Korean J Physiol Pharmacol
Vol 2: 233–239, April, 1998

Inducible Nitric Oxide Synthase mRNA Expression and Nitric Oxide Production in Silica-Induced Acute Inflammatory Lung Injury

Jihee Lee

Department of Physiology, College of Medicine, Ewha Womans University and Division of Cell Biology, Ewha Medical Research Center, Seoul 158–056, Korea

Stimulated alveolar macrophages and neutrophils produce nitric oxide, a free radical by an inducible nitric oxide synthase(iNOS), which reacts with superoxide anion to form peroxynitrite, a more highly reactive toxic species. The objectives of the present study were to evaluate acute inflammatory lung injury and to determine iNOS mRNA induction and nitric oxide production by rat broncho-alveolar lavage cells following intratracheal treatment of silica. After 4 h exposure to silica, differential counts of broncho-alveolar lavage cells and lactate dehydrogenase(LDH) activity as well as total protein in the broncho-alveolar lavage fluid were determined. Broncho-alveolar lavage cells were also assayed for iNOS mRNA and the productions of nitrite and nitrate measured in the cells cultured. Differential analysis of broncho-alveolar lavage cells showed that the number of alveolar macrophages slightly decreased following silica treatment; however, red blood cells, lymphocytes, and neutrophils significantly were increased by 9-, 14-, and 119-fold following silica treatment, respectively, compared with the saline control. It was also found significant increases in the LDH activity and total protein in the lavage fluid obtained from silica-treated rats, indicating silica-induced acute lung injury. Northern blot analysis demonstrated that the steady state levels of iNOS mRNA in broncho-alveolar lavage cells were increased following silica treatment. The productions of nitrite and nitrate in the cultured cells were significantly increased by 2-fold following silica treatment, respectively, which were attenuated by the NOS inhibitor Nω-nitro-L-arginine-methyl ester(L-NAME) and partially reversed by L-arginine. These findings suggest that nitric oxide production in alveolar macrophages and recruited neutrophils is increased in response to silica. Nitric oxide may contribute in part to acute inflammatory lung injury.

Key Words: iNOS mRNA, Nitric oxide, Silica, Acute inflammatory lung injury

INTRODUCTION

Interstitial lung disease caused by exposure to silica is the consequence of damage to lung cells and the resulting lung scarring associated with activation of the fibrotic process. Several mechanisms for the development of silicosis have been proposed: first, direct tissue damage by silicon-based radicals on the surface of silica (Fubini, 1987; Vallyathan et al, 1988); second, stimulation of alveolar macrophages by silica to release oxidants or enzymes which cause lung damage (deShazo, 1982; Heppleston, 1982; Davis, 1986); third, stimulation of alveolar macrophages to secrete inflammatory factors which recruit and activate polymorphonuclear neutrophils causing further lung damage (Futone & Ward, 1982; Martin et al, 1972); fourth, stimulation of alveolar macrophages to secrete factors which induce fibroblast proliferation and stimulate collagen synthesis (Schmidt et al, 1984; Prostlethwaite et al, 1984; Goldring & Krane, 1986; Bronson et al, 1987). However, the molecular mechanisms by which silica causes lung disease are not known.

Several studies have shown that inducible nitric

Corresponding to: Jihee Lee, College of Medicine, Ewha Womans University and Division of Cell Biology, Ewha Medical Research Center, 911-1 Mok-6-dong, Yangcheon-ku, Seoul 158-056, Korea (Tel) 82-2-650-5719, (Fax) 82-2-654-4971, e-mail: jihee@mm.ewha.ac.kr

oxide synthase (iNOS) mRNA expression, protein, and nitirc oxide (NO ·) production can be induced in alveolar macrophages and polymorphonuclear neutrophils by specific stimuli. For instance, iNOS mRNA expression in murine macrophages is increased by interferon γ (INF γ) and lipopolysaccharide (Lyons et al, 1992). Jorens et al(1992) demonstrated that nitrite production in IFN γ -stimulated alveolar macrophages was enhanced by granulocyte macrophages-colony- stimulating factor (GM-CSF) and muramyl dipeptide, a constituent of the bacterial cell wall. Neutrophils have also been shown to produce nitric oxide in response to LPS (Yui et al, 1991). Not only inflammatory cells, structural cells such as airway epithelial cells (Munakata et al, 1990) and lung fibroblasts (Jorens et al, 1992_b) are but also capable of a source of iNOS dependent-nitric oxide following exposure to cytokines such as TNF α and IFN γ .

Nitric oxide production in small amounts locally by constitutive NOS (cNOS) activation may be beneficial in relaxing airway smooth muscle in airway, but much higher concentrations of nitric oxide from iNOS may have deleterious effects on the airway and lung parenchyma. High concentrations of nitric oxide contributed to the epithelial shedding observed in asthmatic patients (Radomski et al, 1990). N ω -nitro-L-arginine methylester (L-NAME), NOS specific inhibitor, protected rats against lung injury induced by injection of immune complex and suppressed the rise in nitrite levels in broncho-alveolar lavage fluid of these animals (Mulligan et al, 1991), providing further evidence for the involvement of nitric oxide in this process.

Recent evidence suggests that alveolar macrophages- and neutrophils-derived nitrogen free radicals may have important roles in mediating cytotoxicity through damping DNA and inhibiting the mitochondrial respiratory cycle (Stuehr & Nathan, 1989; Wink et al, 1993). Nitric oxide may also combine with superoxide anion to produce an even more cytotoxic molecule, peroxynitrite ($ONOO^-$)(Koppenol et al, 1992) which is a long-lived, reactive oxidant that contributes to inflammatory tissue damage.

Since damage to the lung parenchyma may result from silica-induced activation of pulmonary phagocytes to release reactive species that in turn, injure lung cells, it is hypothesized that silica may induce iNOS mRNA expression and increase nitirc oxide in these cells. In the present study, this hypothesis was tested by evaluating acute inflammatory lung injury following exposure to silica and measuring iNOS mRNA expression and nitric oxide production in broncho-alveolar lavage cells.

METHODS

Experimental design

The experimental design consisted of two groups of rats (specific pathogen-free male Sprague-Dawley rats weighing 260~290 g): (1) a control group that received an intratracheal treatment of 1 ml of LPS-free saline (0.9% NaCl); (2) a silica-treated group that received an intratracheal treatment of 5 mg silica (Crystalline Min-U-Sil; U.S. Silica Corp.; 98% of the quarts <5 μm in diameter) in 1 ml LPS-free saline.

Intratracheal instillation

Rats were anesthetized with intraperitoneal injections of 0.5 ml of a 1% solution of sodium methohexital. Once the rats were anesthetized, rats were intratracheally instilled with saline or silica using 20-gauge, 4-inch ball-tip animal feeding needle (Brain et al, 1976).

Isolation of broncho-alveolar lavage cells and cell counts

4 h after treatment, the rats were anesthetized with 0.2 g/kg body weight of sodium pentobarbital. The trachea was then cannulated and the lungs were lavaged 10 times with 8 ml aliquots of Ca^{++}, Mg^{++} -free Hanks balanced salt solution (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, and 5.5 mM dextrose; pH=7.4). The broncho-alveolar lavagage was centrifuged ($500\times g$ for 5 min), and the broncho-alveolar cells were washed and resuspended in Hepes-buffered solution (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 5.5 mM dextrose, and 1.0 mM $CaCl_2$; pH=7.4). Cell counts and differentials were determined using an electronic coulter counter with a cell sizing analyzer (Coulter Model ZBIwith a channelizer 256; Coulter Electronics, FL, USA) as described by Lane and Mehta (1990). Red blood cells (RBC), lymphocytes, polymorphonuclear neutrophils, and alveolar macrophages were distinguished by their characteristic cell volumes (Castranova et al, 1990).

Lactate dehydrogenase (LDH) activity and total protein measurement

The LDH activity and total protein were measured in the first aliquot of the acellular broncho-alveolar lavage fluid. The activity of LDH, a cytosolic enzyme used as a marker for cytotoxicity, was measured by the method of Cabaud & Wroblewski (1958). Using bovine serum albumin as the standard, the measurement of total protein was performed according to the method of Hartreea (1972) to assess the permeability of the bronchoalveolar-capillary barrier.

Total RNA isolation and northern blot analysis

The broncho-alveolar lavage cells from individual rats were centrifuged (500×g for 10 min). Total cellular RNA was extracted by a modification of the acidic guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). The RNA pellet was vacuum-dried for 0.5 h and resuspended in 0.5% sodium dodecyl sulfate (SDS). The RNA was quantified in a spectrophotometer at 260 nm. Total cellular RNA (20 μg) was diluted in loading buffer (N-morpholinopropane-sulfonic acid [MOPS], 40% formamide, and 15% formaldehyde). The RNA was electrophoresed on a 5.6% formaldehyde denaturing 1% agarose gel. The RNA was vacuum-transferred onto a Nytran membrane. The membrane was washed with 2×SSC, dried at 65°C, and then UV crosslinked. The membrane was prehybridized at 68°C for 30 min with 5 ml Quickhyb® hybridization buffer plus 200 μl of 10 mg/ml herring sperm DNA boiled for 10 min. Subsequently, the ^{32}P-α-dCTP labeled murine macrophage iNOS ssDNA probe (5×10^7 cpm) was added, and the hybridization was performed for 1 h at 68°C. The membrane was washed at low stringency 3 times with 50 ml of 2×SSC, 0.1% SDS at room temperature; washed again at high stringency for 30 min with 50 ml of 0.2×SSC, 0.1% SDS at 60°C; and finally apposed to Kodak X-ray film at −70°C.

Nitrite and nitrate assay in cultured broncho-alveolar lavage cells

Broncho-alveolar lavage cells were suspended in MEM (medium essential medium) with 10% fetal bovine serum, 2 mM glutamine, 100 u/ml penicillin, and 100 μg/ml streptomycin at a final concentration of 1×10^6 alveolar macrophages/ml. From cell suspension, 1 ml aliquots were added to 24 well plates (Castar, MA, USA) and incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2. The nonadherent cells were then removed with two 1 ml washes of the frech MEM. The adherent cells were further incubated in 1 ml of the MEM in the presence or absence L-NAME (1 mM), L-NAME (1 mM)+ L-arginine (10 mM). After incubating for 24 h, the cell cultures were centrifuged at 500 g for 15 min and the supernates frozen at −70°C until they were assayed.

Nitrite was assayed after adding 100 μl Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 50 μl samples of cell culture. Optical density at 550 nm (OD_{550}) was measured using a microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in cell culture medium and presented as nmole/mg protein. To measure total nitrate (nitrite plus nitrate), nitrate in the samples was first reduced to nitrite with nitrate reductase, and the nitrite reaction was then determined with Greiss reagent.

Statistical analysis

Data were expressed as means±standard errors of

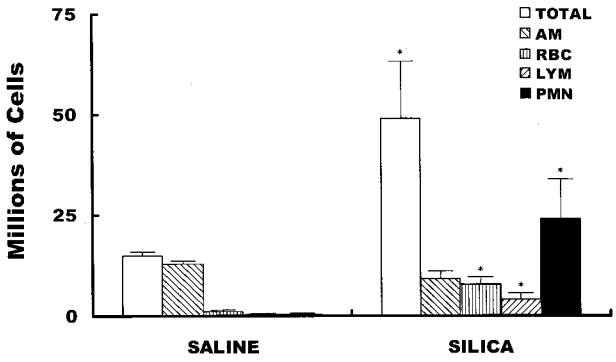

Fig. 1. Cell counts and differentials in broncho-alveolar lavage cells. The lavage cells were obtained 4 h after intratracheal treatment of saline (1 ml saline) or silica (5 mg in 1 ml saline). Using an electronic coulter counter with a cell sizing analyzer, total cells (TOTAL) were counted and alveolar macrophages (AM), red blood cells (RBC), lymphocytes (LYM), and polymorphonuclear neutrophils (PMN) distinguished by their characteristic cell volumes. Values represent means±SEM (n=4). *$p<0.05$ for significant difference compared with the saline control.

separate experiments. Statistical significance was determined using a Student t-test with significance set at $p<0.05$.

RESULTS

The number of total cells isolated in broncho-alveolar lavage fluid was significantly increased by 3-fold in the silica-treated rats compared with saline-treated rats ($p<0.05$)(Fig. 1). In addition, differential analysis of these cells showed that the number of RBC, lymphocytes and polymorphonuclear neutrophils were also significantly increased by 9-, 14, and 119-fold in the silica-treated group, respectively, above the level of saline control ($p<0.05$). In contrast, the number of alveolar macrophages harvested by lavage slightly declined after exposure to silica

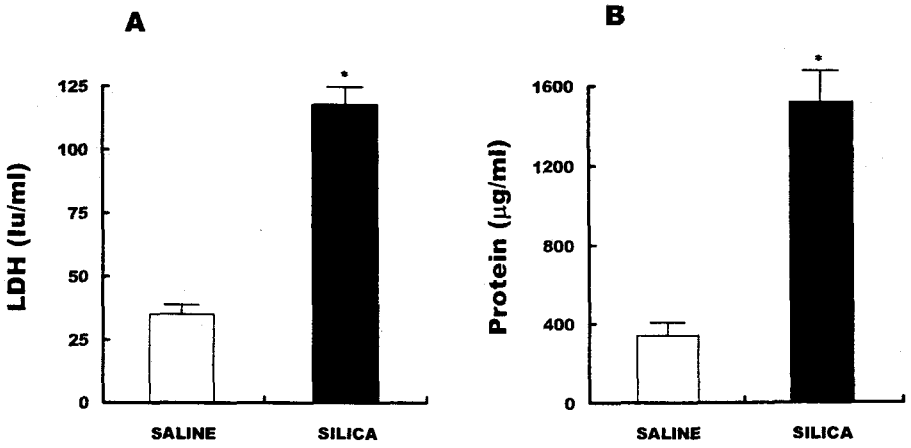

Fig. 2. The activity of LDH (A) and total protein (B) present in broncho-alveolar lavage fluid. Broncho- alveolar lavage fluid was recovered from the lungs 4 h after the intratracheal treatment of saline (1 ml saline) or silica (5 mg in 1 ml saline). Values represent means±SEM (n=4). *$p<0.05$ for significant difference compared with the saline control.

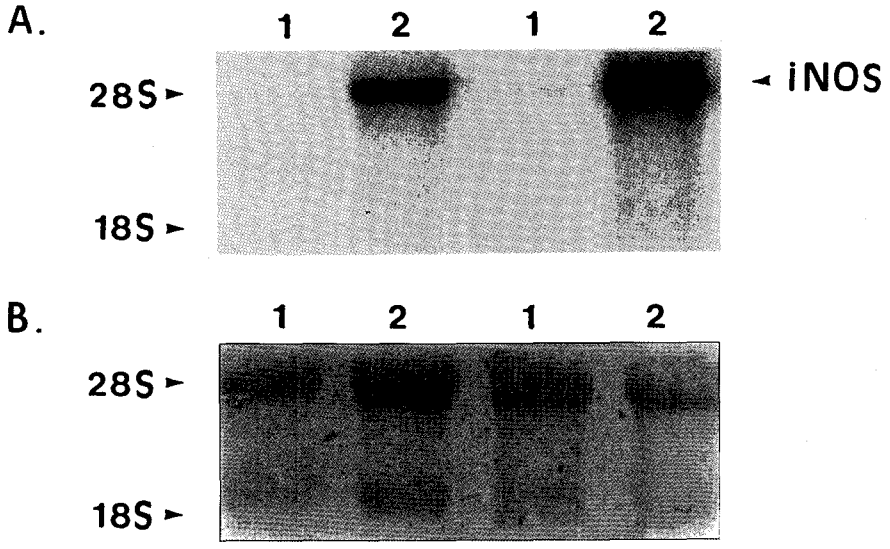

Fig. 3. Northern analysis in broncho-alveolar lavage cells showing autoradiographs of blots probed with murine macrophage iNOS ssDNA probe (A) and methylene blue staining of the rRNA (B). Intratracheal treatment groups include the following: saline (1 ml saline; 1 lane) and silica (5 mg in 1 ml saline; 2 lane).

below the the saline control.

The LDH activity and total protein in the lavage fluid were measured to evaluate acute lung injury following a 4 h exposure to silica. As shown Fig. 2, the LDH activity and total protein were significantly increased by 3- and 4-fold in silica-treated group, respectively, compared with the saline control ($p<0.05$).

Fig. 3 shows a Northern blot demonstrating an increased iNOS-specific mRNA expression after silica treatment, indicating up-regulation of iNOS mRNA

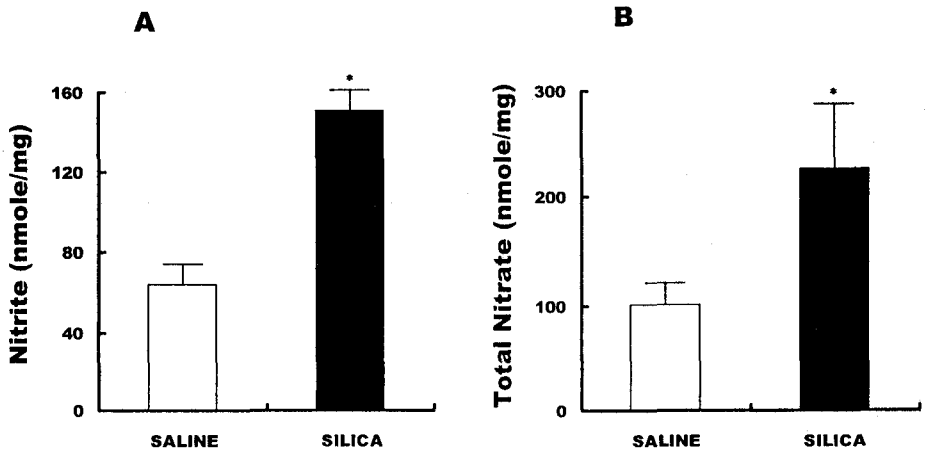

Fig. 4. The productions of nitrite (A) and total nitrate (B) in broncho-alveolar lavage cells cultured for 24 h. Rats were given an intratracheal treatment of saline (1 ml saline) or silica (5 mg in 1 ml saline). After a 4 h exposure, broncho-alveolar lavage cells including alveolar macrophages (1×10^6/ml) were incubated for 24 h and the supernates assayed for the presences of nitrite and nitrate as described in "Materials and Methods." Values represent means±SEM (n=4). * $p<0.05$ for significant difference compared with the saline control.

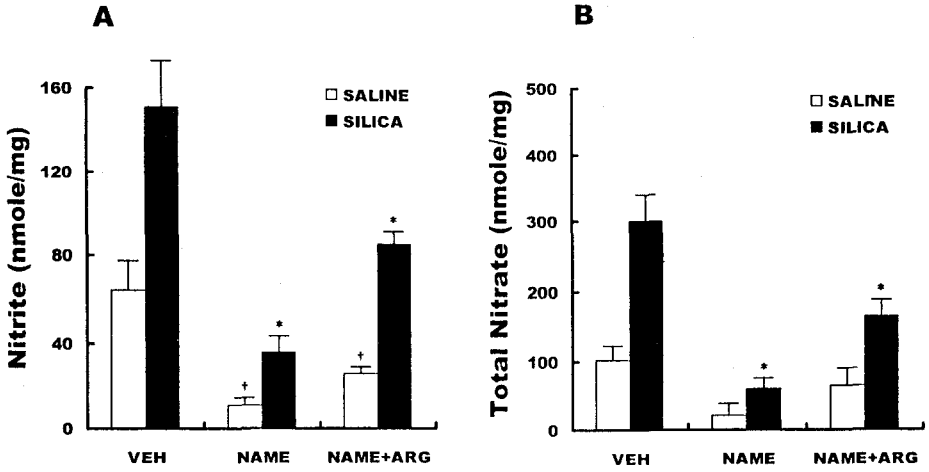

Fig. 5. The productions of nitrite (A) and total nitrate (B) in broncho-alveolar lavage cells cultured for 24 h. Rats were given an intratracheal treatment of saline (1 ml saline) or silica (5 mg in 1 ml saline). After a 4 h exposure, broncho-alveolar lavage cells including alveolar macrophages (1×10^6/ml) were incubated for 24 h in the presence or absence of L-NAME (1 mM), L-NAME (1 mM)+L-arginine (10 mM) and the supernates assayed for the presence of nitrite and nitrate as described in "Materials and Methods." Values represent means±SEM (n=4). * $p<0.05$ for significant difference compared with the silica vehicle and † $p<0.05$ for significant difference compared with the saline vehicle.

steady state levels in broncho-alveolar lavage cells.

Nitrite and nitrate which are oxidation end products of nitric oxide, were measured in broncho-alveolar lavage cells cultured after exposure to saline or silica. Silica treatment significantly increased nitrite as well as total nitrate by 2-fold above the levels of saline control ($p<0.05$)(Fig. 4). These silica-induced productions were completely inhibited by iNOS inhibitor, L-NAME, and furthermore, addition of L-arginine to the culture with L-NAME partially reversed L-NAME-induced suppression of nitrite and total nitrate, indicating L-arginine-dependent productions of nitrite and nitrate in the lavage cells (Fig. 5).

DISCUSSION

The results show that intratracheal treatment of silica caused increases in the number of RBC, lymphocytes, and polymorphonuclear neutrophils harvested by lavage. These findings are consistent with an inflammatory injury to the respiratory air-blood barrier (Beck et al, 1982). However, there was a decrease in the number of alveolar macrophages obtained by broncho-alveolar lavage in silica-treated rats, since activated macrophages after exposure to silica may adhere more tightly to the air ways and be more difficult to remove by broncho-alveolar lavage (Willoughby et at, 1986). It was also found that the LDH activity and total protein were significantly increased in broncho-alveolar lavage fluid obtained from silica-treated rats, indicating silica-induced damage to the lung parenchyma and the alveolar-capillary barrier.

An increase in iNOS mRNA steady-state levels in the lavage cells including mainly alveolar macrophages and polymorphonuclear neutrophils was found. Furthermore, the productions of nitrite and nitrate in these 24 h cultured cells were significantly increased and this pathway was L-arginine dependent. The parallel increases in the parameters indicating inflammatory lung injury and nitric oxide production suggests that a relationship may exist between inflammatory lung injury and nitric oxide production. Nitric oxide has been implicated in the inflammatory response following exposure to specific stimuli. For instance, the production of iNOS protein as well as nitric oxide by alveolar macrophages have been shown following the inhalation of the pulmonary irritant ozone (Pendino et al, 1993). Mulligan et al (1991) demonstrated that immune complex-induced lung injury was protected by the presence of L-arginine analogue, N^G-monomethyl-L-arginine (N-MeARG). They also observed increases in the productions of nitrite and nitrate in broncho-alveolar lavage fluid from animals undergoing immune complex deposition in lung. Nitrite production from activated alveolar macrophages following intratracheal treatment of bleomycin has been also reported by Huot & Hacker (1990). L-arginine analogue, N^6- monomethylarginine (NMA) suppressed either DNA synthesis inhibition or aconitase activity reduction induced in alveolar macrophages from bleomycin-treated rats. Corticosteroids have been exhibited to potently inhibit the expression of iNOS and nitric oxide production (Di Rosa et al, 1990), suggesting part of the anti-inflammatory and immunosuppressive actions of glucocorticoids due to their inhibition of the induction of iNOS.

In conclusion, this study has been shown that intratracheal treatment of silica induces iNOS mRNA expression and results in nitric oxide production in alveolar macrophags and polymorphonuclear neutrophils. Nitric oxide as a reactive free radical may contribute in part to acute inflammatory lung injury.

ACKNOWLENDGEMENTS

This work was supported by a grant from Ewha Womans University.

REFERENCES

Beck BD, Brain JD, Bohannon DE. An in vivo hamster bioassay to assess the toxicity of particulates for the lungs. *Toxicol Appl Pharmacol* 66: 9−29, 1982

Brain JO, Knudson DE, Sorokin SP, Davis MA. Pulmonary distribution of particles given by intratracheal instillation or by aerosol inhalation. *Environ Res* 11: 13−33, 1976

Bronson R, Bertolami C, Siebert E. Modulation of fibroblast growth and glycosaminoglycan synthesis by interleukin-1. *Coll Relat Res* 7: 323−332, 1987

Cabaud PG, Wroblewski F. Colormetric measurement of lactic acid dehydrogenase activity of body fluids. *Am J Clin Path* 40: 234−236, 1958

Castranova V, Jones T, Barger MW, Afshari A, Frazer DJ. Pulmonary responses of guinea pigs to consecutive exposures to cotton dust. In: Jacobs RR, Wakelyn PJ,

Domelsmith LN ed, *Proceedings of the 14th Cotton Dust Research Conference.* National Cotton Council, Memphis, TN, p 131−135, 1990.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156−159, 1987

Davis GS. Pathogenesis of silicosis: current concepts and hypotheses. *Lung* 164: 139−154, 1986

deShazo RD. Current concepts about the pathogenesis of silicosis and asbestosis. *J Allergy Clin Immunol* 70: 41−49, 1982

Di Rosa M, Radomski M, Carnuccio R, Moncada S. Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. *Biochem Biophys Res Commun* 172: 1246−1252, 1990

Fubini B. The surface chemistry of crushed quartz dust in relation to its pathogenicity. *Org Chem Acta* 183: 193−197, 1987

Futone JC, Ward PA. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *Am J Pathol* 107: 397−418, 1982

Goldring MB, Krane SM. *Modulation of collagen synthesis in human chondrocyte cultures by interleukin 1.* J Bone Mineral Res 1 (suppl. 1), p56, 1986

Hartree GF. Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal Biochem* 48: 422−427, 1972

Heppleston AG. Silicotic fibrogenesis: a concept of pulmonary fibrosis. *Ann Occup Hug* 26: 449−462, 1982

Huot AE, Hacker MP. Role of reactive nitrogen intermediate production in alveolar macrophage-mediated cytostatic activity induced by bleomycin lung damage in rats. *Cancer Resear* 50: 7863−7866, 1990

Jorens PG, van Overveld FJ, Bult H, Vermeire PA, Herman AG. Muramyl dipeptide and GM-CSF enhance interferon-γ-induced nitric oxide production by rat alveolar macrophages. *Agents Actions* 36: 243−247, 1992_a

Jorens PG, van Overveld FJ, Bult H, Vermeire PA, Herman AG. Synergism between interleukin-1 β and the nitric oxide synthase inducer interferon-γ in rat lung fibroblasts. *Eur J Pharmacol* 224: 7−12, 1992_b

Koppenol WH, Moreno JJ, Dryor WA, Ischiropoulos H, Beckman JS. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem Res Toxicol* 5: 834−842, 1992

Lane FC, Mehta JR. In vitro human tumor sensitivity assay using cell counting and sizing. *AM Biotech Lab* 8: 12−27, 1990

Lyons CR, Orloff GJ, Cunningham JM. Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J Biol Chem* 267: 6370−6374, 1992

Martin JC, Daniel-Moussard H, LeBouffant L, Policard A. The role of quartz in development of coal workers pneumoconiosis. *Ann NY Acad Sci* 200: 122−141, 1972

Mulligan MS, Hevel JM, Marletta MA, Ward PA. Tissue injury caused by deposition of immune complexed in L-arginine-dependent. *Proc Natl Acad Sci USA* 88: 6338−6342, 1991

Munakata M, Masaki Y, Saxuma I, Vkita H, Obuka Y, Homma Y. Pharmacological differentiation of epithelium-derived relaxing factor from nitric oxide. *J Appl Physiol* 69: 665−670, 1990

Pendino JK, Laskin JD, Shuler RL, Punjabi CJ, Laskin DL. Enhanced production of nitric oxide by rat alveolar macrophages after inhalation of a pulmonary irritant is associated with increased expression of nitric oxide synthase. *J Immunol* 151: 7196−7205, 1993

Prostlethwaite AE, Lachman LB, Kang AH. Induction of fibroblast proliferation by interleukin-1 derived from human monocytic leukemia cells. *Arthritis Rheum* 27: 995−1001, 1984

Radomski MW, Palmer RMJ, Moncada S. Gluco-corticoids inhibit the expression of an inducible, but not the constitute nitric oxide synthase in vascular endothelial cells. *Prod Natl Acad Sci USA* 87: 10043−10047, 1990

Schmidt JA, Oliver CN, Lepe-Zuniga JL, Green I, Gery I. Silica-stimulated monocytes release fibroblast proliferation factors identical to interleukin 1: a potential role for interleukin 1 in the pathogenesis of silicosis. *J Clin Invest* 73: 1462−1472, 1984

Stuehr DJ, Nathan CF. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* 169: 1543−1555, 1989

Vallyathan V, Shi X, Dalal NS, Irv W, Castranova V. Generation of free radicals from freshly fractured silica dust: Potential role in acute silica-induced lung injury. *Am Rev Respir Dis* 138: 1213−1219, 1988

Willoughby WF, Willoughby GF, Gerberick GF, Jaffe HA. Mechanism of pulmonary injury caused by inhaled Enterobacter agglomerans lipopolysaccharide. In: Jacobs RR, Wakelyn PJ ed, *Proc Tenth Cotton Dust Res Conf.* National Cotton Council, Memphis, TN, USA, p 78−81.

Wink DA, Osawa Y, Darbyshire F, Jones CR, Eshenaur SC, Nims RW. Inhibition of cytochromes p450 by nitric oxide and a nitric oxide-releasing agent. *Arch Biochem Biophys* 300: 115−123, 1993

Yui YR, Hattori R, Kosuga K, Eizuwa H, Miki K, Ohkawa S, Ohnishi K, Terao S, Kawai C. Calmodulin-independent nitric oxide synthase from rat polymorphonuclear neutrophils. *J Biol Chem* 266: 3369−3371, 1991