Molecular and Cellular Biochemistry **168:** 125–132, 1997.

Oxidative stress in silicosis: Evidence for the enhanced clearance of free radicals from whole lungs

Val Vallyathan,[1] Stephen Leonard,[1] Periannan Kuppusamy,[2] Donna Pack,[1] Michael Chzhan,[2] Sherry P Sanders[2] and Jay L. Zweir[2]

[1]*Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia;* [2]*The Department of Medicine, The Johns Hopkins Medical Institutions, Baltimore, Maryland, USA*

Received 11 April 1996; accepted 13 September 1996

Abstract

We hypothesized that reactive oxygen species (ROS) may be involved in the pathogenesis of silicosis. To investigate ROS' dependent pathophysiological processes during silicosis we studied the kinetic clearance of instilled stable nitroxide radicals (TEMPO). Antioxidant enzymes' superoxide dismutase (SOD) and glutathione peroxidase (GPx), and lipid peroxidation were also studied in whole lungs of rats exposed to crystalline silica (quartz) and sham exposed controls. Low frequency L-band electron spin resonance spectroscopy was used to measure the clearance of TEMPO in whole-rat lungs directly. The clearance of TEMPO followed first order kinetics showing significant differences in the rate for clearance between the diseased and sham exposed control lungs. Comparison of TEMPO clearance rates in the sham exposed controls and silicotic rats showed an oxidative stress in the rats exposed to quartz. Studies on the antioxidant enzymes SOD and GPx in the lungs of silicotic and sham exposed animals supported the oxidative stress and accelerated clearance of TEMPO by up regulated levels of enzymes in quartz exposed animals. Increased lipid peroxidation potential in the silicotics also supported a role for enhanced generation of ROS in the pathogenesis of silica-induced lung injury. These *in vivo* experiments directly demonstrate, for the first time, that silicotic lungs are in a state of oxidative stress and that increased generation of ROS is associated with enhanced levels of oxidative enzymes and lipid peroxidation. This technique offers great promise for the elucidation of ROS induced lung injury and development of therapeutic strategies for the prevention of damage. (Mol Cell Biochem **168:** 125–132, 1997)

Key words: oxidative stress, silicosis, antioxidant enzymes, lipid peroxidation

Introduction

Reactive oxygen species (ROS) have been well recognized as important intermediates in biologic reactions and as a primary cause of cell injury and cell death in a variety of pathophysiologic processes [1–4]. In recent years considerable research evidence has also implicated ROS in pulmonary diseases caused by air pollutants, occupational exposures and tobacco smoke [5–10]. Quantitation of ROS in living systems presents a formidable task because of low concentrations, short half-lives and a fast rate of reaction with biological molecules. Although spin traps and other biologic reaction products formed from cellular components such as lipids, proteins and nucleic acids are often used as markers of ROS induced injury, they all have inherent limitations. For example, in widely used techniques with the aid of spin traps, the spin adducts detected are often modulated by cellular oxidation and metabolism [11]. On the other hand, direct on-line electron spin resonance ESR measurements of free radicals would provide a kinetic view of cellular events occurring at an organ or whole animal level.

Earlier studies using ESR with the aid of spin-traps to de-

Address for offprints: V. Vallyathan, National Institute for Occupational Safety and Health, Pathology Section, 1095 Willowdale Road, Morgantown, WV 26505, USA

tect free radicals have provided indirect evidence that ROS production occurs in animal models of lung injury caused by the exposure of carbon tetrachloride, endotoxin, cigarette smoke and ozone [12–14]. However, these studies overall reflect radical generation in the whole body and have not delineated the target organ. To overcome the limitations and inadequacies of conventional ESR techniques, in this study we used L-band low-frequency (1–2 GAZ) ESR spectroscopy. This enabled high sensitivity ESR measurements of whole organs adapting to large lossy samples, such as the lung. Using the L-band low-frequency ESR spectroscopy we were able to make simultaneous measurements of free radical concentrations and *in vivo* kinetic changes in radical metabolism within the whole lung. To impose an oxidative stress in the lungs we used an experimental animal model with progressive silicosis. To provide a state of radical overburden and subsequent clearance we used TEMPO (2,2,6,6-tetramethyl piperidine-N-oxyl), a nitroxyl radical with an antioxidant property as a superoxide dismutase (SOD) mimic. Using this technique we studied the kinetics of decay and metabolism of known amounts of TEMPO introduced into the lungs of an animal model with active inflammatory processes leading to silicosis. The goal of the present study was to detect whether the oxidative stress induced by the instillation of quartz would alter the ability of the lung to clear an additional burden of TEMPO free radicals. We also studied whether an up regulation of antioxidant enzymes accompanies the oxidative stress in silicosis.

Materials and methods

Reagents

Min-U-Sil (quartz) was obtained from Pennsylvania Sand and Glass Corporation, Pittsburgh, PA and fractionated to < 5 μm using an Accucut Particle Classifier (Donaldson-Majal Division, St. Paul MN). Scanning electron microscopy with the aid of automated image analyses and x-ray spectrometric analyses, was carried out to verify the particle mass median aerodynamic diameter (3.5 μm) and quartz purity (98.7%). Catalase, SOD, xanthine, xanthine oxidase, TEMPO and other laboratory chemicals were obtained from Sigma Chemical Company, St. Louis, MO.

Animals

Adult Sprague Dawley 60–70 day old male rats weighing 280–300 gm, were exposed to quartz or normal sterilized saline intratracheally. We anesthetized experimental and sham exposed control animals with Brevitol intra peritoneally and each animal in the treatment group received a single intratracheal instillation of 0.5 ml sterilized saline containing 10 mg quartz. Control, sham exposed animals, received 0.5 ml sterile saline. We sacrificed animals at two and eight days after quartz or saline exposure. These sacrifice times represented peak periods in inflammation and early stages of granuloma development [15].

At sacrifice, each animal received an intraperitoneal sodium pentobarbital overdose and was exsanguinated by severing the vena cava. Lungs were exposed and perfused with normal saline through the pulmonary artery until the lungs turned visibly pale and were without blood. After tracheal cannulation, the lungs were lavaged twice each with 8 ml calcium and magnesium free Hanks' balanced salt solution to remove phagocytic cells and surfactant. The lungs were then removed *in toto* with the trachea, weighed, and instilled with 1 ml of 2 mM TEMPO.

ESR measurements

After the instillation of 1 ml of 2 mM TEMPO kinetic ESR measurements were performed at room temperature using a custom-built L-band (1.2 GHz.) microwave bridge and a 3-loop two-gap reentrant resonator [16, 17]. The TEMPO was instilled through the trachea and the lungs were transferred to 20 mm glass tubes, which were centered at the active volume of the resonator (Fig. 1). Spectral acquisitions were started, within 40–60 sec after the instillation of TEMPO using a series of 15 sec sweeps during the first five min, followed by 30 sec sweeps for the next 10 min. The data acquisition parameters were: sweep width 40 G, microwave power 20 mW, modulation frequency 100 kHz and modulation amplitude 1.0 G. The receiver gain and time constant were set according to the signal intensity and sweep time, respectively. Double integration was performed on the measured first-derivative ESR spectra to obtain relative intensity of the TEMPO spectra.

Further, to learn whether ROS or antioxidant enzymes present in the lung interact with the TEMPO clearance, we monitored the peak intensity of TEMPO spectrums in the isolated lung. The *in vitro* interactions of TEMPO with specific commercial antioxidant enzymes and oxygen radicals were also studied. Xanthine-xanthine oxidase (0.4 μM X and 0.0005 U XO/ml) generated the superoxide radicals. Hydrogen peroxide and iron in varying concentrations of 0.8, 1.6, and 3.2 mM were used to generate hydroxyl radicals. For these reactions a typical reaction mixture in a total volume of one ml contained 100 μl of 2 mM TEMPO, 200 μl of ROS generating system with or without 100 μl of scavengers or metal chelators. The reaction mixture in Hanks' balanced buffer solution at pH 7.4 was mixed well and transferred to flat quartz cells. ESR measurements were made in a Varian

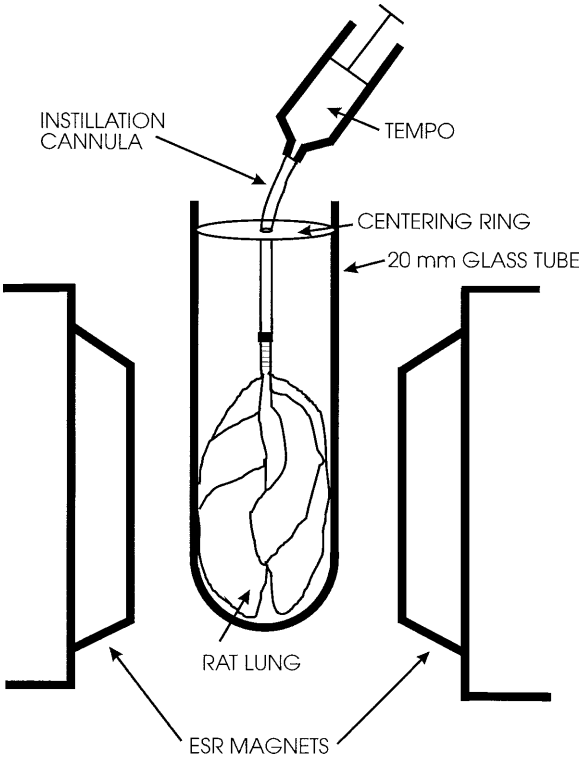

Fig. 1. Diagram of a lung preparation in the 20 mm glass tube centered in the active region of the L-band ESR resonator with the instillation cannula attached to trachea.

E109 ESR spectrometer operating at the X-band (~9.4 GHz.) frequencies. We made all measurements in 250 µl samples at room temperature at a microwave power of 50 mW. In a magnetic field of 3470 ± 50 G, we used a field modulation amplitude of 2 G with 100 sec scan time and 1 sec time constant. Using dedicated acquisition software the intensity decays were continuously monitored for 10 min. The peak intensity of the middle line of the triplet spectrum of TEMPO was monitored as a function of time and used as a measure of TEMPO radical clearance.

Enzyme measurements

Total enzyme activities of SOD and glutathione peroxidase (GPx) was measured in lung supernatant of animals exposed to quartz or sham saline exposed controls. Lungs were homogenized in Hanks' buffered medium and spun at 10,000 × g for 10 min to remove the cellular debris. SOD and GPx were estimated using an automated Cobas Fara II Analyzer (Roche Diagnostic Systems, Montclair, NJ). All the reagents and samples placed in the instrument were automatically pipetted, diluted, mixed, incubated, spectrophotometrically analyzed and calculated according to programmed instructions. SOD was estimated in the supernatant according to the well-characterized method of McCord and Fridovich [18] with modifications as reported by Elstner *et al*. [19]. The final reaction mixture in a total volume of 400 µl contained 16 mM cytochrome *C*, 0.4 µM xanthine in 31 mM carbonate buffer with 0.06 mM EDTA. Simultaneous absorbance of inhibited and uninhibited values of cytochrome *C* reduction were made after one min at 20 sec intervals at 550 nm. GPx was assayed according to the method of Flohe and Gunzler by measuring changes in the absorbance at 340 nm [20]. The final reaction mixture in a total volume of 400 µl contained 50 µl sample, 0.11 mM NADPH, 0.23 mM glutathione, 1.8 mM sodium azide and 0.8 U/ml glutathione reductase in 25 mM Tris-buffer with 0.05 mM EDTA. The decreases in the absorbance were measured at 10 sec intervals at 25°C.

Lipid peroxidation

Malondialdehyde, an end product of lipid peroxidation, reacts with thiobarbuturic acid to produce a colored substance. Lipid peroxidation was measured according to the method of Hunter *et al*. [21] by monitoring the thiobarbituric acid (TBA) reactive substance formed in the incubation mixture containing thin slices of pulmonary tissue for 1 h with and without iron. Thin lung slices (350–450 mg) were incubated in phosphate-buffered medium at pH 7.4 in a shaking water bath with or without 100 mM $FeSO_4$. Addition of 300 µl 5 N HCl and 625 µl 40% trichloroacetic acid terminated the reaction and the vials were mixed and 625 µl 2% thiobarbituric acid was added. The mixture was then heated for 20 min at 95–100°C. TBA reactive substance in the supernatant developed a color, which was monitored after cooling and centrifugation, at 540 nm using a Beckman DU-650 model spectrophotometer. We compared the absorbance of TBA reactive substance with known concentrations of malondialdehyde reacted with the same reagents. Control experiments were carried out simultaneously without lung tissues, and in the presence of antioxidant butyl hydroxy toluene, to inhibit lipid peroxidation.

Statistical analysis

Data presented are the means and standard errors. Analysis of the variance or students *t*-test also made comparisons between treatment groups where appropriate to test the significance of differences of means between the quartz exposure group and the sham exposed controls. A probability value of less than $p = 0.05$ was considered significant. Computing linear correlations or least squares' best-fit polynomials using Sigma Stat analyzed kinetic decay of TEMPO radicals.

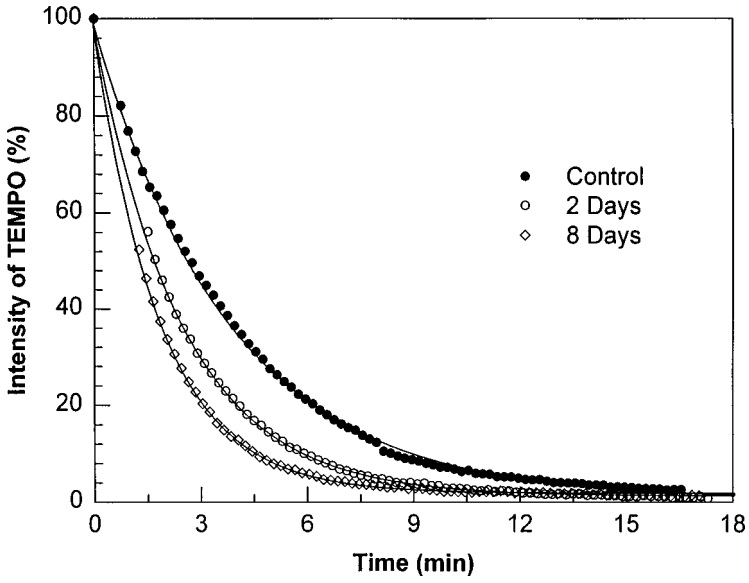

Fig. 2. Graph showing the kinetics of radical clearance in isolated rat lungs instilled with one ml 2mM TEMPO. Sham exposed control lungs (slope 1), quartz exposed and sacrificed after two days (slope 2) p – 0.0001, and quartz exposed and sacrificed after eight days (slope 3) p – 0.0001. Logarithmic plot of the TEMPO radical clearance was non-linear in all the three groups.

Results

Clearance of TEMPO radicals

Clearance of TEMPO radicals from the lungs after the instillation of TEMPO was observed to follow a single exponential decay process, both in sham exposed controls and silicotics. A plot of ESR intensity vs. time clearly shows distinct differences in the exponential decay in each group of animals (Fig. 2). In the sham exposed control rat lungs, the initial rapid decay accounted for a 23% loss of TEMPO radicals within 1 min as compared with a 33 and 41% loss of TEMPO radicals in 2 and 8 day quartz exposed animals. In 2 and 8-day quartz exposed animals more than 70% of the TEMPO radicals were cleared within three min. After the instillation of TEMPO, the sham exposed control lungs cleared the TEMPO radicals at a rate of 150 count/sec in the first two min. In contrast the 2 and 8-day quartz exposed animals cleared the radicals from lungs 134 and 160% faster than the sham exposed controlled lungs in the first 2 min (0.0001). The data were fitted using a first order decay kinetic curve to obtain the following rate constants: 0.22 ± 0.03 min for sham exposed control lungs, 0.35 ± 0.09 for 2 day quartz exposed lungs (p0.0001) and 0.54 ± 0.09 (0.0001) for 8 day quartz exposed lungs.

Figures 3a, $3a_1$ and $3a_2$ show typical ESR spectra obtained from sham exposed control rat lungs containing 1 ml 2 mM TEMPO placed in a 20 mm glass tube at 1 min, 2 min and 3 min. Figures 3b, $3b_1$ and b_2 show ESR spectra obtained from rat lungs 2 days post exposure to quartz containing 1 ml 2 mM TEMPO at 1 min, 2 min and 3 min. Figures 3c, c_1 and c_2 show ESR spectra obtained from rat lungs after 8 days post exposure to quartz containing 1 ml 2 mM TEMPO at 1 min, 2 min and 3 min. The peak-to-peak width of the ESR spectra was 1.60 G and it did not change during the TEMPO radical clearance process. Furthermore, the shape of the triplet lines did not change during clearance, suggesting that there was no immobilization of the nitroxide molecule during the process. Addition of hydrogen peroxide and Fe^{2+} resulted in a reduction of the TEMPO spectral intensity. The decrease was significant by Fe^{2+} alone without hydrogen peroxide. When EDTA was present in the medium, Fe^{2+} induced a decrease in intensity and when DETAPAC was present in the medium, the TEMPO peaks were totally abolished.

Antioxidant enzyme levels

To learn if the enhanced TEMPO radical clearance observed in silicotic animals is directly correlated with an up regulation of antioxidant enzyme activity, we carried experiments out using rat lungs exposed to quartz and subsequent assessment of SOD and GPx levels. Figure 4 shows SOD and Fig. 5 shows GPx levels in lungs after intratracheal exposure of rats to quartz after 2 days, 8 days and sham exposed control. Instillation of the highly inflammatory and fibrogenic quartz caused significant ($p < 0.05$) increases in SOD levels in rat lungs at 2 days (46%) and 8 days (46%). We observed mod-

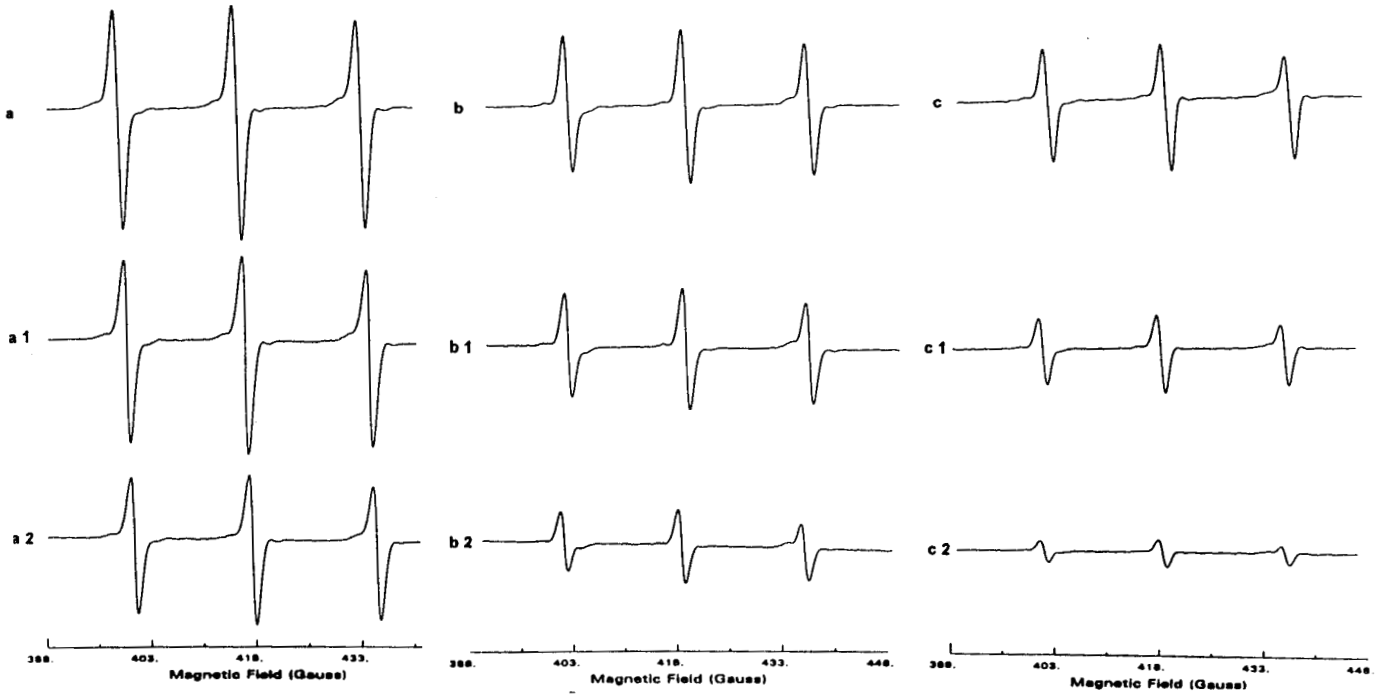

Fig. 3. Typical ESR spectra obtained from sham exposed control, and quartz exposed whole lungs containing one ml of 2 mM TEMPO solution showing the rapid clearance of TEMPO radicals. (a) Spectra obtained from sham exposed control lungs at 1 min (a), 2 min (a_1), 3 min (a_2) after starting the TEMPO infusion. (b) Spectra obtained from quartz exposed (2 days) rat lungs at 1 min (b), 2 min (b_1), 3 mi_n (b2) after starting the TEMPO instillation. (c) Spectra obtained from quartz exposed (8 days) rat lungs at 1 min (c), 2 min (c_1), 3 min (c_2) after instilling TEMPO.

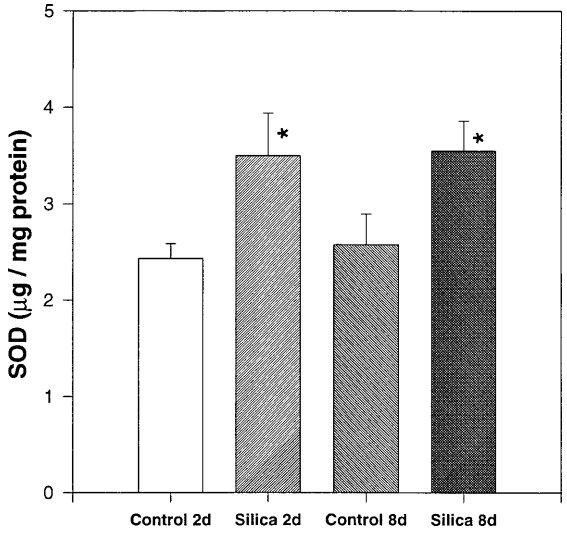

Fig. 4. Up regulated levels of superoxide dismutase in the lung homogenate supernatant of quartz exposed and sham exposed controls. *Denotes the value is significant above the sham exposed group at $p < 0.05$.

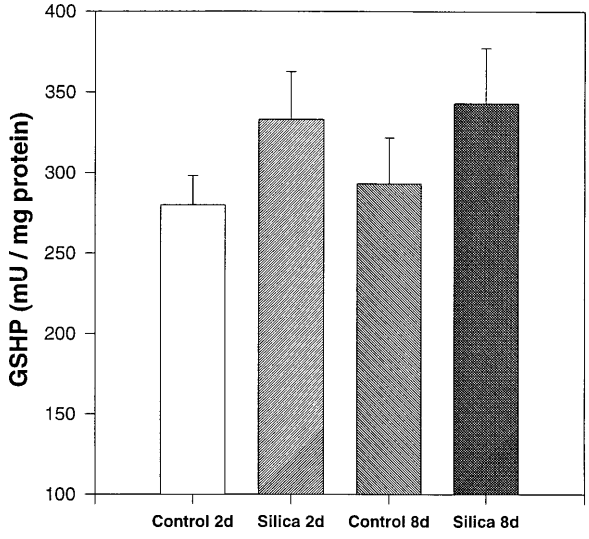

Fig. 5. Glutathione peroxidase levels in the lung homogenate supernatant of sham exposed, quartz exposed rats. *Denotes the value is significant above the sham exposed group at $p < 0.05$.

erate statistically insignificant increases in GPx levels at 2 days (23%) and 8 days (19%) after quartz exposure. However, the levels of these antioxidant enzymes remained unchanged in sham exposed controls at 2 days and eight days.

We also evaluated the lipid peroxidation potential as a measure of ROS-induced biological correlation in the lungs of rats instilled with quartz and the sham exposed control rats. Results presented in Fig. 6 show that the lipid peroxidation potential in the lungs of animals exposed to quartz after eight days is enhanced significantly ($p < 0.05$) compared with sham

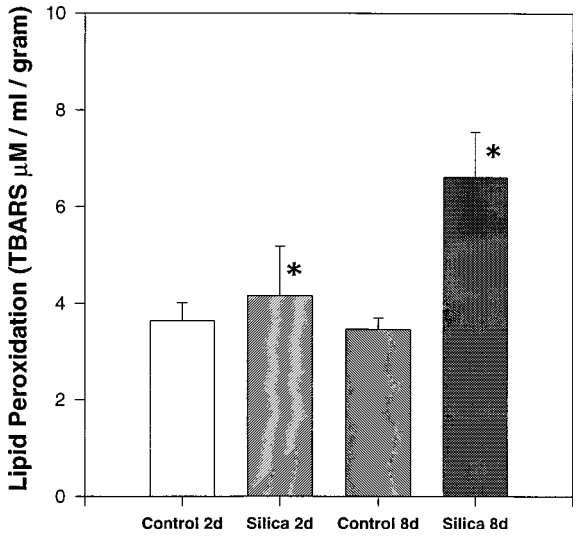

Fig. 6. Bar graph showing the enhanced lipid peroxidation thiobarbituric acid reactive substance formed in rats exposed to quartz. *Denotes the value is significant above the sham exposed control group at $p < 0.05$.

exposed control animals. Addition of iron further enhanced the lipid peroxidation process (data not shown) providing additional support for the mechanistic nature of this process.

Discussion

Pulmonary tissues are constantly exposed to ROS derived from endogenous and exogenous sources. To combat the unpredictable exposure to ROS the lungs are uniquely equipped with antioxidant enzymes and nonenzymatic defenses. In healthy lungs, a delicate balance exists between ROS generation and antioxidant defenses. When the delicate balance between antioxidant defenses and generation of ROS is overwhelmed by either an excessive production of ROS or loss of antioxidant defenses, an oxidative stress is induced resulting in cell injury.

Chronic and acute exposure to quartz is associated with the provocation of an inflammatory response and triggering of extensive host defense mechanisms [22–25]. These inflammatory reactions result in the secretions of cytokines, growth factors, platelet activating factor, eicosanoids, lytic enzymes, chemotactic factors and ROS. Inflammatory reactions are sustained in quartz exposed animals resulting in the continuum of secretions of cellular products, repeated phagocytosis of quartz and the enhanced generation of ROS [8]. This quartz induced potentiation of ROS results in an oxidative stress and up regulation of antioxidant enzymes [25]. It is important to note in this respect that glutathione is an important pulmonary defense in lessening particle induced lung injury and exposure to particles has been documented to alter the glutathione levels of the lung [26]. In the present study during silicosis the elevated levels of SOD and GPx observed provided a biochemical link to this increased oxidative stress. We could interpret the increase in the antioxidant enzymes as a compensatory mechanism in response to increased ROS generation in silicosis. Besides the biochemical response, a functional disturbance in the cell membrane structure is implicated in view of the ROS specific attack on unsaturated lipids as suggested by the increased lipid peroxidation in silicosis.

The importance of oxidative stress in the regulation and induction of antioxidant enzymes and mRNA message levels are well documented in the mineral induced inflammatory processes [27, 29]. In asbestosis and silicosis a complex sequence of events is involved in the perpetuation of phagocytic and inflammatory processes and continued production of ROS [8]. The combination of greater radical generation by surface chemistry and iron, coupled with repeated phagocytosis and augmented inflammatory processes, may lead to an oxidative stress that can lead to detrimental effects in the lung if the antioxidant defenses are insufficient.

In pulmonary tissues increased generation of ROS occurs after exposure to ozone [6], asbestos [5, 8, 24], quartz [8], coal [9], bleomycin [30], paraquat [31], toluene diisocyanate [32], hyperoxia [33] and cigarette smoke [34]. Concomitant increases in antioxidant enzymes has been reported in some models of oxidative lung injury [27, 28]. The generalized response to oxidative stress may show some variability in response because the antioxidant enzymes are often coordinated differently at the molecular level by independent mechanisms.

We need conclusive studies to document that the up regulated antioxidant enzymes are sufficient to protect the lungs during oxidative stress. In several instances it has been shown that the induction of antioxidant enzymes occurs after a lag time subsequent to an oxidative injury [28]. Irreversible lung injury can probably occur during this lag time with an acute exposure, as in this animal model of silicosis. Besides, sustained ROS generation resulting from the phagocytosis of indestructible quartz can result in a continuum of phagocytosis and cell death with the induction of irreversible lung injury. The data presented here on TBA reactive substances show that increased free radical chain reactions have occurred in silicosis leaving behind a 'footprint' of ROS damaged products. It is evident from the results that concurrent up regulation of antioxidant enzyme levels were inadequate to protect the lungs from the spontaneous ROS mediated acute insult.

TEMPO is a low-molecular weight, stable, water soluble, nitroxide free radical. It can oxidize reduced metal ions [35] and react with carbon-centered radicals [36]. TEMPO can maintain iron in the Fe^{3+} form, thereby preventing the Haber Weiss reaction and generation of hydroxyl radicals [35]. It is also lipophilic and cell membrane permeable and thus can be readily compartmentalized within the cells [37]. TEMPO is known to have antioxidant activity that mimics that of SOD

[38–40]. It was also shown to be effective in protecting radiation induced lung injury in a murine experimental animal model [41]. All these studies suggest that TEMPO prevents biological ROS mediated oxidative damage at the molecular, cellular and whole-animal level, effectively interacting with ROS at the site of generation [37, 39, 41].

Inside the cells, TEMPO undergoes one-electron reduction to hydroxylamine, which is non-paramagnetic. This cellular decay of TEMPO is the major pathway for the loss of nitroxide in biological tissues and cells. Therefore, we can describe the mechanism of clearance of the infused TEMPO radicals in the silicotic lungs as due to a combination of several processes, including intracellular reduction. While intracellular reduction may be occurring at equal rates in both sham exposed controls and silicotic lungs, the enhanced rate of clearance in the silicotic lungs may be due to the SOD mimicking activity of TEMPO, thus suggesting that superoxide radicals are generated at an increased rate in silicosis. Furthermore, the ability of TEMPO to oxidize Fe^{2+} would prevent the formation of hydroxyl radicals and by that lipid peroxidation. All these mechanisms may have played a role in the enhanced clearance of TEMPO radicals.

In conclusion, the results presented in this study show that direct measurement of free radicals in whole animal lungs is feasible and that the results can be correlated with other independent biochemical and metabolic processes. With the use of this direct *in vivo* ESR technique evaluating the role of ROS generation in a broad range of pathological conditions, which results in tissue injury or disease may be possible.

References

1. Halliwell B, Cross CE: Oxygen-derived species: Their relation to human disease and environmental stress. Environ Health Perspect 102: (10) 5–12, 1994
2. Bast A, Haellen G, Doleman C: Oxidants and antioxidants: state of the art. Am J Med 91: 2S–13S. 1991
3. Southorn P, Powis G: Free radicals in medicine. II. Involvement in human disease. Mayo Clin Proc 63: 390–408, 1988
4. Halliwell B, Gutteridge JHC, Cross CE: Free radicals, antioxidants, and human disease: where are we now? J Clin Med 119: 598–620, 1992
5. Mossman BT, Marsh JP: Evidence supporting a role for active oxygen species in asbestos-induced toxicity and lung disease. Environ Health Perspect 81: 91–94, 1989
6. Mustafa MG: Biochemical basis of ozone toxicity. Free Rad Biol Med 9: 245–65, 1990
7. Frei B, Forte TM, Ames BN, Cross CE: Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in the lipoprotein properties in human blood plasma. Biochem J 277: 133–38, 1991
8. Vallyathan V, Mega JF, Shi X, Dalal NS. Enhanced generation of free radicals from phagocytes induced by mineral dusts. Am J Respir Cell Mol Biol 6: 404–13, 1992
9. Dalal NS, Newman J, Pack D, Leonard S, Vallyathan V: Hydroxyl radical generation by coal mineral dust: Possible implication to coal workers' pneumoconiosis (CWP). Free Rad Biol Med 18: 11–20, 1995
10. Kamp DW, Graceffa P, Pryor WA, Weitzman SA: The role of free radicals in asbestos-induced diseases. Free Rad Biol Med 12: 293–315, 1992
11. Samuni A, Carmicheal AJ, Russo A, Mitchell JB, and Riese P. On the spin trapping and ESR detection of oxygen-derived radicals generated inside cells. Proc Nat Acad Sc 83: 7593–7597, 1986
12. Davies MJ: Direct detection of free radical generation in an in vivo model of acute lung injury. Free Rad Res Comm 15: 167–76, 1991
13. Lai EK, McCay PB, Noguchi T, Fong KL: *In vivo* spin-trapping of trichloromethyl radicals formed from CC14. Biochem Pharmacol 28: 2231–35, 1979
14. Kennedy CH, Hatch GE, Slade R, Mason RP: Application of the EPR spin-trapping technique to the detection of radicals produced in vivo during inhalation exposure of rats to ozone. Toxicol Applied Pharmacol 114: 41–46, 1992
15. Vallyathan V, Schwegler D, Reasor M, Stettler L, Clere J, Green FHY: Comparative in vitro cytotoxicity and relative pathogenicity of mineral dusts. Ann occup Hyg 32: 279–289, 1988
16. Zweier JL, Kuppusamy P: Electron paramagnetic resonance measurements of free radicals in the intact beating heart: A technique for detection and characterization of free radicals in whole biological tissues. Proc Nat Acad Sci 85: 5703–7, 1988
17. Chzhan M, Shteynbuk M, Kuppusamy P, Zweier JL: An optimized L-band resonator for EPR imaging of biological samples. J Magn Reson A105: 49–53, 1993
18. McCord JM, Fridovich I: The reduction of cytochrome C by milk xanthine oxidase. J Biol Chem 243: 5733–60, 1968
19. Elstner EF, Youngman RJ, Osswald W: In: HU Bergmeyer (ed). Methods of Enzymatic Analysis. 3rd ed, 3: Verlag Chemic, Deerfield Beach, FL, 1983, pp 293–302
20. Flohe L, Gunzler WA: In: L Packer (ed). Methods in Enzymology. 105: Academic Press, New York, 1984, pp 114–121
21. Hunter FE Jr, Gebicki JM, Hoffsten PE, Weinstein J, Scott A: Swelling and lysis of rat liver mitochondria induced by ferrous ions. J Biol Chem 238: 828–32, 1963
22. Craighead JE, Kleinerman J, Abraham JL, Gibbs AR, Green FHY, Harley RA, Ruettner JR, Vallyathan NV, Juliano EB: Diseases associated with exposure to silica and nonfibrous silicate minerals. Arch Pathol Lab Med 112: 673–720, 1988
23. Davis GS: Pathogenesis of silicosis: current concepts and hypothesis. Lung 164: 1339–54,1986
24. Kane AB. Pathogenesis of silicosis: current concepts. In: V Castranova, W Wallace and V Vallyathan V (eds). Silica and Silica-Induced Lung Diseases: Current Concepts. CRC Press. Boca Raton, FL, 1996, pp 121–136
25. Vallyathan V, Castranova V, Pack D, Leonard S, Shumaker J, Hubbs AF, Shoemaker DA, Ramsey DM, Pretty JR, McLaurin JL, Khan A, Teass A: Freshly fractured quartz inhalation leads to enhanced lung injury and inflammation: Potential role of free radicals. Am J Respir Crit Care Med 152: 1003–9, 1995
26. Lombard-Gillooly K, Hubbard AK: Modulation of silica-induced lung injury by reducing lung non-protein sulfhydryls with buthionine sulfoximine. Toxicol Letters 66: 305–15, 1993
27. QuinlanT, Spivack S, Mossman T: Regulation of antioxidant enzymes in lung after oxidant injury. Environ Health Persp 102: Suppl 2, 79–87, 1994
28. Janssen YM, Marsh JP, Driscoll KE, Born PJ, Oberdorster G, Mossman BT: Increased expression of manganese-containing superoxide dismutase in rat lungs after inhalation of inflammatory and fibrogenic minerals. Free Rad Biol Med 16: 315–22, 1994
29. Janssen YM, Marsh JP, Absher MP, Hemenway D, Vacek PM, Leslie KO, Born PJ, Mossman BT: Expression of antioxidant enzymes in rat lungs after inhalation of asbestos or silica. J Biol Chem 267: 10625–30, 1992

30. Hay J, Shahzeidi S, Laurent G: Mechanism of bleomycin-induced lung damage. Arch Toxicol 65: 81–94, 1991
31. Bus JS, Gibson JE: Paraquat: model for oxidant-initiated toxicity. Environ Health Perspect 55: 37–46, 1984
32. Dedhia HV, Banks DE, Castranova V, Vallyathan V, Masri F, Burrell R, Billie M: Alteration in lung lavagate and macrophage function in rats due to acute toluene diisocyanate exposure. Am J Respir Crit Care Med 150: A768, 1995
33. Jackson RM: Molecular, pharmacologic and clinical aspects of oxygen induced lung injury. Clin Chest Med 11: 73–86, 1990
34. Church DF, Pryor WA: Free-radical chemistry of cigarette smoke and its toxicological implications Environ Health Perspect 64: 111–126, 1985
35. Mitchell JB, Samuni A, Krishna CM, DeGraff WG, Ahn MS, Samuni U, Russo A: Biologically active metal-independent superoxide dismutase mimics. Biochemistry 29: 2802–2807, 1990
36. Belkin S, Mehlhorn RJ, Hideg K, Hankovsky O, Packer L: Reduction and destruction rates of nikoxide spin probes. Arch Biochem Biophys 256: 232–243, 1987
37. Swartz HM, Sentjurc M, Morse PD: Cellular metabolism of water-soluble nitroxides: Effect on rate of reduction of cell/nitroxide ration, oxygen concentrations and permeability of nitroxides. Biochim Biophys Acta 888: 82–90, 1986
38. Krishna MC, Grahame DA, Samuni A, Mitchell JB, Russo A: Oxuammonium cation intermediate in the nitroxide catalysis dismutation of superoxide. Proc Nat Acad Sc 89: 5537–5541, 1992
39. Samuni A, Mitchell JB, DeGraff W, Krishna CM, Samuni U, Russo A: Nitroxide SOD-mimic: Modes of action. Free Rad Res Commun 12: 187–94, 1991
40. Voest EE, Van Faassen E, Marx JJM: An electron paramagnetic resonance study of the antioxidant properties of the nitroxide free radical TEMPO. Free Rad Biol Med 15: 589–95, 1993
41. Hahn SM, Tochner Z, Krishna CM, Glass J, Wilson L, Samuni A, Sprague M, Venzon D, Glatstein E, Mitchell JB, Russo A: Tempol, a stable free radical, is a novel murine radiation protector. Cancer Res S2: 1750–1753, 1992