

# Enhancement of Nitric Oxide Production by Pulmonary Cells following Silica Exposure

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*In vivo* exposure of rat lungs to crystalline silica either by intratracheal instillation or by inhalation results in an increase in mRNA levels for inducible nitric oxide synthase (iNOS) in bronchoalveolar lavage cells (BALC), elevated nitric oxide (\*NO) production by BALC, and an increase in \*NO-dependent chemiluminescence (CL) from alveolar macrophages (AM). Induction of iNOS message occurs in both AM and polymorphonuclear leukocytes (PMN) harvested from silica-exposed lungs but is not significantly elevated in lavaged lung tissue. *In vitro* exposure of AM to silica does not stimulate \*NO production or enhance iNOS message. However, treatment of naive AM with conditioned media from BALC harvested from silica-exposed rats does increase iNOS message and \*NO production by these AM. The potency of this conditioned medium is dependent on interaction between AM and PMN. In the rat model, a relationship exists between the ability of various dusts to cause PMN recruitment or protein leakage into the alveolar space and the induction of iNOS message in BALC, i.e., silica > coal mine dust > carbonyl iron > titanium dioxide. Similarly, a comparison of BALC from a healthy volunteer, a silica-exposed coal miner with a normal chest radiograph, and a silica-exposed coal miner with an abnormal chest radiograph shows a correlation between pathology and both the level of iNOS message in BALC and the magnitude of \*NO-dependent CL from AM. These data suggest that \*NO may play a role in silicosis and that human pulmonary phagocytes exhibit enhanced \*NO production in response to an inflammatory insult. — *Environ Health Perspect* 106(Suppl 5):1165-1169 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1165-1169castranova/abstract.html>

Key words: silica exposure, nitric oxide production, inducible nitric oxide synthase, alveolar macrophages, coal miners

## Introduction

Exposure to crystalline silica can result in inflammation, lung damage, and fibrosis (1). It has been proposed that reactive oxygen species (ROS) play an important role in the silica-induced cycle of pulmonary inflammation and damage (2). There appear to be two major sources of ROS from silica exposure. The first is the direct generation of hydroxyl radicals (\*OH) from silica particles, and the second is the silica-induced production of ROS from pulmonary phagocytes.

Occupations such as sandblasting, silica flour milling, rock drilling, and tunneling are associated with relatively high risks of pulmonary fibrosis. Common to these operations is the fracturing of Si-O bonds forming the silica crystalline lattice. Cleavage of Si-O bonds would result in the generation of silicon radicals (\*Si and Si\*O) on the fracture planes. The existence of such surface radicals in freshly ground silica has been demonstrated by electron spin resonance

(3). The half-life of these silicon radicals is approximately 30 hr in air. Upon contact with aqueous solution, these surface radicals generate \*OH (3,4). The Fenton reaction has been implicated in the generation of these \*OH, as the \*OH signal can be inhibited by the Fe<sup>2+</sup> chelator desferal (5). The ability of fractured silica to generate \*OH decreases with aging, exhibiting a half-time of approximately 24 hr (3). A direct relationship has been demonstrated between the generation of \*OH by fractured quartz and silica-induced lipid peroxidation *in vitro* (6).

Exposure of alveolar macrophage (AM) to silica *in vitro* stimulates the production of ROS, measured as an increase in oxygen consumption (3-fold), superoxide anion radical (O<sub>2</sub><sup>-</sup>) production (2-fold), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) release (4-fold), and chemiluminescence (CL) (10-fold). This silica-induced activation of ROS production by AM is rapid, occurring within 1 to 2 min of *in vitro* exposure (7). Similarly, *in vitro* exposure of polymorphonuclear leukocytes (PMN) to silica increases CL by 9-fold (7). *In vivo* silica exposure via intratracheal instillation or inhalation primes ROS release, resulting in an increase in zymosan-stimulated H<sub>2</sub>O<sub>2</sub> release and CL by AM (7-9). Both *in vitro* activation and *in vivo* priming of ROS production by AM is greater upon exposure to freshly fractured silica than to aged silica (9,10). Induction of ROS from AM has also been reported in silicotic patients. Indeed, both basal O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> release are elevated in AM from patients with chronic silicosis (11), whereas both basal and zymosan-stimulated CL are increased 12-fold in AM from a rock driller with acute silicosis (12).

Recent evidence indicates that reactive nitrogen species such as nitric oxide (\*NO) are produced by bronchoalveolar lavage cells (BALC) in response to inflammatory exposures. For example, increased mRNA levels for inducible nitric oxide synthase (iNOS) and/or elevated \*NO release have been reported for BALC harvested from animals exposed to ozone, cotton dust, endotoxin, or diisocyanate (13-15). Once formed, \*NO can combine with O<sub>2</sub><sup>-</sup> to produce peroxynitrite (OONO<sup>-</sup>), which is a highly reactive oxidant (16). It has been proposed that OONO<sup>-</sup> is the reactive species responsible for damage to the blood-air barrier in reaction to challenge with immune complexes (17) and that it can stimulate the production of proinflammatory mediators (18).

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Abbreviations used: AM, alveolar macrophages; BALC, bronchoalveolar lavage cells; CL, chemiluminescence; IFN- $\gamma$ , interferon gamma; ILO, International Labor Organisation; iNOS, inducible nitric oxide synthase; L-NAME, N<sub>ω</sub>-nitro-L-arginine methylester; LPS, lipopolysaccharide; \*NO, nitric oxide; \*OH, hydroxyl radicals; OONO<sup>-</sup>, peroxynitrite; O<sub>2</sub><sup>-</sup>, superoxide anion radical; PCR, polymerase chain reaction; PMN, polymorphonuclear leukocytes; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; WVU, West Virginia University.

Recent evidence from our laboratory indicates that \*NO may play a role in the pulmonary response to silica. This manuscript summarizes these results.

## Methods

### Institutional Approval

Protocols for the exposure of rats by intratracheal instillation or inhalation to silica or other dusts and for the use of rats as a source of primary AM for *in vitro* studies were approved by the respective Animal Care and Use Committee at the National Institute for Occupational Safety and Health or West Virginia University (WVU). Rats were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility and given water and food *ad libitum*.

Procedures for bronchoalveolar lavage of human subjects were approved by the Human Institutional Review Board at WVU and were conducted after receiving informed consent in writing from the subjects.

### Intratracheal Instillation

Specific pathogen-free male Sprague-Dawley or Fischer 344 rats (175–225 g) were anesthetized by intraperitoneal injection of sodium methohexital. Rats were intratracheally instilled with 0.5 ml sterile saline (control) or dust suspended in 0.5 ml saline (5–10 mg/100 g bw) via a 20-gauge feeding needle, as described previously (19). Animals were sacrificed 1 day postexposure unless otherwise noted.

### Inhalation Exposure

Freshly fractured silica was generated in a jet mill fitted with a polyurethane liner and stainless steel jets at a P-jet pressure of 70 psi and an O-jet pressure of 100 psi. This milled dust was fed directly to the exposure generator in the case of exposure to fresh silica. In the case of aged silica exposure, the milled dust was collected and stored at room temperature for 2 months before use in the exposure system. Rats were exposed to 20 mg silica/m<sup>3</sup> for 5 hr/day, 5 days/week for 2 weeks. Animals were sacrificed for evaluation 1 to 3 days postexposure. Further details were given previously (9).

### Isolation of Bronchoalveolar Lavage Cells

Rats were anesthetized with sodium pentobarbital and exsanguinated. Bronchoalveolar lavage was performed through a tracheal cannula with 10 lavages of 8 ml each using ice-cold Ca<sup>2+</sup>, Mg<sup>2+</sup>-free phosphate-buffered

medium (pH 7.4) consisting of 145 mM NaCl, 5 mM KCl, 1.9 mM NaH<sub>2</sub>PO<sub>4</sub>, 9.35 mM Na<sub>2</sub>HPO<sub>4</sub>, and 5.5 mM dextrose. BALC were pelleted, washed, and resuspended in a HEPES-buffered solution (pH 7.4) consisting of 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM CaCl<sub>2</sub>, and 5.5 mM dextrose. Cell counts and differentials were determined using an electronic cell counter equipped with a cell-sizing attachment as described previously (20).

### Measurement of iNOS Message

RNA was harvested and iNOS mRNA measured using Northern blot analysis as described previously (20). Briefly, total cellular RNA was extracted by a modification of a guanidinium thiocyanate-phenol-chloroform method (21). The RNA was then size-fractionated on a 1.0% denaturing agarose gel containing 5.6% formaldehyde and blotted onto a Nytran membrane. RNA bands were stained with methylene blue and optical density measured using a PC-based Optima imaging system (Bioscan, Edmonds, WA). The membrane was then washed to remove the methylene blue. iNOS mRNA was then indexed using a <sup>32</sup>P-labeled hybridization probe derived from a plasmid containing a 4100-bp cDNA fragment for murine macrophage iNOS (gift of S. Snyder and C. Lowenstein, Johns Hopkins University, Baltimore, MD). The iNOS fragment was amplified from the plasmid construct by the polymerase chain reaction (PCR) technique (Gene Amp DNA Amplification Kit, Perkin-Elmer Cetus, Norwalk, CT) using 20-bp synthetic DNA oligonucleotide primers to produce a double-strand cDNA template. Subsequently, a single-strand DNA hybridization probe was generated by PCR using the antisense primer and <sup>32</sup>P- $\alpha$ -labeled deoxycytidine-triphosphate (dCTP) (ICN Biochemicals, Costa Mesa, CA). Northern blot hybridization for iNOS mRNA was performed using Quikhyb hybridization buffer (Stratagene, La Jolla, CA). The autoradiographic signal was obtained by exposing the membrane to Kodak XAR film at -80°C.

### Determination of \*NO Production

Cells were suspended in minimum essential medium supplemented with 1 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 100  $\mu$ g/ml kanamycin with 10% fetal bovine serum and 10 mM HEPES, and incubated at 37°C in a humidified atmosphere (95% air, 5% CO<sub>2</sub>). Cells were cultured for 20 hr and

cell-free media collected for analysis. Media nitrate was converted to nitrite using a nitrate reductase enzyme isolated from *Escherichia coli* according to the method of Bartholomew (22). \*NO production was determined as total nitrite measured spectrophotometrically by the Greiss reaction (23).

### Measurement of \*NO-Dependent Chemiluminescence

CL generated from AM was measured at 37°C in the presence of 0.008  $\mu$ g/ml luminol using a Berthold LB953 luminometer. AM ( $7.5 \times 10^5$  cells) were suspended in 0.75 ml HEPES-buffered medium and preincubated for 15 min at 37°C prior to being placed into the luminometer. Zymosan-stimulated CL (cpm with zymosan minus cpm at rest) was measured in the presence of 2 mg/ml unopsonized zymosan added just prior to measuring CL. Note that PMN do not respond to unopsonized zymosan (9). \*NO-dependent CL (counts per minute with zymosan minus counts per minute with zymosan plus N<sup>ω</sup>-nitro-L-arginine methylester [L-NAME]) was determined as the amount of zymosan-stimulated CL that was inhibitable by 1 mM L-NAME (i.e., an inhibitor of NOS) added during the preincubation period.

### Studies with Human Bronchoalveolar Lavage Cells

One coal mine dust-exposed worker with an abnormal chest radiograph (International Labor Organisation [ILO] category 1/0), one coal mine dust-exposed worker with a normal chest radiograph, and one healthy volunteer subject without coal mine dust exposure underwent bronchoalveolar lavage. Both coal mine workers had worked more than 20 years underground as roof bolters, beltmen, motormen, and long-wall operators. These coal mine operations have been associated with high levels of exposure to silica-containing dusts and workers performing these operations have been reported to exhibit increased incidence of silicotic lung lesions (24).

All three subjects were male nonsmokers without respiratory symptoms. Each had normal pulmonary function measured by spirometry, and carbon monoxide diffusing capacity. Briefly, subjects underwent bronchoscopy with bronchoalveolar lavage in a subsegment of the right middle lobe. The procedure was performed with atropine premedication to reduce secretions and with local anesthesia using topical lidocaine.

Lavage was performed with warmed saline solution instilled in 50-ml aliquots and removed with gentle suction. The total lavage volume instilled was constant at 200 ml. Variable amounts of lavage fluid were recovered by gentle suction. The mean return volume was approximately 60 to 65% of that instilled in both the control subject and the underground coal miners. BALC were pelleted, washed, and resuspended in HEPES-buffered medium. Cell counts and distribution were determined using an electronic cell counter equipped with a cell-sizing attachment as described above. Zymosan-stimulated and  $\text{NO}$ -dependent CL were determined as described above. The level of iNOS mRNA was determined using reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, cellular RNA was extracted and cDNAs were synthesized with a commercial kit (Cetus, Norwalk, CT). cDNA equivalents of 1  $\mu\text{g}$  RNA were amplified for 35 cycles in the presence of commercially available PCR primers for human iNOS (Clontech Laboratories Inc., Palo Alto, CA). The PCR products were visualized by ultraviolet illumination after gel electrophoresis.

## Results and Discussion

Intratracheal instillation of rats with silica upregulates  $\text{NO}$  production by BALC. This upregulation involves induction of mRNA for iNOS, enhanced production of  $\text{NO}$ , and increased generation of  $\text{NO}$ -dependent CL from zymosan-stimulated AM. Table 1 shows the magnitude of this silica-induced up regulation in BALC 24 hr postexposure. The time course of the induction of iNOS message is given in Figure 1. After a single instillation of silica, message levels in BALC are significantly elevated at 4 hr postexposure, peak at 8 hr, and return toward control levels over 1 week when the iNOS message is not significantly elevated. The production of  $\text{NO}$ , measured as

**Table 1.** Nitric oxide induction in response to intratracheal instillation of silica.<sup>a</sup>

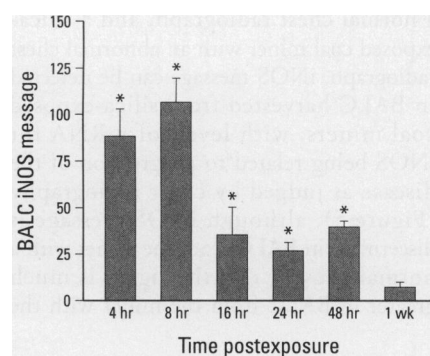
Treatment	iNOS message	$\text{NO}$ production	$\text{NO}$ -dependent
Saline	100%	100%	100%
Silica	300 $\pm$ 50%*	600 $\pm$ 15%*	570 $\pm$ 142%*

Data modified from Blackford et al. (20) and Huffman et al. (25). <sup>a</sup>BALC were harvested 24 hr after intratracheal instillation of saline (control) or silica (10 mg/100 g bw). Values are means  $\pm$  SEM of at least three experiments and expressed as percent of control. \*Significant increase above control ( $p \leq 0.05$ ).

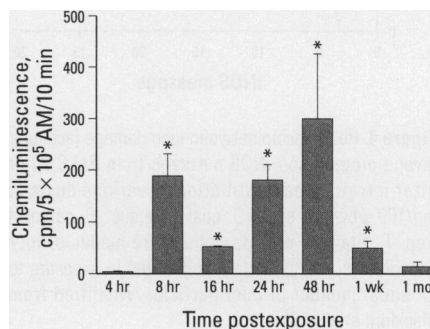
$\text{NO}$ -dependent CL, is somewhat delayed in time, not being significantly elevated until 8 hr postexposure, peaking at 48 hr, and returning toward normal until at 1 month values are not different than those of control (Figure 2). Similar upregulation of  $\text{NO}$  production is also observed after inhalation of silica (9). These results (not presented here) indicate that freshly fractured silica is a more potent stimulant of  $\text{NO}$ -dependent CL than aged dust. Fresh silica induced a 64-fold increase above control compared to 19-fold for aged dust.

As described above, *in vivo* exposure results in upregulation of  $\text{NO}$  in BALC. Analysis of bronchoalveolar lavage preparations from silica-exposed rats indicates that two types of pulmonary phagocytes predominate; AM and PMN represent 99% of leukocytes harvested 24 hr after silica exposure, with the ratio of AM to PMN in the lavage of approximately 1 to 2 (20). Enriched preparations of PMN and AM can be obtained by differential adherence. AM are enriched in the adherent cell fractions by 3.5-fold, whereas PMN represent 93% of the nonadherent cells. Analysis of iNOS mRNA in these enriched fractions indicates that message is increased 6.4-fold in AM and 14.8-fold in PMN following intratracheal instillation of silica (20). Analysis of mRNA obtained from lavaged lung tissue indicates a small (37%) but nonsignificant induction of iNOS message in response to silica exposure (20). These results suggest the silica induction of iNOS message is not a generalized pulmonary response but rather a reaction specific to certain cell types such as pulmonary phagocytes.

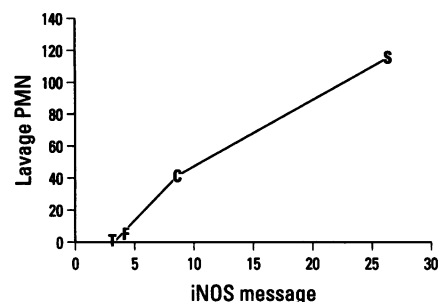
The question now becomes how to determine the role of this upregulation of  $\text{NO}$  in the inflammatory and cytotoxic reaction to dust exposure. To investigate this, we intratracheally instilled rats with dusts exhibiting a range of pathogenicity, i.e., silica, coal mine dust, carbonyl iron, and titanium dioxide. When exposure is normalized on an equal particle number basis, a correlation between inflammation or lung damage and iNOS message in BALC is apparent (Figures 3, 4). A direct relationship is noted between iNOS mRNA levels and lavagable PMN or acellular lavage protein following dust exposure, with silica exhibiting the greatest reaction and titanium dioxide inducing the least. To investigate further the relationship between pathology and  $\text{NO}$ , we evaluated human BALC from a healthy volunteer, a silica-exposed coal miner with



**Figure 1.** Time course of induction of iNOS message following a single intratracheal instillation of silica (5 mg/100 g bw). Values are means  $\pm$  SEM of three experiments expressed as the optical density of the iNOS autoradiograph signal minus the control. \*Significantly different from the saline control ( $p \leq 0.05$ ).

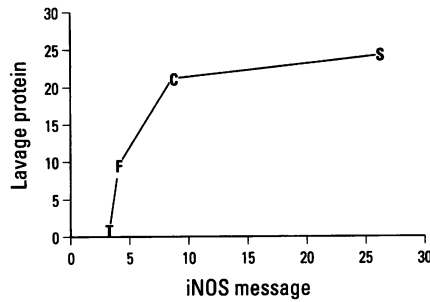


**Figure 2.** Time course of  $\text{NO}$ -dependent CL from zymosan-stimulated AM following a single intratracheal instillation of silica (5 mg/100 g bw).  $\text{NO}$ -dependent CL was determined by measuring the amount of CL that was inhibited by 1 mM L-NAME, an NOS inhibitor. Values are means  $\pm$  SEM of three experiments expressed as the L-NAME-inhibited CL minus the control. \*Significantly different from the saline control ( $p \leq 0.05$ ).

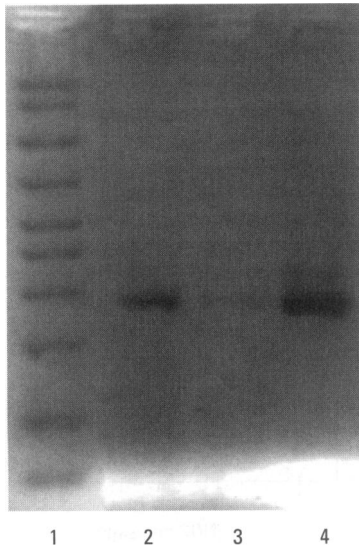


**Figure 3.** Relationship between inflammation (lavagable PMN) and iNOS message levels of BALC 24 hr after intratracheal instillation of various dusts (5 mg/100 g bw). S, silica; C, coal mine dust; F, carbonyl iron; and T, titanium dioxide. Values are means from four experiments. Responses are normalized to exposure to an equal number of dust particles. Modified from Blackford et al. (19).

a normal chest radiograph, and a silica-exposed coal miner with an abnormal chest radiograph. iNOS message can be detected in BALC harvested from silica-exposed coal miners, with levels of mRNA for iNOS being related to progression of the disease as judged by chest radiography (Figure 5); although iNOS message is discernible in BALC from the miner with a normal chest X ray, the signal is much greater in BALC from the miner with the



**Figure 4.** Relationship between lung damage (acellular lavage protein) and iNOS message from BALC 24 hr after intratracheal instillation of various dusts (5 mg/100 g bw). S, silica; C, coal mine dust; F, carbonyl iron; T, titanium dioxide. Values are means of four experiments. Responses are normalized to exposure to an equal number of dust particles. Modified from Blackford et al. (19).



**Figure 5.** Analysis of iNOS mRNA in BALC from silica-exposed coal miners using RT-PCR. 1, molecular weight marker (Bio-Rad). 2, message from BALC harvested from a silica-exposed coal miner with an abnormal radiograph (ILO category 1/0). 3, message from BALC harvested from a silica-exposed coal miner with a normal chest radiograph. 4, positive control (Clontech Laboratories).

abnormal chest X ray. A relationship also exists between pathology and the amount of <sup>•</sup>NO-dependent CL generated by zymosan-stimulated human AM; AM from the nonexposed subject produce the least <sup>•</sup>NO-dependent CL, whereas the silica-exposed coal miner with the abnormal X ray exhibits the most <sup>•</sup>NO production (Table 2).

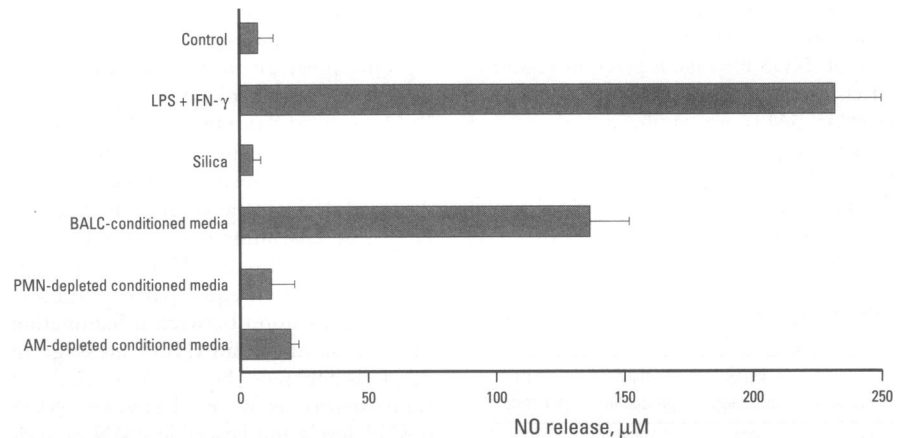
As published previously, silica is a direct stimulant of ROS from AM *in vitro* as well as a primer of ROS release *in vivo* (7-9). In contrast, silica does not stimulate <sup>•</sup>NO production from AM *in vitro* even after a 20-hr exposure of cultured primary AM to silica (0.1-100 µg/ml). The lack of an *in vitro* activation of <sup>•</sup>NO release from cultured AM is shown in Figure 6. In contrast to silica, rat AM are very responsive to *in vitro* stimulation by lipopolysaccharide (LPS) plus interferon γ (IFN-γ), which increases <sup>•</sup>NO production 35-fold. Interestingly, <sup>•</sup>NO production is increased 20-fold when

naive AM are treated with conditioned media from BALC harvested from silica-exposed rats. Interaction (most likely cytokine signaling) between AM and PMN seems important to the stimulatory potency of this conditioned media, as BALC obtained from PMN-depleted silica-exposed rats (pretreated with a PMN antibody) or PMN purified by centrifugal elutriation from BALC obtained from silica-exposed rat both fail to produce factors that activate <sup>•</sup>NO production in naive AM (Figure 6). Our laboratory has attempted to identify the active mediator(s) in conditioned media of BALC harvested from silica-exposed rats (25). The effects of several cytokines (IFN-γ, interleukin-1, tumor necrosis factor α, granulocyte macrophage-colony-stimulating factor) alone and in combination have been investigated. Unfortunately, none was found to induce iNOS message or cause <sup>•</sup>NO production from naive AM treated with

**Table 2.** Evaluation of reactive species production from human alveolar macrophages using luminol-enhanced chemiluminescence.

Case	Zymosan-stimulated CL	<sup>•</sup> NO-dependent CL
Coal mine dust-exposed, abnormal chest radiograph	184.4	40.3
Coal mine dust-exposed, normal chest radiograph	39.3	19.8
Nonexposed control	4.9	1.3

Values are expressed as cpm × 10<sup>-5</sup>/0.75 × 10<sup>6</sup> AM/30 min. AM (0.75 × 10<sup>6</sup>/0.75 ml) were incubated for 20 min at 37°C in the absence or presence of 1 mM L-NAME to determine NO-dependent CL, i.e., the CL inhibited by NOS inhibitor. After the incubation period, the luminol-enhanced CL response to unopsonized zymosan (2 mg/ml) was measured. Values reflect the integrated response over 30 min.



**Figure 6.** <sup>•</sup>NO production from naive primary rat AM cultured for 20 hr in the absence or presence of LPS (100 µg/ml) + IFN-γ (10 U/ml), silica (100 mg/ml), or conditioned media. Conditioned media were obtained from BALC, harvested from silica-exposed rats, and cultured for 20 hr. PMN-depleted conditioned media were obtained from BALC harvested from silica-exposed rats pretreated (24 hr prior) with rabbit anti-rat PMN antibody (4 ml/kg bw intraperitoneally). AM-depleted conditioned media were obtained from PMN purified by centrifugal elutriation from BALC harvested from silica-exposed rats. Values are means ± SEM of three to four experiments. Modified from Huffman et al. (25).

cytokine(s) in culture for 24 hr. Therefore the active mediator is still unidentified.

Data presented indicate that iNOS message and \*NO production are induced after *in vivo* exposure of rat pulmonary

phagocytes to silica. In contrast, *in vitro* exposure, although effective in activating ROS, has no effect on \*NO production. Induction of iNOS message and \*NO production can also be demonstrated in alveolar

phagocytes from humans exposed to silica-containing dusts. In both the rat and human, a relationship exists between induction of \*NO and the degree of lung pathology or inflammation.

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