lung, employing a total volume of 50 ml of PBS containing 3 mM EDTA. Cells were separated from the lavage fluid by centrifugation and were then combined with the pelleted cells from the first lavage. The number of total cells recovered by lavage and the percentage of differential cell counts were then determined by standard procedures (Warr and Jakab, 1983).

**Enzyme analyses.** Analysis for SOD and CAT activities were separately determined on unlavaged lungs that were first perfused free of blood, trimmed of extraparenchymal tissue, weighed, and then frozen at ~80°C prior to analysis. Enzyme assays were determined on postmitochondrial fractions prepared by differential centrifugation (20,000g) of tissue homogenates prepared in phosphate-buffered saline. The protein content of the resulting fraction was determined by the dye-binding assay of Bradford (1976). SOD activity was determined by the method of McCord and Fridovich (1969) based on the inhibition of xanthine oxidase-generated superoxide anion-induced reduction of cytochrome c. One unit of SOD activity is defined as the amount of SOD required to inhibit the maximal rate of cytochrome c reduction by 50%. CAT activity was determined by the method of Beer and Sizer (1952), based on the rate of hydrogen peroxide oxidation monitored spectrophotometrically at 240 nm in a 0.05 M phosphate buffer at pH 7.0. One unit of CAT activity is defined as 1 ymol HzO₂ removed per minute.

**Endothelial cell culture.** Bovine pulmonary arterial endothelial cells (ATCC CCL-207) were obtained from the American Type Culture Collection (Bethesda, MD). Cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 2 mM glutamine and 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY), in a humidified chamber maintained in 5% CO₂ in air at 37°C. On reaching confluence, the cells were incubated for 24 hr with increasing concentrations of EDU (0, 0.25, 0.5, and 1 mM) or with fresh medium only (control). EDU was dissolved in DMSO and diluted to 100 mM with distilled water, with further dilution to required concentrations with Dulbecco’s medium without added fetal bovine serum. The final DMSO concentration in culture was <0.1%. Following incubations, cells were rinsed twice with PBS prior to removal from the culture flasks with 0.25% trypsin treatment. Cells were rewash with PBS, counted, and then disrupted by pulse sonication (Branson Sonifier, Model 350) as previously described (Stevens et al., 1988). The resulting sonicate was then centrifuged for 10 min at 100,000g (Model TL100 mini-ultracentrifuge, Beckman Instruments, Palo Alto, CA) prior to analysis for protein and SOD and CAT activities.

**Statistical analysis.** Data were evaluated by two-way analyses of variance and were compared by Duncan’s new multiple range test using a level of significance of \( P < 0.05 \) (Dowdy and Wearden, 1983). Logarithmic transformations of the data were carried out in those cases where variances were found not to be homogeneous according to Hartley’s test (Dowdy and Wearden, 1983).

**RESULTS**

**Anti-oxidant Enzyme Activities**

The effect of EDU pretreatment on pulmonary artery endothelial cell anti-oxidant enzyme activities was demonstrated by incubating confluent cell cultures for 24 hr with increasing concentrations of EDU (0, 0.25, 0.5, and 1 mM) (Fig. 1). Significant increases in cell SOD activity were observed, giving a value that was nearly threefold greater than the control at the highest EDU concentration studied (1 mM) (Fig. 1A). EDU incubation also increased endothelial cell CAT activity, but a statistical significant increase was only observed with 1 mM EDU (Fig. 1B). It should be noted that glutathione peroxidase activity was also measured in these experiments and was found not to be significantly altered from a control value of 28.4 ± 4.5 U/mg protein (±SEM, \( n = 6 \)) by EDU incubation.

Two days of EDU pretreatment of rats significantly increased total lung SOD activity by 39% from 636 ± 53 to 882 ± 68 U/lung (±SEM, \( n = 4 \)) (\( P < 0.05 \)). Lung tissue CAT activity was also enhanced by EDU pretreatment from 599 ± 42 to 856 ± 54 U/lung.

**Changes in Animal Weights following Ozone Exposure**

The pretreatment of rats with EDU for 2 days prior to ozone and air exposure had no significant effect on body weights. The initial body weights for the two different exposure protocols were 254 ± 2 g (±SEM, \( n = 48 \)) for acute exposures to 2 ppm ozone for 3 hr and 283 ± 2 g (±SEM, \( n = 14 \)) for continuous exposure to 0.85 ppm ozone for 2 days. De-
increases in body weights of $-13 \pm 2$ and $-12 \pm 2 \text{ g (±SEM, n = 12)}$ were observed 24 hr following exposure to 2 ppm ozone in control and EDU-pretreated rats, respectively. No significant changes of body weight were observed in air-exposed rats from either control or EDU-pretreated groups. EDU pretreatment also did not alter the decreases in body weight of $-38 \pm 6$ and $-33 \pm 6 \text{ g (±SEM, n = 4)}$, respectively, observed in control and EDU-pretreated animals as a result of 2 days of exposure to 0.85 ppm ozone. Air-exposed animals gained an average of $5 \pm 2 \text{ g (±SEM, n = 8)}$ during the same period of time. These losses of body weight resulting from ozone exposure have previously been linked to decreases in food and water intake (Bassett et al., 1988b).

**Twenty-four Hours Following Exposure to 2 ppm Ozone**

EDU pretreatment alone did not significantly alter the measurements of unlavaged lung tissue wet weights that were made immediately following perfusion to remove blood components from the vasculature. However, at 24 hr following acute exposure to 2 ppm ozone for 3 hr, lung wet weights were increased from $1.10 \pm 1.27 \pm 0.03 \text{ g for control and from 1.07 \pm 0.05 g to 1.24 \pm 0.06 g (±SEM, n = 4)}$ for EDU-pretreated rats. A separate study demonstrated that EDU pretreatment had no effect on control unlavaged lung tissue dry to wet weight ratios of $0.173 \pm 0.001$ and $0.174 \pm 0.003$ for air- and ozone-exposed lungs, respectively.

Bronchoalveolar lavage analysis of air-exposed rats demonstrated that EDU pretreatment did not result in any observable lung damage, as indicated by the unaltered lavage fluid albumin content (Table 1). Ozone-induced lung damage was indicated by a greater than fourfold increase in lavage fluid albumin concentration in control lungs 24 hr after an exposure of 2 ppm for 3 hr. A similar increase in the lavage fluid albumin content was observed in EDU-pretreated rats after ozone exposure (Table 1), suggesting that EDU did not protect the lung from the increased permeability associated with this degree of ozone-induced lung damage.

In this series of experiments, although the
total numbers of inflammatory cells recovered by bronchoalveolar lavage were not changed by either EDU pretreatment or by subsequent ozone exposure (Table 1), the relative numbers of the different types of recovered cell were significantly altered (Fig. 2). Bronchoalveolar lavage of unexposed control rats yielded 3.3 million cells (Table 1) that were identified as being 96% macrophages (Fig. 2A), less than 1% PMNs (Fig. 2B), and 3% lymphocytes (Fig. 2C). Twenty-four hours following acute ozone exposure, lavages from untreated control rats were associated with a 35% decrease in the numbers of recovered macrophages (Fig. 2A) and with the appearance of PMNs that represented 36% of the total number of recovered cells (Table 1, Fig. 2B). Ozone exposure also increased the number of lymphocytes recovered in the lavage fluid by 140% (Fig. 2C).

No significant differences were observed in the relative numbers of recovered inflammatory cells in air-exposed lungs as a result of EDU pretreatment. On the other hand, a significant decrease of 42% in PMN recovery was observed on lavaging the ozone-exposed lungs of EDU-pretreated rats. The observed ozone-induced increase in lavage fluid lym-

### TABLE 1

**Lavage Fluid Analysis 24 hr Following 3-hr Exposure to 2 ppm Ozone**

|                    | Control          | EDU pretreated  |
|--------------------|------------------|-----------------|
|                    | Air  | Ozone| Air    | Ozone|
| Albumin (µg/ml lavage fluid) | 90 ± 7 | 378 ± 56* | 88 ± 4 | 406 ± 20* |
| Total recovered cells (millions of cells) | 3.3 ± 0.3 | 3.4 ± 0.4 | 3.3 ± 0.3 | 3.3 ± 0.5 |

*Note: Results represent the mean ± SEM with n = 4 for albumin analyses and n = 8 for measurements of total recovered cells.

*Significantly different on comparison of ozone-exposed animals with corresponding air-exposed values with \( P < 0.05 \). No significant effects of EDU pretreatment were observed.

**Fig. 2.** The number of (A) macrophages, (B) polymorphonuclear leukocytes (PMNs), and (C) lymphocytes recovered by bronchoalveolar lavage of rat lungs following pretreatment with either carrier (control) or EDU, 1 day following 3 hr of exposure to either air or 2 ppm ozone. Results represent the means as millions of cells per lung ± SEM for eight rats exposed under each condition. *Significantly different on comparison between air and ozone-exposed lungs with \( P < 0.05 \). †Significantly different than untreated control rat lungs.
TABLE 2

LAVAGE FLUID ANALYSIS FOLLOWING 2 DAYS EXPOSURE TO 0.85 ppm OZONE

|                         | Control       | EDU pretreated |
|-------------------------|---------------|----------------|
|                         | Air           | Ozone          | Air           | Ozone          |
| Albumin (μg/ml lavage fluid) | 106 ± 21     | 1854 ± 340<sup>a</sup> | 112 ± 24     | 873 ± 119<sup>b</sup> |
| Total recovered cells (millions of cells) | 3.0 ± 0.5   | 12.7 ± 1.5<sup>a</sup> | 4.9 ± 0.4   | 5.5 ± 0.8<sup>b</sup> |

Note: Results represent the mean ± SEM with n = 4 for albumin analyses and n = 8 for measurements of total recovered cells.

<sup>a</sup> Significantly different on comparison of ozone-exposed animals with corresponding air-exposed values with P < 0.05.

<sup>b</sup> Significantly different on comparison of EDU-pretreated animals with corresponding untreated control values.

Two days Continuous Exposure to 0.85 ppm Ozone

In a second series of experiments, bronchoalveolar lavage analyses for albumin content and for inflammatory cells were conducted on control and EDU-pretreated rats following 2 days of continuous exposure to either air or 0.85 ppm ozone. A greater degree of lung damage was achieved with this ozone exposure protocol as indicated by a 17.5-fold increase in lavage fluid albumin recovered from the lungs of ozone-exposed control rats (Table 2). In contrast to the acute ozone studies, EDU pretreatment reduced the ozone-induced increase in lavage fluid albumin by 56% (Table 2). These data indicate that EDU pretreatment provided some protection against the greater degree of lung damage associated with this longer exposure to ozone.

Two days exposure of control rats to ozone in these experiments resulted in a significant increase in the number of total cells recovered by bronchoalveolar lavage (Table 2). This greater than threefold enhancement was accounted for by increases in all cell types recovered. Macrophage numbers were significantly elevated by 217% (Fig. 3). The appearance of 5.5 million PMNs in ozone-exposed lungs accounted for 44% of the total cells recovered, which compared with a value of less than 2% for air-exposed control rat lungs (Fig. 3B). Lymphocytes were also elevated by 450% following ozone exposure (Fig. 3C). Although EDU pretreatment increased the total number of lavageable cells recovered as macrophages from unexposed lungs (Table 2, Fig. 3A), statistical analysis demonstrated this increase not to be significant (P > 0.05). On the other hand, EDU pretreatment did significantly prevent the increase in total numbers of cells associated with the ozone exposure (Table 2). EDU decreased the number of PMNs recovered from ozone-exposed lungs by 62% and prevented the ozone-induced increases in lavaged macrophages and lymphocytes (Fig. 3).

DISCUSSION

No adverse toxicological effects on whole lung tissue were observed as a result of the EDU pretreatment. No alterations were observed in the lavage fluid inflammatory cell.
and albumin contents. EDU pretreatment did consistently reduce the appearance of polymorphonuclear leukocytes in the bronchoalveolar lavage fluid recovered from lungs following both acute and continuous exposures to ozone. It should be noted that in the case of 2 days of continuous exposure to 0.85 ppm ozone, EDU pretreatment also prevented the ozone-induced increase in lavageable lymphocytes and macrophages.

The observed increases in lung SOD and CAT activities were similar to those previously reported by Stevens et al. (1988). Since EDU pretreatment alone did not result in any alterations in lavaged cell recoveries, the observed increases in lung tissue SOD and CAT activity could not be accounted for by infiltration of inflammatory cells. Although the mechanism by which EDU increases these tissue anti-oxidant enzymes is unclear, previous studies using gingival fibroblasts have suggested that EDU increases SOD activity at the level of cytosolic translation (Stevens et al., 1988). One potential site for the increase in these enzyme activities in the whole lung was suggested by the enhancement of cultured endothelial cell SOD and CAT on exposure to EDU (Fig. 1).

One might speculate that the decrease in the ozone-induced infiltration of PMNs following EDU pretreatment might have resulted from alterations in the interaction between the capillary endothelium and circulating PMNs. The involvement of reactive oxygen intermediates in the adherence and subsequent migration of PMNs is suggested by the observation that hyperoxic exposure of cultured endothelial cells (Bowman et al., 1983) and perfused rabbit lungs (Krieger et al., 1985) increase adherence of PMNs. It is therefore possible that increased endothelial SOD and CAT activities decrease both endogenous and inflammatory cell productions of superoxide anion and hydrogen peroxide and so decrease PMN adherence and subsequent infiltration. It should be noted that the numbers of lavageable macrophages are diminished during the initial stages of lung ozone exposure (Fig. 2) (Bassett et al., 1988a, Coffin et al., 1968). These cells, together with epithelial cells, represent possible sources of chemotaxins that initially call in the PMNs (Fox et al., 1981). EDU-induced increases in SOD and CAT activities of these resident lung cells might therefore alter the chemotactic responses that initiate infiltration of the

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**Fig. 3.** The number of (A) macrophages, (B) polymorphonuclear leukocytes (PMNs), and (C) lymphocytes recovered by bronchoalveolar lavage of rat lungs following pretreatment with either carrier (control) or EDU, following 2 days of continuous exposure to either air or 0.85 ppm ozone. Results represent the means as millions of cells per lung ± SEM for four rats exposed to air and three rats exposed to ozone for each treatment condition. *Significantly different on comparison between air and ozone-exposed lungs with P < 0.05. †Significantly different than untreated control rat lungs.
circulating PMNs, macrophages, and lymphocytes.

Although elevation of these lung anti-oxidant enzymes has previously been correlated with increased protection of the lung from the damaging effects of oxidants (Denke and Fanburg, 1980; Frank et al., 1978), EDU pretreatment did not alter the increase in lung permeability following the acute ozone exposure used in this study and observed as changes in lavage fluid albumin (Table 1). SOD and CAT would be expected to protect the lung from oxygen-free radical-mediated damage, but might not prevent the cellular damage resulting from ozonide formation (Rabinowitz and Bassett, 1988), or from the inhibition of such key metabolic enzymes as glyceraldehyde-3-phosphate dehydrogenase (Menzel, 1971), previously associated with acute ozone exposures.

The infiltration of inflammatory cells into oxidant-damaged lungs has previously been linked to the amplification of injury as a result of their potential to generate reactive oxygen intermediates (Fox et al., 1984; Repine, 1985; Shasby et al., 1982). However, in the present acute exposure studies where rats were exposed for 3 hr to 2 ppm ozone, the observed EDU-induced decrease in PMN infiltration could not be correlated with any decrease in lavage fluid albumin. This observed permeability damage could therefore only be attributed to the direct interaction of ozone with lung tissue components and not to any contribution by the infiltrating PMNs. In contrast, the greater degree of permeability damage associated with 2 days of continuous exposure to 0.85 ppm ozone was diminished by EDU pretreatment and could also be correlated with decreases in both macrophage and PMN content of the lungs (Fig. 3, Table 2). This latter observation suggests that these inflammatory cells are contributing to the observed lung damage during the prolonged exposures to ozone. The degree of PMN infiltration associated with the acute ozone exposures might therefore have been insufficient to differentiate between ozone and possible inflammatory cell-mediated damage. On the other hand, inflammatory cells are constantly infiltrating into the lungs of rats during the first 3 days of continuous exposure to 0.85 ppm ozone (Bassett et al., 1988b). Their oxidant-generating capacity might make a considerable contribution to the permeability damage observed after 2 days of ozone exposure, especially if they are maintained in an activated state. EDU pretreatment might therefore reduce the lung permeability damage associated with these prolonged ozone exposures, by decreasing inflammatory cell infiltration, and so diminish the exposure of the lung to reactive oxidant metabolites (Fox et al., 1984; Repine, 1985; Shasby et al., 1982).

It is clear that future experiments are required to more clearly define the relationship between EDU-induced increases in lung SOD and CAT and the observed decrease in oxidant-mediated inflammation. The present study has demonstrated that EDU decreases initial PMN infiltration into oxidant-exposed lungs and prevents the subsequent macrophage and lymphocyte accumulations. EDU therefore represents a useful agent that could potentially prevent the amplification of acute lung injuries associated with infiltration of inflammatory cells and the subsequent development of adult respiratory distress syndrome (Repine, 1985).

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