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INHALATION OF COTTON DUST IS ASSOCIATED WITH INCREASES IN NITRIC OXIDE PRODUCTION BY RAT BRONCHOALVEOLAR LAVAGE CELLS

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The inhalation of cotton or other organic dusts can cause alterations in pulmonary function, and these pulmonary effects appear, in part, due to endotoxin contamination of the dusts. Since endotoxin is a potent stimulus for the induction of nitric oxide (NO) synthesis, we examined whether the inhalation of cotton dust might also be associated with increases in NO production. Rats were exposed to normal air, cotton dust aerosol (40.6 ± 3.7 mg/m^3), or a nebulized aerosol of endotoxin (2.2 × 10^4 EU/m^3) for 3 h, and responses were studied 18 h postexposure. Increases in inducible NO synthase (iNOS) production by bronchoalveolar lavage cells (BALC) from rats occurred following exposure to cotton dust or endotoxin as evidenced by increases in iNOS mRNA levels and in vitro nitrate and nitrite production. However, a contribution of NO to oxidant species generation by BALC, as indexed by luminol-dependent chemiluminescence, was observed only in endotoxin-exposed rats. These results indicated that while the inhalation of either cotton dust or endotoxin may be associated with a number of similar responses, the pulmonary consequences can be somewhat different.

Acute exposure to cotton or other organic dusts can result in organic dust toxic syndrome (ODTS), which is characterized by alterations in pulmonary function. Specifically, bronchoconstriction can occur in exposed individuals with attendant increases in airway resistance and decreases in forced expiratory volume (Chan-Yeung et al., 1992). ODTS is also associated with an inflammatory reaction as evidenced by increases in the number of polymorphonuclear leukocytes in bronchoalveolar lavage samples from exposed individuals (Lecours et al., 1986; Von Essen et al., 1990). Recently, inhalation exposures of animal models to cotton, silage, hay, or compost dust have been found to mimic ODTS and, generally, to be predictive of human responses to these potential irritants (Castranova et al., 1996).

In humans, a significant correlation exists between the decline in forced expiratory volume following cotton dust exposure and the endo-

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toxin level of the dust (Castellan et al., 1987). This relationship also exists in terms of the pulmonary effects of cotton dust in animal models (Karol et al., 1985). These findings suggest that endotoxin contamination is an important determinant of the response to cotton dust exposure. This concept is further supported by observations that inhalation exposure to cotton dust or endotoxin results in a number of common events that include acute pulmonary inflammatory responses and a similar pattern of the release of mediators, such as tumor necrosis factor alpha (TNFα) and platelet-activating factor (Beijer & Rylander, 1985; Rochemonteix-Galve et al., 1991; Ryan & Karol, 1991; Rylander & Beijer, 1987).

Recently, endotoxin has been shown to be a potent stimulus for the induction of nitric oxide (NO) synthesis in a number of organ systems, including the lung (Nussler & Billiar, 1993; Wizemann et al., 1994). NO is a free radical that may combine with superoxide anion to produce peroxynitrite (Beckman et al., 1990). This latter, more toxic, oxidant has been implicated as playing a substantial role in the pathophysiologic responses to endotoxin exposure (Wizemann et al., 1994). The purpose of the present study was to investigate whether the inhalation of cotton dust might also result in an induction of NO synthesis and to evaluate the contribution of NO to oxidant generation following cotton dust exposure.

METHOD

Animals and Exposures

Specific pathogen-free male Sprague-Dawley rats (225–305 g body weight; Hilltop, Scottdale, PA) were used in experiments. The animals were exposed to normal air, cotton dust aerosol (40.6 ± 3.7 mg/m^3; Frazer et al., 1991), or aerosolized endotoxin (2.2×10^4 EU/m^3 generated from a stock solution of endotoxin, 100 μg/ml; Frazer et al., 1992) for 3 h, and responses were studied 18 h postexposure. In the exposure system used, the cotton dust aerosol leaving the acoustical generator passed through a static charge neutralizer (TSI model 3054), then through a 25-L cylindrical settling chamber. As the cotton dust passed through this system, the larger nonrespirable cotton dust particles were removed by gravitational forces. The count median diameter of the cotton dust aerosol was 1.5 μm (Frazer et al., 1987). A count median diameter of 1.0 μm was obtained for the endotoxin aerosol generated with an ultrasonic nebulizer system (Frazer et al., 1992; Frazer, personal communication). Endotoxin deposited upon filters placed within either the cotton dust or endotoxin exposure chambers was extracted for 6 h with pyrogen-free water, measured using the *Limulus* amebocyte lysate (LAL) assay (BioWhittaker, Walkersville, MD),

and expressed as endotoxin units (EU) per cubic meter (Olenchock et al., 1987).

Collection of Bronchoalveolar Samples

To obtain bronchoalveolar lavage fluid and cells, the rats were first anesthetized with sodium pentobarbital (65 mg, ip; Butler, Columbus, OH) and then exsanguinated by cutting the left renal artery. A tracheal cannula was inserted and an initial bronchoalveolar lavage was performed with 6 ml of cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline solution (145 m*M* NaCl, 5 m*M* KCl, 9.4 m*M* Na_2HPO_4, 1.9 m*M* NaH_2PO_4, and 5.5 m*M* dextrose, pH 7.4). Subsequent bronchoalveolar lavages were performed with volumes of phosphate-buffered saline solution up to 8 ml each until a total volume of 74 ml was collected. The initial and subsequent lavage samples were then centrifuged (500 × g, 5 min, 4°C). The supernatant from the initial lavage was processed for analyses of protein, lactate dehydrogenase (LDH), and nitrate/nitrite levels. The cell pellets from the initial and subsequent lavages were then combined and resuspended in a HEPES-buffered solution (145 m*M* NaCl, 5 m*M* KCl, 10 m*M* HEPES, 1 m*M* $CaCl_2$, and 5.5 m*M* dextrose, pH 7.4). Differential cell counts were determined using an electronic cell counter equipped with a cell-sizing attachment (Coulter model ZBI with a 256 Channelizer, Coulter Electronics, Hialeah, FL).

Bronchoalveolar Lavage Fluid Analyses

Protein levels in the initial lavage fluid sample were analyzed according to the method of Bradford (1976) using purchased reagents (BioRad, Hercules CA). Lactate dehydrogenase (LDH) determinations were made using a commercially available kit (Sigma, St. Louis, MO). Nitrates in the lavage fluid were first converted to nitrite using a nitrate reductase enzyme generated by *Escherichia coli* (Bartholomew, 1984). Total nitrite levels (NOx) were then determined using a spectrophotometric assay based upon the Greiss reaction (Green et al., 1982).

Determination of Cellular Reactive Species Production

The production of reactive species from bronchoalveolar lavage cells (1×10^6 alveolar macrophages/ml/sample) was determined using luminol-enhanced chemiluminescence under basal (resting) and stimulated conditions. Cells were stimulated with either unopsonized zymosan (2 mg/ml) or opsonized zymosan (2 mg/ml). Opsonized zymosan was prepared by incubating this particulate with equal volumes of rat serum and HEPES-buffered solution for 30 min at 37°C in a shaking water bath. The particles were then washed two times with HEPES-buffered solution before use. The NO synthase (NOS) dependent reactive species component was assessed by measuring *N*-ω-nitro-L-arginine

methyl ester (L-NAME; 1 m*M*) inhibitable responses as described previously in detail (Blackford et al., 1994).

Measurement of NO Production by Cultured Bronchoalveolar Lavage Cells

Following the harvest of bronchoalveolar lavage cells (BALC) and cell differential determinations, a portion of the cells was resuspended in medium (minimum essential medium; BioWhittaker, Walkersville, MD) supplemented with 1 m*M* glutamine, 100 U/ml each penicillin, streptomycin, and kanamycin, with 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and 10m*M* HEPES before culture at 37°C in a humidified atmosphere (95% air, 5% CO_2). The cells were plated on the basis of 1×10^6 phagocytes/ml/well in 24-well plates. After 18 h of culture, the media was harvested for NOx determinations.

RNA Analyses

Total cellular RNA was extracted using a guanidinium thiocyanate-based procedure (Chomczynski & Sacchi, 1987; Huffman et al., 1992) and quantified spectrophotometrically (E260 = 40 µg/ml/OD). The RNA was then size-fractioned on a 1.5% agarose gel containing 2 *M* formaldehyde and blotted onto a Duralose membrane (Stratagene, La Jolla, CA) using capillary-mediated bulk flow transfer. Inducible NOS (iNOS) mRNA was indexed using a ^{32}P nick-translated double-stranded cDNA probe derived from a plasmid containing a 4100-bp cDNA fragment for murine macrophage iNOS (Lowenstein et al., 1992), which was a gift from Dr. S. H. Snyder and Dr. C. Lowenstein (Johns Hopkins University, Baltimore, MD). The cDNA probe for use was amplified from the plasmid construct by the polymerase chain reaction technique (Gene Amp DNA Amplification Kit, Perkin Elmer Cetus, Norwalk, CT) using 20-bp synthetic DNA oligonucleotide primers (Blackford et al., 1994). Northern blot hybridization for iNOS mRNA was performed using Quikhyb hybridization buffer (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Subsequently, the blot was boiled in RNase-free distilled water for 5 min to remove hybridized probe and the amount of 28S ribosomal RNA loaded onto the blot was assessed (Barbu & Dautry, 1989). The relative optical densities of the hybridization signals were quantified using a computerized image analysis system (Optimas, Edmonds WA). Individual iNOS mRNA sample signal levels were then divided by the corresponding 28S rRNA sample signal level to normalize values for the amount of RNA loaded.

Statistics

Analyses of variance were performed. Following any overall significant analysis of variance result, differences from the control group were assessed using specific comparison procedures (SAS Institute, Inc., 1985). Significance was set at $p \leq .05$.

RESULTS

Endotoxin analyses of filters collected from the chambers following cotton dust exposure averaged $3.2 \pm 0.4 \times 10^5$ EU/m^3 ($n = 17$), whereas those for filters from chambers used for endotoxin exposure were $2.2 \pm 1.1 \times 10^4$ EU/m^3 ($n = 23$). The rats responded to cotton dust or endotoxin exposure with an acute pulmonary inflammatory response, reflected by increases in red blood cells, lymphocytes, and polymorphonuclear leukocytes in the bronchoalveolar lavage cell populations that were harvested 18 h postexposure (Figure 1). In general, these cellular responses were slightly more pronounced following endotoxin inhalation than following cotton dust exposure. Cotton dust and endotoxin also tended to increase bronchalveolar lavage fluid levels of LDH and protein, with the increase being significant after endotoxin exposure (Table 1).

Following the inhalation of either cotton dust or endotoxin, NOx levels in the bronchoalveolar lavage fluid were elevated (Table 1). This suggested that pulmonary NO production was increased in response to

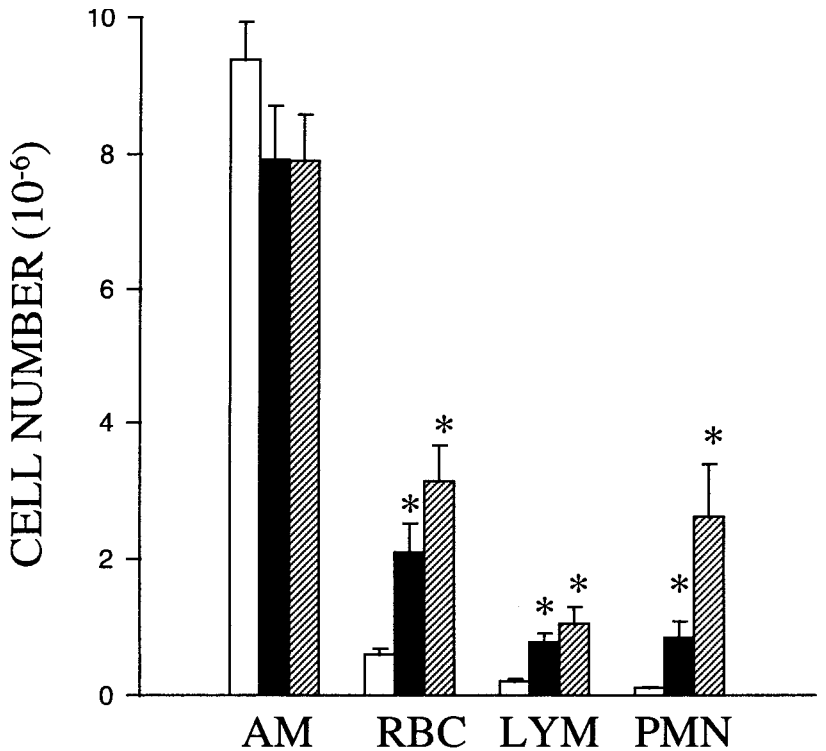

FIGURE 1. Bronchoalveolar lavageable cell populations from rats exposed in vivo to air (open bars), cotton dust (closed bars), or endotoxin (shaded bars). AM, alveolar macrophages; RBC, red blood cells; LYM, lymphocytes; and PMN, polymorphonuclear leukocytes. Results are presented as mean ± SEM, $n = 15$. Asterisk indicates significant at $p \leq .05$ vs. air.

TABLE 1. Bronchoalveolar lavage fluid levels of LDH, protein, and NOx following inhalation exposure to air, cotton dust, or endotoxin

Inhalation	LDH (IU/ml)	Protein (μg/ml)	NOx (μ*M*)
Air	44.2 ± 2.2	126 ± 5	3.83 ± 0.38
Cotton dust	53.6 ± 5.0	164 ± 21	4.88[a] ± 0.21
Endotoxin	68.4[a] ± 3.0	206[a] ± 7	6.57[a] ± 0.46

Note. Results are presented as mean ± SEM, $n = 6$.
[a]Significant, $p \leq .05$, vs. air.

these stimuli. To explore this possibility in more detail, we assessed iNOS mRNA levels in bronchoalveolar lavage cells. A marked increase in iNOS mRNA levels in harvested bronchoalveolar lavage cells was observed following either cotton dust or endotoxin exposure (Figure 2). In vitro NOx production by these cells was also increased (Table 2). The induction of NOS activity following cotton dust or endotoxin exposure appeared to be confined to the bronchoalveolar lavage cell population, since we observed no detectable hybridization signal for iNOS mRNA following Northern blot analysis of trachea, extrapulmonary bronchi, lung, or pulmonary artery tissue from air-, cotton dust-, or endotoxin-exposed rats (data not presented).

Following endotoxin inhalation, both the basal and stimulated production of oxidant species by bronchoalveolar lavage cells was increased as monitored by chemiluminescence (Figure 3). Furthermore, a significant amount of this chemiluminescence was dependent upon NOS activity (Figure 4). In contrast, exposure to cotton dust tended to elevate zymosan-stimulated and NOS-dependent chemiluminescence, but these increases did not reach significance (Figures 3 and 4).

DISCUSSION AND CONCLUSIONS

A body of evidence suggests that endotoxin contamination of baled cotton is responsible for the inflammatory reactions that are characteristic of acute byssinosis (Castellan et al., 1987). In the present study, the inhalation of either cotton dust or endotoxin by rats was associated with acute pulmonary inflammation, as evidenced by increases in polymorphonuclear leukocytes and lymphocytes harvested by bronchoalveolar lavage. Disruption of the alveolar air–blood barrier also appeared to occur, since elevated numbers of red blood cells and increased acellular protein levels were observed in the bronchoalveolar lavage fluid following cotton dust or endotoxin exposure. These findings complement those of others who have observed significant similarities in the pattern of cellular responses to endotoxin or cotton dust inhalation (Castranova et al., 1996).

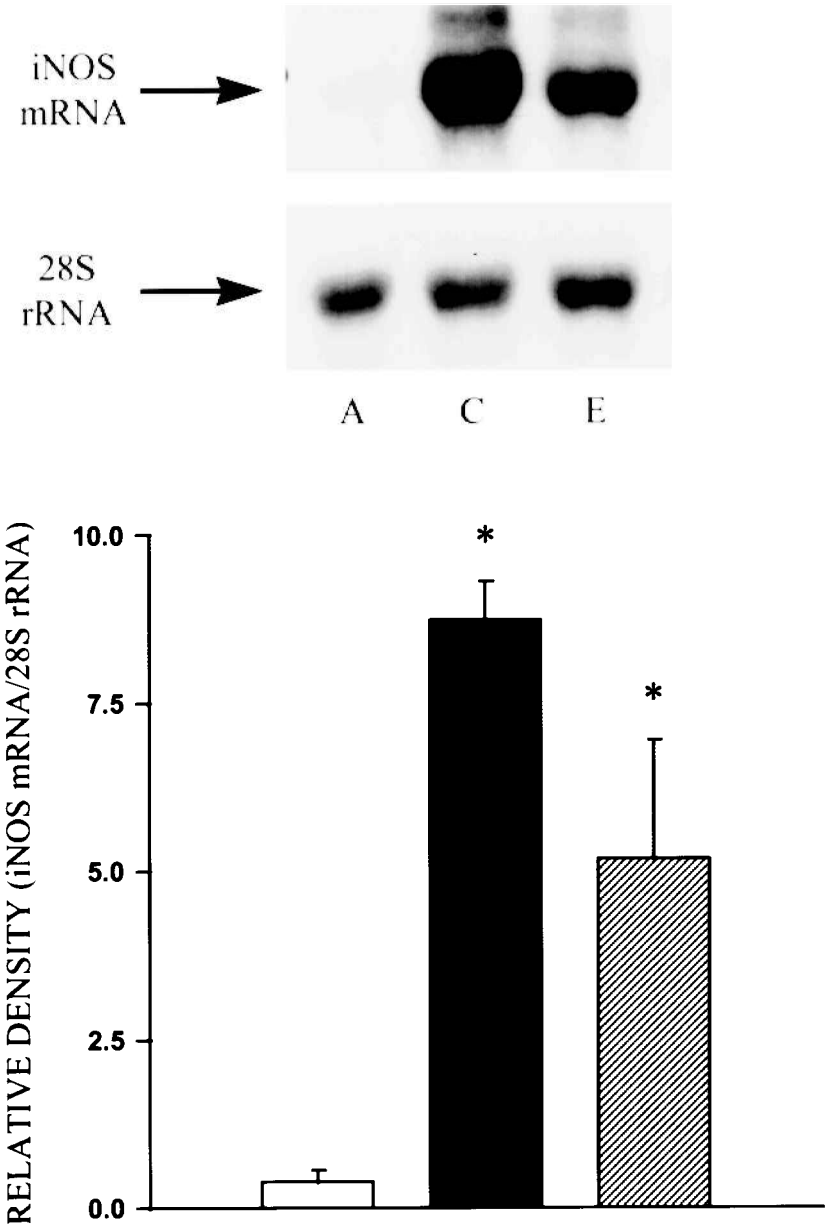

FIGURE 2. (Upper panel) Northern blot analyses of iNOS mRNA and 28S rRNA from representative bronchoalveolar lavage cell samples from air- (A), cotton dust- (C), or endotoxin (E)–exposed rats. (Lower panel) Quantification of iNOS mRNA levels in bronchoalveolar lavage cells from rats exposed to air (open bars), cotton dust (closed bars), or endotoxin (shaded bars). Results are presented as mean ± SEM, $n = 3$. Asterisk indicates significant at $p \leq .05$ vs. air.

TABLE 2. In vitro NOx production by bronchoalveolar lavage cells harvested from rats exposed in vivo to air, cotton dust, or endotoxin

	In vivo treatment		
	Air	Cotton dust	Endotoxin
NOx (μ*M*)	35.7 ± 7.6	96.8[a] ± 16.1	69.5[a] ± 17.4

Note. Results are presented as mean ± SEM, $n = 6$.
[a]Significant, $p \leq .05$, vs. air.

In our study, the magnitude of these cellular responses was slightly more pronounced in endotoxin-exposed animals, a phenomenon that was also reflected in the levels of LDH and protein detected in initial lavage fluid samples. These observations seemingly contrast with those regarding the analysis of endotoxin content on sampling filters from

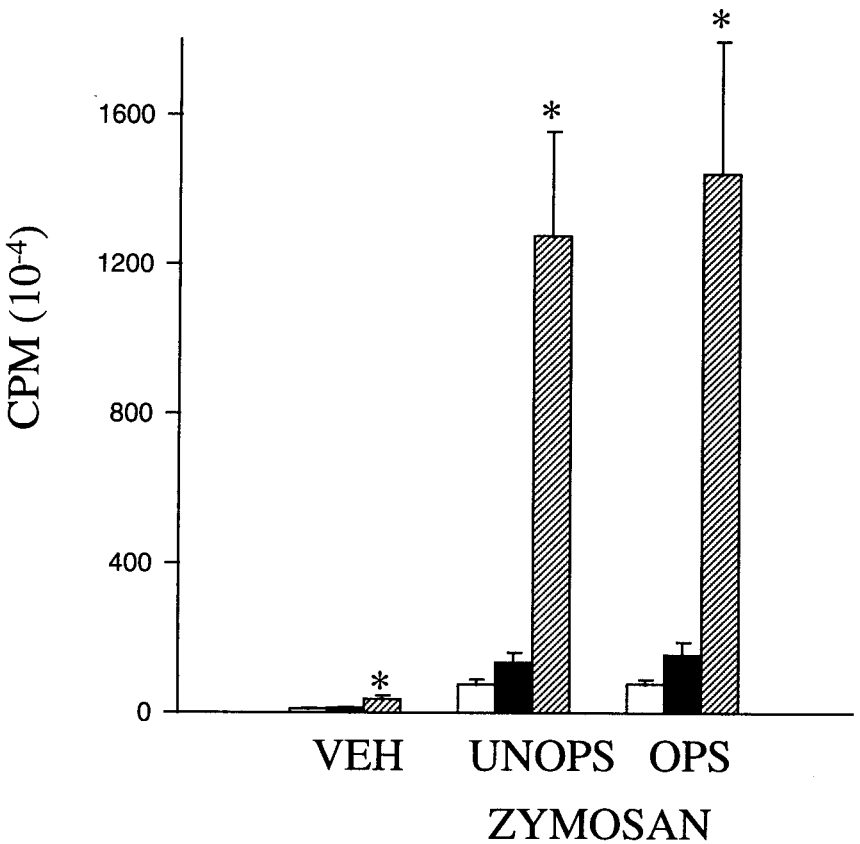

FIGURE 3. Chemiluminescence of bronchoalveolar lavage cells exposed in vitro to vehicle (veh), unopsonized (unops) zymosan, or opsonized (ops) zymosan. Cells were harvested from rats exposed in vivo to air (open bars), cotton dust (closed bars), or endotoxin (shaded bars). Results are presented as mean ± SEM, $n = 6$. Asterisk indicates significant at $p \leq .05$ vs. air.

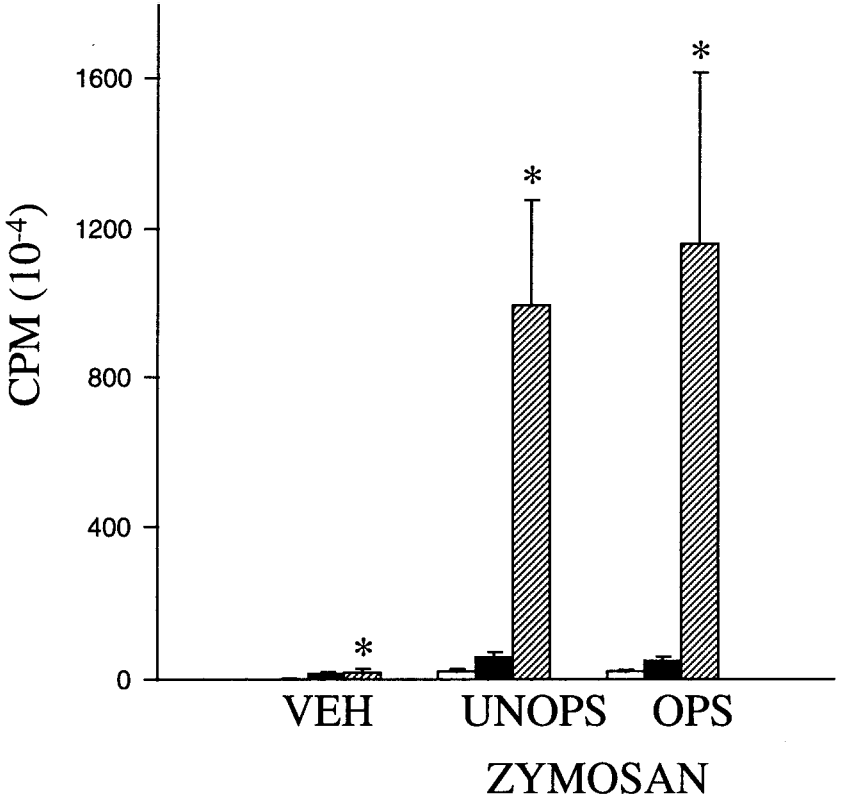

FIGURE 4. NOS-dependent chemiluminescence of bronchoalveolar lavage cells. Legend as described in Figure 3.

the exposure chambers. In the latter case, the measured endotoxin content of the cotton dust was 10-fold greater than that of the aerosolized endotoxin. However, it should be noted that we did not directly measure the actual biologically available dose of endotoxin during these exposures; that is, extraction of endotoxin from cotton dust within the lung may be incomplete when compared to the rigorous extraction (i.e., large fluid volume and continuous mixing) used with the filter samples from the cotton dust exposure chambers. Furthermore, it should also be noted that at least two components of cotton dust, β-glucans and formyl-methionyl-leucyl-phenylalanine, have been shown to reduce the cellular response of animals to endotoxin when these components have been given in combination with endotoxin (Frazer et al., 1996; Robinson et al., 1996). Collectively, these results suggest that the endotoxin content of aerosolized cotton dust per se cannot be compared directly to aerosolized endotoxin to predict the severity of all pulmonary effects.

As hypothesized, an increase in NO production occurred following the inhalation of either cotton dust contaminated with endotoxin or

purified aerosolized endotoxin. This was evidenced by elevations in lavage fluid levels of the stable decomposition products of NO, that is, nitrate and nitrite, following cotton dust or endotoxin exposure. This phenomenon was apparently due to an induction of NOS activity in bronchoalveolar lavage cells, since mRNA levels of the iNOS isoform were elevated in these cells and increases in media nitrate and nitrite levels were observed following in vitro culture of cells from cotton dust- or endotoxin-exposed rats. The increase in iNOS activity appeared to be localized to bronchoalveolar lavage cells, since iNOS mRNA could not be detected in tracheal, extrapulmonary bronchi, or lung tissue samples from any of the animals. Endotoxin is a potent stimulus, in vivo and in vitro, for the induction of NO by rat alveolar macrophages (Wizemann et al., 1994; Jorens et al., 1991). Increases in NO production by polymorphonuclear leukocytes can also be observed following endotoxin stimulation (Kolls et al., 1994). Therefore, an induction of NO synthesis could conceivably occur in both of these bronchoalveolar cell types following exposure to endotoxin-contaminated cotton dust.

Results from the present study complement those of others who have shown that the cascade of proinflammatory substances that are released following cotton dust exposure is very similar to that observed following endotoxin challenge (Beijer & Rylander, 1985; Rochemonteix-Galve et al., 1991; Ryan & Karol, 1991; Rylander & Billiar, 1987). A pivotal role for TNFα in the acute toxic alveolitis resulting from cotton dust exposure is supported by the finding that administration of anti-TNFα antiserum prior to dust exposure resulted in an attenuation of cellular indices of inflammation (Shvedova et al., 1994). While endotoxin itself can stimulate the production of NO by lung cells, TNFα and other cytokines can also induce NO production in bronchoalveolar lavage cells (Warner et al., 1995). Thus, a number of potential mediators could participate in the upregulation of NO production following cotton dust exposure.

An increasing number of studies suggest that NO production is induced during inflammatory reactions (Nussler & Billiar, 1993). What is less clear, however, is the role that NO plays in the inflammatory cascade. NO has the potential to be cytotoxic and cytostatic, and many of these actions have been attributed to ability of NO to combine with superoxide anion to form peroxynitrite, a toxic oxidant (Beckman & Crow, 1993). In the present study, we examined NO-dependent oxidant production by bronchoalveolar lavage cells following cotton dust or endotoxin exposure using luminol-enhanced chemiluminescence. Specifically, we assessed responses following in vitro stimulation with unopsonized or opsonized zymosan in the presence or absence of L-NAME to evaluate the contribution of NOS activity to

this chemiluminescence. It has been proposed that much of this luminol-enhanced chemiluminescence is due to peroxynitrite (Van Dyke et al., 1994). In fact, unlike the case for peroxynitrite, the presence of either NO or superoxide alone is associated with little or no chemiexcitation of luminol (Radi et al., 1993). Unexpectedly, we detected a marked difference in the luminol-enhanced chemiluminescence response of bronchoalveolar lavage cells following in vivo cotton dust or endotoxin exposure. Endotoxin exposure was associated with a small increase in chemiluminescence generation by unstimulated cells and a marked rise in the production of oxidants following opsonized zymosan stimulation. Since rat alveolar macrophages, in contrast to polymorphonuclear leukocytes, respond to zymosan without the need for opsonization, we also evaluated luminol-enhanced chemiluminescence in the presence of unopsonized zymosan (Castranova et al., 1987b). Much of the reactive species production appeared to derive from the alveolar macrophage population, since responses following unopsonized or opsonized zymosan stimulation were similar. Furthermore, a substantial portion of the luminol-enhanced chemiluminescence of the bronchoalveolar lavaged cell population following in vivo endotoxin treatment was dependent upon NOS enzyme activity. In contrast, we could detect only a small enhancement of reactive species generation by bronchoalveolar lavage cells harvested following cotton dust exposure. An explanation for the apparent differences in luminol-enhanced chemiluminescent responses of bronchoalveolar lavage cells following cotton dust or endotoxin exposure may lie in the relative amounts of NO and superoxide anion that are produced. The results from the present study (Tables 1 and 2 and Figure 2) suggest that relatively similar amounts of NO were generated following cotton dust or endotoxin exposures. However, it appears that although the inhalation of cotton dust is associated with an increase in superoxide generation by alveolar macrophages, the levels of this free radical that are produced are relatively low compared to those that can be generated following the inhalation of endotoxin (Castranova et al., 1987a; J. Milanowski, personal communication). Consequently, it may be that less peroxynitrite and thus less chemiluminescence would be generated from the combination of NO with superoxide anion from bronchoalveolar lavaged cells following cotton dust inhalation.

Thus, although the inhalation of either cotton dust or endotoxin appears to be associated with many similar responses, including an induction of NO production, the sequelae of these events may be substantially different. These results support the findings of others that suggest that while endotoxin may be a major etiologic agent in the response to cotton or other organic dusts, it is not the sole contributing factor in ODTS (Castranova et al., 1996).

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