Free Radical Research, March 2005; 39(3): 305–316

Age-related sensitivity to lung oxidative stress during ozone exposure

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Accepted by Professor H.E. Poulsen

(Received 26 February 2004; in revised form 25 June 2004)

Abstract

As immature and aged rats could be more sensitive to ozone (O_3)-linked lung oxidative stress we have attempted to shed more light on age-related susceptibility to O_3 with focusing our interest on lung mitochondrial respiration, reactive oxygen species (ROS) production and lung pro/antioxidant status. For this purpose, we exposed to fresh air or O_3 (500 ppb 12 h per day, for 7 days) 3 week- (immature), 6 month- (adult) and 20 month-old rats (aged). We determined, in lung, H_2O_2 release by mitochondria, activities of major antioxidant enzymes [superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)], heat shock protein (HSP_{72}) content and 8-oxodG and dG-HNE nDNA contents, as DNA oxidative damage markers. In adult rats we did not observe alteration of pro/antioxidant status. In contrast to adults, immature rats exposed to O_3 higher nDNA 8-oxodG content and HSP_{72} and without antioxidant enzymes modification. Aged rats displayed mild uncoupled lung mitochondria, increased SOD and GPx activities, and higher 8-oxodG content after O_3 exposure. Thus, in contrast to adults, immature and aged rats displayed lung oxidative stress after O_3 exposure. Higher sensitivity of immature to O_3 was partly related to ventilatory parameters and to the absence of antioxidant enzyme response. In aged rats, the increase in cytosolic SOD and GPx activities during O_3 exposure was not sufficient to prevent the impairment in mitochondrial function and accumulation in lung 8- oxodG. Finally, we showed that mitochondria seem not to be a major source of ROS under O_3 exposure.

Keywords: *8-OxodG, antioxidant enzymes, HSP, mitochondria, H_2O_2, ROS*

Introduction

Ozone (O_3) is a strong photochemical oxidant and the principal air pollutant in many urban areas during the summer months. Numerous studies demonstrated pulmonary[1,2] and extra-pulmonary O_3 effects[3,4]. In addition to changes in lung function, acute effects of O_3 exposure on pulmonary tissue include oedema, cell necrosis, cell proliferation, increase in epithelial thickness and inflammation. However, the response of various regions of respiratory system is not homogeneous[1,5]. The sensitive sites evaluated included the nose, trachea, lobar bronchus and distal conducting airways (terminal bronchioles and proximal alveolar ducts) of the central pulmonary acinus[1,5]. The magnitude of O_3 effects generally correlates well with O_3 exposure concentration. Although O_3 is not a radical species ($O^-O{=}O$), most of O_3 toxic effects are mediated through free radical reactions[5,6] but O_3 is too reactive to penetrate far into tissue suggesting that cellular damage is not perceived as being a consequence of ozone *per se*, but rather as being mediated through a cascade of secondary, free radical derived, ozonation products[7]. These reactions provoke lung

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/10715760400011098

inflammatory response, which is then an important source of radical species that sustain oxidative chain reactions[5]. To fight against radical species overproduction, lung antioxidant systems are increase to limit the propagation of oxidative chain reactions[8,9]. However, when the amount of oxidant gas inhaled exceeds the lung capacities of detoxification, lung injury will occur.

Whereas acute exposures to O_3 in humans and animals have been reported to cause inflammation and decrements in lung function, the inflammatory response is attenuated during multiday O_3 inhalation[10,11] partly due to increased antioxidant defense capacities[7,9,12,13]. Surprisingly, O_3-induced biochemical and morphological changes in lung tissue do not show this attenuated response and may even progress with ongoing exposure[13]. This could be particularly harmful for pulmonary mitochondria that are affected by O_3 exposure[14–17]. Thus, mitochondrial O_2 consumption is severely reduced by acute and high exposure to O_3, but chronic exposure resulted in an increase of lung mitochondrial O_2 consumption[16]. Changes in O_2 consumption through the mitochondrial electron transport chain (ETC) during O_3 exposure could be involved in the propagation of oxidative damages. In fact, during the course of normal oxidative phosphorylation, 0.1–0.5% of all oxygen consumed is converted into reactive oxygen species (ROS) as the superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2)[18] and mitochondria are particularly susceptible to damage induced by ROS[19]. It has been previously believed that O_3 toxicity was only the result of ROS formation by oxidative products derived from O_3 degradation[7]. In the present study, we hypothesized that O_3 could also be able to enhance active lung mitochondrial ROS production.

Relatively few studies have evaluated the effect of age on lung responses to O_3. Whereas ventilatory response to O_3 in adult rats is a decrease in minute ventilation, immature rats are characterized by the lack of this response which leads to greater injury[20,21]. Paradoxically, under severe oxidative stress induced by hyperoxia, young rats (<44 days) are more resistant than sexually mature rats[22]. This greater oxygen tolerance could be linked to a favorable balance between ROS production and antioxidant defenses in lung of young rats[23]. On the other hand, a limited number of studies have investigated the effects of ageing on pulmonary O_3 responses in modulating pulmonary oxidant toxicity. Aged rat have different strategy of lung antioxidant response to oxidative stress (hyperoxia) than young adult rat[24]. Specifically, aged rats exposed acutely to O_3 showed greater initial lung injuries than younger rats[25] and significant greater mortality were found in aged mice and rats[26,27]. Thus, the evidence suggested that old rats would be more sensitive to pulmonary oxidant than adult animals.

In the present study, we have attempted to shed more light on age-related susceptibility to O_3 (500 ppb for 12 h per day during 7 days) with focusing our interest on lung mitochondrial respiration and ROS production. In addition, because lung antioxidant protection has been suggested to explaining the difference in survival between immature and adult rats exposed to oxidative stress, we determined both activities of the main antioxidant enzymes [superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)] and heat shock protein (HSP_{72}) content as complementary defense against oxidative stress. The assessment of pro/antioxidant balance in lung was evaluated by determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) content, a specific marker of DNA oxidative damage. Additionally, it has been shown that 4-hydroxynonenal (4-HNE) a toxic compound produced during lipid peroxidation is reactive with nucleic acids, preferentially with deoxyguanosine to form HNE-deoxyguanosine (HNE-dG)[28]. Therefore, we evaluated the impact of O_3 exposure on lung HNE-dG accumulation.

Experimental procedures

Animals

The present investigation was carried out according to the ethical principles laid down by the French (Ministère de l'Agriculture) and the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe No. 123, Strasbourg, 1985).

Sprague–Dawley pathogen free male rats of 3 week (immature), 6 (adult) and 20 month-old (aged) were purchased from Harlan (Gannat, France). The following rationale was used for the selection of age groups. At 4 weeks of age, the animals have reached about one-fourth the maximal alveolar surface area and parenchymal tissue volume of adults. Therefore, we refer to the 3 week-old Sprague–Dawley rats as immatures. The lifespan of male Sprague–Dawley rats fed *ad libitum* is 36 months, whereas the median survival age (50% survival) is 27 months (Charles River, Domaine des Oncins, France). Old age can be defined as past the midpoint to the maximal survival age, and thus 20 month-old rats can be conveniently defined as aged. The animals were maintained under conventional conditions: food (AO4, UAR Villemoisson, France) and water *ad libitum*, at room temperature 22–25°C on a 12:12 h light–dark cycle. For each age group, animals were randomly assigned to ozone (O_3) or control (C) group ($n = 9$ for adult and aged groups and $n = 36$ for immature, in both conditions).

Ozone exposure

Rats were kept in a Plexiglas chamber (0.3 m^3) supplied with a constant airflow (6 $m^3.h^{-1}$). O_3 rats were exposed to 500 ± 50 ppb of O_3, for 12 h per day during the night for 7 days. O_3 was generated by passing filtered air across an ultraviolet light generator. O_3 concentration was continuously assessed using a calibrated ozone analyzer (Environment S.A., France) connected to the outlet line of the chamber. Control groups were simultaneously placed in a similar chamber provided with filtered air at the same flow rate with controlled humidity (50–60%) and temperature (22–25°C). Rats were accustomed to the new environmental condition by placing them in the chamber 1 week prior to the O_3 exposure. Food and water were daily changed during the non-exposure period, and the individual rat body weight was determined.

Basal ventilation monitoring

The physiological impact of O_3 inhalation on lung was evaluated on resting ventilation. At the sixth day of exposure, ventilation was measured in awake rats using a barometric plethysmograph around 1–2 h after the last exposure[29]. A plexiglas plethysmographic chamber flushed with humidified air was connected to a reference box of the same size. Both boxes were saturated with water vapor. Temperature, CO_2 and O_2 concentration inside the animal chamber were continuously monitored. When animal was quiet, the flow was interrupted and the inlet and outlet tubes of the chamber were closed. After calibration of the animal box by injecting 1 ml of air, pressure fluctuations related to breathing were recorded with a differential pressure transducer (Celesco, CA, USA). Minute ventilation ($\dot{V}e$) was calculated over 50–80 consecutive breaths by computer analysis of spirogram using standard methods as described previously[29].

Tissue preparation

At the end of O_3 exposure, rats were euthanized shortly after the last night exposure. Lungs were quickly removed, carefully rinsed and divided into two parts: a small portion was quickly frozen in liquid nitrogen for biochemical analyses whereas the major portion was used for mitochondrial extraction. Because analyses required large amount of tissue, lungs from four immature rats were pooled.

Isolation of lung mitochondria

After careful and repeated rinsing, lung tissue was rapidly placed into cold isolation buffer (Sucrose 250 mM, TrisBase 20 mM, EGTA 10 mM, pH 7.4) and homogenized with a Potter-Elvehjem homogenizer. The homogenates were freed from debris and nuclei by centrifugation at 800*g* for 10 min. Resting supernatant was filtered through cheesecloth and centrifuged at 10,000*g* for 10 min to obtain mitochondria pellet. This pellet was washed twice in isolation buffer. Final pellet was resuspended in a minimal volume of buffer. After proteins determination by the Biuret method with BSA as standard, mitochondria were diluted to 20 mg.ml^{-1}. All centrifugation steps were performed at 4°C.

Mitochondrial O_2 consumption

Respiration of isolated mitochondria (0.5 mg mitochondrial protein.ml^{-1}) was determined polarographically with a Clark oxygen electrode (oxygraph Gilson 5/6 H), in a glass cell of 1.5 ml thermostated at 30°C with a constant stirring as reported previously[30]. The respiration reaction medium, saturated with room air, contained 200 mM Sucrose, 10 mM KH_2PO_4, 20 mM TrisBase, pH 7.4, with fatty acid free albumin (FFA-BSA) concentration of 0.1% (w/v). The control state of respiration (state IV) was initiated by addition of succinate (5 mM), in presence of rotenone (5 μM), while the active state of respiration (state III) was initiated by addition of 100 μM ADP. The respiratory control ratio (RCR) was calculated as the state III to state IV ratio.

Mitochondrial H_2O_2 production

The rate of mitochondrial H_2O_2 release was measured at 30°C following the linear increase in fluorescence (λ_{ex} 320 nm and λ_{em} 412 nm) due to enzymatic reaction of Homovanilic acid (HVA) by H_2O_2 in the presence of horseradish peroxidase (HRP), as previously described[30]. Reaction conditions were 0.2 mg of mitochondrial protein.ml^{-1}, 6 U.ml^{-1} of HRP, 0.1 mM HVA and 5 mM succinate in the same incubation buffer used for oxygen consumption measurements. H_2O_2 release was calculated from standard calibration curve. As reported by St-Pierre *et al.*[31], H_2O_2 release from mitochondria with NAD-linked substrate or succinate + rotenone was undetectable. The mitochondrial ROS linked to electron back flow to complex I plus intrinsic production of complex III can be determined by incubating mitochondria with succinate alone.

Antioxidant enzymes activities

A portion of lung tissue was homogenized with a potter Elvehjem at 4°C, in buffer containing KH_2PO_4 (100 mM), DTT (1 mM) and EDTA (2 mM), pH 7.4. After centrifugation (3000*g*, for 5 min), the supernatant was used for enzymatic assays.

Cytosolic and mitochondrial SOD activities were assayed as reported previously.[32] One activity unit of SOD is defined as the amount of enzyme that inhibits the rate of acetylated cytochrome c reduction by 50%. The assay for total activity of GPx coupled the reduction of cumene hydroperoxide to the oxidation of NADPH by glutathione reductase, and this coupled reaction was monitored at 340 nm.[33] The activity of CAT was determined by the method of Aebi.[34] After measurement of total protein content, all enzyme activities are expressed in U. mg^{-1} of proteins.

HSP_{72} content

For HSP quantification, we performed polyacrylamide gel electrophoresis and immunoblotting as previously described[35]. Briefly, 200 µl of lung homogenate was mixed with 200 µl of buffer containing 40 mM Tris(hydroxymethyl)aminomethane pH 6.8, 1% SDS, 6% glycerol and 1% β-mercaptoethanol. This mixture was then heated at 100°C for 10 min, and subjected to one-dimensional sodium dodecyl sulfate (SDS)-PAGE with a 5% stacking and 12.5% resolving gels for 12 h. After electrophoretic separation, proteins were transferred at a constant voltage to nitrocellulose membranes. After protein transfer, the membranes were blocked for 2 h, then incubated for 2 h with a monoclonal antibody specific for HSP_{72} (SPA 810, StressGen) and then reacted with the secondary antibody (goat anti-mouse immunoglobulin G conjugated to HRP, Bio-Rad). HSP_{72} were visualized by the enhanced chemiluminescence detection method (RPN 2106, Amersham). Scanning with a densitometer performed quantification of bands from blots and the data were expressed numerically as integrated optical density arbitrary units. In order to reduce variability results were reported as percentage of HSP_{72} content from adult control rats.

nDNA oxidative damage: 8-OxodG and HNE-dG contents

As previously described[36] determination of 8-oxodG needs some precautions to limit artefactual oxidation during DNA extraction.

Nuclei isolation. Lung samples (~150 mg) were homogenized with a potter glass homogenizer in 1.2 ml buffer (320 mM sucrose, 10 mM Tris, 5 mM $MgCl_2$, 0.1 mM desferroxamine mesylate, 1% Triton-100, pH 7.5). After homogenization, the sample was centrifuged at 1500*g* for 5 min at 4°C. The supernatant was discarded and the pellet was washed in homogenization buffer before centrifugation at 1500*g* for 5 min at 4°C.

DNA Isolation. Nuclear pellets were resuspended in 600 µl extraction buffer (10 mM Tris, 5 mM EDTA, 0.15 mM desferroxamine mesylate, pH 8) with addition of SDS (10%). RNase A (3 µl, 100 $mg.ml^{-1}$) and RNase T1 (8 U) were then added and the samples were incubated for 15 min at 50°C. The samples were then incubated with 30 µl Qiagen proteinase (20 $mg\,ml^{-1}$) for 60 min at 37°C. Isolation of nucleic acids was achieved by adding a NaI solution (1.2 ml, 7.6 M NaI, 40 mM Tris, 20 mM EDTA, pH 8), and isopropanol (100%). The samples were centrifuged for 15 min at 5000*g* at room temperature. The nucleic acids pellets were initially rinsed with isopropanol (40%) and then with ethanol (70%). Following the last centrifugation (5000*g*, 10 min.), the DNA pellet was solubilized into deionized water containing 0.1 mM desferroxamine mesylate.

Digestion. DNA solutions were incubated for 2 h at 37°C with 10 U of nuclease P1 (Sigma, St Louis, MO) in solution (10 µl, 300 mM sodium acetate, 1 mM $ZnSO_4$, pH 5.3). Then, 4 U of alkaline phosphatase in buffer (10 µl, 500 mM Tris, 1 mM EDTA, pH 8) was added. After incubation for 1 h at 37°C, proteins were precipitated by addition of chloroform. The samples were centrifuged and the aqueous layer collected. Content in 8-oxodG was determined by HPLC-EC whereas HNE-dG was determined by HPLC associated with tandem mass spectrometry (HPLC-MS/MS).

HPLC-EC analyses. The analytical system consisted of a model 305 Gilson pump connected to a 231 XL Gilson autosampler equipped with an Interchrom Uptisphere ODSB (particle size 5 µm) octadecylsilyl silica gel column (250 × 4.6 mm^2 I.D.) (Interchim, Montluçon, France). The isocratic eluent was a 25 mM aqueous solution of potassium phosphate (pH 4.7) containing 8% methanol. The temperature was maintained at 28°C. Coulometric detection was provided by a Coulochem II detector equipped with a 5011 cell (ESA, Chelmsford, MA) with the potential of the two electrodes set at 200 and 450 mV. The retention time of 8-oxodG was 20 min. Elution of normal nucleosides was simultaneously monitored by a Waters 484 UV variable wavelength spectrophotometer set at 280 nm. Both EC and UV signals were collected on a D7500 Hitachi integrator (Tokyo, Japan). The amount of DNA analyzed was inferred from the area of the peak of dGuo after appropriate calibration.

HPLC-MS/MS analyses. DNA samples were then analyzed for their content in 4-HNE adducts to 2′-deoxyguanosine (HNE-dG) by HPLC-MS/MS

using the approach described previously for oxidized nucleosides[28]. The vials used for the HPLC-EC analysis were freeze-dried. The resulting residue was solubilized in 30 µl of water containing 200 fmol of the [$^{15}N_5$]-labeled derivatives of HNE-dG adduct. The latter compound was used as internal standard for isotopic dilution quantification. Samples were then analyzed by reverse phase HPLC associated with an API 3000 mass spectrometer (Perkin–Elmer/SCIEX, Thornhill, Canada) used in the multiple reaction monitoring mode. HNE-dG was quantified by HPLC-MS/MS in the positive mode.

Statistical analysis

All values reported are means ± SEM. Effects of immaturity, aging and ozone exposure were determined using a two way ANOVA followed by a *post hoc* test (Fisher) with Statview® software. To test the effects of O_3 on content in HNE-dG non-parametric test (Mann–Whitney) was used. The level of significance was set at $P < 0.05$.

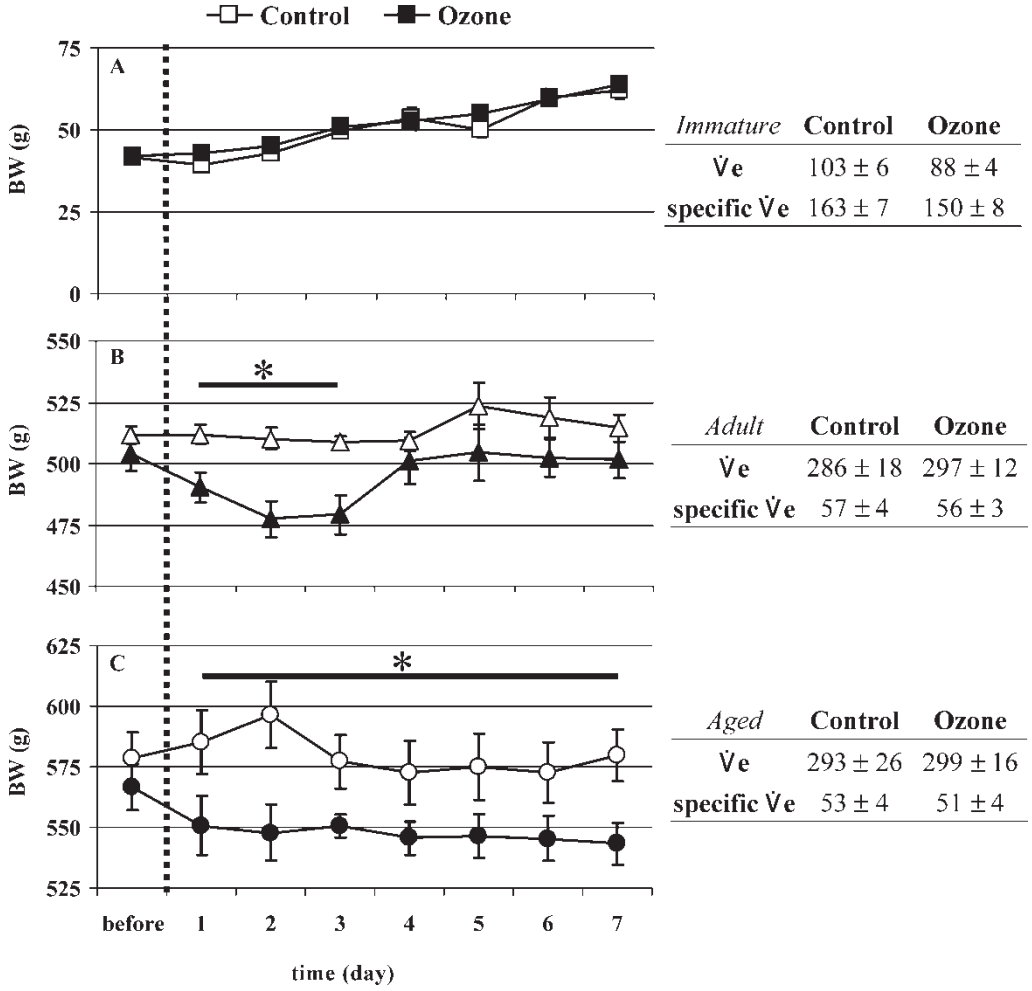
Results

Evolution of body weight (Figure 1)

Whereas growth rate of immature rats was not affected by O_3 exposure, adult and aged rats displayed a significant body weight reduction on the first day of O_3 inhalation. Body mass was completely recovered by the end of the fourth day of exposure in adult but not in aged rats.

Basal ventilation (Figure 1)

After 6 days of O_3 exposure, $\dot{V}e$ and specific $\dot{V}e$ were unchanged in the three groups of age. Minute ventilation ($\dot{V}e$, ml.min^{-1}) was 3-fold lower in immature than adult and aged rats ($P < 0.001$). Nevertheless, specific $\dot{V}e$ (ml.min^{-1}.100 g^{-1}) was



Immature	Control	Ozone
$\dot{V}e$	103 ± 6	88 ± 4
specific $\dot{V}e$	163 ± 7	150 ± 8

Adult	Control	Ozone
$\dot{V}e$	286 ± 18	297 ± 12
specific $\dot{V}e$	57 ± 4	56 ± 3

Aged	Control	Ozone
$\dot{V}e$	293 ± 26	299 ± 16
specific $\dot{V}e$	53 ± 4	51 ± 4

Figure 1. Effect of O_3 on body weight (in g) and basal ventilation in (A) immature, (B) adult and (C) aged rats. Basal ventilation was measured at the sixth day of O_3 exposure, during the day time (non-exposure period). $\dot{V}e$: minute ventilation (ml.min^{-1}); specific $\dot{V}e$ (ml.min^{-1}.100 g^{-1}). Data are represented in mean ± SEM. *$P < 0.05$, significantly different from control at the same age. $n = 9$ in each group.

Table I. Effect of O_3 exposure on lung mitochondrial O_2 consumption and H_2O_2 release in immature, adult and aged rats.

	Immature		Adult		Aged		ANOVA		
	Control	Ozone	Control	Ozone	Control	Ozone	Age	O_3	Interaction
O_2 consumption									
State IV	15.7 ± 1.7*†	13.9 ± 1.0	10.8 ± 1.0	13.3 ± 1.2	11.5 ± 0.6	15.0 ± 0.8‡	$P < 0.05$	NS	$P < 0.05$
State III	49.6 ± 6.0*†	50.7 ± 1.6	36.0 ± 2.4	42.6 ± 2.6	36.0 ± 2.4	40.5 ± 1.5	$P < 0.05$	NS	NS
Respiratory parameter									
RCR	3.5 ± 0.3	3.7 ± 0.2	3.4 ± 0.2	3.3 ± 0.2	3.2 ± 0.1	2.7 ± 0.2‡	NS	NS	NS
H_2O_2 release									
State IV	16.3 ± 3.2†	15.0 ± 3.3	20.4 ± 2.4	17.7 ± 3.9	10.9 ± 0.3*	13.6 ± 0.6‡	$P < 0.05$	NS	NS
H_2O_2/O_2 consumed									
State IV	0.97 ± 0.19*	1.34 ± 0.24	2.12 ± 0.43	1.58 ± 0.38	0.91 ± 0.06*	0.86 ± 0.08	$P < 0.05$	NS	NS

Measurements were performed with succinate as substrate. State IV, with succinate; State III, with succinate + ADP; RCR, respiratory control ratio. Data of O_2 consumption were expressed in ng Atom $O.min^{-1}$.mg of $protein^{-1}$; H_2O_2 release by lung mitochondria was expressed in pmole of $H_2O_2.min^{-1}$.mg of $protein^{-1}$; H_2O_2/O_2 consumed was expressed in pmole of H_2O_2.ng Atom O^{-1}. Data are expressed as means ± SEM. *$P < 0.05$, significantly different from adult; †$P < 0.05$, significantly different from old; ‡$P < 0.05$, significantly different from control at the same age. $n = 9$ in each group.

significantly higher in immature than adult and aged rats (by +180 and +200%, respectively).

Lung mitochondrial respiratory parameters (Table I)

In the presence of succinate, mitochondrial oxygen consumption in state IV, III and RCR were unchanged by O_3 exposure in immature and adult rats. By contrast, O_2 consumption in state IV of aged rats exposed to O_3 was significantly increased in comparison to aged control rats (+30%) thereby decreasing RCR by 15%.

In control condition, O_2 consumption with and without ADP was significantly higher in immature than in adult and aged rats (IV: +50 and +40%, respectively and III: +40% in both groups). Adult and aged groups have similar O_2 consumption both in absence or presence of ADP. The RCR was not different in control groups irrespective of their age.

H_2O_2 release by lung mitochondria (Table I)

In immature and adult rats, lung mitochondrial H_2O_2 release with succinate alone was not affected by O_3 exposure. Succinate supported H_2O_2 release by lung mitochondria of aged rats was increased after O_3 exposure (+24%), but remained unchanged when expressed by unit of oxygen consumed.

In presence of succinate alone, lung mitochondrial H_2O_2 production in immature and adult control rats was similar when expressed in $pmole.min^{-1}.mg\,prot^{-1}$. However, the amount of H_2O_2 produced per unit of O_2 consumed was significantly lower in immature than in adult rats (−54%). H_2O_2 production ($pmole.min^{-1}.mg\,prot^{-1}$) by mitochondria of immature control rats was significantly higher in comparison to aged rats. H_2O_2 release by aged control rats mitochondria was 2-fold lower than adult rats even expressed in $pmole.min^{-1}.mg\,prot^{-1}$ or $pmole.min^{-1}$ per unit of oxygen consumed (Table I).

Pulmonary cells antioxidant enzyme activities (Table II)

Ozone exposure did not modify SOD, GPx and CAT activities in immature and adult rats. On the other hand, aged rats exposed to O_3 displayed a significant increase in activities of cytosolic SOD (+90%) and GPx (+20%) as compared to aged control rats whereas lung CAT activity was slightly decreased (−20%).

In control conditions, immature rats had a 2-fold higher pulmonary total SOD activity than adult and aged rats ($P < 0.05$). This was essentially due to higher activity of the cytosolic SOD isoform ($P < 0.05$). On the other hand, GPx activity was significantly lower in immature compared to adult and aged rats (−70%), whereas CAT activity was

Table II. Effect of O_3 exposure on lung antioxidant enzyme activities in immature, adult and aged rats

	Immature		Adult		Aged		ANOVA		
	Control	Ozone	Control	Ozone	Control	Ozone	Age	O_3	Interaction
SOD TOT	2.30 ± 0.32*†	2.07 ± 0.36	1.26 ± 0.17	1.71 ± 0.18	1.32 ± 0.14	1.94 ± 0.33	$P < 0.05$	NS	NS
SOD C YTO	1.50 ± 0.33*†	1.49 ± 0.28	0.86 ± 0.18	1.02 ± 0.21	0.62 ± 0.86	1.19 ± 0.16‡	NS	NS	NS
SOD M ITO	0.80 ± 0.23	0.58 ± 0.10	0.40 ± 0.31	0.69 ± 0.15	0.58 ± 0.65	0.53 ± 0.11	NS	NS	NS
GPx	0.022 ± 0.002*†	0.025 ± 0.004	0.082 ± 0.007	0.086 ± 0.004	0.068 ± 0.004*	0.081 ± 0.004‡	$P < 0.001$	NS	NS
CAT	0.041 ± 0.004	0.046 ± 0.005	0.044 ± 0.004	0.044 ± 0.002	0.048 ± 0.002	0.040 ± 0.003‡	NS	NS	NS

SOD TOT, total superoxide dismutase; SOD CYTO and MITO, cytosolic and mitochondrial superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase. Data are expressed in U.mg^{-1} of proteins as means ± SEM. * $P < 0.05$, significantly different from adult; † $P < 0.05$, significantly different from old; ‡ $P < 0.05$, significantly different from control at the same age. $n = 9$ in each group.

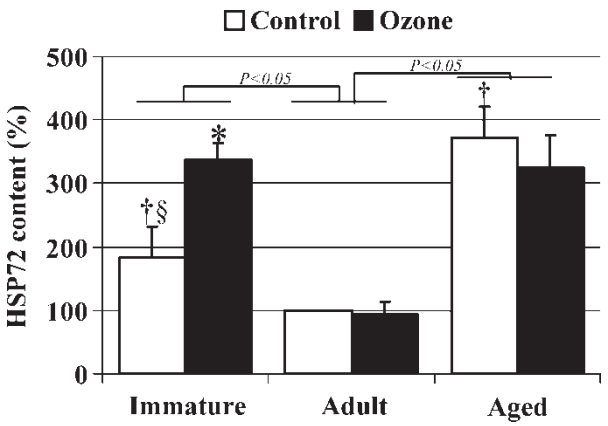


Figure 2. Effect of ozone exposure on lung HSP_{72} content (in % of adult control) in immature, adult and aged rats. Data are represented in mean ± SEM. †: $P < 0.05$, significantly different from adult. §: $P < 0.05$, significantly different from aged. *: $P < 0.05$, significantly different from control at the same age. Further significant differences are indicated by brackets. $n = 9$ in each group.

unchanged. Contrary to activities of cytosolic and mitochondrial SOD, and CAT which were unchanged with aging, GPx activity was significantly lower in the aged than in adult rats (−20%).

Lung HSP_{72} content (Figure 2)

In response to O_3 exposure lung HSP_{72} content was significantly increased in immature rats (+80%, $P < 0.001$), but remained unchanged in adult and aged rats.

In control conditions, adult rats displayed a lower content in lung HSP_{72} than immature and aged rats (−50 and −70%, respetively, $P < 0.05$). In addition, lung HSP_{72} content in aged rats was 2-fold higher than in immature rats.

Lung nDNA 8-OxodG and HNE-dG contents (Figure 3A,B)

Contents of 8-oxodG measured in the present study were in the same range as those previously reported.[37] Contrary to adult rats displaying similar 8-oxodG levels in control and O_3 condition, immature and aged rats, exhibited significant increase in 8-oxodG content after O_3 exposure (+110 and +80%, respectively). Interestingly, lung 8-oxodG content was significant lower in immature than in adult rats (−40%) in control conditions.

Lung HNE-dG content was unchanged after O_3 exposure irrespective of the age of the rats. HNE-dG content was significantly higher in aged than adult rats (+75%) in control conditions.

Discussion

The effects of ozone exposure on pulmonary oxidant/antioxidant status were examined in immature

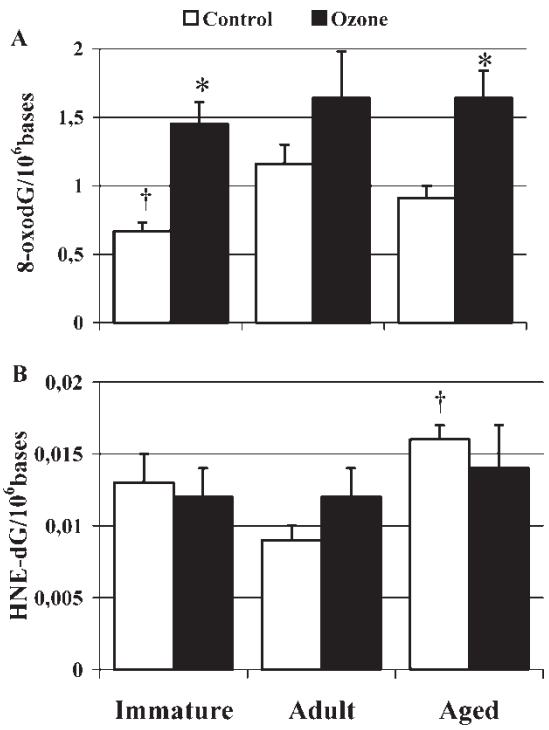


Figure 3. Effect of ozone exposure on lung 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (A) and 6-(1-hydroxyhexanyl)-8-hydroxy-1,N^2-propano-2′-deoxyguanosine (HNE-dG) (B) content (per 10^6 bases) in immature, adult and aged rats. Data are represented in mean ± SEM. †: $P < 0.05$, significantly different from adult. ⋆: $P < 0.05$, significantly different from control at the same age. $n = 9$ in each group for 8-oxodG, $n = 4$ for HNE-dG.

(3 week), adult (6 month) and aged (20 month) rats. We focused our interest on mitochondria as a source of ROS in relation to O_2 consumption. Adults were found to be resistant to intermittent O_3 exposure for 7 days, whereas immature and aged rats displayed significant lung oxidative damages.

Adults were resistant to O_3 exposure

In adult rats we have shown that apart from a short-lasting reduction in body weight, O_3 exposure did not have another adverse effect considering the array of endpoint markers examined in this study. Actually, specific ventilation in basal condition and lung mitochondrial oxygen consumption were not affected by O_3 exposure. The level of nuclear 8-oxodG which is considered as the most abundant DNA lesion generated by ROS[38] and HNE-dG content were similar in adult control and exposed to O_3. Moreover, major antioxidant enzyme activities (SOD, GPx and CAT) and HSP_{72} content were unaltered after O_3 exposure in adult rats. Finally, it seems therefore that our O_3 exposure protocol (500 ppb, 12 h per day for 7 days) was moderate and did not result in massive lung oxidative stress in adult rats. It has to be kept in mind that O_3 effects are closely dependent upon dose and duration of O_3 exposure[12]. Indeed, mitochondria from young adult rats acutely exposed to O_3 (2–3 ppm for 8 h) displayed a 25% depression of respiration, whereas subchronic O_3 exposure (0.8 ppm for 10–20 days) resulted in 30–45% increase in O_2 lung cellular utilization[16,17]. It appears that during recovery from O_3-mediated lung injury, the yield of isolated lung mitochondria was increasingly greater for O_3--exposed lungs relative to control, this increase being primarily due to an increase in population of mitochondria[14]. This proliferation can be linked to type I and II cells hyperplasia reported after chronic O_3 exposure[9]. We can make the assumption that our protocol of intermittent exposure to O_3 (restricted to the night period exclusively) permitted the normalization of O_2 consumption. Our hypothesis is reminiscent of the phenomena of adaptation to O_3 reported by many authors[11,13,39,40]. In fact, ventilatory alterations by O_3 were attenuate when exposure is repeated[13,40]. Adaptation affects also lung biochemical markers in bronchoalveolar lavage fluid as observed by van Bree *et al.*[11] who reported that a single exposure to 0.4 ppm O_3 causes marked permeability and inflammatory responses in lower airways of rats whereas 5 days of exposure to O_3 for 12 h/night resulted in a complete disappearance of these responses. The present study does not allow to discriminate precisely the cellular mechanisms implicated in O_3 adaptation in adult rats. However, the lack of measurable changes in mitochondrial function and ROS production (Table I), DNA damages (Figure 3), antioxidant defense (Table II), and pulmonary function (Figure 1) are consistent with the disappearance of marked permeability and inflammatory responses with repeated daily O_3 exposure[11].

O_3 induced lung oxidative stress in immature rats

While adult rats were relatively insensitive to mild subchronic O_3 exposure, some populations like immature rats were found to be more sensitive.[20,21] Because the inhaled dose of O_3 is the product of O_3 concentration, exposure time and ventilation, the higher specific ventilation rate measured in younger rats as compared to adults (Figure 1) could partly explain their greater susceptibility to O_3 exposure.[20] Immature rats are known to be more susceptible to developing airways hyperresponsiveness than are adults and this enhanced response was recently attributed to a weaker interdependence between airways and parenchyma in young animals compared to mature animals[41]. Lastly, it has been shown that the ventilatory responses to O_3 (marked decrease in $\dot{V}e$ in adult rats) is absent in 2–4-week-old rats, and this lack of response, in conjunction with a greater specific ventilation, leads to greater lung injuries[20]. Consistent with an increased dose, immature mice displayed increased bronchoalveolar lavage protein content but a reduced capacity to

release some cytokines like IL6 or TNFα[42]. These data suggest that there are differences in age-related susceptibility to O_3 depending on the outcome indicator examined (airways hyperresponsiveness, lung injury). In the present study, we reported a large increase in nuclear 8-oxodG levels (Figure 3) and in HSP_{72} (Figure 2) content after O_3 exposure in immature rats. These data clearly evidence that pro/antioxidant status in pulmonary cells from immature rats was disturbed by O_3 inhalation.

It did not seem that O_3 exposure affected the mitochondrial release of H_2O_2 in younger rats. It has to be mentioned however that, in control conditions, lung mitochondria from immature rats displayed a 50% reduction in H_2O_2 release (Table I) jointly with a significant increment in state IV O_2 consumption (+50%) in comparison to adult rats. Mechanisms of mitochondrial ROS production have been largely evaluated by using inhibitors that block electron flow within the respiratory chain at specific sites, particularly complexes I and III[43]. In the present study, the ROS production was drastically lower in lung mitochondria respiring on complex I substrates (glutamate + malate, data not shown) and almost completely abolished by rotenone when respiring on succinate (data not shown). It is well established that the rate of mitochondrial ROS production is linked to the degree of reduction of the respiratory carriers and/or the transmembrane electric potential difference[18]. In this sense, moderate uncoupling and decrease in the membrane potential may protect mitochondria from damage due to free radical production. From the data reported in the present study it can be hypothesized that the reduced rate of mitochondrial ROS production in immature rats exposed to O_3 was likely due to some "uncoupling" which will be evaluated in future studies. Therefore, increases in HSP_{72} and 8-oxodG levels observed in immature rats after O_3 exposure were not linked to enhanced ROS production by lung mitochondria.

Oxidative stress tolerance, at any age or in any species, is thought to be dependent on the ability of the lung to actively increase antioxidant enzyme activity, although there are arguments to the contrary[44]. In the present study, we found that total SOD activity was significantly higher in immature rats, this increase being exclusively due to an enhanced activity of the cytosolic (Cu, Zn) isoform (Table II). By contrast GPx activity was drastically reduced in immature rats (−75%), and CAT activity was similar in immature and adult rats. It has been previously reported that young (3 week-old) rats are able of rapidly increasing their pulmonary antioxidant-enzyme activities on exposure to hyperoxia, in contrast to adult rats[23]. In the present study, intermittent O_3 exposure was unable to enhance the antioxidant enzyme activities (Table II) in contrast to the higher SOD, CAT and GPx mRNA levels and greater enzyme activities reported after 5 days of continuous exposure to O_3[8]. Interestingly, it was shown that O_3 exposure for only 24 h decreased SOD, CAT and GPx activity but did not affect corresponding mRNA[8]. Therefore, it is likely that our protocol of intermittent exposure to O_3 was not severe enough (or permitted a full recovery with air exposure during the daytime period) to increase antioxidant enzyme expression.

In addition to antioxidant enzymes, HSP are known to exert protective function under stress conditions in virtually all cell types studied[45]. Recently, enhanced HSP_{72} levels in lung tissue extracts were reported following acute and chronic exposure to O_3[46,47]. The mechanism(s) underlying the induction of HSP_{72} by *in vivo* O_3 exposure is not known, but some authors speculate that oxidative stress may play a role[47]. The indirect induction of HSP_{72} by ozone-initiated inflammation may be critical because direct O_3 exposure of alveolar macrophages and epithelial cells did not elicit HSP expression[48]. Recently, Hamilton et al.[49] suggested that secondary reactive species (e.g. 4-HNE) formed after O_3 have a potential role in HSP_{72} response in human lung cells to ozone. In this respect, the higher lung HSP_{72} content measured in immature rats exposed to O_3 as compared to adults (Figure 2) suggests that the young animals are better able to detect specific form of damage and to activate the expression of gene products which presumably function in either repairing or increasing the resistance of cells to further damage. Interestingly, we found that, in control conditions the HSP_{72} content was significantly higher in immature than adult rats (Figure 2). The influence of maturation on HSP_{72} levels has never been examined in lung tissue, but it has been shown that, in rat heart, both HSP_{72} and HSP_{25} displayed a significant decrease from birth to adulthood[50] suggesting a specific, development-dependent function of HSP_{72} in lung tissue.

Lastly, we cannot exclude the assumption that the increase in 8-oxodG was related to an impairment of base excision repair activity in rat lungs during the earlier phase of O_3 exposure. Indeed, it has been shown that 8-oxodG levels in lung tissue was significantly increased after 1 week of inhaled sodium chromate but returned to basal levels with prolonged exposure[51]. This normalization in DNA adducts was coincident with full recovery of 8-oxodG repair enzyme activity.

Lung oxidative stress in aged rats exposed to O_3

In a large ozone project initiated by the Health Effects Institute, rats were exposed for 20 months to various levels of ozone (0, 0.12, 0.5 and 1.0 ppm) for 6 h per day, 5 days per week and it was concluded that prolonged exposure to ozone had mild to moderate effects to various regions of the respiratory tract except

the nose where some of the most striking effects were observed[1]. Nevertheless, early studies have indicated an age-related sensitivity of the lungs to oxidant exposure[25,26]. Thus, in response to a single acute exposure to O_3 senescent Fisher 344 rats display cellular proliferation in terminal bronchioles and alveolar ducts as compared to adult rats[25]. Aged animals could be more sensitive to O_3 due to deficiencies at the levels of basal antioxidant status, the regeneration of reduced equivalents, or even the kinetics of induction of enzymatic defense during oxidative stress[52].

In this study, we found a higher nuclear 8-oxodG level in aged O_3-exposed rats than in control rats (+80%, Figure 3) suggesting that O_3 inhalation in aged rats provoked lung oxidative stress with direct DNA oxidation. In addition, increase in nDNA 8-oxodG content (Figure 3) was accompanied by alterations in mitochondrial function, characterized by an increase in O_2 consumption and H_2O_2 release so that the amount of ROS generated by mitochondria was closely related to O_2 respiration (same H_2O_2 to O_2 ratio, Table I). To our knowledge, this is the first study that examined lung mitochondrial ROS production following O_3 exposure. Appearance of lung oxidative stress after O_3 exposure in aged rats (Figure 3) cannot be explained by a higher inhaled dose of O_3 since the ventilatory rate was similar in aged rats control and exposed to O_3 (Figure 1). On the other hand, HSP_{72} content was not modified by O_3 exposure. However, it is worth mentioning that lung HSP_{72} content was significantly greater ($\times 4$) in aged than in adult rats. These data contrast with the decreased cellular expression of HSP_{72} generally reported in response to stress during mammalian aging[53]. Recently, it has been shown that reactive aldehydes from lipid peroxidation as (4-HNE) are able to interact with the amino groups of bases to form 6-(1-hydroxyhexanyl)-8-hydroxy-1,N^2 -propano-2′-deoxyguanosine (HNE-dG), another relevant class of oxidative damage to DNA[37] and HNE could be implicated in HSP_{72} expression during oxidative stress[49]. In the present study, we found that HNE-dG was significantly higher in aged rats as compared to adults in conjunction with a higher HSP_{72} content (Figs. 2 and 3). The coincidental increases in HNE-dG and HSP_{72} do not necessarily mean that they are related, but some biological effects (e.g. HSP_{72} response) can be reproduced *in vitro* by incubating cells with 4-HNE[49].

The lung's ability to respond to oxidative stress depends largely on its capacity to upregulate protective antioxidants and prolonged exposure to hyperoxia results in a large increase in CAT, GPx and SOD activities[54]. In the present study, we found that intermittent exposure to O_3 in aged rats resulted in a specific antioxidant response. In fact, activity of total SOD was strongly increased due to a strong enhancement of cytosolic SOD isoform (+100%). On the other hand, we observed a slight increase in GPx activity with a mild decrease of CAT activity following to O_3 exposure (Table II). The slight reduction in activity of CAT confirms that GPx and SOD are major antioxidant enzymes to fight against ROS in lung[54]. Similar changes in rat lung antioxidant enzyme induction by ozone was previously reported in adult rats[8] suggesting that aging did not impair lung ability to fight against O_3 exposure. It appeared that ozone-induced lung injury in rats is site-specific, with the primary target sites being the distal trachea and the central acinus[12]. However, by using more sensitive methods (immunogold labeling) to determine O_3-induced changes in antioxidant enzymes on a per cell basis, the same group[9] reported that Cu,ZnSOD was not increased in any cell type in either terminal bronchioles or alveolar ducts including Clara cells, type I and II epithelial cells and interstitial fibroblasts even though rats were exposed to similar O_3 concentration (1 ppm) and duration (3 months) in this latter study. These contrasting data suggest that care must be taken for evaluating the antioxidant lung response to O_3, which could lead to opposite conclusions depending upon the methodology used for assessing antioxidant status. We can hypothesize that the increases in cytosolic SOD activity with mitigate increase in capacities to eliminate H_2O_2 by GPx and CAT explain the higher lung 8-oxodG content and mitochondrial impairments in aged rats exposed to O_3.

Limitation of the methodology

Several findings from this study suggest that caution should be taken for extrapolating these results to human populations sporadically exposed or seasonally to the high ozone concentrations found in many cities. It might appear that the concentration of O_3 used in the present study (0.5 ppm) was relatively high as compared to the level of that could be encountered on a day of high urban air pollution. Nevertheless, in several studies human subjects were exposed to O_3 levels close (0.2–0.4 ppm) to the one used in the present study[10,55]. Interestingly, in the collaborative ozone project supported by the Health Effects Institute[1] it was shown that chronic exposure to O_3 below 0.5 ppm has no significant effect on lungs, including surface epithelium, mucus or inflammation. We deliberately choose an intermittent O_3 exposure to take into account the day-to-night variations that generally characterize O_3 pollution in urban areas.

The array of endpoint markers of oxidative stress examined in this study limits the conclusions that can be drawn as to which mechanisms produce age-related O_3-induced responses in lung. Even though it has

been shown a site- and cell-specific alteration of lung injury by O_3 exposure[9,12], studies on mitochondria require relatively large amount of tissue. Therefore, our study was performed on lung homogenates, without distinction of cell type. This precludes any extrapolation of the O_3 response to the site of greatest damage found at the bronchiole-alveolar duct junction[9].

It is well known that short-term O_3 exposure causes lung inflammation[1,10,11] and no particular attention was paid in the present study to the inflammatory response to O_3 exposure. However, on the basis of an attenuation of O_3-induced inflammation with intermittent O_3 exposure[10,11], we deliberately excluded examination of the inflammatory response. In addition, we could have also extended our measurements to other antioxidant enzymes or oxidative products but it must be kept in mind that no major alterations in mitochondrial function, DNA damages, antioxidant defense, and pulmonary function were found in adult rats exposed intermittently to O_3. Therefore, it is unlikely that additional measurements would have contradict our conclusion that intermittent exposure to low O_3 levels did not provoke massive oxidative stress in adult rats but for frail populations like immature and aged animals, the same mild oxidative stress resulted in pulmonary DNA damage as assessed from accumulation of nuclear 8-oxodG.

Acknowledgements

S. Servais was supported by the Agence de l'Environnement et de la Maitrise de l'Energie (ADEME). This study was supported by the Ministère de l'Aménagement du territoire et de l'Environnement (Grant Primequal Predit), the Association de Prevention de la Pollution Atmosphérique (APPA Lyon) and the Comité pour le contrôle de la Pollution Atmosphérique sur le Rhône et la région Lyonnaise (COPARLY). Authors gratefully acknowledge J.M. Cottet-Emard, K. Couturier, J. Pequignot and B. Semporé for their technical assistance.

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