LUNG PERMEABILITY, ANTIOXIDANT STATUS AND NO_2 INHALATION: A SELENIUM SUPPLEMENTATION STUDY IN RATS

C.Y. de Burbure, J.-F. Heilier,[1] J. Nève,[2] A. Becker, C. Albrecht, P.J.A. Borm,[3] J. Gromadzinska, W. Wasowicz, K. Rydzynski,[4] A.M. Bernard[1]

ABSTRACT

Little is known about antioxidant status, selenium status in particular, and lung response to NO_2 which acts as proinflammatory air pollutant. The effects of a low selenium diet (1.3 µg Se/day) with or without selenium supplementation were therefore studied in 128 two-month-old male Wistar rats exposed to either acute (50 ppm, 30 minutes), intermittent subacute (5 ppm, 6 h/d, 5 days), intermittent long-term NO_2 (1 ppm, 10 ppm, 6 h/d, 5 d/wk, 28 days) or normal atmospheric air (controls). Following sacrifice, measurements of lipid peroxidation (thiobarbituric acid reactive substances, chemiluminescence), antioxidative protective enzymes (glutathione peroxidase GPx, superoxide dismutase SOD, glutathione S-transferase GST, ceruloplasmin), lung damage (lactate dehydrogenase, alkaline and acid phosphatases), lung permeability (total protein, albumin), inflammation (cell populations), along with the determination of new biomarkers such as CC16 (Clara cell protein) were performed in serum and broncho-alveolar lavage fluid (BALF). While selenium supplemented animals had increased GPx activity in serum prior to inhalation experiments, they also had decreased BALF-CC16, blood-SOD and GST levels. Nevertheless, the protective role of normal selenium status with respect to NO_2 lung toxicity was evident both for long-term and acute exposures, as the increase in BALF-total proteins and corresponding decrease in serum (indicating increased lung permeability) was significantly more pronounced in selenium-deficient animals. During the various inhalation experiments, serum CC16 demonstrated its key role as an early marker of increased lung permeability. These findings corroborate the important role of selenium status in NO_2 oxidative damage modulation, but also indicate, in view of its negative impact on CC16, a natural anti-inflammatory and immunosuppressor, that caution should be used prior to advocating selenium supplementation.

[1] Université Catholique de Louvain, Brussels, Belgium.
[2] Université Libre de Bruxelles, Brussels, Belgium.
[3] Institut für Umweltmedizinische Forschung gGmbH (IUF), Düsseldorf, Germany.
[4] The Nofer Institute of Occupational Medicine, Lodz, Poland.

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INTRODUCTION

Although the effects of NO_2 on the lung of both humans and animals have been widely documented, little is known about antioxidant status and particularly selenium status on the lung response to NO_2, which acts as a proinflammatory air pollutant. The objective of the present study was therefore to first evaluate the pulmonary effects of a low selenium diet (1.3 μg Se/day) with or without selenium supplementation in 128 two-month old male Wistar rats, then expose them to various concentrations of NO_2 and analyze the changes in lung response.

Nitrogen dioxide (NO_2) has become a major cause of concern not only as one of the most common man-induced air pollutants, but also more critically as a key mediator of the photochemical formation of ozone. This combustion product is mainly released into the environment in diesel exhaust fumes, tobacco smoke, by industries of nitric acid, fertilizers or explosives, or simply by cooking and heating with unventilated gas appliances. Its outdoor concentrations usually average 0.04-0.06 mg/m^3 i.e. 0.02-0.03 ppm, rising amidst dense traffic (0.05-0.1 ppm), while indoor concentrations average 0.02-0.08 mg/m^3, peak values being found in homes with gas stoves (average 0.5 ppm, peak values 1-2 ppm (Blomberg et al., 1999)). The occupational exposure limit (OEL) advised by the European Commission for NO_2 is 0.4 mg/m^3, i.e. 0.2 ppm (time weighted average TWA, 8h), its short-term exposure limit (STEL) being 1 mg/m^3 i.e. 0.5 ppm (TWA, 15 min). The WHO air quality guidelines currently recommend a maximal mean annual value of 40 $\mu g/m^3$ and a one-hour value of 200 $\mu g/m^3$ (WHO, 2000).

The pulmonary effects of NO_2 exposure are mainly reported on the lower respiratory tract, and are due to its capacity to react with water to produce HNO_3 and/or HNO_2, highly corrosive and irritant substances. Acute responses described vary from small dose-dependent reversible morphological changes in the trachea (reduced mucociliary transport, altered permeability, Kakinoki et al., 1998), bronchioles (changes in Clara cell shape, (Barth and Muller, 1999)) and alveoli (increased number of macrophages and type II epithelial cells (Chang et al., 1986), enhanced permeability (McElroy et al., 1997)) to important inflammatory responses resembling those of acute bronchitis at higher doses (influx of neutrophils and eosinophils, microvascular leakage, smooth muscle hyper-responsiveness (Papi et al., 1999)) and finally death from pulmonary edema reported in all species at much larger doses (Gray et al., 1954). A degree of morphological adaptation of the airways to oxidative stress has also been reported following prolonged exposure (Barth and Muller, 1999) or even repeated exposure (Blomberg et al., 1999) with the conclusion that NO_2 acts as a proinflammatory air pollutant.

The reported biochemical responses to NO_2 exposure have mainly concentrated on changes in lipid peroxidation and antioxidative protective systems, demonstrating wide interspecies differences in sensitivity to NO_2. Lipid peroxide (thiobarbituric acid reactant substances, TBARS) levels thus appear to be inversely related to the ability of the species to induce antioxidative protective factors in the lung such as the enzymes glutathione peroxidase, Glucose-6-phosphate dehydrogenase, Glutathione S-transferase, superoxide dismutase or ceruloplasmin and to regenerate reducing factors including non-protein sulfhydryls, Vitamin E and Vitamin C. Hence a direct interest in the potential protective role that antioxidant status may play in influencing the lung response to air pollutants such as NO_2 and ozone, which have indeed often been studied jointly (Ichinose et al., 1988; Ichinose and Sagai, 1989; Sagai et al., 1987; Sagai and Ichinose, 1991).

Interestingly, although the roles of vitamins A, C and E have already been investigated in the lung exposed to NO_2 (Dogra et al., 1983; Tom et al., 1985; Hatch et al., 1986; Sevanian et al., 1982), with the exception of one study on respiratory pentane (Dillard et al., 1980), the influence of selenium (Se) on the lung response to NO_2 exposure has, to the best of our knowledge, not yet been reported, although numerous selenium supplementation studies can be found in the literature. The antioxidant role of selenium is dependent on the action of three selenium-containing enzyme classes, cytosolic glutathione peroxidase, membrane-bound phospholipid hydroperoxide glutathione peroxidase and thioredoxin reductase, which in turn control the redox tone and the levels of cellular hydroperoxides and may also be responsible for the effects of selenium on the immune system. Its various and complex qualitative and quantitative effects on the cell cycle, viability, protein synthesis, DNA integrity and cellular metabolism are, however, mediated by a whole variety of other cellular selenium compounds which also interact with the antioxidant pathways (Spallholz, 2001). As selenium is already well known to play a protective role in vitro against oxidative damage to several organs (Chen and Tappel, 1995) as well as against apoptosis induced by superoxide anion (Guo et al., 2001) the hypothesis that selenium may play a protective role in vivo in the case of NO_2 exposure was therefore investigated in this inhalation study on Wistar rats, which are known to be able to induce antioxidant enzymes in response to NO_2.

The other innovative aspect of this study lies in the determination of the newly discovered and applied biomarker of lung response, the Clara cell secretory protein (CC16), which has been shown to be a major secretory product of human and animal bronchiolar Clara cells and controls lung sensitivity to pollutants (Bernard et al., 1992). CC16 is indeed a natural immunosuppressor and anti-inflammatory agent inhibiting both monocyte and polymorphonuclear neutrophil chemotaxis and phagocytosis *in vitro* (Lesur et al., 1995). It also appears to decrease the activity of interferon-γ, a potent multifunctional cytokine produced in the course of viral infections and inflammation (Dierynck et al., 1995) and its synthesis is stimulated by tumor necrosis factor-α (TNF-α) (Yao et al., 1998). As CC16 has already been shown to be a sensitive non-invasive biomarker of lung epithelium damage in case of ozone exposure (Broeckaert et al., 2000) we therefore included its study along standard measurements of lipid peroxidation (TBARS, chemiluminescence), antioxidative enzymes (Glutathione peroxidase, Superoxide Dismutase, Glutathione S-Transferase, Ceruloplasmin), lung damage (lactate dehydrogenase, alkaline and acid phosphatases), lung permeability (total protein, albumin) and inflammation markers (cell populations), both in serum and in broncho-alveolar lavage fluid (BALF).

MATERIALS AND METHODS

Study design

Following approval of the Ethical Committee of the Nofer Institute of Occupational Medicine, two month-old male Wistar rats (Outbred IMP, Wist, n=128) were divided at the start of the study into two separate groups and fed for 14 weeks either a special low selenium diet (group Se-, n=64) or the same low selenium diet supplemented with selenium as sodium selenite in drinking-water (Na_2SeO_3, 225 μg/l) *ad libitum* (group Se+, n=64). The low Se diet, containing 0.016 μg Se/g of fodder, was composed of bread (37%), cottage cheese (14%), sunflower oil (3%), vegetables (43.5%) and flax grain (2.5%). Fodder and water intake was checked daily. Estimated intake of the Se- group fed this diet was 1.3 μg Se/day, whereas the Se+ group animals were provided with 6.0 μg Se/day. Glutathione peroxidase (GPx) activity in erythrocytes was measured in blood at the beginning of the experiment and then at 3-4 week intervals for 14 weeks, as an indicator of Se status. Once a week the animal body weight gain was controlled. When GPx activity was stable and differentiated in both groups, NO_2 exposure began.

Groups of Se-normal and Se-deficient Wistar rats were placed in dynamic inhalation chambers and were exposed to 1 or 10 ppm NO_2 6 h/day, 5 days/week for 4 weeks (Groups I and II respectively, long-term exposures), 5 ppm for 6 h/day for 5 days (Group III, sub-acute exposure), 50 ppm for 30 minutes (Group IV, acute exposure) or used as controls breathing normal atmospheric air (Group V). Rats were sacrificed immediately after the experiment or 48 hours later in order to estimate recovery (see Table 1). Actual NO_2 concentrations were 1.04 ± 0.40 ppm, 5.45 ± 1.02 ppm, 10.40 ± 1.60 ppm and 45.55 ± 5.62 ppm. The control groups were fed either the low selenium or the selenium enriched diet. They were also kept in toxic chambers but breathed atmospheric air.

Table 1. Exposure conditions to NO_2 and Selenium (Se) status of animals in the inhalation study.

Exposure conditions to NO_2	**Groups**	**Total exposure to NO_2**		**Se-status**	**Recovery**
		ppm	**ppm.days**		
1 ppm, 28 days, 6 hrs/day, 5 d/wk	I (4x n=8)	28	5	Se+/Se-	0/48 hrs
10 ppm, 28 days, 6 hrs/day, 5 d/wk	II (4x n=8)	280	50	Se+/Se-	0/48 hrs
5 ppm , 5 days, 6 hrs/day	III (4x n=8)	25	6.25	Se+/Se-	0/48 hrs
50 ppm, 30 min	IV (2x n=8)	1.04	1.04	Se+/Se-	0 hrs
Controls	V (2x n=8)	0	0	Se+/Se-	0 hrs

Se-, diet with low selenium (1.3μg Se/day); Se+, diet with normal selenium content (6.0μg Se/day).

At the end of their exposure time, half of the rats of each exposed group were injected intraperitoneally with sodium pentobarbital (20 mg/250 g body weight) immediately after exposure and the other half 48 hours later in order to be able to evaluate potential recovery responses. The rats were exsanguinated via the abdominal aorta. Blood was collected into heparinised tubes, 200 μl of heparinised whole blood was immediately placed into a cold box away from sunlight and kept there no longer than 2 h (Habig et al., 1974) prior to analysis, while the remaining blood was centrifuged to separate plasma. In order to collect the bronchoalveolar lavage fluid (BALF)

from each rat, the trachea was cannulated, lungs were lavaged twice with 5 ml of 0.9% NaCl and lavage fluid collected from the 2 washes. BALF recovery in all investigated groups of animals was about 70%. 500 µl of BALF samples were stored in a cold box away from sunlight until analysis. Smears were prepared from BALF samples, stained according to May-Grünwald and Giemsa, and the total and differential cell counts were calculated with the use of light microscopy (Chapin, 1995). The rest of BALF samples were centrifuged at 600 g for 10 min to separate BALF cells. The remaining supernatant was stored at -20°C for further biochemical analysis which was conducted within six months of collection.

Biochemical analysis

Concentrations of nitric oxides in the inhalation chambers were measured using the colorimetric method after reduction of nitrates to nitrites, diazo reaction and conjugation of diazo compounds with chloride N-(1-naphthyl)-ethylenediamine to diazo dye. Selenium concentration was measured by graphite furnace atomic absorption spectrometry with Zeeman background correction (Neve et al., 1987). Quality assessment included participation in interlaboratory comparison trials and determination of commercial reference samples (Seronom trace elements from Sero AS).

The activity of GPx in BALF, plasma and red blood cell (RBC) lisate was measured using t-butylhydroperoxide as substrate (Paglia & Valentine, 1967). Superoxide dismutase (SOD) activity was determined with the use of xanthine oxidase and nitroblue tetrasolium (Beauchamp & Fridovich, 1971). Glutathione S-transferase (GST) activity was measured using 1-chloro2,4,-dinitrobenzene (Habig et al., 1974). Oxidase activity of ceruloplasmin (Cp) in BALF was measured by the spectrophotometric method (Sunderman, Jr. and Nomoto, 1970). The extent of lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) both in plasma and in BALF by the method optimized by Wasowicz et al. (1993).

The measurement of chemiluminescence (CL) was performed using a Luminometer 1251 linked to an IBM PC AT. After the addition of whole blood or BALF, CL was defined as the area under the emission curve as a function of time, calculated over 30 min (Kantorski and Tchorzewski, 1992). The value of CL was obtained from the following: measurement **x** value of hemoglobin / per 10^3 neutrophils in blood or macrophages in BALF.

Clara cell protein (CC16) was determined by latex immunoassay both in serum and in BALF (Halatek et al., 1998). Total protein and albumin, lactate dehydrogenase (LDH), acid phosphatase (ACP) and alkaline phosphatase (ALP) activities were determined in BALF (Technicon RA system, Bayer diagnostic, Domont, France), epithelial lining fluid volume was calculated as follows: ELF volume = total BALF urea/plasma urea.

Statistical analysis

Statistical analysis was performed with JMP, Version 5. (SAS Institute Inc., Cary, NC, 1989-2002). Data are described by mean and standard error on the mean. Data were checked for normality and by means of the Dixon Q test (Dixon, 1950; 1951), outliers were removed. Comparison between exposed and unexposed control animals was performed by Student t test. A systematic comparison of the groups for biomarkers of NOx exposure (BALF and serum CC16 level) was done in an ANOVA (one way), separating unexposed and NOx exposed and normal and Se-deficient rats (Sall et al., 2001). Statistical significance (*, $p<0.05$) was tested using ANOVA.

RESULTS

Effects of selenium status on lung parameters in control rats

The two different selenium concentrations in the fodder (Se-: 1.3 µg Se/day and Se+: 6 µg Se/day) affected the studied lung parameters in several ways. As expected and monitored during the 14 weeks prior to the inhalation studies, blood selenium concentrations rose significantly in parallel with GPx concentrations in plasma and red blood cells in those rats fed the 6 µg Se/ day diet compared with those fed the 1.3 µg Se/day (each $p<0.0001$). In BALF, however, GPx concentrations showed the reverse tendency, the low selenium control group tending to have higher levels than the normal selenium group, though not significantly so ($p=0.12$). Interestingly, some other parameters also varied unexpectedly between control groups fed a different selenium diet, such as for example the Clara cell secretory protein (CC16), and Alkaline phosphatase activity in BALF. The Clara cell protein showed a lower concentration in serum ($p<0.0001$) and a higher concentration in BALF in Se-animals. Alkaline phosphatase activity in BALF was also found to be lower in selenium deficient animals. Other

antioxidant parameters such as the zinc and copper dependent SOD enzyme also showed unexpected statistically significant variations in serum, increasing in Se- animals just as GST activity, demonstrating thereby compensatory opposite tendencies to GPx in serum (see Figure 1). None of the other studied parameters showed any impact of selenium status (plasma TBARS, BALF TBARS, total protein, albumin, ACP, LDH and Cp, CL and stimulated CL in blood and in BALF, hematocrit, hemoglobin, blood and BALF leukocyte, polymorphonuclear, neturophil, eosinophil, lymphocyte and monocyte counts, ELF and BALF volume).

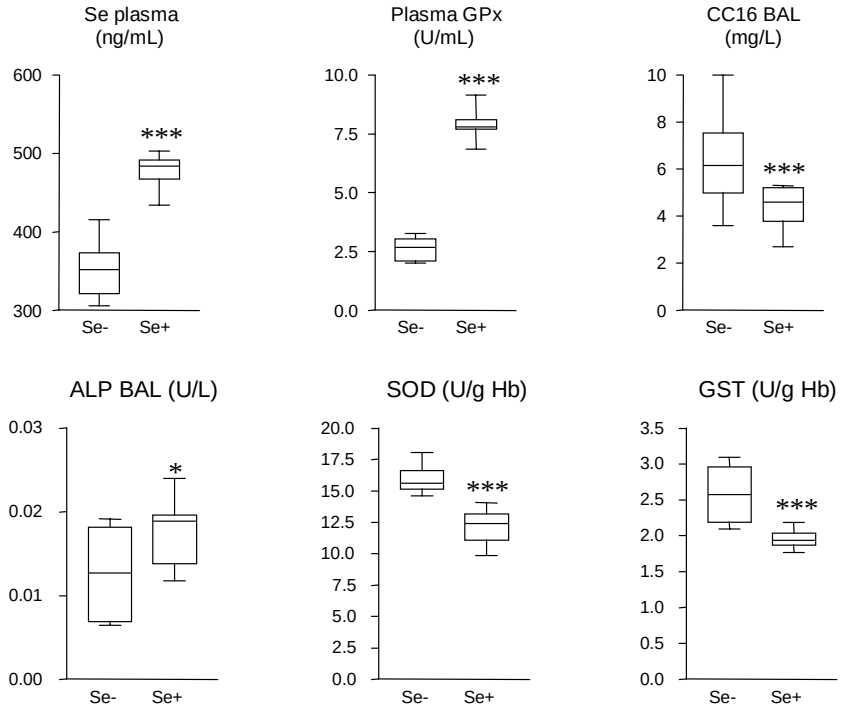

Figure 1. Changes in plasma and bronchoalveolar lavage (BAL) composition according to Selenium status in control rats (n=8 in each group). Comparison between Se+ and Se-: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Se: selenium (Se-: 1.3 µg Se/d, Se+: 6.0 µg/d), GPx glutathione peroxidase, CC16: Clara cell protein, ALP: alkaline phosphatase, SOD: superoxide dismutase, GST: glutathione S-transferase, Hb: hemoglobin.

NO_2 inhalation studies

The changes observed in three categories of studied end-points during the various inhalation experiments and their degree of statistical significance in comparison to control animals can all be found in Table 2.

A. Biomarkers of increased lung permeability and cellular damage

Total proteins and albumin. As illustrated in Figure 2, the increase in BALF-total proteins, indicating increased permeability of the lung epithelial barrier, was significantly more pronounced in Se- animals compared to rats receiving adequate selenium both for long-term exposure to 10 ppm and acute exposures to 50 ppm. As expected, changes in total protein and albumin concentrations were mainly and markedly significant for the acute exposure experiments at 50 ppm NO_2, increasing by a factor of 4 in both Se- and Se+ animals in the case of albumin, reflecting acute pulmonary damage and leakage of plasma into the airways. Interestingly, this did not seem to apply for the subacute exposure to 5 ppm, where total protein in BALF curiously actually decreased significantly in both Se- and Se+ animals, whereas albumin levels were only significantly reduced by 48 h ($p=0.0195$).

Table 2. Table of comparison to control values of the various parameters within each set of NO_2 inhalation. Increases compared to control values are indicated by +, decreases by -. NS: not statistically significant. Statistically significant variations are indicated as follows: +/-: $p<0.05$; ++/--: $p<0.01$; +++/---: $p<0.001$.

NO_2	1ppm (intermittent, 28 days)				5ppm (intermittent, 5 days)				10ppm (intermittent, 28 days)				50ppm (½hr)	
Diet	Se-		Se+		Se-		Se+		Se-		Se+		Se-	Se+
Vs. Ctl	Imm	48h	Imm	48h	Imm	48h	Imm	48h	Imm	48h	Imm	48h	Imm	Imm
A. Biomarkers of increased lung permeability and cellular damage														
CC16-S	NS	++	--	NS	+	+	NS	NS	+	++	---	NS	+	NS
CC16-BAL	NS	-	NS	++	---	NS	NS	NS	NS	NS	NS	+++	NS	NS
Tprot-BAL	NS	NS	NS	NS	--	NS	--	-	++	NS	NS	NS	+++	+++
Alb-BAL	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	+	NS	+++	+
ACP-BAL	NS	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	NS	---	NS
ALP-BAL	++	+++	+++	+	NS	NS	NS	NS	NS	NS	+	NS	+	NS
LDH-BAL	NS	NS	++	NS	NS	NS	NS	NS	NS	NS	++	NS	++	++
B. Selenium levels, antioxidant enzymes and oxidant production														
Se-Pl	--	NS	---	---	NS	NS	---	---	NS	NS	---	---	NS	---
GPx-Pl	NS	NS	---	---	NS	NS	---	---	+	NS	NS	+	NS	---
GPx-RBC	+++	+++	NS	+	++	+	++	+++	NS	+++	+++	+	+++	++
GPx-BAL	NS	NS	+++	+++	+++	NS	+++	+++	+++	+++	+++	+	+	+++
CP-BAL	+	NS	NS	NS	NS	NS	NS	NS	NS	+	NS	NS	NS	NS
SOD	+	---	+++	NS	---	---	NS	+++	--	NS	+++	NS	---	NS
GST	+	+++	+++	+++	NS	+++	+++	+++	NS	+	+++	+	NS	NS
CL-Bd	+	---	NS	NS	NS	+++	+++	NS	NS	NS	+	NS	NS	NS
CL-Bd+	NS	NS	NS	NS	NS	++	NS	NS	NS	NS	+	NS	NS	NS
CL-BAL	NS	+	-	NS	NS	+	NS	NS	NS	NS	+++	-	NS	NS
CL-BAL+	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	++	NS	NS	NS
TBAR-Pl	++	NS	++	-	NS	NS	NS	NS	NS	NS	NS	-	NS	NS
TBAR-BAL	NS	---	NS	NS	+++	NS	+++	---	NS	NS	++	---	NS	++
C. Parameters of blood and BALF cell populations														
Ht-Bd	NS	NS	---	-	NS	NS	---	NS	NS	NS	NS	---	-	NS
Hb-Bd	NS	NS	---	-	NS	NS	---	NS	NS	NS	NS	--	-	NS
Leu-Bd	NS	+	NS	NS	NS	--	NS	-	NS	NS	NS	-	-	NS
PMN-Bd	-	NS	NS	NS	NS	-	-	NS	NS	NS	NS	NS	NS	NS
Neu-Bd	NS	NS	NS	NS	NS	NS	NS	NS	-	-	NS	NS	NS	NS
Eosino-Bd	-	NS	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS
Lymph-Bd	++	NS	NS	NS	NS	+	+	NS	NS	NS	NS	NS	NS	NS
Mono-Bd	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS
Leu-BAL	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
PMN-BAL	NS	NS	NS	NS	NS	NS	+	NS	NS	NS	NS	NS	NS	NS
Lymph-BAL	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	+	NS	NS	NS
Mono-BAL	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	NS

BAL: broncho-alveolar lavage, Bd: blood; Pl: plasma; RBC: red blood cells, CC16 (µg/l): Clara cell protein, Tprot (µg/ml): total protein in BALF; Alb (µg/ml): albumin in BALF; ACP (IU/l): acid phosphatase, ALP (IU/l): alkaline phosphatase, LDH (IU/l): lactate dehydrogenase, CL: chemiluminescence in area under curve, Se (ng/ml): Selenium; GPx (IU/ml): glutathione peroxidase, GST (IU/gHb): glutathione S-transferase, SOD (IU/gHb): superoxide dismutase, TBAR (nmol/ml): thiobarbituric acid reactive substances, Cp (mg/l): ceruloplasmin, Ht (%): hematocrit, Hb (g/dl): hemoglobin, Leu (10^6/ml): leukocytes, PMN (%): polymorphonuclear neutrophils, Neu (%): neutrophils; Lymph (%): lymphocytes; Mono (%): monocytes.

CC16. The changes in CC16 in both serum and BALF in rats exposed to intermittent 1 ppm NO_2 6 hrs/day, 5 days/week for 28 days can be visualized in Figure 3, with the statistically significant variations compared to control values and their exact p values. The significant decrease in BALF-CC16 was mirrored by an increase in serum CC16 in the Se- rats, whereas those rats with normal selenium levels actually showed the reverse tendency. These changes even continued to increase in Se- rats at 48 hrs post-inhalation, whilst rats with

sufficient selenium intake appeared to recover faster, as serum CC16 levels returned to normal at 48 hrs although production of CC16 still appeared increased in the lung according to the higher BALF concentrations. Similar significant variations in CC16 were to be found in the short term exposure experiments at 5 ppm for 6 hrs/day for 5 days. However, at higher intermittent chronic exposure to 10 ppm NO_2 for 28 days, CC16 variations in BALF in the Se- rats indicated a different type of response. Indeed, CC16 in BALF did not decrease as for 1 ppm or 5 ppm, it appeared to have adapted in the same way as in the Se+ rats, its concentration tending to remain the same if not higher than prior to the inhalation experiments, whilst still leaking significantly into serum. Interestingly, in the acute exposure to 50 ppm NO_2, CC16 only appeared very markedly in serum in Se- rats, indicating major leakage across the lung epithelial barrier, whilst its levels did not diminish in BALF. Two-way analysis of variance for CC16 in BALF in the control, 1 ppm and 10 ppm NO_2 inhalation experiments indicated that the effects of exposure to NO_2 took precedence ($p=0.0493$) over the effects of the selenium status ($p=0.5390$) in affecting the BALF-CC16 variations in the model, both immediately after inhalation and at 48h ($p=0.0166$ and $p=0.0537$ respectively).

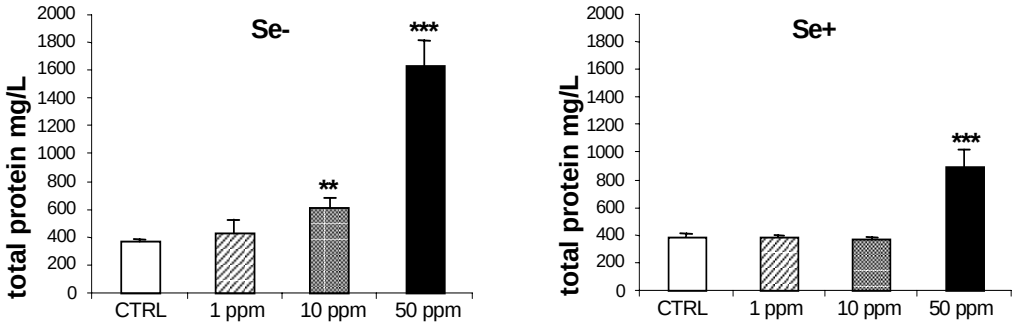

Figure 2. Total protein concentrations found in BALF immediately following NO_2 inhalation experiments at 1 ppm and 10 ppm NO_2 for 28 days (6 hrs/d, 5 d/week), and at 50 ppm NO_2 for 30 minutes, according to Selenium status (n=8 in each group except Se- 50 ppm n=6). Comparison to controls: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

CC16. The changes in CC16 in both serum and BALF in rats exposed to intermittent 1 ppm NO_2 6 hrs/day, 5 days/week for 28 days can be visualized in Figure 3, with the statistically significant variations compared to control values and their exact p values. The significant decrease in BALF-CC16 was mirrored by an increase in serum CC16 in the Se- rats, whereas those rats with normal selenium levels actually showed the reverse tendency. These changes even continued to increase in Se- rats at 48 hrs post-inhalation, whilst rats with sufficient selenium intake appeared to recover faster, as serum CC16 levels returned to normal at 48 hrs although production of CC16 still appeared increased in the lung according to the higher BALF concentrations. Similar significant variations in CC16 were to be found in the short term exposure experiments at 5 ppm for 6 hrs/day for 5 days. However, at higher intermittent chronic exposure to 10 ppm NO_2 for 28 days, CC16 variations in BALF in the Se- rats indicated a different type of response. Indeed, CC16 in BALF did not decrease as for 1 ppm or 5 ppm, it appeared to have adapted in the same way as in the Se+ rats, its concentration tending to remain the same if not higher than prior to the inhalation experiments, whilst still leaking significantly into serum. Interestingly, in the acute exposure to 50 ppm NO_2, CC16 only appeared very markedly in serum in Se- rats, indicating major leakage across the lung epithelial barrier, whilst its levels did not diminish in BALF. Two-way analysis of variance for CC16 in BALF in the control, 1 ppm and 10 ppm NO_2 inhalation experiments indicated that the effects of exposure to NO_2 took precedence ($p=0.0493$) over the effects of the selenium status ($p=0.5390$) in affecting the BALF-CC16 variations in the model, both immediately after inhalation and at 48h ($p=0.0166$ and $p=0.0537$ respectively).

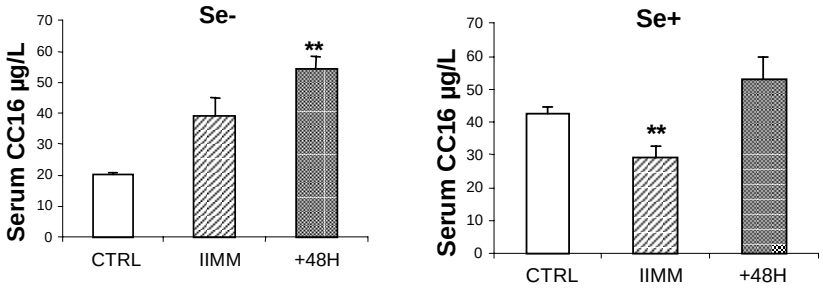

Figure 3. CC16 levels in BALF and serum following exposure to 1 ppm NO_2 for 28 days (6 hrs/d and 5 d/week) and according to Selenium diet. Comparison to controls: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and acid phosphatase (ACP). Interestingly, none of these markers indicated significant changes during the subacute exposure experiment at 5 ppm in either sets of rats. While ACP decreases significantly at higher levels of exposure (10 & 50 ppm) in selenium deficient rats, curiously LDH only increases significantly in rats with normal selenium levels, except during the acute high level of exposure to 50 ppm where LDH rises significantly in both groups. ALP appears much more sensitive to NO_2 inhalation, increasing significantly in all rats at 1 ppm exposure levels, only in Se+ animals at 10 ppm and in Se- animals at 50 ppm.

B. Selenium levels, antioxidant enzymes and oxidant production

Selenium levels. Selenium levels were found to fall dramatically during NO_2 exposure in all acute, short or long-term inhalation studies, but curiously only in Se+ animals, receiving adequate amounts of selenium in their diet. These levels even continued to fall significantly over the next recovery period of 48 hrs following inhalation ($p<0.0001$ for all exposures compared to controls). In those rats on very low selenium diets, however, the levels only fell significantly for the least aggressive regimen, the long-term 1 ppm NO_2 exposure ($p=0.0081$). The exact reason for selenium levels to drop only in one group of rats and not the other would require further investigations, as in humans there appear to be no threshold values for selenium, some populations, for example in China, being known for their extremely low and almost non-existent selenium levels.

GPx in BALF, in plasma and in red blood cells. GPx levels rose in BALF in all animals during all experiments, strikingly more so in Se+ animals. The increases observed in GPx in BALF were mirrored in the Se+ animals by corresponding significant decreases in GPx in plasma at all experimental levels, implying leakage across the lung epithelial barrier of this selenodependent antioxidant in response to the oxidative stress. The Se- animals never quite reached the levels of GPx attained by selenium-supplemented rats, even in the 50 ppm acute exposure experiment, and these animals did not show any significant GPx changes in either BALF or serum at 1 ppm. GPx in RBC, however, increased significantly in almost all animals at all inhalation exposure levels.

Ceruloplasmin. Cp levels, which as we saw above, were unaffected by Selenium status in the controls, did however show some changes in response to the oxidative stress caused by NO_2 inhalation. This was particularly the case for the chronic experimental protocols, where Cp levels appeared elevated in Se- rats immediately following the intermittent 28-day exposure to 1 ppm and at 48 hrs post-exposure to 10 ppm, but not in Se+ rats, which maintained their levels constant in BALF.

SOD. Red blood cell superoxide dismutase activity showed statistically significant changes at all levels of NO_2 exposure, indicating thereby its sensitivity to oxidative stress. The changes were all consistent with a significant increase in activity in response to NO_2 inhalation at 1 ppm, 5 ppm and 10 ppm in the Se+ rats. Surprisingly, the response was quite the opposite in Se- rats. Indeed, except at 1 ppm where SOD increased, SOD dropped significantly in Se- animals at 5 ppm, 10 ppm and 50 ppm.

GST. Glutathione S-Transferase levels increased significantly in Se+ and Se- animals during the 1 ppm and 5 ppm exposure experiments and continued to rise for 48 hrs after exposure. The increase in GST levels was significantly greater in Se+ than in Se- rats immediately following exposure (1 ppm: Se- $p=0.0422$, Se+ $p<0.0001$; 5 ppm: Se- $p=0.0753$ while Se+ $p<0.0001$). The same effect of selenium status was observed at 10 ppm NO_2, where GST levels rose immediately post-inhalation in Se+ but not in Se- animals whilst all 48 h-levels

post-exposure were significantly raised. There were, however, no GST-level variations at 50 ppm acute NO_2 exposure.

TBARS. In plasma, TBARS rose significantly for both Se- and Se+ animals during the long-term 1 ppm inhalation studies, but at 48 h returned to normal (Se-) or significantly below control levels (Se+). There were no significant changes in plasma TBARS at 5 and 50 ppm, and only a significant decrease in Se+ rats immediately after exposure to 10 ppm for 28 days. In BALF, there were significant increases in TBARS at 5 ppm. The only other significant changes in TBARs in BALF were increases observed in Se+ rats at 10 ppm and 50 ppm.

Chemiluminescence (CL). Both spontaneous and phorbol-myristate acetate (PMA)-stimulated oxidant release were measured in BALF-cells and blood leukocytes. The responses observed to the various levels of oxidative stress appeared to move in similar ways for both Se- and Se+ rats, demonstrating an overall increase in oxidant formation in response to NO_2. However, if oxidant formation measured in blood significantly increased after 28 days 1 ppm NO_2 inhalation then rapidly decreased over 48 hours in Se- and not in Se+ animals (increase $p=0.0380$, 48h decrease $p<0.0001$), at 5 and 10 ppm exposures it only rose in Se+ rats ($p=0.0147$ and $p=0.0206$ respectively), returning to normal levels at 48 hours whilst Se- rats then showed a significant increase in oxidant formation ($p=0.0006$). The increases in both blood and BALF CL observed at 50ppm did not reach statistical significance in either Se+ or Se- rats. Increases in BALF CL were significant for Se+ animals during the long-term exposures at 1 ($p=0.0121$) and 10 ppm ($p=0.0008$) but not at 5 ppm, whilst they were only significant in Se- animals at 48 h post-exposure to 1 ($p=0.0217$) and 5 ppm (0.0123). Stimulated CL variations were not significant.

C. Parameters of blood and BALF cell populations

Results in polymorphonuclear counts (PMN) were interesting, particularly following short-term 5 ppm NO_2 inhalation, where PMN count was reduced in blood, and correspondingly increased in BALF for both groups of rats, significantly so in Se+ rats (decrease in blood PMN $p=0.0481$; increase in BALF PMN $p=0.0490$). For Se+ rats, however, the PMN count showed signs of recovering, both in BALF and in blood, 48 hrs post-exposure, while still diminishing from the circulation in Se- rats. Another interesting finding concerned the number of lymphocytes recovered in BALF which increased in Se- rats following 5 ppm NO_2 exposure ($p=0.0535$) and in Se+ rats following the chronic 10 ppm exposure experiments ($p=0.0133$). There were no consistent findings that would otherwise suggest a role of selenium on cell counts and differentials in animals exposed to NO_2.

DISCUSSION

Selenium diet. When assessing daily amounts of fodder consumed by rats, daily Se intake in the Se- group was estimated at 1.3 μg Se/day/animal. According to the literature, a truly Se-deficient diet provides <0.12 μg Se/day/animal, so the animals used in this experiment were not entirely deficient in selenium but simply on a low Se diet. A Selenium-free diet is based on Torula yeast, cultivated on special selenium-free cultures, as a main source of protein (Beilstein & Whanger, 1988), while a standard Murigran diet for rodents contains 0.176 μg Se/g/fodder and provides rats with about 3.5 μg Se/day. According to the literature data this amount of selenium is recognized as appropriate and thus used in most experiments (Jenkinson et al., 1987). The range of Se concentrations applied in experiments with rats is very wide, and the supply of even 20 μg Se/day falls within limits of correct values. However, at such high doses of selenium, concentration and activity of selenoproteins do not increase proportionally to the applied dose (Sun et al., 1998). The changes observed on the various lung parameters in this study in control rats are most interesting and demonstrate the importance of appropriate selenium supply in the diet. Furthermore, most markers studied indicated a positive protective effect to NO_2 in Se+ compared to Se- animals. The unexpected decreased CC16 secretion in BALF observed in Se+ rats, further corroborated in the human studies of the BIOART project (unpublished data), indicates however that one should exert caution when advocating selenium supplementation and that further studies on the complex role played by selenium on the lung and the Clara cell in particular are recommended.

Lung permeability markers. Changes in total protein and albumin in BALF clearly indicate an enhanced permeability of the lung epithelial barrier, known to increase following NO_2 inhalation studies (McElroy et al., 1997). Selenium status, however, also had considerable effects on the concentrations observed in the various groups: as clearly shown in Figure 1, Se- rats had a statistically significant increase in BALF-total protein concentrations at 10 ppm, when this was not the case for the Se+ rats. Moreover, the mean total protein in BALF observed in Se+ rats were much lower than those observed in Se- animals, indicating a greater protection of their

epithelial barrier to oxidative stress. Interestingly, this did not seem to apply for the subacute experimental conditions, where total protein increased in both Se+ and Se- animals, indicating increased permeability of the lung epithelium to some proteins at least, such as demonstrated by the mirrored responses in serum and BALF of CC16. Concerning CC16, it is remarkable to notice how the changes in BALF were mostly mirrored by those in serum, confirming the increased permeability (Hermans and Bernard, 1999). The unchanged total protein and albumin concentrations in BALF during the chronic 1 ppm experiment, whilst CC16 moved from BALF into serum, demonstrated once more the selective increase in permeability changes previously described which characterize CC16 as an early marker of increased permeability (Broeckaert et al., 2000). At 10 ppm exposure, the higher CC16 levels in BALF in both Se+ and Se- animals could sign an adaptative response to the higher oxidative stress (increased CC16 production, increased Clara cell numbers) with or without increased cell destruction and spilling of CC16 contents. These results are consistent with the findings reported by Barth and Muller, 1999 who described in rats maximal proliferation of the bronchiolar epithelium following short term exposure to 5 ppm (three day exposure), when long term exposure to 5 ppm did not increase epithelial proliferation. Exposure to higher doses NO_2 long term (10 and 20 ppm for 25 days) increased bronchial and bronchiolar cell proliferation in a linear dose-response fashion (Barth and Muller, 1999). However, the increased serum CC16 levels indicate that the epithelial barrier still has an increased permeability, as it had for 1 and 5 ppm, as evidenced by the countercurrent of total protein leakage into BALF in Se- rats.

Why the CC16 levels are not diminished in BALF at 50 ppm could be explained by the acute exposure circumstances to the high level of NO_2 and hence by the destruction of the epithelial and Clara cells in the respiratory tree, inducing raised levels of CC16 originating from the destroyed Clara cells. These changes could indeed be linked to the morphological report by Kawakami et al. (1989) describing almost total loss of apical projections of Clara cells in terminal bronchioles following inhalation of 50 ppm for 5 hours. Although the other epithelial cells showed signs of recovery from day 3 onwards, new cilia and secretory granules reappearing on day 5 in major bronchi, day 7 in terminal bronchioles, the Clara cells did not return to normal over the next seven days of intermittent high level exposure experiments (Kawakami et al., 1989). The responses of selenium-supplemented rats with regard to CC16 levels all confirm that selenium appears to have some protective effect on the Clara cells and their production of the antioxidant CC16 protein. As seen from the recovery period results at 48 h, they also appear to recover faster than Se- rats. However, these interesting protective effects, which warrant further investigations, do not prevent injury by very high doses NO_2 such as the 50 ppm burst exposure.

Indicators of cellular damage. The above results confirm alkaline phosphatase to be one of the most sensitive markers of pulmonary damage, as previously reported (Pauluhn, 2000). The decrease in acid phosphatase observed at 50 ppm had also been noted by Pauluhn who found it associated to increased phophatidylcholine in alveolar macrophages, linking it to protracted lysosomal catabolism due to increased phospholipid phagocytosis from oxidative damage to the airways epithelium (Pauluhn, 2000).

Antioxidants and oxidant formation. It is obvious from the results that selenium status influenced GPx levels in response to NO_2 inhalation. Concerning the 1ppm long-term exposure of Se- rats to NO_2, GPx levels in BALF are not significantly raised, yet selenium in plasma is significantly decreased. Although GPx in plasma has not moved either, GPx in red blood cells has increased significantly and one hypothesis could be that selenium is being used for its synthesis, as part of the long-term adaptative response one month after the start of the inhalation studies, corroborated by its noted increase at all exposure levels.

Ceruloplasmin results were most interesting, showing marked differences according to selenium levels, rising mostly in Se- rats at 1 ppm long-term NO_2 exposures. This could be interpreted, in the light of the unchanged total protein and albumin in BALF and the raised alkaline phosphatase levels, as an argument in favor of the theory that Cp does indeed stem from respiratory epithelial cells, as the increase noted does not correspond to an increase in plasma leakage across the alveolocapillary barrier at this level of exposure. Ceruloplasmin has indeed an even larger molecular weight than albumin (132kDa vs 68kDa) and its increase in BALF following NO_2 exposure and oxidative stress is therefore corroborating the hypothesis that it could indeed originate from within the airway and be synthesized and released by epithelial cells. These findings in rats cannot be extrapolated to humans, as there is to date no evidence to the synthesis and release of Ceruloplasmin by human respiratory epithelial cells (Baker et al., 2000). Results concerning SOD levels also appear to indicate it can be upregulated and adapted over longer periods of exposure, and that selenium may play a part in this regulatory mechanism. On the other hand, the GST post-exposure results at 48h indicate that this isoenzyme family takes a while to be induced in response to oxidative stress, and that its induction is also favored by a healthy selenium status, the

precise mechanism of which would also need further investigations as those described in the lung, the alpha isoenzymes, are supposed to have selenium-independent GPx activity (Morrison et al., 1998).

Unexpectedly, while a certain degree of protection from selenium status was evident in the chemiluminescence results at 1 and 10 ppm, the increases in TBARs in BALF were clearly more marked in Se+ than Se- animals at all exposure levels (acute 50 ppm, subacute 5 ppm and chronic 10 ppm NO_2), except for the 1 ppm exposure levels where TBARs were only raised in plasma. These findings do not seem to match others to be found in the literature, such as the reports by Ichinose and Sagai (Ichinose et al., 1988; Ichinose & Sagai, 1989; Sagai et al., 1987; Sagai & Ichinose, 1991), Sevanian's studies concerning Vitamin E (Sevanian et al., 1982) or Tom's report on vitamin A status (Tom et al., 1985) who all agree that when GPx levels in the lung are decreased there is a corresponding increase in lipid peroxidation, in particular in the presence of oxidative stress. However, measurements of TBARs in those reports were made on lung homogenates, so any comparison should only be made with caution. NO_2 is thought to act directly by inducing peroxidation on the lipid cell membranes through oxidative stress, and increased TBAR levels can therefore imply either a greater rate of lipid peroxidation, or a lesser catabolism of the molecules, the mechanism of which is complex and studied elsewhere (Giulivi et al., 1995). Why the changes are more marked in selenium-supplemented animals for the higher doses of exposure is unclear and requires further investigations. It could also simply be a reflection of a degree of aspecificity of TBAR measurements, which are well recognized (Moore & Roberts, 1998).

CONCLUSIONS

These findings confirm the important role of antioxidant status, and selenium status in particular, in the lung's own defense mechanisms such as the integrity of the alveolo-capillary barrier and the production of anti-oxidants, among others CC16, when challenged by air pollutants such as NO_2.

However, in view of the unexpected decreased CC16 levels in BALF observed in selenium-supplemented rats, further corroborated in the human studies of the BIOART project, one should exert caution when advocating selenium supplementation.

Further studies on the complex interactions of selenium and CC16 in their protective antioxidant properties in the lung are required as these would shed light on the important fields of lung disease, asthma and allergy.

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