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Silica-Induced Pulmonary Inflammation in Rats: Activation of NF-κB and Its Suppression by Dexamethasone

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The goal of this study was to examine the relationship of the transcriptional regulatory factor nuclear factor-kappaB (NF-κB) to the early inflammatory events involved with silica exposure. Male F-344 rats received an intratracheal (i.t.) instillation of silica (100 mg/kg in a volume of 1 ml/kg) of saline. At 1, 3, 6, and 18 h postinstillation, and the rats were sacrificed and underwent bronchoalveolar lavage (BAL) for functional analysis of inflammation. Beginning at 1 h postinstillation, the silica-instilled (Si) rats displayed significant increases in neutrophils in BAL fluid compared to the saline controls. BAL cells from the Si group displayed a significant increase in luminol-dependent chemiluminescence (LDCL) compared to the controls. NF-κB activation was measurable at 3 h postinstillation, and this activation continued throughout the 18-h time course. Treatment with dexamethasone (5 mg/kg) at −3 h prior to silica instillation, at the time of instillation (0 h), and +1.5 h postinstillation resulted in both a reduction in NF-κB expression (by 70%) at 3 h postinstillation and corresponding reductions in LDCL, BAL cell count, and BAL neutrophils. These results show that activation of NF-κB is associated with silica-induced pulmonary inflammation, and the inhibition of its activation correlates temporally with suppression of inflammation.

Inhalation of respirable particles can present an occupational hazard to human lungs. This toxicity results from inflammation/damage to the respiratory epithelium and interstitial matrix thereby decreasing efficiency of blood-oxygen exchange (1–3). Silica (SiO_2) and asbestos are prime examples of such toxic, respirable particles. The best example of silica toxicity is the Hawk's Nest Incident (4) which occurred in the early 1930's in West Virginia. Miners were exposed to extremely high concentrations of crystalline silica dust while drilling a tunnel which was poorly ventilated. Within several months, workers died of silicosis or tuberculosis which result from this exposure thus causing the greatest industrial accident in U.S. history (4).

Exposure to silica can result in this inflammation characterized by a tremendous influx of neutrophils and other inflammatory cells as well as massive increases in inflammatory cytokine secretions, increased adhesion proteins and molecular receptors on the inflammatory cells (5–8). Inducible proteins and oxidases such as NO synthase (i-nos, type II), tumor necrosis factor alpha (TNF-α), and interleukin-one (IL-1) (8–10) play an important role in the inflammatory process.

These inflammatory secretions as well as others such as interleukin-two (IL-2), interleukin-six (IL-6) and interleukin-eight (IL-8) are all under control of a common genetic transcription factor known as nuclear factor-kappaB (NF-κB) (11). NF-κB is a heterodimeric protein complex containing two members of the rel family of transcription factors, p50 and p65. In the cytoplasm of most cells, the heterodimeric NF-κB complex is bound to an inhibitory factor, I KappaB. Upon stimulation or challenge with a foreign substance, IκBα is separated and becomes vulnerable to proteolytic degradation. Free NF-κB heterodimer then translocates into the nucleus where it binds to the kappaB promoter regions of selected inflammatory genes and upregulates or inhibits transcription of these genes (12).

NF-κB expression in macrophage cell lines has been shown to increase upon silica challenge *in vitro* (13, 16), but we are not aware of any studies that have examined this process in the lungs *in vivo.* If NF-κB is a key to the common control of many inflammatory genes, a drug which blocks the induction of this mech-

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anism would be useful in treatment of the pulmonary damage. Anti-inflammatory steroids, e.g., dexamethasone, act by binding with a steroid receptor in the cytoplasm which translocates to the nucleus and blocks the promoter site of kappaB promoters. This prevents the NF-κB complex from binding and stimulating or depressing transcription of these inflammatory genes *in vitro* (17). The steroids may stimulate the production and/or attachment of Ikβ to the heterodimer (p50, p65) as well (18).

The goals of the present study were twofold. First, we measured the effects of intratracheal instillation of silica to rats on the inflammatory response and NF-κB expression. Secondly, we treated rats with dexamethasone prior to and following silica administration, and examined whether silica-induced pulmonary inflammation and NF-κB expression could be inhibited by this anti-inflammatory corticosteroid.

MATERIALS AND METHODS

Chemicals and reagents. The silica particles (alpha-quartz) were originally from Pennsylvania Sand and Glass Co. (Pittsburgh, PA). The size of the particles was 5 microns or less, with silica content 98.05%. Luminol, Hepes, and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Hanks' Balanced Salt Solution was obtained from Gibco Lab., Life Technologies, Inc. (Grand Island, NY). Sodium pentobarbital was obtained from The Butler Company, Columbus, OH. Brevital was obtained from Eli Lilly & Co. (Indianapolis, IN). Dexamethasone was bought from (Fujisawa, USA); the final concentration of the drug was 4 mg/ml. Lipopolysaccharide (from *E. coli* 0111:B4) was purchased from Sigma.

Animals and treatment. All rats used in this experiment were male F-344 from Hilltop Labs (Scottdale, PA), weighing approximately 250-300 g. After arrival, animals were allowed to acclimate in the animal quarters for one week. Animals were randomly divided into four groups with four to six rats per group. The rats were anesthetized with Brevitol (0.7 ml, 10 mg/ml), suspended by the maxillary incisors on a wire and the tongue was moved to the side. At time 0, silica was administered intratracheally (i.t.) at a dose of 100 mg/kg (1 ml/kg). Controls received an equal volume of sterile saline. At times -3, 0, and $+1.5$ h, saline- and silica-treated rats received dexamethasone (5 mg/kg, i.p.) or an equal volume of sterile saline. The experimental protocol resulted in four groups. Group 1 (saline/i.t.–saline/i.p.); Group 2 (saline/i.t.–dexamethasone/i.p.); Group 3 (silica/i.t.–saline/i.p.); Group 4 (silica/i.t.–dexamethasone/i.p.). As a positive control, LPS was administered (i.t.) at a dose of 5 μg/kg (10 μg/ml).

Bronchoalveolar lavage (BAL) procedure. At the appropriate time point, rats were anesthetized with 0.7 ml of sodium pentobarbital (64.8 mg/ml) and exsanguinated by cutting the abdominal aorta. A lavage tube (PE 160) was placed into the trachea and the diaphragm was cut. While massaging the lungs, 2 ml/100 g body wt of calcium and magnesium-free HBSS were instilled into the lungs. Fluid was withdrawn and placed in a 50-ml tube. Lavage continued until 100 ml of fluid had been collected. After lavage, tubes were centrifuged at 2400*g* for 7 min. All pellets were combined for each rat while discarding remaining supernatant fluids. Cells were recentrifuged and the pellet resuspended in 1.0 ml HBSS for cell counting. Cells were counted via hematocytometer. Cellular differentials (alveolar macrophages, neutrophils [PMNs], and lymphocytes) were determined on slides stained with Wright–Giemsa stain. Four hundred cells from each animal were counted.

Luminol-dependent chemiluminescence (LDCL) of bronchoalveolar cells. Cell number was adjusted to 10^6/ml. Luminol (10^{-4} M) was first dissolved in DMSO and then diluted to 10^{-5} M in calcium-containing Hepes buffer (0.1 M, pH 7.4). The volume in each cuvette was adjusted to 500 μl with the Hepes buffer. There were 100 μl of cells, 100 μl of luminol and 100 μl of calcium-containing Hepes buffer in each cuvette as common components. Calcium-containing Hepes buffer was made by adding $CaCl_2$ to regular Hepes buffer to a final concentration of 4 mM. The reaction was started by adding 100 μl phorbol myristate acetate (PMA) (10 mg/ml) or 100 μl of Hepes buffer. Chemiluminescence was followed for 20 min at 37°C using a Berthold LB9505C Luminometer (Wildbad, Germany). The integrated response was determined with a KINB program supplied with the luminometer.

Nuclear protein extractions. Nuclear protein extracts were prepared from BAL cells from the different groups. Aliquots of cells were mixed with liquid nitrogen, ground to a powder with mortar and pestle. Four milliliters of solution A (0.6% Nonidet P-40 detergent, 150 mM NaCl, 10 mM Hepes, pH 7.9, 1 mM EDTA, and 0.5 mM PMSF) were added to the mortar. The contents from the mortar were placed in a Dounce tissue homogenizer (Vineland, NJ) and the cells lysed with five strokes of the pestle. After transfer to a 15-ml tube, debris was pelleted at 2000 rpm for 30 s. The supernatant solution containing intact nuclei was transferred to 50-ml Corex tubes, incubated on ice for five minutes and centrifuged for 10 min at 5000 rpm. Nuclear pellets were resuspended in 300 μl solution B (25% glycerol, 20 mM Hepes, pH 7.9, 420 mM, NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDNA, 0.5 mM ETT, 0.5 mM PMSF, 2 mM leupeptin and 0.5 μg/ml aprotinin) and incubated on ice for 30 min. The mixture was transferred to microcentrifuge tubes, and nuclei were pelleted by centrifugation at 14,000 rpm for 1 min. Supernatant solutions containing nuclear proteins were saved, aliquoted and stored at -70°C. Protein quantitation was performed using the assay of Bradford (Pierce, Rockford, IL).

Electrophoretic mobility shift (EMSA). The oligonucleotide used as a probe for EMSA is a 25-bp double standard construct (5′TGTGCTCCGGGAATTTCCCTGGCCT) corresponding to the sequence (-70 to -45) in the CINC proximal promoter region containing the NF-κB motif (underlined). End labeling of the complimentary strand was accomplished by treatment with T4 kinase in the presence of [^{32}P]ATP. Labeled oligonucleotides were column purified on a Sephadex G-25M column (Pharmacia Biotech., Inc., Piscataway, NJ).

Labeled double stranded probe (approx. 40,000 cpm) was added to 5 microgram of nuclear protein in the presence of a nonspecific blocker, salmon testes DNA (0.1 μg/μl). The binding reaction also contained 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40 and 0.5 mM dithiothreitol (DTT). This mixture was incubated at 25°C for 20 min and separated by electrophoresis on a 5% polyacrylamide gel in 1× Tris glycine EDTA buffer. Gels were vacuum dried and subjected to phosphoroimager (CyQuant). The phosphoroimager was subjected to densitometry using the CyQuant program. Cold competition was performed by adding 100 ng of specific unlabeled double-stranded probes to the reaction mixture. Nonspecific competition was performed by adding 100 ng of unlabeled double-stranded mutant CINC oligonucleotide (5′-CCTGTGCTCCAATTTCCCTGGCCTGGA-3′) that does not bind NF-κB.

Statistics. Data were analyzed using ANOVA and Tukey's protected "*t*" post-hoc test using the GBStat statistical program. Statistical significance was set at $p < 0.05$.

RESULTS

Cell Count and Cell Differentials Without and With Dex Treatment

The intratracheal instillation of silica led to a significant increase in PMNs in BAL fluid (Fig. 1). This increase was prevented by the treatment with dexa-

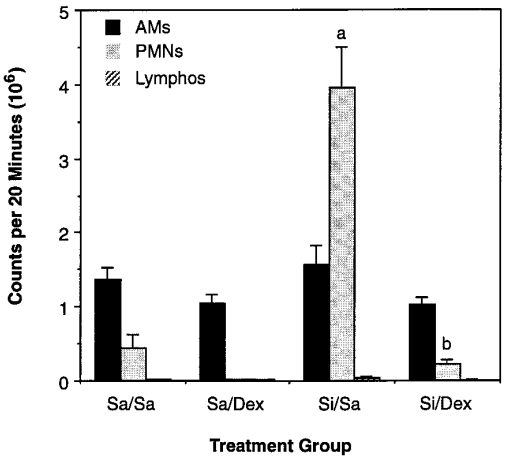

FIG. 1. Differential counts of cells in BAL recovered from rats 3 h after silica instillation. The groups represent rats treated as follows: Sa/Sa, saline-i.t./saline-i.p.; Sa/Dex, saline-i.t./dexamethasone-i.p.; Si/Sa, silica-i.t./saline-i.p.; Si/Dex, silica-i.t./dexamethasone-i.p. Following treatments, cells were collected by BAL, counted and stained with Wright–Giemsa. AMs, alveolar macrophages; PMNs, neutrophils; Lymphos, lymphocytes. Values represent means ± SEM (n = 3). [a]Significantly different from Sa/Sa and Sa/Dex ($p < 0.05$). [b]Significantly different from Si/Sa ($p < 0.05$).

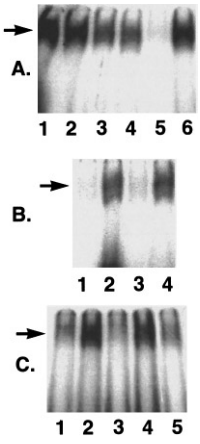

FIG. 2. (A) *In vivo* activation of NF-κB in BAL cells. Lane 5 is from a saline-instilled rat (negative control) at 1 h postinstillation of silica. Lane 6 is from an LPS-instilled rat (positive control) at 1 h postinstillation. Lane 1–18 h postinstillation of silica; Lane 2, 6 h postinstillation; Lane 3, 3 h postinstillation; Lane 4, 1 h postinstillation. NF-κB band (arrow). (B) Confirmation of NF-κB expression. Lanes 2 and 4 are NF-κB expression from BAL cells at 3 h postinstillation of silica; Lanes 1 and 3 are same as Lanes 2 and 4 with cold competitor added. NF-κB band (arrow). (C) Suppression of NF-κB expression in BAL cells by dexamethasone. Lanes 2 and 4 are silica-induced NF-κB expression in BAL cells at 3 h postinstillation; Lanes 1, 3, and 5 are silica-induced NF-κB expression in BAL cells at 3 h postinstillation from rats treated with dexamethasone. NF-κB band (arrow).

methasone. There was no significant change in the recovery of alveolar macrophages or lymphocytes with any of the treatments.

In Vivo Activation of NF-κB and Suppression by Dexamethasone

In BAL cells from saline-treated rats, there was essentially no detectable NF-κB expression (Fig. 2A, lane 5). With silica instillation, activation of NF-κB was detected in BAL cells by 1 h and was apparent as long as 18 h postinstillation (Fig. 2A, lanes 1–4). LPS was instilled in a rat, i.t. (5 μg/kg) to serve as a positive control (Fig. 2A, lane 6).

To ensure that we were measuring NF-κB, we also ran samples at 3 h with cold competition (Fig. 2B). An unlabeled NF-κB, DNA sequence that is 1000× the concentration of the ^{32}P labeled sequence, was added to the binding reaction. The addition of the cold competitor inhibited labeling (lanes 1 and 3) and, therefore, the signal response induced by silica instillation (lanes 2 and 4) was specific for NF-κB.

After confirming NF-κB activation by silica instillation, we tested whether dexamethasone treatment could affect this activation (Fig. 2C). Lanes 1, 3, and 5 show that dexamethasone treatment reduced NF-κB expression in BAL cells. This reduction was approximately 70% according to densitometric scans (data not shown).

LDCL

Finally, inflammation as measured by redox-based light production during PMA-stimulation of BAL cells is shown in Fig. 3. BAL cells in the silica/saline (Si/Sa) group produced over a 100-fold increase in chemiluminescence compared to its control (Sa/Sa),

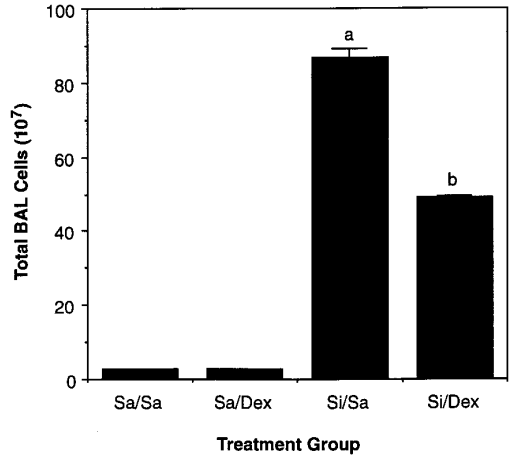

FIG. 3. Luminol-dependent chemiluminescence of PMA-stimulated BAL cells from rats recovered 3 h after silica instillation. The groups represent rats treated as follows: Sa/Sa, saline-i.t./saline-i.p.; Sa/Dex, saline-i.t./dexamethasone-i.p.; Si/Sa, silica-i.t./saline-i.p.; Si/Dex, silica-i.t./dexamethasone-i.p. Following treatments, cells were collected by BAL and subjected to Luminol-dependent chemiluminescence. Values represent means ± SEM (n = 3). [a]Significantly different from Sa/Sa and Sa/Dex ($p < 0.05$). [b]Significantly different from the other three groups.

whereas cells in the silica/dexamethasone (Si/Dex) treatment group showed a significant reduction in light production compared to those in the Si/Sa group.

DISCUSSION

The work presented in this report was performed in order to investigate and characterize the events that occur in the lungs as a result of acute silica exposure. In our system, intratracheally instilled silica resulted in an inflammatory response as assessed by inflammatory cell influx into the alveoli and inflammatory cell chemiluminescence. This has been reported previously by us (7) and others (19). We found that, indeed, NF-κB activation is closely associated with the inflammatory response. This is significant in two ways. First this is the first time that we are aware that NF-κB has been shown to be activated *in vivo* in the lungs as a result of silica exposure. Second, inhibition of the activation of NF-κB by dexamethasone was associated with a decrease in inflammation.

Silica-induced NF-κB has been shown to be inhibitable in a macrophage cell line *in vitro* by tetrandrine (16), a Chinese herbal compound used to treat silicosis. This effect was attributed to tetrandrine's ability to suppress the silica-induced degradation of IκBα. Our results demonstrate that NF-κB activation in the lungs can be inhibited with systemic administration of dexamethasone.

The rationale for this study was that inflammatory factors such as i-nos and TNF-α, which have been shown to play an integral role in silica-induced pulmonary inflammation (20, 21), are under transcriptional control of the kappaB promoter region (12). Therefore, by a reduction in NF-κB expression, the downstream consequences of inflammation which appear to lead to pulmonary fibrosis, the pathology associated with silicosis (8), will also be inhibited. Corticosteroids act by binding with a steroid receptor in the cytoplasm. This steroid-receptor complex translocates to the nucleus and blocks the kappaB promoter region. This prevents the NF-κB dimer from being able to bind and upregulate transcription of the various inflammatory genes. Alternatively, it has been proposed that corticosteroids prevent degradation of IκBα (18), a process which would prevent activation of NF-κB (22). Recent evidence (23) however, suggests that this is not the mechanism by which activation occurs. Furthermore, NF-κB makes a particularly inviting therapeutic target because it has been shown to be activated by an increase in the oxidative state of the cell (24).

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