Bull Environ Contam Toxicol (2010) 84:662–666
DOI 10.1007/s00128-010-0021-1

Dual Role of Vitamin C Utilization in NO_2-Induced Oxidative Stress in Lung Tissues of Mice

Yingyi Zhang · Chunyan Ma · Yuhuan Xiao · Chang Liu · Qiang Xu · Yan Wang · Xin Xu · Lin Hao

Received: 19 October 2009 / Accepted: 21 April 2010 / Published online: 30 April 2010

Abstract Earlier studies with in vitro models have revealed that application of vitamin C can act as a primary NO_2 absorption substrate to contribute to NO_2-induced cellular injury. In the present study, we showed that the pharmacological application of vitamin C had dual role in lungs of mice exposed to NO_2, with an exacerbated oxidative stress occurring at low concentrations, as indicated by excessive reactive oxygen species production and lipid peroxidation. However, at high concentrations, vitamin C functioned as an antioxidant removing reactive oxygen species and maintaining a reducing status in cells, alleviating NO_2-induced oxidative toxicity.

Keywords Mice · Nitrogen dioxide · Vitamin C · Lipid peroxidation · Antioxidant defense

As one of the most serious oxidizing air pollutants, nitrogen dioxide (NO_2) has been well documented to cause ecosystem deterioration and induce airway diseases (Han and Naeher 2006; Poynter et al. 2006; Brandsma et al. 2008). However, the specific mechanisms for initiating toxicity are not yet fully understood, and remain intensive research. Undoubtedly, the detrimental effects of NO_2 are directly related to its oxidizing potential (Velsor and Postlethwait 1997). Inhalation of NO_2 causes pulmonary cellular injury that includes site-specific epithelial damage, lipid and protein oxidation, impairment of gas exchange, and initiation of an inflammatory response (Velsor et al. 2003 and references here). Exposure of mice to NO_2 results in enhanced protein s-glutathionylation in parenchymal regions of lung tissues, which is proposed to be involved in redox signal transduction pathway and translocation (Shelton et al. 2005; Aesif et al. 2009). In spite of the substantial evidence indicating that vitamin C, a classical antioxidant, can prevent or attenuate NO_2-induced lipid oxidation and lung disease (Menzel 1992, 1994), it is also shown that vitamin C at low concentrations strongly increases NO_2-iduced lipid oxidation in in vitro experimental systems (Velsor and Postlethwait 1997; Velsor et al. 2003), suggesting that vitamin C seems to have two attributes in animal response to NO_2 exposure.

Vitamin C is well established biochemically as an antioxidant that not only scavenges neutrophil oxidants but also reduces oxidized vitamin E, thus restoring the antioxidant capability of this important lipid antioxidant (Packer et al. 1979). The lungs and respiratory tract are target tissues for vitamin C (Willis and Kratzing 1976), where it is present in the airway surface liquid and forms a critical interface between the respiratory tract epithelial cells and the external environment. Well established in vitro experimental model demonstrates that vitamin C as the primary NO_2 absorption substrates in the epithelial lining fluid (ELF) drives the rapid and irreversible reactions between NO_2 and ELF, and forces the continued net flux of this relatively aqueous-insoluble gas into the ELF. Thus the deleterious effects of inhaled NO_2 are likely mediated by products of these initial NO_2-ELF reactions rather than NO_2 per se (Velsor and Postlethwait 1997). To explain the dual attributes of vitamin C in animal response to NO_2 exposure, we carried out this experiment using mice, and showed that low concentrations of vitamin C

Yingyi Zhang and Chunyan Ma contributed equally to this work.

Y. Zhang · C. Ma · Y. Xiao · C. Liu · Q. Xu · Y. Wang · X. Xu · L. Hao (✉)
College of Life and Chemistry Sciences, Shenyang Normal University, 110034 Shenyang, People's Republic of China
e-mail: haolinwj2001@yahoo.com.cn

promoted the NO_2-induced oxidative stress, whereas vitamin C at high concentrations attenuated the oxidative stress.

Materials and Methods

Five week-old healthy male albino mice weighing 20 ± 2 g were used as animal subjects in the present experiment. The mice were randomly divided into normal control group and NO_2 treated groups supplied with different concentrations of vitamin C solutions and each treatment group consists of six animals. Vitamin C was supplied every other day before 3 days of NO_2 exposure until the exposure end. In details, the animals were administered by oral gavage of 0 (physiological saline), 25, 50, 100, and 200 mg kg^{-1} body weight of sodium-L-ascorbate, respectively. The animals were caged under a 12 h light/dark cycle in humidity (60 ± 10%) and temperature (25 ± 2°C) controlled rooms, and had free access to food and water. For NO_2 exposure, the mice of different groups were fumigated with 20 μL L^{-1} NO_2 in a chamber for 4 h (8:00–12:00 a.m.) per day for 10 days. The concentration of NO_2 used was derived from related literatures (Poynter et al. 2006; Brandsma et al. 2008). The NO_2 gas was supplied directly from cylinders, into a dilution reservoir into which charcoal filtered air was drawn. The concentration was controlled by means of individual needle valves and flow meters for each chamber. Chamber NO_2 concentration was monitored during treatment using an NO_2 analyzer. For the control group, charcoal filtered air alone was supplied. At the end of the NO_2 exposure, animal lung samples were harvested and washed three times with ice-cold physiological saline, weighted, wrapped with aluminum foil and stored in liquid nitrogen. All animal procedures were approved by the Shenyang Medical College Animal Investigational Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health, People's Republic of China.

Relative body weight increment of animals of NO_2 exposed groups was expressed as a percentage of the control. For the determination of biochemical indices, tissues at 0.5 g were homogenized in liquid nitrogen with pestle/mortar and the powder was added to a test tube containing 5 mL of ice-cold extract solution consisting 50 mM phosphate buffer, pH 7.0, 1 mM dithiothreitol. After mixing, the solution was incubated for 15 min on ice. The homogenate was centrifuged at 12,000×*g* for 15 min, and the supernatant was collected to determine the following indices. SOD (EC 1.15.1.1) activity was assayed using the method of Sun et al. (1988). One unit of SOD is defined as the amount of enzyme necessary to inhibit the rate of NBT reduction by 50%. CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm for 3 min as described by Aebi (1983). POD (EC 1.11.1.7) activity was estimated according to Hemeda and Klein (1990). The level of lipid peroxidation was determined by measuring the level of thiobarbituric acid reactive substance (TBARS) following Esterbauer and Cheeseman (1990) and expressed as nM of malondialdehyde (MDA) formed. The reduced glutathione (GSH) content was determined as described by Griffith and Meister (1979). The oxidized glutathione (GSSG) content was calculated from the difference between total glutathione from DTT-treated samples and GSH from non-DTT-treated samples. The hydrogen peroxide was measured by the Fluoro H_2O_2 kit from Cell Technology (Mountain View, CA). In all the enzyme preparations, protein concentration was estimated by the method of Lowry et al. (1951) using bovine albumin as standard. For assessment of lung histopathology, the left lung lobe of the animals was fixed at a constant inflation pressure, embedded in paraffin and stained with hematoxylin and eosin according to the method described by Tuder et al. (2003).

All data were obtained from at least three separate experiments and expressed as mean ± standard deviation, and analyzed with one-way ANOVA. The differences were considered significant at $p < 0.05$.

Results and Discussion

Figure 1a shows that the exposure of NO_2 remarkably reduced the body weight increment of mice compared to the control group. Application of vitamin C had complicated effect on the animal growth, with seemly exacerbated growth inhibition at a dose of 25 mg kg^{-1}, while completely removing of the NO_2-induced growth retardation occurring upon supplement of 100 mg kg^{-1} or higher dose of vitamin C. The determination of H_2O_2 content in lung tissues indicated that the NO_2 exposure resulted in excessive H_2O_2 accumulation relative to control (Fig. 1b). Interestingly, vitamin C used at 25 mg kg^{-1} further promoted the NO_2-caused H_2O_2 accumulation, while a dose of 100 mg kg^{-1} nearly counteracted the accumulation (Fig. 1b), which was correlated well to the animal growth state (Fig. 1a), confirming that the excessive ROS production initiated by NO_2 inhalation was responsible for the NO_2 toxicity to animals.

Increasing evidence demonstrates that a balance between oxidant production and antioxidant defenses plays a critical role in animal or human response to deleterious stresses (Valko et al. 2007; Nadeem et al. 2008; Li et al. 2009). Thus, we investigated the changes of antioxidative enzyme activity and antioxidant content. Figure 1c revealed that NO_2 inhalation increased SOD activity,

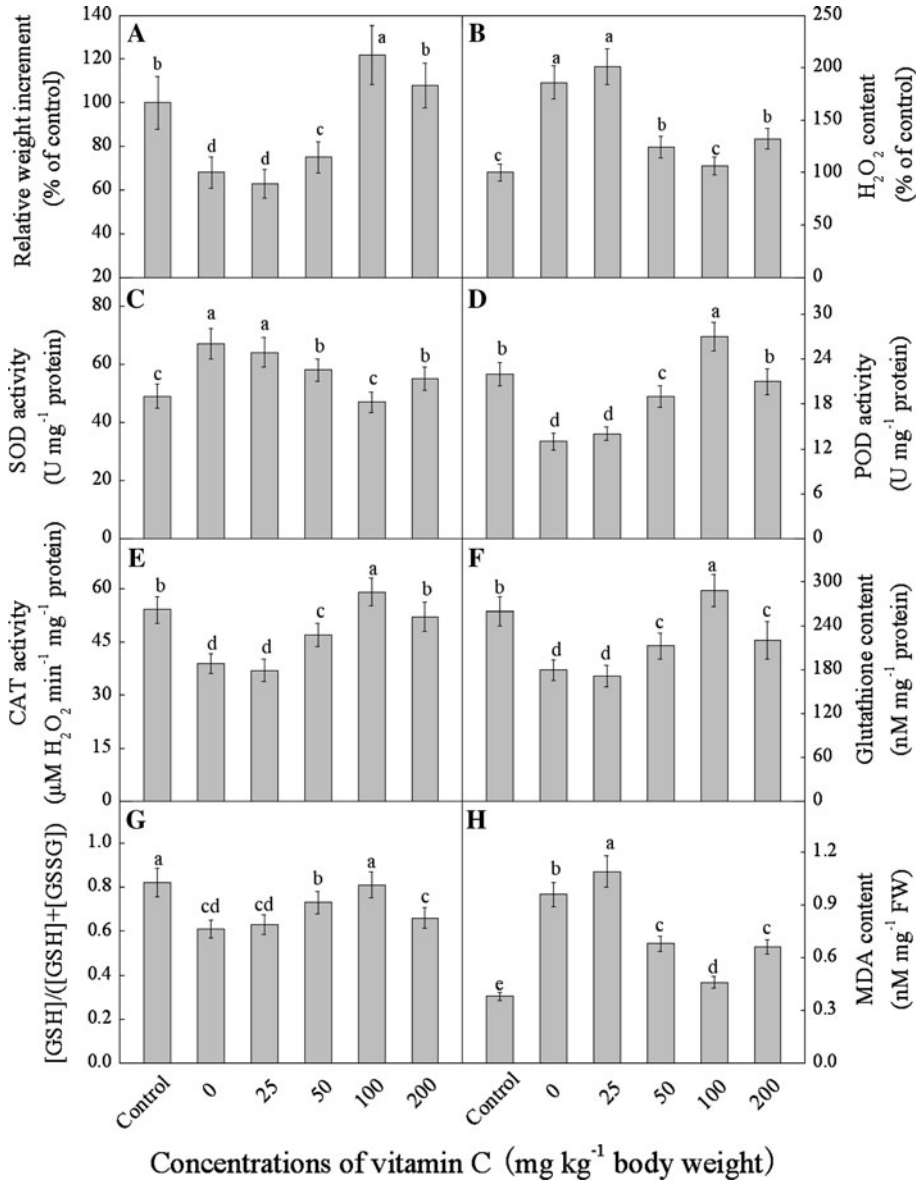

Fig. 1 Effect of NO_2 exposure and vitamin C application on relative body weight increment of mice (**a**), hydrogen peroxide (**b**), superoxide dismutase (**c**), peroxidase (**d**), catalase (**e**), reduced glutathione (**f**), ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) (**g**) and malondialdehyde (MDA) content (**h**) in the lung tissues. The data shown here from three replicated experiments and represent the mean ± SD. The different letters (*a, b*, etc.) indicate a significant difference at $p < 0.05$

together with the fact that SOD is normally induced by moderate intensity of oxidative stress (For reviews, see Kinnula and Crapo 2003; Mak 2008), therefore preventing an increase in oxidative burden (Siedlinski et al. 2009), further indicating NO_2 inhalation resulting in oxidative stress. Vitamin C application at 100 mg kg^{-1} maintained the SOD activity to the control level, suggesting that the concentration of vitamin C effectively protected the animals against NO_2-induced oxidative stress. Nevertheless, neither did vitamin C at 25 mg kg^{-1} further increase the SOD activity, consideration of its putative prooxidant in the present study. Dramatic decreases of POD and CAT activity were observed in the lung tissues exposed to NO_2 compared to control, and the vitamin C supplements except the dose of 25 mg kg^{-1} counteracted the inhibition to some extent, especially with a dose of 100 mg kg^{-1} the POD and CAT activity even exceeding the control level (Figs. 1d and 1e), which was correlated essentially to the changes of H_2O_2 level in the lung tissues (Fig. 1b), indicating that those two enzymes likely contributed to H_2O_2 scavenging in lung tissues. Glutathione is a major water-soluble antioxidant thiol in the lung ELF, and an efficient scavenger of hydrogen peroxide, and oxidant injury always links with glutathione deficiency in lung ELF (Cantin et al. 1987, 1989). Figure 1f, g showed that NO_2 inhalation significantly lowered reduced glutathione (GSH) level and the ratio of GSH to oxidized glutathione (GSSG), whereas vitamin C used at 100 mg kg^{-1} effectively protected the GSH level and the ratio to GSSG. With a similarity to the antioxidative enzymes, vitamin C applied at 25 mg kg^{-1} did not have effect on the glutathione indices (Fig. 1f, g). However, this dose of vitamin C indeed exacerbated

NO_2-induced lipid peroxidation, as indicated by increased malondialdehyde (MDA) formation, while other doses of vitamin C, especially 100 mg kg^{-1} effectively alleviated the NO_2-induced MDA elevation (Fig. 1h). These data suggested that low concentration of vitamin C (such as 25 mg kg^{-1}) augmented oxidative stress by promoting reactive oxygen species (ROS) production, but not decreasing the antioxidant defenses, whereas high vitamin C (e.g. 100 mg kg^{-1}) enhanced the antioxidant capability to protect lung tissues against NO_2-induced oxidative injury.

To further address the contribution of NO_2 to pulmonary inflammation, as well as the role of exogenous application of vitamin C, We examined the lung tissues of NO_2-exposed mice by histopathology (Figs. 2 and 3). In parallel with the changes of the biochemical indices mentioned above, lungs harvested from mice exposure to NO_2 displayed alveolar space augmentation (Fig. 2b) and cellular infiltration within terminal bronchioles (Fig. 3b), and vitamin C application at 25 mg kg^{-1} apparently exacerbated the inflammation response, with swelling of the alveolar septa (Fig. 2c) and severely desquamated bronchial epithelium (Fig. 3c), while the lung tissues of 100 mg kg^{-1} vitamin C treated animals were similar to control (Fig. 2a vs. d and Fig. 3a vs. d, respectively). The

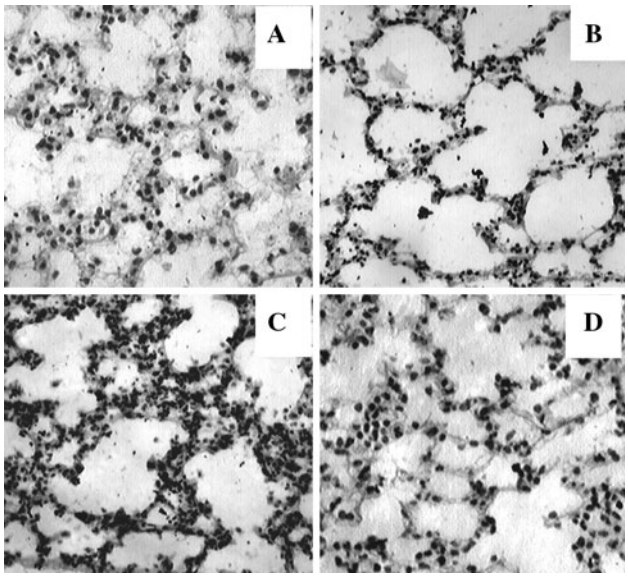

Fig. 2 Hematoxylin and eosin-stained lung sections of four animals to show alveolar space. Original magnification, ×200. **a** Lung tissue of a control animal. **b** Representative lung tissue of an animal exposed to 20 μL L^{-1} NO_2. **c** Representative lung tissue of an animal treated with 25 mg kg^{-1} of vitamin C every other day before 3 days of 20 μL L^{-1} NO_2 exposure until the exposure end. **d** Representative lung tissue of an animal treated with 100 mg kg^{-1} of vitamin C instead of 25 mg kg^{-1} in "C". Three mice in each experimental group were examined and a representative result is shown

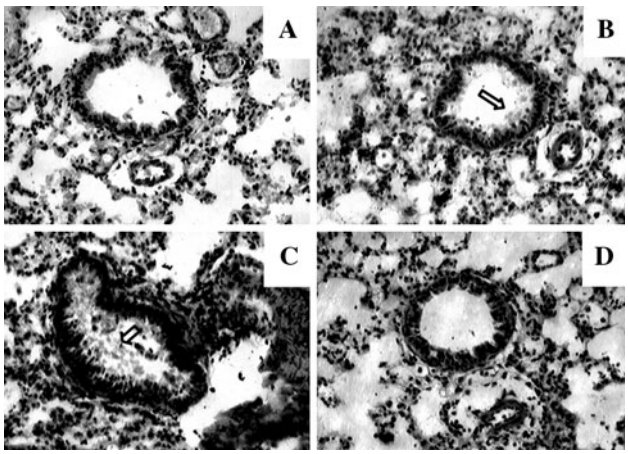

Fig. 3 Hematoxylin and eosin-stained lung sections of four animals to show terminal bronchioles. Original magnification, ×150. **a** Lung tissue of a control animal. **b** Representative lung tissue of an animal exposed to 20 μL L^{-1} NO_2. The *arrow* indicates cellular infiltration within terminal bronchioles. **c** Representative lung tissue of an animal treated with 25 mg kg^{-1} of vitamin C every other day before 3 days of 20 μL L^{-1} NO_2 exposure until the exposure end. The *arrow* indicates cellular infiltration within terminal bronchioles. **d** Representative lung tissue of an animal treated with 100 mg kg^{-1} of vitamin C instead of 25 mg kg^{-1} in "C". Three mice in each experimental group were examined and a representative result is shown

NO_2-caused histopathological changes were in agreement with other similar study (Poynter et al. 2006).

A series of elaborately established in vitro animal experimental systems have undoubtedly documented that application of vitamin C promotes NO_2 absorption, therefore resulting in oxidative injury (Postlethwait et al. 1990, 1995; Velsor and Postlethwait 1997; Velsor et al. 2003). In the present experiment, we used mice as subject to demonstrate that application of low concentrations of vitamin C could promote NO_2-induced ROS production in lung tissues, thus leading to exacerbated lipid peroxidation and animal growth retardation, while high concentrations of vitamin C efficiently removed ROS by enhancing antioxidant capability, protecting animals against NO_2-caused oxidative injury. The histopathological changes were also correlated well to the biochemical indices.

Acknowledgments We would like to thank Dr. Sheng T Hou (Institute for Biological Sciences, National Research Council of Canada) for comments on the manuscript. This research was partly supported by the National Natural Science Foundation of China (30570445) and Director Foundation of Experimental centre, Shenyang Normal University (SY200802).

References

Aebi HE (1983) Catalase. In: Bergmeyer HU (ed) Methods of enzymatic analyses. Verlag Chemie, Weinheim, pp 273–282

Aesif SW, Anathy V, Havermans M, Guala AS, Ckless K, Taatjes DJ, Janssen-Heininger YMW (2009) In situ analysis of protein S-glutathionylation in lung tissue using glutaredoxin-1-catalyzed cysteine derivatization. Am J Pathol 175:36–45

Brandsma CA, Hylkema MN, Luinge MA, Geerlings M, Klok PA, Cassee FR, Timens W, Postma DS, Kerstjens HAM (2008) Nitrogen dioxide exposure attenuates cigarette smoke-induced cytokine production in mice. Inhal Toxicol 20:183–189

Cantin AM, North SL, Hubbard RC, Crystal RG (1987) Normal alveolar epithelial lining fluid contains high levels of glutathione. J Appl Physiol 63:152–157

Cantin AM, Hubbard RC, Crystal RG (1989) Glutathione deficiency in the epithelial lining fluid of the lower respiratory tract in idiopathic pulmonary fibrosis. Am Rev Respir Dis 139:370–372

Esterbauer H, Cheeseman KH (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol 186:407–421

Griffith OW, Meister A (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (s-n-butylhomocysteine sulfoximine). J Biol Chem 254:7558–7560

Han XL, Naeher LP (2006) A review of traffic-related air pollution exposure assessment studies in the developing world. Environ Pollut 32:106–120

Hemeda HM, Klein BP (1990) Effects of naturally occuring antioxidants on peroxidase activity of vegetable extracts. J Food Sci 55:184–185

Kinnula VL, Crapo JD (2003) Superoxide dismutases in the lung and human lung diseases. Am J Respir Crit Care Med 167:1600–1619

Li RG, Li TT, Hao L, Xu X, Na J (2009) Hydrogen peroxide reduces lead-induced oxidative stress to mouse brain and liver. Bull Environ Contam Toxicol 82:419–422

Lowry OH, Rosenbrough NJ, Far AL, Randel RJ (1951) Protein measurement with folin–phenol reagent. J Biol Chem 193:265–275

Mak JC (2008) Pathogenesis of COPD. Part II. Oxidative-antioxidative imbalance. Int J Tuberc Lung Dis 12:368–374

Menzel DB (1992) Antioxidant vitamins and prevention of lung disease. Ann N Y Acad Sci 669:141–155

Menzel DB (1994) The toxicity of air pollution in experimental animals and humans: the role of oxidative stress. Toxicol Lett 72:269–277

Nadeem A, Masood A, Siddiqui N (2008) Review: Oxidant–antioxidant imbalance in asthma: scientific evidence, epidemiological data and possible therapeutic options. Ther Adv Respir Dis 2:215–235

Packer JE, Slater TF, Willson RL (1979) Direct observation of a free radical interaction between vitamin E and vitamin C. Nature 278:737–738

Postlethwait EM, Langford SD, Bidani A (1990) Reactive absorption of nitrogen dioxide by pulmonary epithelial lining fluid. J Appl Physiol 69:523–531

Postlethwait EM, Langford SD, Jacobson LM, Bidani A (1995) NO_2 reactive absorption substrates in rat pulmonary surface lining fluids. Free Radic Biol Med 19:553–563

Poynter ME, Persinger RL, Irvin CG, Butnor KJ, van Hirtum H, Blay W, Heintz NH, Robbins J, Hemenway D, Taatjes DJ, Janssen-Heininger Y (2006) Nitrogen dioxide enhances allergic airway inflammation and hyperresponsiveness in the mouse. Am J Physiol Lung Cell Mol Physiol 290:L144–L152

Shelton MD, Chock PB, Mieyal JJ (2005) Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation. Antioxid Redox Signal 7:348–366

Siedlinski M, van Diemen CC, Postma DS, Vonk JM, Boezen HM (2009) Superoxide dismutases, lung function and bronchial responsiveness in a general population. Eur Respir J 33:986–992

Sun Y, Oberley LW, Li YA (1988) A simple method for clinical assay of superoxide dismutase. Clin Chem 34:497–500

Tuder RM, Zhen L, Cho CY, Taraseviciene-Stewart L, Kasahara Y, Salvemini D, Voelkel NF, Flores SC (2003) Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade. Am J Respir Cell Mol Biol 29:88–97

Valko M, Leibfritz D, Moncola J, Cronin MTD, Mazura M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84

Velsor LW, Postlethwait EM (1997) NO_2-induced generation of extracellular reactive oxygen is mediated by epithelial lining layer antioxidants. Am J Physiol (Lung Cell Mol Physiol) 237:L1265–L1275

Velsor LW, Ballinger CA, Patel J, Postlethwait EM (2003) Influence of epithelial lining fluid lipids on NO_2-induced membrane oxidation and nitration. Free Radic Biol Med 34:720–733

Willis RJ, Kratzing CC (1976) Extracellular ascorbic acid in lung. Biochim Biophys Acta 444:108–111