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To cite this article: Mirjana Poljakovic , Dale W. Porter , Lyndell Millecchia , Diane Kepka-Lenhart , Christopher Beighley , Michael G. Wolfarth , Vincent Castranova & Sidney M. Morris Jr. (2007) Cell- and Isoform-Specific Increases in Arginase Expression in Acute Silica-Induced Pulmonary Inflammation , Journal of Toxicology and Environmental Health, Part A, 70:2, 118-127, DOI: [10.1080/15287390600755075](https://doi.org/10.1080/15287390600755075)

To link to this article: <https://doi.org/10.1080/15287390600755075>



Published online: 24 Feb 2007.



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## Cell- and Isoform-Specific Increases in Arginase Expression in Acute Silica-Induced Pulmonary Inflammation

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Arginase induction was reported in several inflammatory lung diseases, suggesting that this may be a common feature underlying the pathophysiology of such diseases. As little is known regarding arginase expression in silicosis, the induction and cellular localization of arginase were elucidated in lungs of Sprague-Dawley rats 24 h following exposure to varying doses of silica by intratracheal instillation. Arginase expression was evaluated by activity assay, quantification of arginase I and arginase II mRNA levels using real-time polymerase chain reaction (PCR), and immunohistochemistry. Analyses of cells and fluid obtained by bronchoalveolar lavage (BAL) showed that markers of pulmonary inflammation, tissue damage, activation of alveolar macrophages (AM) and NO production were significantly increased by all silica doses. Arginase activity was increased

also in AMs isolated from BAL fluid of silica-treated rats. Silica produced two- and three-fold increases in arginase activity of whole lung at doses of 1 and 5 mg/100 g body weight, respectively. Levels of arginase I mRNA, but not of arginase II mRNA, were similarly elevated. In control lungs, arginase I immunoreactivity was observed only in AMs sparsely dispersed throughout the lung; no inducible nitric oxide synthase (iNOS) immunoreactivity was detected. In silica-treated lungs, arginase I and iNOS were co-expressed in most AMs that were abundantly clustered at inflammatory foci. The rapid induction of arginase I expression in inflammatory lung cells, similar to induction of arginase in other inflammatory lung diseases, implicates elevated arginase activity as a factor in the development of lung damage following exposure to silica.

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Accepted 3 February 2006.

The authors thank Dr. Robert Mercer for helpful discussion and Jason Devlin and Stuart Shand for assistance with microscopy. We thank Dr. Simon Watkins, Director of the Center for Biologic Imaging, for providing access to the instrumentation used for the immunohistochemical studies. This research was supported in part by NIH grants RO1 GM57384 and RO1 GM064509 to S. M. Morris.

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The findings and conclusion in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Silica inhalation through mining, tunneling, rockdrilling, sand blasting, or working with concrete has been linked to silicosis, a pulmonary disease characterized by a severe decline in respiratory function and premature death (Banks, 1996; Shi et al., 1998). In animal models, acute inhalation of silica produces sustained pulmonary inflammation, characterized by activated alveolar macrophages (AMs), increased number of polymorphonuclear (PMN) leukocytes in bronchoalveolar spaces, production of free radicals (Blackford et al., 1994; Huffman et al., 1998, 2003; Sacks et al., 1998), and damage of lung tissue (Zhang et al., 1996). However, the precise mechanisms underlying many of the pathophysiologic changes remain unknown. Many studies of the pulmonary toxicity of silica focussed on the role of nitric oxide (NO) because of

the greatly increased expression of inducible NO synthase (iNOS) and NO production in lung following exposure to silica (Blackford et al., 1994; Porter et al., 2002b; Srivastava et al., 2002; Zeidler et al., 2003, 2004). However, the recent discoveries of increased arginase expression in asthma (Meurs et al., 2002; Zimmermann et al., 2003), bleomycin-induced pulmonary fibrosis (Endo et al., 2003), and hyperoxic lung injury (Que et al., 1998) raised the possibility that changes in arginase expression may occur also in silicosis and thus play roles in the pathophysiology of this disease. Apart from a single recent report (Misson et al., 2004), little was known regarding the impact of silica exposure on arginase activity and expression in lung prior to the present study. In particular, nothing to our knowledge was known regarding the dose dependence of silica-induced changes in arginase activity or localization of arginase-expressing cells within lungs following acute exposure to silica, nor had it been determined whether silica exposure affected expression of only one or both arginase isoforms.

Mammals express two arginase isozymes which are encoded by different genes and have different subcellular localizations, tissue distribution, and patterns of regulation (Iyer et al., 1998; Morris, 2002). Arginase I is a cytosolic enzyme that is highly expressed in the liver as a component of the urea cycle, while arginase II is a mitochondrial protein expressed in various nonhepatic tissues (Iyer et al., 1998; Morris, 2002). The expression of both arginases is induced by a variety of cytokines and inflammatory stimuli in many cell types, including macrophages (Mori & Gotoh, 2000; Morris, 2000; Morris et al., 1998). Because both arginase and iNOS utilize L-arginine as substrate, elevated levels of arginase activity can inhibit NO production via substrate limitation (Mori & Gotoh, 2000; Morris, 2000; Morris et al., 1998). Under some circumstances, elevated arginase activity also inhibits iNOS expression (Lee et al., 2003). Ornithine produced from L-arginine by arginase is metabolized to polyamines and proline (Wu & Morris, 1998), which are essential for cell proliferation and collagen synthesis, respectively. Thus, increased expression of the arginases during pulmonary inflammation might affect disease progression not only by reducing NO production due to substrate depletion but also by promoting long-term airway remodeling and fibrosis via enhanced synthesis of proline and polyamines (Meurs et al., 2003; Ricciardolo, 2003).

Understanding early pulmonary responses to inhaled particulates may lead to prevention and intervention approaches to help eliminate or mitigate the development of irreversible occupational lung disease. Because early changes in arginase expression in lung may play roles in the initiation of silica-induced lung damage, in the present study experiments were performed to determine the dose dependence of expression and cellular localization of the arginases in lungs of rats following acute exposure to silica.

## METHODS

### Animal Maintenance

Male Sprague-Dawley [Hla:(SD) CVF] rats (200–225 g) were obtained from Hilltop Lab Animals (Scottsdale, PA). The animals were maintained as described previously (Porter et al., 2002a). The program of animal use was accredited by AAALAC International, and all procedures involving animals were performed under protocols approved by the NIOSH Institutional Animal Care and Use Committee (IACUC).

### Intratracheal Instillation

Prior to use, silica (Min-U-Sil 5; U.S. Silica Corporation, Berkeley Springs, WV) was heated dry for 90 min at 160°C to inactivate any contaminating endotoxin. Suspensions of silica were prepared in 0.9% sterile saline (Baxter, Deerfield, IL). For intratracheal instillations, rats were anesthetized by ip injection of 30–40 mg/kg body weight (BW) sodium methohexital (Brevital, Eli Lilly and Company, Indianapolis, IN) and were intratracheally instilled using a 20-gauge 4-inch ball-tipped animal feeding needle. Rats received either 0.1, 1, or 5 mg silica/100 g BW dose or an equivalent volume of vehicle control.

### Bronchoalveolar Lavage

Rats were euthanized 24 h post intratracheal exposure with an i.p. injection of sodium pentobarbital (>100 mg/kg BW). After exsanguination by cutting the abdominal vena cava/abdominal aorta, a tracheal cannula was inserted and bronchoalveolar lavage (BAL) was performed as described previously (Porter et al., 2001). Briefly, the first lavage (6 ml) was kept separate from the rest of the lavage fluid. Subsequent lavages used 8 ml phosphate-buffered saline (PBS) until a total of 80 ml lavage fluid was collected. BAL cells were isolated by centrifugation, and an aliquot of the acellular supernatant from the first BAL fluid was transferred to tubes for analysis of lactate dehydrogenase (LDH) activity and albumin levels. The acellular supernatants from the remaining lavage samples were discarded. BAL cells isolated from the first and subsequent lavages for the same rat were pooled after resuspension in HEPES-buffered medium (10 mM HEPES, 145 mM NaCl, 5.0 mM KCl, 1.0 mM CaCl<sub>2</sub>, 5.5 mM D-glucose; pH 7.4), centrifuged, and the BAL cell pellet was then resuspended in HEPES-buffered medium and placed on ice.

To assess pulmonary inflammation, cell counts of AMs and PMN cells were obtained using an electronic cell counter with a cell sizing attachment (Coulter Electronics, Hialeah, FL). Cytospin preparations of BAL cells were also made using  $0.1 \times 10^6$  total phagocytes (AM+PMN cells) with a cyto-centrifuge (Shandon Elliot Cyto-centrifuge, London, UK) and

stained with modified Wright–Giemsa stain (Hema-Tek 2000, Bayer Corporation, Elkhart, IN).

### Analyses of BAL Fluid

BAL fluid albumin concentrations were determined as an indicator of the integrity of the air-blood barrier. BAL fluid albumin was measured colorimetrically at 628 nm, based on albumin binding to bromocresol green (Doumas et al., 1971), using a commercial assay kit (Sigma Chemical Company, St. Louis, MO) and a Cobas MIRA analyzer (Roche Diagnostic Systems, Montclair, NJ). BAL fluid lactate dehydrogenase (LDH) activities were determined as a marker of cytotoxicity and were measured by monitoring the LDH-catalyzed oxidation of pyruvate coupled with the reduction of NAD at 340 nm (Gay et al., 1968), using a commercial assay kit and a Cobas MIRA analyzer (both from Roche Diagnostic Systems).

### Chemiluminescence Assays

Alveolar macrophage chemiluminescence was determined as described previously (Porter et al., 2002b). Briefly, resting AM chemiluminescence was determined by incubating  $1.0 \times 10^6$  AM/ml at 37°C for 20 min, followed by the addition of luminol and the measurement of chemiluminescence. To determine zymosan-stimulated chemiluminescence, unopsonized zymosan was added immediately prior to the measurement of chemiluminescence. All chemiluminescence measurements were made with an automated luminometer (Berthold Autolumat LB 953, EG& G, Gaithersburg, MD) at 390–620 nm for 15 min. The integral of counts per minute (cpm) versus time was calculated. Zymosan-stimulated chemiluminescence was calculated as the integral cpm in the zymosan-stimulated assay minus the integral cpm in the resting assay. NO-dependent chemiluminescence was determined by subtracting the zymosan-stimulated chemiluminescence from cells pre-incubated with 1 mM of the iNOS inhibitor 1400W (Axxora, San Diego, CA), from the zymosan-stimulated chemiluminescence without 1400W. The use of unopsonized zymosan in the chemiluminescence assay allowed only AM chemiluminescence to be measured, because unopsonized zymosan stimulates AM chemiluminescence (Castranova et al., 1987) but not PMN cell chemiluminescence (Allen, 1977; Hill et al., 1977).

### Separation of BAL AM and PMN Cells

AMs and PMN cells were isolated by density gradient as described (Huffman et al., 2003). Briefly, at 24 h after intratracheal exposure, rats were euthanized with an i.p. injection of sodium pentobarbital (>100 mg/kg BW), followed by exsanguination. BAL cell isolation and differentials were determined as described earlier. BAL cells from groups of three rats that

received vehicle control were pooled. BAL cells from silica-exposed rats were not pooled. AM and PMN fractions from the density gradient were washed twice in PBS, and the final pellets were resuspended in PBS and aliquots removed for determination of cell differentials; the remaining cells were pelleted by centrifugation, snap frozen in liquid nitrogen and stored at –80°C.

The AM-enriched fraction of BAL cells isolated from saline-exposed rats was  $94.7 \pm 0.88\%$  AM ( $n=3$ ). No PMN cell-enriched fraction was obtained from saline-exposed rats, consistent with the determination that saline-exposed rats have essentially no BAL PMN cells. For silica-exposed rats, the AM-enriched fraction was  $64.3 \pm 3.71\%$  AM ( $n=3$ ) and the PMN cell-enriched fraction was  $66.0 \pm 1.53\%$  PMN cells ( $n=3$ ). Within the AM-enriched cellular fraction, the major cellular contaminant was PMNs. Within the PMN-enriched cellular fraction, AMs were the major cellular contaminant.

### Immunohistochemistry

Rats were euthanized 24 h post intratracheal exposure as described for BAL. Next, the lungs were removed and inflated via the trachea by instillation of 6 ml 2% paraformaldehyde in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS. A suture was tied around the trachea to retain the fixative, and the lungs were placed in fixative for 4–6 h. The lobes were separated, trimmed, rinsed in PBS, and placed in 30% sucrose solution (w/v, prepared in PBS) overnight at 4°C. Each lobe was embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Torrance, CA), frozen in dry-ice-cooled 2-methyl butane (Fisher, Pittsburgh, PA), and stored at –80°C until sectioning. Sections of 10  $\mu\text{m}$  thickness were cut in a cryostat (MICROM International GmbH, Walldorf, Germany) and preincubated with PBS containing 1% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) for 10 min at room temperature. Tissue sections were washed and then blocked with 5% normal goat and/or donkey serum (both from Sigma) in PBS containing 0.15% glycine (Bio-Rad Laboratories, Hercules, CA) and 0.5% bovine serum albumin (BSA) (Sigma) for 45 min at room temperature. After washing, the sections were co-incubated with primary antibodies (all diluted with PBS containing 0.15% glycine and 0.5% BSA) in different combinations: chicken polyclonal antibody raised to recombinant human arginase I (1:500; Morris et al., 1998), rabbit polyclonal antibody raised to murine iNOS (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-human prosurfactant protein C (prosp-C, 1:2000; Chemicon International, Temecula, CA), or mouse monoclonal anti-rat CD68 (ED-1; 1:50; Serotec Ltd, Oxford, UK), for 1 h in a moisture chamber at room temperature. The sections were rinsed and then incubated for 1 h with Alexa488-conjugated goat anti-rabbit immunoglobulin (Ig) G (1:3000; Molecular Probes, Eugene, OR), Cy-5-conjugated donkey anti-chicken IgY (1:1000), and/or Cy-3-conjugated donkey anti-mouse IgG (1:3000; both from Jackson Immuno-research Laboratories, Inc., West Grove, PA) in PBS containing

0.15% glycine and 0.5% BSA. Tissue sections were rinsed and mounted in gel/mount (Biomedica Corp., Foster City, CA) to prevent fading. Control experiments established that no immunoreactivity was detected in sections incubated with only the secondary antibodies.

All micrographs of the immunolabeled sections were obtained using a digital camera system (Olympus Provis AX70 microscope and Olympus Digital camera Olympus America; Olympus Optical Co, Tokyo), and the pictures were captured using appropriate filter settings for Alexa488, Cy-5, and Cy-3. Adobe® Photoshop was used for image handling, and the three-color channels were handled separately. Only the background level, contrast and brightness of the entire image were changed in the final picture.

### Arginase Assay

In some experiments, BAL cells and lung and spleen tissue were isolated for analyses of arginase activity. For these studies, rats were exsanguinated by cutting the abdominal vena cava/abdominal aorta. The left lung lobe was then clamped off with a hemostat and was removed below the clamp. The spleen was also removed, and both lung and spleen samples were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Bronchoalveolar lavage of the right lung was done as already described except that all volumes of PBS used were halved. First, BAL fluid isolation and analyses, BAL cell isolation, and BAL cell differentials were done as described earlier. After BAL cell differentials were determined, the remaining BAL cells were centrifuged ( $650 \times g$ , 5 min,  $4^{\circ}\text{C}$ ), the supernatant was decanted, and the cell pellet was snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

All arginase activity assays were performed on lysed BAL cells or on lung homogenates. Lungs were homogenized in 0.1% Triton X-100 containing 2 mM Pefabloc (Boehringer Mannheim, Mannheim, Germany), 2  $\mu\text{g}/\text{ml}$  pepstatin A, and 10  $\mu\text{g}/\text{ml}$  leupeptin (both from Sigma) using a tapered tissue grinder (Wheaton, Millville, NJ). BAL cells were lysed on ice for 20 min in 0.1% Triton X-100 containing 2 mM Pefabloc, 2  $\mu\text{g}/\text{ml}$  pepstatin A, and 10  $\mu\text{g}/\text{ml}$  leupeptin. The lung homogenates or BAL cell lysates were centrifuged for 2 min at  $13,600 \times g$  at  $4^{\circ}\text{C}$  to remove insolubles. Supernatants were used for assays of protein concentration and arginase activity. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL). Arginase activity was determined as the conversion of [ $^{14}\text{C}$ -guanidino]-L-arginine (50 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) to [ $^{14}\text{C}$ ]urea, which was converted to  $^{14}\text{CO}_2$  by urease and trapped as  $\text{Na}_2^{14}\text{CO}_3$  for scintillation counting as previously described (Morris et al., 1998).

### Real-Time PCR

For real-time PCR, rat lungs were isolated as described for arginase activity assay, and total RNA was isolated from

snap-frozen lungs. Briefly, lungs were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA), using a Polytron homogenizer (Kinematica GmbH, Kriens, Switzerland). Following chloroform extraction and isopropanol precipitation, RNA was precipitated by incubation in 0.15 M NaCl in 100% ethanol. The RNA was washed with 70% ethanol and treated with RNase inhibitor (Applied Biosystems, Foster City, CA) for 45 min. Following heat inactivation treatment at  $65^{\circ}\text{C}$  for 15 min, the RNA was cleaned and treated with deoxyribonuclease using the Qiagen RNA isolation kit, as directed by the manufacturer (Qiagen, Valencia, CA). Four micrograms of total RNA was reverse transcribed using random primers and Superscript II enzyme (both from Invitrogen Life Technologies), as directed by the manufacturer. For real-time PCR, the relative gene expression method (Liu & Saint, 2002) was used. 18S rRNA was used as the normalizer, and RNA isolated from saline-treated lung served as the calibrator. Predesigned Assays-on-Demand TaqMan probes and primers for arginase I (NM 017134), arginase II (NM 019168), and eukaryotic 18S rRNA (X03205) were obtained from Applied Biosystems (Foster City, CA). Real-time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems), and reactions were conducted in 50  $\mu\text{l}$  volume. All samples were run in duplicate and with no-reverse-transcriptase controls on an ABI Prism sequence detector 7700. Relative gene expression was calculated as  $2^{-\Delta\Delta\text{Ct}}$ , where  $\Delta\text{Ct} = \text{Ct}_{\text{gene of interest}} - \text{Ct}_{\text{normalizer}}$ , and  $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{calibrator}}$ . Arginase mRNA levels are expressed relative to the levels for saline-treated lungs.

### Statistical Analysis

Data are expressed as mean  $\pm$  standard error (SE). The effect of silica dose on each toxicological endpoint (Table 1 and Figure 1) was assessed using one-way analysis of variance using SAS Proc Mixed (SAS Institute, Cary, NC), with experiment used as a block effect. In all cases, tests were performed for homogeneity of variances between each dose level. In cases of unequal variances, analyses accounted for the heterogeneous variances, enabling efficient inferences to be made. Pairwise comparisons were performed for each dose level using the Tukey-Kramer method to adjust for multiple comparisons. Two-sided tests were used. All other data (Figures 2–4) were analyzed by analysis of variance (ANOVA), followed by the Bonferroni post hoc test for multiple comparisons. The criterion for significance was set at  $p < .05$ .

## RESULTS

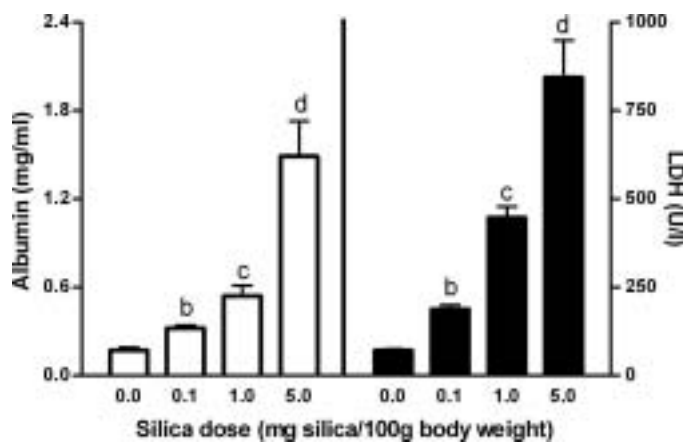
### Pulmonary Inflammation Following Silica Treatment

Several markers of pulmonary inflammation and tissue damage were quantified to evaluate the responses to varying amounts of silica. Although there were no changes in the number of AM in BAL fluid with increasing amounts of silica,

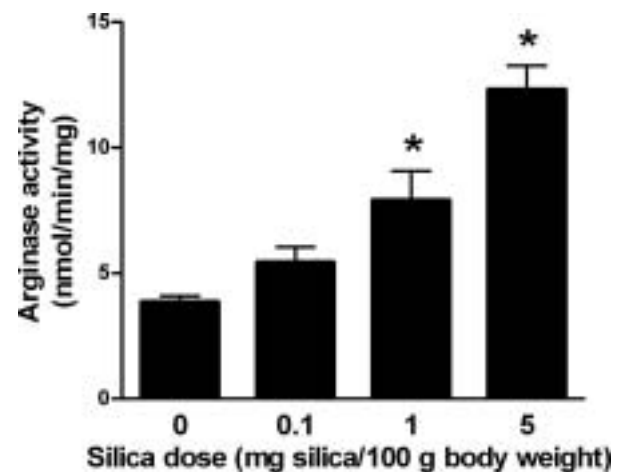
**TABLE 1**  
Markers of Pulmonary Inflammation in Control and Silica-Treated Rats

Silica dose (mg silica/ 100g body weight)	BAL cells		AM chemiluminescence	
	AM ( $\times 10^6$ /rat)	PMN ( $\times 10^6$ /rat)	Total (cpm $\times 10^5/0.25 \times$ $10^6$ AM/15 min)	NO-dependent (cpm $\times 10^5/0.25 \times$ $10^6$ AM/15 min)
0	9.69 $\pm$ 0.86 <sup>a</sup>	0.15 $\pm$ 0.04 <sup>a</sup>	15.32 $\pm$ 2.98 <sup>a</sup>	2.43 $\pm$ 0.70 <sup>a</sup>
0.1	10.19 $\pm$ 1.25 <sup>a</sup>	6.20 $\pm$ 1.27 <sup>b</sup>	103.43 $\pm$ 18.72 <sup>b</sup>	55.34 $\pm$ 11.72 <sup>b</sup>
1	10.78 $\pm$ 1.35 <sup>a</sup>	18.42 $\pm$ 2.70 <sup>c</sup>	155.15 $\pm$ 17.75 <sup>b</sup>	75.37 $\pm$ 11.77 <sup>b</sup>
5	10.37 $\pm$ 0.94 <sup>a</sup>	20.13 $\pm$ 3.20 <sup>c</sup>	147.63 $\pm$ 23.66 <sup>b</sup>	57.50 $\pm$ 11.57 <sup>b</sup>

Note. Data are expressed as means  $\pm$  SE ( $n=7-8$ ). Four experiments, each consisting of two rats/dose, were conducted. <sup>a-c</sup>Values within the same column with different superscripts are significantly different.



**FIG. 1.** Albumin concentration and LDH activity in BAL fluid of rat lungs as a function of silica dose. Scales on y axes have been set to allow direct comparison of the magnitude of relative increases in (left) albumin concentration and (right) LDH activities with increasing silica dose. Within each panel, values (means  $\pm$  SE;  $n=6-7$ ) identified by different lower case letters are significantly different ( $p < .05$ ).



**FIG. 2.** Arginase activity in rat lungs as a function of silica dose. Rats were intratracheally instilled with the indicated amounts of silica or an equivalent volume of vehicle. After 24 h, arginase activity was determined in lung homogenates. Values are expressed as mean  $\pm$  SE ( $n=5-22$ ). Asterisk indicates significant difference,  $p < .05$ .

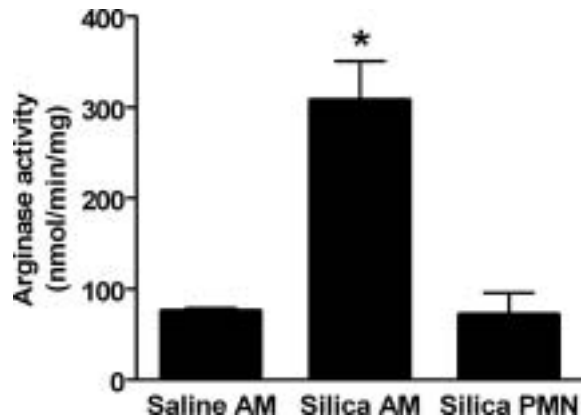
AM became maximally activated at all doses of silica used in this study, as determined by chemiluminescence (Table 1). In contrast to AM, there were marked increases in numbers of PMNs, which reached maximal levels by 1 mg silica/100 g BW (Table 1). Cell and tissue damage, as indicated by levels of albumin and LDH activity in BAL fluid, showed progressive enhancement with increasing amounts of silica (Figure 1).

### Arginase Activity and Gene Expression in Silica-Treated Lungs

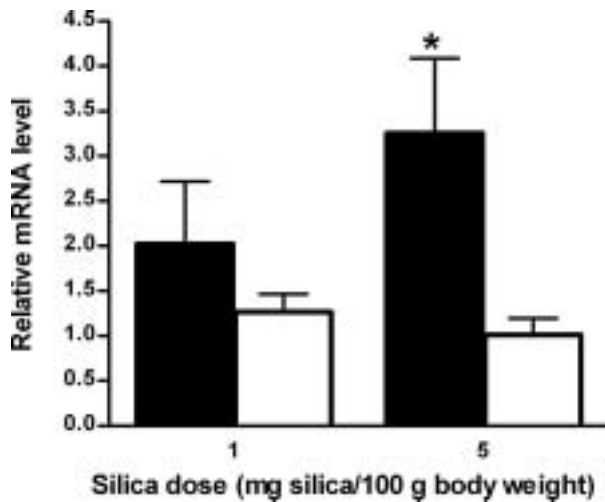
Exposure to silica resulted in a dose-dependent increase in arginase activity in whole lung tissue, with statistically significant 2.1- and 3.2-fold rise at 1 and 5 mg silica/100 g BW, respectively (Figure 2). The profile of enhanced arginase

activity with increasing amounts of silica is similar to the profiles of albumin and LDH activity in BAL fluid (Figure 1).

To determine which cell types may be contributing to the increased arginase activity in lungs of silica-treated rats, arginase activity was determined in AMs and PMNs isolated from BAL fluid of rats exposed to saline or 5 mg silica/100 g BW (Figure 3). Arginase specific activity was elevated 4.1-fold in AMs from silica-treated rats, relative to the specific activity in AMs from saline-treated rats. Owing to the very low abundance of PMNs in BAL fluid of saline-treated rats (Table 1), it was not possible to isolate sufficient PMNs from control rats to measure arginase activity. Arginase specific activities in PMN from silica-treated rats were indistinguishable from the basal specific activities in AMs from saline-treated rats (Figure 3). Taking into account the arginase specific activities (AM activity fourfold greater than PMN) and the total numbers of AM and PMN present in BAL fluid after silica treatment



**FIG. 3.** Arginase activity in AMs and PMN cells isolated by BAL. Arginase activity was determined in cell lysates of AMs and PMN cells isolated by BAL 24 h after intratracheal instillation of vehicle or 5 mg silica/100 g BW. No PMN cells were obtained from control rats. Values are expressed as mean  $\pm$  SE ( $n=3$ ). Asterisk indicates significant difference,  $p < .05$ .



**FIG. 4.** Levels of arginase I and II mRNAs in lungs of control and silica-treated rats. Rats were intratracheally instilled with either 1 or 5 mg silica/100 g BW or an equivalent volume of vehicle. After 24 h, total mRNA was isolated from rat lungs as described in Materials and Methods. Levels of arginase I (filled bars) and arginase II (open bars) mRNAs (mean  $\pm$  SE;  $n=5-8$ ) are expressed relative to the levels in saline-treated lungs. Asterisk indicates significant difference,  $p < .05$ .

(PMN count twice that of AM), it was concluded that both cell types contribute significantly to total cellular arginase activity in BAL cells harvested from silica-treated rats.

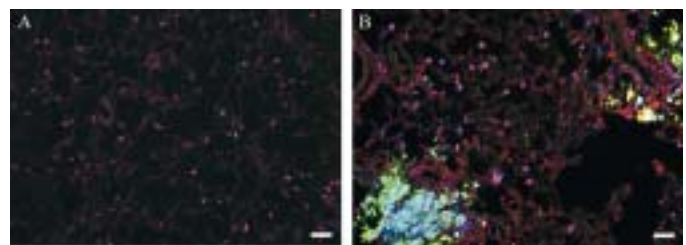
Arginase activity measurements reflect the sum of the activities of arginases I and II. In order to determine whether enhanced arginase activity in silica-treated lungs reflected increased expression of one or both arginase isozymes, the levels of arginase I and II mRNAs in whole lung were measured by real-time PCR. Paralleling the increases in arginase activity

(Figure 2), 2- and 3.3-fold elevations in arginase I mRNA levels were observed in lungs of rats exposed to 1 and 5 mg silica/100 g BW, respectively, but there were no changes in levels of arginase II mRNA (Figure 4). These results indicate that elevated arginase I expression accounts for the increased arginase activity in lungs after acute exposure to silica.

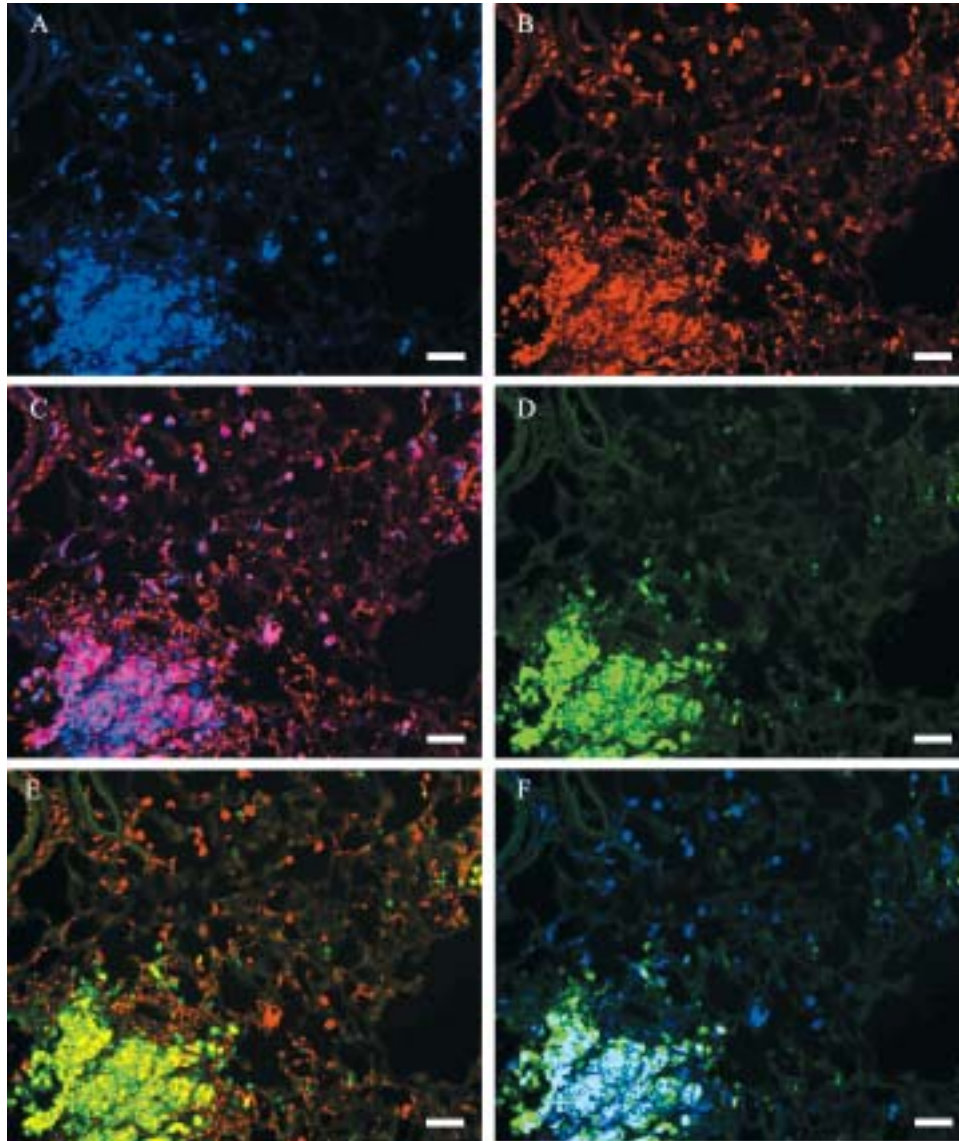
### Immunohistochemical Analyses of Silica-Treated Lungs

Within 24 h of exposure to silica, multiple foci of inflammatory cells were seen in lungs of silica-treated rats (Figure 5B), but no such foci were found in lungs of saline-treated controls (Figure 5A). In control lungs, many, but not all, cells that were positive for the monocyte/macrophage marker ED-1 were also arginase I immunopositive (Figure 5A). The arginase I-positive AMs were mainly localized to individual alveoli (Figure 5A). No arginase I immunoreactivity was observed in alveolar type II epithelial cells (identified by immunostaining with antibody to pro-surfactant protein C; data not shown); nor were any iNOS-positive cells detected in saline-treated lungs (Figure 5A), consistent with previous reports (Blackford et al., 1994, 1997; Porter et al., 2002b; Srivastava et al., 2002).

Numerous macrophages (identified by expression of the monocyte/macrophage marker ED-1 (Dijkstra et al., 1985)) were localized at focal sites of inflammation in lungs of silica-treated rats (Figure 6B). The number of inflammatory foci per lung rose with increasing amounts of silica treatment (data not shown), consistent with elevation in arginase activity (Figure 1). Within the foci, intense arginase I immunostaining (Figure 6A) was seen in ED-1 positive cells (Figure 6C) whereas iNOS staining was seen in both ED-1 positive and ED-1 negative cells (Figure 6, D and E). Many, but not all, arginase I-immunopositive AMs in the inflammatory foci were also iNOS positive (Figure 6F). Only AMs immunopositive



**FIG. 5.** Immunohistochemical comparison of control and silica-treated rat lungs. Immunohistochemistry was performed as described in Materials and Methods. Results are shown for rats treated with (A) vehicle or (B) 5 mg silica/100 g BW. Key to antibody staining: arginase I (blue), iNOS (green), and the macrophage marker ED-1 (red). Arginase I-positive macrophages are pink; iNOS-positive macrophages are yellow; cells expressing both arginase I and iNOS are turquoise. See Figure 6 for more detailed analysis. Photomicrographs are representative of multiple sections examined for each of eight rats in both treatment groups. Scale bar = 50  $\mu$ m.



**FIG. 6.** Identification of cells expressing arginase I and iNOS in silica-treated rat lung. Results are shown for rats treated with 5 mg silica/100 g BW. The tissue section was stained with anti-arginase I (blue), anti-iNOS (green), and the macrophage marker ED-1 (red). (A) Identification of arginase I-positive cells. (B) Identification of macrophages with the macrophage marker ED-1. (C) Identification of arginase I-positive macrophages by colocalization of immunoreactivity for arginase I and ED-1 (pink). (D) Identification of iNOS-positive cells. (E) Identification of iNOS-positive macrophages by colocalization of immunoreactivity for iNOS and ED-1 (yellow). (F) Identification of cells expressing both arginase I and iNOS by colocalization of immunoreactivity for arginase I and iNOS (turquoise). Photomicrographs are representative of multiple sections examined for each of eight silica-treated rats. Scale bar = 50  $\mu$ m.

for arginase I but not for iNOS were observed in areas outside inflammatory foci in silica-treated lungs (Figure 5B).

## DISCUSSION

This is the first detailed analysis of arginase activity, expression, and cellular localization in rat lung following an acute exposure to silica. Arginase activity in whole lung increased in a dose-dependent manner within 24 h of a single exposure to silica, similar to the dose-dependent rise in indices of lung

damage (i.e., albumin concentration and LDH activity in BAL fluid) accompanying pulmonary inflammation. In contrast to the absence of any change in arginase II mRNA levels, increases in arginase I mRNA levels in whole lung correlated with elevation in arginase specific activity, indicating that increased expression of the arginase I isozyme accounted for enhanced activity. Arginase specific activity also increased in AMs isolated from BAL fluid following silica exposure, consistent with elevated arginase expression. Although

arginase activity was present also in PMNs isolated from BAL fluid of silica-treated rats, it was not possible to determine whether this also represented increased expression because of the inability to obtain sufficient PMNs from BAL fluid of control rats for analysis. The presence of arginase activity in rat PMNs confirms previous reports of arginase activity and expression in PMNs (Munder et al., 2005; Reyer & Dorner, 1975).

Arginase I-expressing cells in lungs of controls and silica-treated rats were almost exclusively AMs, based on colocalization of immunoreactivity with antibodies to arginase I and the macrophage marker ED-1. No colocalization of immunoreactivity with antibodies to arginase I and pro-surfactant protein C, a marker for alveolar type II epithelial cells, was observed in lungs of control or silica-treated rats. Small numbers of individual arginase I-positive cells were present throughout the lungs of saline-treated rats, mainly localized in the alveoli. In marked contrast to control lungs, large numbers of arginase I-positive cells were clustered at inflammatory foci in lungs of silica-treated rats. Most of the cells clustered at the inflammatory foci were macrophages, based on ED-1 immunoreactivity. Unlike the arginase I-positive cells in lungs of saline-treated rats, most of the arginase I-positive cells at inflammatory foci also expressed iNOS. Taking into account the fact that arginase specific activity was significantly greater in AMs isolated from BAL fluid of silica-treated rats, relative to AMs from saline-treated controls, the increased arginase activity in whole lung of silica-treated rats probably reflects elevated arginase I expression per cell as well as increased numbers of arginase I-expressing cells.

Prior to this report, little was known regarding effects of silica exposure on arginase expression in lung. A recent study examined some aspects of arginase activity and expression in mouse lung following exposure to a single large dose of silica (~13 mg/100g BW) (Misson et al., 2004). As the earliest time examined was 3 d posttreatment, it could not be ascertained whether increases in arginase activity and expression represented a rapid or delayed response to treatment. Although arginase activity of whole lung was not reported, arginase I mRNA levels were more than twofold above control values at 3 d posttreatment but returned to control levels at 30 and 60 d posttreatment. Significant increases in arginase activity and levels of both arginase I and iNOS mRNAs were found in AMs isolated from murine BAL fluid at 3 d after silica treatment, similar to results presented here. Unlike the present study, however, neither expression of arginase II nor immunohistochemical localization of arginase I and iNOS expression within the lung was determined. One study reported increased catabolism of arginine to urea in lungs of rats 3–14 d after exposure to approximately 16.7–20 mg silica/100 g BW (Nelin et al., 2002), but enhanced catabolism correlated with increased pulmonary expression of arginine transporters and arginase activity was not determined. Consequently, it could not be

determined whether the increased conversion of arginine to urea reflected enhanced arginine uptake, increased arginase activity, or some combination of the two.

The role of elevated arginase expression in inflammatory lung disease is still unresolved. One possibility is that increased arginase activity inhibits NO production (Mori & Gotoh, 2000; Morris, 2000; Morris et al., 1998)—and possibly also iNOS expression (Lee et al., 2003)—by reducing the availability of L-arginine, thus limiting NO-dependent cytotoxicity of activated macrophages. Arginase-dependent reductions in arginine availability may exacerbate cytotoxic effects of activated macrophages because NOS produces superoxide instead of NO when arginine is sufficiently limiting (Bronte et al., 2003; Xia et al., 1996).

It should be emphasized that the role of arginases in inflammatory lung diseases may not be simply to regulate availability of L-arginine for NOS but also to regulate polyamine and proline synthesis by producing L-ornithine, thus contributing to tissue remodeling and fibrosis (Meurs et al., 2003). Chronic silicosis develops over time, as retained intrapulmonary silica induces a sequence of events including inflammation, fibrogenesis, and ultimately end-stage pulmonary fibrosis (Adamson, 1992; Velan et al., 1993). Since lung tissues were analyzed only at 24 h post silica instillation, the role of arginase I expression in development of pulmonary fibrosis could not be investigated in the present study. However, increases in arginase activity or arginase I mRNA levels were not seen in a previous study of mouse lung or BAL cells at 30 and 60 d post silica treatment, suggesting that sustained rise in arginase expression was not required for eventual development of pulmonary fibrosis in mice. However, it should be cautioned that this study did not rule out the possibilities that enhanced arginase activity and expression at early times (i.e., <30 d) following silica exposure played an essential role in the eventual development of fibrosis or that increased activity of arginase I or II in a small subpopulation of cells within the lung occurred at longer times posttreatment. Therefore, further studies are warranted in order to better understand the roles of the arginases in silica-induced fibrosis.

Although it has not been determined whether the increased arginase activity in AMs from silica-treated lungs represents a direct response to the silica particles or an indirect response to inflammatory mediators produced in response to silica, the tight clustering of arginase I-positive cells at inflammatory foci, coupled with the previous finding that iNOS expression in rat AMs is induced by conditioned medium from a mixture of AMs and PMNs cultured in the presence of silica but not by silica itself (Castranova et al., 1998), suggests that induction of arginase I also reflects an indirect response via inflammatory mediators rather than a direct response to silica. This possibility is supported by the rather promiscuous induction of the arginases in response to a wide variety of cytokines and other inflammatory stimuli (Mori & Gotoh, 2000; Morris, 2000; Morris et al., 1998). Further studies will be required

to identify the mediators involved in induction of arginase expression following exposure to silica.

In summary, this study demonstrated that a single exposure of rat lung to varying doses of silica results in dose-dependent increases in arginase activity within 24 h in whole lung and in AMs isolated from BAL fluid and enhanced expression of arginase I but not of arginase II in lung. Dose-dependent elevation in arginase activity correlate well with dose-dependent increases in indices of cell and tissue damage. Our results indicate that the increased arginase activity in whole lung is probably due to greater numbers of arginase I-expressing cells as well as to increases in arginase I expression per cell. Immunohistochemistry demonstrated coexpression of arginase I and iNOS in macrophages localized at inflammatory foci in silica-treated lungs. The high concentration of arginase I-expressing cells at inflammatory foci raises the possibility that arginase may be inhibiting NO production at those sites. The increases in arginase activity and expression in silica-treated lungs resemble the elevation in arginase activity and expression reported for a wide variety of inflammatory lung diseases, suggesting that increases in arginase expression may play common roles in the pathophysiology of multiple lung diseases, particularly with regard to processes of airway remodeling and fibrosis.

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