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Pulmonary Effects of Inhaled Limonene Ozone Reaction Products in Elderly Rats

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Abstract

Background—d-limonene is an unsaturated volatile organic chemical found in cleaning products, air fresheners and soaps. It is oxidized by ozone to secondary organic aerosols consisting of aldehydes, acids, oxidants and fine and ultra fine particles. The lung irritant effects of these limonene ozone reaction products (LOP) were investigated.

Methods—Female F344 rats (2 mo and 18 mo) were exposed for 3 h to air or LOP formed by reacting 6 ppm d-limonene and 0.8 ppm ozone. BAL fluid, lung tissue and cells were analyzed 0 h and 20 h later.

Results—Inhalation of LOP increased TNF-α, cycloxygenase-2, and superoxide dismutase in alveolar macrophages (AM) and Type II cells. Responses of older animals were attenuated when compared to younger animals. LOP also decreased p38 MAP kinase in AM from both younger and older animals. In contrast, while LOP increased p44/42 MAP kinase in AM from younger rats, expression decreased in AM and Type II cells from older animals. NF-κB and C/EBP activity also increased in AM from younger animals following LOP exposure but decreased or was unaffected in Type II cells. Whereas in younger animals LOP caused endothelial cell hypertrophy, perivascular and pleural edema and thickening of alveolar septal walls, in lungs from older animals, patchy accumulation of fluid within septal walls in alveolar sacs and subtle pleural edema were noted.

Conclusions—LOP are pulmonary irritants inducing distinct inflammatory responses in younger and older animals. This may contribute to the differential sensitivity of these populations to pulmonary irritants.

Keywords

LOP; lung; aging; PM; TNF-α; SOD; MAP kinase; transcription factor

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Introduction

Epidemiological studies indicate that the elderly (≥ 65 yr old) are generally more susceptible to the health effects of air pollution (Donaldson *et al.*, 2001; Pope *et al.*, 2002; USEPA, 2004). Air pollutants found indoors are of particular concern since on average people spend 85-90% of their time indoors (USEPA, 1996), and this may be especially important among older individuals. Many household products are sources of volatile organic compounds (VOCs), an important class of air pollutants that are typically found in concentrations several fold higher indoors when compared to outdoors (Wallace, 1991). It has been suggested that the elderly may have a higher exposure to VOCs due to more frequent cleaning activities in their residences, including nursing homes (USEPA, 1996). Unsaturated VOCs, such as the terpenes, limonene and pinene, are found in many common household products including cleansers and air fresheners. Ozone, infiltrating from outdoors or generated directly indoors by electrostatic air cleaners, printers and photocopiers, reacts rapidly with terpenes to form secondary organic aerosols (SOA) containing formaldehyde, free radical species and fine and ultrafine organic particles (Wainman *et al.*, 2000; Weschler, 2000). The health effects of exposure to SOA are unclear. Whereas, we found that short-term exposure of young healthy volunteers to SOA generated by the reaction of ozone with a mixture of VOCs including terpenes caused no changes in lung function or nasal irritation/inflammation (Laumbach *et al.*, 2005), Wolkoff and colleagues (Wolkoff *et al.*, 2000) reported respiratory and mucus membrane irritation from terpene-ozone SOA. The health effects of SOA have not been investigated in elderly humans or animals.

Following inhalation of smog or nitrogen dioxide, greater structural injury is observed in the lungs of older when compared to younger animals (Bils *et al.*, 1967; Cabral-Anderson *et al.*, 1977; Bowes *et al.*, 1989; van Bree *et al.*, 2000). Older animals are also more sensitive to the pulmonary and systemic effects of various types of inhaled gases and particulate matter (PM) (Elder *et al.*, 2000; Oberdorster *et al.*, 2000; Zelikoff *et al.*, 2003; Elder *et al.*, 2004; Nadziejko *et al.*, 2004; Tankersley *et al.*, 2004; Kasagi *et al.*, 2006). Although pre-existing diseases may be important in the increased sensitivity of the elderly to inhaled air pollutants, specific mechanisms underlying their enhanced responsiveness are unknown. AM represent the first line of cellular defense in the lung. These cells play an important role in the inflammatory response to inhaled substances, and their function has been reported to be altered in the elderly (Plackett *et al.*, 2004; Gomez *et al.*, 2005). Antioxidant defenses are also known to be impaired in the elderly (Heffner, 1991; Nel *et al.*, 1998; Dhalla *et al.*, 2000; Lang *et al.*, 2002). With increasing age, levels of antioxidants and the capacity of oxidative injury to stimulate release of antioxidants declines (Lykkesfeldt *et al.*, 1999; Squier, 2001; Thomas *et al.*, 2001; Servais *et al.*, 2005). Thus alterations in macrophage inflammatory activity and antioxidant defense may be important factors contributing to age-related increases in the toxicity of inhaled PM and this was investigated in the present studies. We found that inhalation of SOA containing LOP by younger adult rats resulted in increased expression of the inflammatory proteins, tumor necrosis factor-alpha (TNFα) and cyclooxygenase-2 (COX-2), as well as the antioxidant enzyme, Cu/Zn superoxide anion dismutase (SOD) in the lung. This was associated with activation of the p44/42 MAP kinase signaling pathway and transcription factors, NF-κB and C/EBP in macrophages. Although similar responses were also observed in elderly rats exposed to LOP, they were significantly attenuated. These data demonstrate that the response to LOP is age dependent. Age related differences in the sensitivity to inhaled LOP may be due to impaired activity of signaling molecules and transcription factors that regulate macrophage activity and antioxidant defenses in the lung.

Methods

Aerosol generation and characterization

The aerosol generation and characterization system is shown in Fig. 1. Organic aerosols were formed by gas-phase reaction of d-limonene and ozone in a Teflon flow tube reactor. Animals were exposed to the resulting aerosol in a Plexiglas whole body exposure chamber immediately downstream (W 20.3 cm × L 41cm × H 17.8 cm). Ozone (0.8 ppm) was generated from oxygen gas via an ultraviolet light generator (Orec Corp., Phoenix, AZ). d-limonene vapor was generated by bubbling compressed purified (charcoal and HEPA filtered) air through a 250 ml glass reservoir containing liquid d-limonene (>97%, Sigma-Aldrich, Allentown, PA). The d-limonene concentration was adjusted to 6 ppm by dilution with partially humidified air (dry mixed with humidified air; membrane humidifier; Perma Pure Inc., Toms River, NJ). Ozone and d-limonene were mixed in the flow tube reactor and then introduced into the chamber. Particle number size distribution, ozone concentration, temperature and relative humidity (RH) were measured continuously (1 min resolution) in the exposure chamber (ozone = 0.79 ± 0.05 ppm; temperature = 25.5 ± 1.9 °C; RH = 21.4 ± 5.3 %; SOA = 2242 ± 183.1 $\mu g/m^3$). Integrated particle mass measurements were also performed. The temperature remained stable over the exposure period (data not shown). RH was largely dependent on animal activity. Thus RH was high while the animals were active and lower when animals were sleeping. Exposures were conducted between 10-30% RH and 22-28 °C (RH/Temp data logger: HOHO Pro, Onset, Burne, MA; monitor: model Testo 625, Hotek Technologies, Tacoma, WA).

The mean residual ozone in the exposure chamber was 0.062 ppm (photometric ozone analyzer, model 1008-AH, Dasabi Environmental Corp, Glendale, CA). Residual d-limonene was not measured. Assuming the reaction of d-limonene and ozone is pseudo-first order (d-limonene levels were much higher than ozone levels), and using the reactor residence time and the rate constant for d-limonene oxidation by ozone (5.1×10^{-6} ppb^{-1} s^{-1} at 23 °C) (Atkinson *et al.*, 1990) the predicted ozone concentration at the exposure chamber entrance is 0.046 ppm. This is comparable to the measured concentration of 0.062 ppm. Applying a comparable loss rate for d-limonene, 5.2 ppm d-limonene is predicted at the entrance of the exposure chamber.

Particle number concentrations in the exposure chamber increased immediately after the initiation of the reaction and reached steady state within 10-30 min (TSI 7610 condensation particle counter, CPC, >0.01 μm in diameter, Shoreview, MN). As shown in Fig. 2, the organic aerosol size distribution contained both “ultrafine” and “fine” size modes. Ultrafine particles (0.01-0.1 μm in diameter) dominated the particle number distribution, whereas accumulation mode particles (0.1-1.0 μm in diameter) dominated the particle mass distribution (Optical particle counter: LASAIR 1002, Particle Measuring System, Boulder, CO, 8 size channels, 0.1-2.0 μm in diameter. Ultrafine particle concentrations were determined by the difference between the CPC and LASAIR. Dilution was used to bring concentrations within the operating range of the LASAIR and CPC).

Animals

Female specific pathogen-free F344 rats (2 mo and 18 mo) were purchased from the National Institute of Aging (MD). Animals were housed in microisolator cages and maintained on sterile food and pyrogen-free water *ad libitum*. All animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. Animals were exposed to ultra-pure air (control) or LOP for 3 h and sacrificed immediately (0 h) or 20 h post exposure (PE). Four sets each of younger and older animals were used for each exposure.

Cell Isolation

Animals were euthanized by ip injection of Nembutal (250 mg/kg). The lungs were perfused with 50 ml of warm (37°C) HBSS containing 25 mM HEPES, 0.5 mM EGTA, and 4.4 mM $NaHCO_3$, pH 7.3, followed by perfusion with Ca^{+2}/Mg^{+2} -free HBSS containing 22 mM HEPES, 4.2 mM $NaHCO_3$, pH 7.3 at a rate of 22 ml/min. AM were isolated from perfused lungs by bronchoalveolar lavage (BAL) 5-6 times with HBSS. Cells were washed 4 times with HBSS containing 2% FBS. Cell viability was 98% as determined by trypan blue dye exclusion, and cell purity greater than 97% as assessed morphologically after Giemsa staining. Type II alveolar epithelial cells were isolated from lavaged lungs as previously described (Sunil *et al.*, 2002). Briefly, after washing twice with 10 ml of buffer (140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 1.3 mM $MgSO_4.7H_2O$, 2 mM $CaCl_2$,10 mM HEPES, pH 7.4), 30 ml of elastase (4.2 U/ml, Worthington Biochemicals, NJ) was infused into the lungs using a 30 cc syringe. The tissue was then incubated at 37°C for 20 min, the trachea and major bronchi removed, the lungs minced in the presence of 4 ml DNAse (1 μg/ml), and then digested for 5 min at 37°C with 10 ml of elastase. The reaction was stopped by the addition of 5 ml FBS. The tissue was then sequentially filtered through 220, 60 and 15 μm nylon mesh. Cells were collected, washed and purified by negative selection (1 h, 37°C) on IgG coated plates. Non-adherent cells were collected and washed with DMEM containing 10% FBS. Cell purity, assessed by modified Papinicolou staining, was 95% and viability, determined by trypan blue dye exclusion, was greater than 98%.

Measurement of BAL protein

Total protein was quantified in the BAL fluid recovered from the first lung lavage using the BCA Protein Assay kit (Pierce Biotechnologies Inc., Rockford, IL) with bovine serum albumin as the standard.

Immunohistochemistry

Lungs were perfused with buffer, removed, fixed in 3% paraformaldehyde in PBS for 4 h on ice, transferred to 50% ethanol and paraffin embedded. Tissue sections (6 μm) were deparaffinized and incubated overnight at 4°C with goat or rabbit IgG, anti-TNF-α (2 μg/ml), anti-COX-2 (2 μg/ml, Santa Cruz Biotechnology), or anti-Cu/Zn SOD (2.5 μg/ml, Stressgen Biotechnologies Inc., San Diego, CA) antibodies, followed by 30 min incubation with biotinylated secondary antibody (Vector Labs, Burlingame, CA). Binding was visualized using a Vectastain Elite ABC kit (Vector Labs). Some sections were stained with H & E to assess lung structure.

Western Blotting

Cytoplasmic extracts of cells were prepared using Nuclear and Cytoplasmic Extraction Reagents (NE-PER), (Pierce, Rockford, IL). Proteins (15-25 μg) were fractionated on 10%-12.5% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific binding was blocked by incubation of the blot for 30 min at room temperature with 5% non-fat dry milk in Enhanced Chemi-Luminescence (ECL) buffer (Amersham Life Sciences, Arlington Heights, IL). Blots were incubated overnight at 4°C with antibodies to p38, p44/42 or JNK mitogen activated protein kinase (1:1000, Cell Signaling Technology, Beverly, MA) prepared in ECL buffer with 1% milk. After 4 washes in ECL buffer, blots were incubated with anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibody (1:2000) for 30 min at room temperature and the bands visualized using an ECL detection system (Amersham).

Electrophoretic Mobility Shift Assays (EMSA)

Assays were performed using nuclear extracts as described previously (Sunil *et al.*, 2002) with some modifications. Binding reactions were carried out at room temperature for 20 min in a total volume of 10-15 μl containing 10 μg of nuclear extracts, 3-6 μl of 5× gel shift binding buffer (20% glycerol, 5mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5), 2 μg poly dI-dC and 3×10^4 cpm/μl γ[^{32}P]ATP (3000 Ci/mmol at 10 mCi/ml) labeled NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG-3′), C/EBP (5′-TGC AGA TTG CGC AAT CTG CA-3′) or CREB (5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′) consensus oligonucleotide (Santa Cruz Biotechnology, CA). Protein-DNA complexes were separated on 5-7% non-denaturing polyacrylamide gels run at 250V in 0.5× TBE and visualized after the gels were dried and autographed. For competitor reactions, 50-fold excess of unlabeled NF-κB, C/EBP or CREB oligonucleotide was added to the mixture prior to the addition of the labeled probe.

Results

Effects of exposure to LOP on inflammatory mediator and antioxidant expression in the lung

In our first series of studies, we compared the effects of inhaled LOP on production of inflammatory mediators in the lungs of younger and older rats. Initially, we focused on the inflammatory protein TNF-α, and the enzyme COX-2, which catalyzes the production of prostaglandins. TNF-α and COX-2 were not detected in the lungs of air-exposed animals (Figs. 3 and 4). Inhalation of LOP caused a rapid increase in expression of both of these proteins in the lung which persisted for at least 20 h. This was evident in AM, as well as in Type II alveolar epithelial cells. LOP-induced expression of TNF-α and COX-2 was greater in younger when compared to older animals.

In the lung, Cu/Zn SOD is considered the first line of antioxidant defense (Kinnula *et al.*, 2003; Rahman *et al.*, 2006). As observed with TNF-α and COX-2, Cu/Zn SOD was not detected in lungs of air-exposed younger or older animals (Fig. 5). Exposure of rats to LOP caused a rapid and time-related increase in Cu/Zn SOD expression which was evident immediately after exposure. LOP-induced expression of Cu/Zn SOD was greater in younger when compared to older animals.

Effects of exposure to LOP on MAP kinase expression in the lung

MAP kinases such as p38, p44/42 (ERK) and JNK are upstream regulators of inflammatory and antioxidant gene expression (Rahman, 2002; Lopez-Neblina *et al.*, 2006). In our next series of experiments, we determined if LOP-induced increases in inflammatory proteins was associated with alterations in expression of MAP kinases. AM from both younger and older animals were found to constitutively express p38 MAP kinase protein (Fig. 6, top panel). Following inhalation of LOP, a rapid and transient decrease in p38 MAP kinase expression was observed, which returned to control levels by 20 h post exposure in both groups. p44/p42 MAP kinase proteins were also detected in AM from air-exposed younger and older animals (Fig. 6, top panel). Inhalation of LOP resulted in a delayed increase in p44/42 MAP kinase expression in AM from younger animals, which was evident at 20 h. In contrast, an immediate and persistent decrease in expression of this protein was noted in AM from older rats.

The JNK kinase antibody detects 3 proteins of molecular weight 56, 54 and 46 kDa which are activated by distinct stimuli (Chan *et al.*, 1997). AM from younger animals were found to constitutively express the p56 and p54 isoforms of JNK, whereas cells from older rats constitutively expressed the p54 and p46 isoforms (Fig. 6, top panel). Treatment of younger animals with LOP caused a significant increase in expression of all three isoforms of JNK which was observed in AM collected immediately and 20 h post exposure. In older animals,

LOP caused a rapid and persistent increase in the p56 JNK isoform, but a transient decrease in the p54 and p46 isoforms.

We also analyzed expression of MAP kinases in Type II alveolar epithelial cells isolated from air and LOP-exposed animals. As observed in AM, Type II cells from both younger and older animals exposed to air control constitutively expressed p38 and p44/42 MAP kinase proteins (Fig. 6, bottom panel). Although levels of p38 were unaffected by LOP exposure in the younger animals, a decrease in both p38 and p44/42 MAPK proteins was observed in cells from older rats. Interestingly, in contrast to AM, in Type II cells, only the p54 and p46 isoforms of JNK kinase were detected. Type II cells isolated from older rats constitutively expressed lower levels of p54 JNK (Fig. 6, bottom panel). While exposure to LOP did not significantly alter expression of p54 JNK in younger animals, a sustained decrease in expression was noted in Type II cells from older animals.

Effects of exposure to LOP on transcription factor activity

Inflammatory and anti-oxidant genes are regulated by transcription factors including NF-κB, C/EBP and CREB (Castranova, 2004; Rahman *et al.*, 2005; Barnes, 2006; Park *et al.*, 2006). We next compared the effects of LOP inhalation on transcription factor activity in AM and Type II cells from younger and older animals. Constitutive DNA binding activity of NF-κB, C/EBP and CREB was noted in AM from both younger and older animals (Fig. 7, left panels). Greater activity was detected in AM from older rats. While exposure to LOP caused a transient increase in NF-κB and C/EBP DNA binding activity in younger animals, there was no significant effect on CREB DNA binding activity. In older rats, exposure to LOP had no effect on the activity of any of these transcription factors in AM. Binding was decreased or eliminated by using 50-fold excess of the NF-κB, C/EBP or CREB unlabeled competitor oligonucleotide, demonstrating the specificity of the probe.

Constitutive NF-κB, C/EBP and CREB DNA binding activity was also detected in Type II cells from both younger and older rats (Fig. 7, right panels). Exposure to LOP had no significant effect on NF-κB DNA binding activity in younger or older rats. In contrast to AM, Type II cells from younger animals exhibited greater C/EBP and CREB DNA binding activity than cells from older rats (Fig. 7). Whereas exposure to LOP had no major effect on C/EBP in younger animals, a significant decrease was noted in Type II cells from older rats. LOP also significantly decreased CREB DNA binding activity in Type II cells from both younger and older animals.

Effects of LOP on BAL protein and cell number

Protein accumulation in BAL fluid is an indication of altered epithelial cell permeability and a measure of injury in the alveolar regions of the lung (Bhalla, 1999). This is typically associated with increased numbers of inflammatory cells in the tissue. In further studies we determined if inhalation of LOP caused lung injury and inflammatory cell accumulation in the lung. Low levels of protein were detected in BAL fluid from both younger and older animals exposed to air control (Fig. 8, upper panel). Exposure of animals to LOP had no significant effect on BAL protein levels in either younger or older rats. Similarly, LOP had no major effect on the number or type of cells recovered in BAL fluid. Thus in both younger and older rats, greater than 97% of BAL cells were identified as AM and the number and percentage of these cells did not change after LOP exposure (Fig 8, middle panel and data not shown). In contrast to AM, significantly fewer Type II cells were recovered from the older animals when compared to the younger animals (Fig. 8, bottom panel). However, this was unaffected by exposure to LOP.

We also analyzed lung sections histologically after LOP exposure. Although no major differences were noted in lung histology between air-exposed younger and older rats (Fig. 9), the response of the rats to LOP was distinct. Thus, while in younger animals, exposure to LOP caused endothelial cell hypertrophy, increased perivascular and pleural edema, and thickening of alveolar septal walls (Fig 9, left panel), in older animals, patchy accumulation of fluid within septal walls in alveolar sacs and subtle pleural edema was noted (Fig. 9, right panel).

Discussion

Alterations in the production of inflammatory mediators and antioxidants, as well as pulmonary epithelial-endothelial barrier dysfunction have been suggested as potential factors mediating increases in the susceptibility of the elderly to air pollutants (Tankersley *et al.*, 2003). Consistent with this idea, the present studies demonstrate that the pulmonary inflammatory response to LOP at levels similar to those found in indoor environments after cleaning (Singer *et al.*, 2006), was significantly attenuated in older when compared to younger rats. This may lead to impaired ability to repair damaged tissue and to protect against infectious agents, thus contributing in part, to increased morbidity and mortality in the elderly.

Cytokines such as TNF-α and eicosanoids have been reported to be generated in the lung following exposure to gaseous and particulate air pollutants (Mundandhara *et al.*, 2006). These mediators play an important role in initiating tissue repair after injury (Wong *et al.*, 1996). We found that acute exposure of younger and older rats to inhaled LOP resulted in a rapid and persistent induction of TNF-α and COX-2 in lung macrophages and Type II cells. However, expression of these proteins was reduced in older, when compared to younger rats. This suggests an impaired ability of geriatric animals to mount a robust pulmonary inflammatory response. These findings are in accord with previous reports of decrements in TNF-α production by AM from elderly rats exposed to silica or in isolated cells treated with LPS (Corsini *et al.*, 1999; Corsini *et al.*, 2003; Corsini *et al.*, 2004). We have previously reported increases in TNF-α production by AM following exposure of animals to fine PM plus peroxides (Morio *et al.*, 2001), and others have demonstrated PM-induced upregulation of TNF-α, as well as COX-2 expression, in macrophages and epithelial cells (van Eeden *et al.*, 2001; Monn *et al.*, 2003; Ishii *et al.*, 2004; Becker *et al.*, 2005). These findings suggest that TNF-α and COX-2 are sensitive markers of exposure to inhaled PM.

Induction of antioxidant enzymes within the lung is critical for limiting oxidant-induced damage. Of particular interest is Cu/Zn SOD which is one of the first antioxidant enzymes to be expressed in tissues undergoing oxidative stress (Tsan, 1993, 1997; Kinnula *et al.*, 2003). The present studies show that LOP exposure caused a rapid induction of SOD in the lung. The fact that there are lower levels of SOD in the lung of older when compared to younger animals after LOP exposure, indicates that antioxidant defenses are also impaired in these animals. Similar deficits in SOD expression have been noted in lungs of elderly rats exposed to $PM_{2.5}$ or diesel exhaust particles (Liu *et al.*, 2005; Sugimoto *et al.*, 2005). Age-dependent decreases in antioxidant enzyme activity following LOP may be due to post-translational chemical modifications of SOD (Machado *et al.*, 1991; Augustyniak *et al.*, 2004). In contrast to our findings with LOP, rats exposed to ozone or oxygen exhibit either increases (Gumuslu *et al.*, 2001; Servais *et al.*, 2005) or no changes in lung SOD (Gomi *et al.*, 2002). Differences between these findings and ours may be due to the distinct exposures and exposure conditions, and/or to the type of SOD analyzed in the studies.

Genes involved in production of inflammatory mediators such as TNF-α and eicosanoids, and antioxidants like SOD, are regulated, in part, by upstream signaling molecules including members of the MAP kinase family (Rahman, 2002; Lopez-Neblina *et al.*, 2006). We found that AM from both younger and older rats constitutively expressed p38 and p44/42 MAP

kinases and JNK proteins, suggesting that these cells are primed to respond to inhaled irritants. However, differences in expression were noted between younger and older animals, as well as in their responses to LOP. In general, cells from older animals constitutively expressed lower levels of these signaling proteins when compared to cells from younger animals. Whereas LOP exposure resulted in increased p44/42 and JNK MAP kinase expression in AM from younger rats, in older animals, expression of these proteins decreased. Exposure to LOP also transiently decreased p38 MAP kinase expression in AM from both younger and older animals. Similar decreases in MAP kinase protein expression have been reported previously in macrophages from older animals following exposure to LPS (Ding *et al.*, 1994; Boehmer *et al.*, 2004; Boehmer *et al.*, 2005). Reduced levels of MAP kinases may account for decreased LOP-induced expression of inflammatory mediators by AM observed in the older animals in the present studies.

Type II cells from both younger and older animals, also constitutively expressed p38, p44/42 and JNK proteins indicating that these cells, like AM, are primed to respond to pulmonary irritants. Moreover, following exposure to LOP, Type II cells from older rats expressed lower levels of each of the MAP kinase proteins when compared to younger animals. This suggests that Type II cells participate in the pulmonary response to LOP, and that MAP kinase and JNK signaling pathways are impaired in these cells in elderly rats. Similar age-associated decreases in p44/42 MAP kinase protein have been observed in rat intestine (Gentili *et al.*, 2000; Pardo *et al.*, 2004), hepatocytes (Ikeyama *et al.*, 2002; Li *et al.*, 2003), and fibroblasts (Park *et al.*, 2000). Our observations that MAP kinase expression is reduced in older when compared to younger rats is consistent with our findings with TNF-α, COX-2 and SOD expression, and suggest that these signaling molecules are important in regulating LOP-induced expression of inflammatory mediators and antioxidants in the lung.

Transcription factors such as NF-κB, C/EBP and CREB regulate the expression of inflammatory and antioxidant genes downstream of MAP kinases (Pourazar *et al.*, 2005; Wang *et al.*, 2005; Dong *et al.*, 2006; Kim *et al.*, 2006; Tsatsanis *et al.*, 2006). We found that DNA binding activity of NF-κB and C/EBP, but not CREB, increased in AM following exposure to LOP. However this was only observed in cells from younger rats. We also noted significantly greater constitutive NF-κB, C/EBP and CREB DNA binding activity in AM from older animals. Moreover, this was not altered by exposure to LOP. These data suggest that activation of these transcription factors is not required for AM responsiveness to inhaled irritants in elderly animals. Alternatively, other transcription factors may play a role in LOP-induced inflammatory and antioxidant responses in these animals. For example, in A549 cells, supernatants from AM exposed to PM caused an increase in AP-1 and SP-1 activity (Jimenez *et al.*, 2002; Ishii *et al.*, 2004). In contrast to our findings, decreased CREB activity has been observed in older rats following carrageenan-induced lung inflammation (Corsini *et al.*, 2005). Differences between these findings and ours may reflect irritant-specific pulmonary responses. Reduced constitutive NF-κB, C/EBP and CREB binding activity was also noted in Type II cells from older when compared to younger animals. Moreover, LOP reduced C/EBP and CREB activity, but only in the older animals. These results are in accord with our findings that expression of TNF-α, COX-2 and SOD, as well as MAP kinases was reduced in Type II cells from older animals. These results suggest that the effects of LOP on transcription factor activity depends on age, as well as the responding cell type.

A characteristic feature of lung injury is the accumulation of protein and inflammatory cells in BAL fluid (Bhalla, 1999). Despite our findings of altered structural integrity in the lungs following LOP exposure, we did not detect increases in BAL protein or inflammatory cell number. This is consistent with our previously reported lack of spirometric or nasal inflammatory changes in young adults (Laumbach *et al.*, 2005). It may be that more sensitive methods are required to identify biochemical markers of lung injury in rodents and humans.

Interestingly, significantly fewer Type II epithelial cells were isolated from the lung of older when compared to younger rats which is consistent with previous reports (Pinkerton *et al.*, 1982). The significance of this deficit is unknown but it may result in reduced surfactant production (Betsuyaku *et al.*, 2004) and increased sensitivity to pulmonary irritants.

The present studies demonstrate that inhalation exposure of rats to a SOA, potentially generated in indoor environments, resulted in increased inflammatory mediator and antioxidant expression in lung macrophages and Type II cells. These findings indicate that these aerosols may be a possible health concern. Of particular interest was our observation that the pulmonary response to LOP was significantly attenuated in the older animals. These findings suggest a potential mechanism that may in part, be important in the heightened responsiveness of the elderly to air pollutants. Further studies are in progress to determine if reduced activation of MAP kinases and/or altered transcription factor activity plays a role in impaired inflammatory and antioxidant responses in elderly animals.

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Abbreviations

LOP	limonene ozone reaction products
TNF-α	tumor necrosis factor-alpha
COX-2	cyclooxygenase -2
SOD	superoxide dismutase
AM	alveolar macrophage
VOC	volatile organic compound
SOA	secondary organic aerosols
PM	particulate matter
BAL	bronchoalveolar lavage

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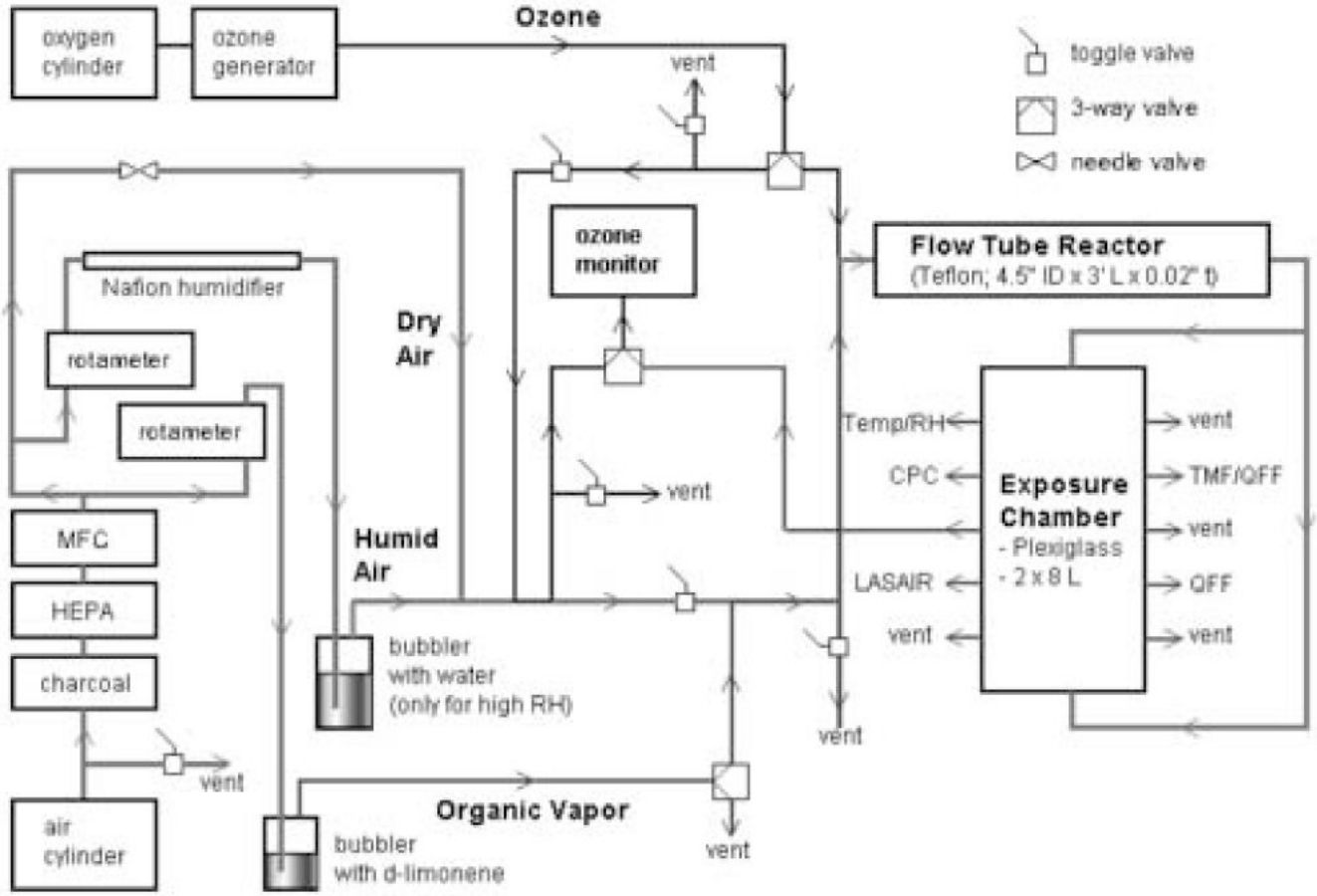

Figure 1.

Flow diagram of aerosol generation and animal exposure system. Mixture of ozone and d-limonene generates a secondary organic aerosol at fixed relative humidity (RH) in the flow tube reactor. Particle size, mass, number, ozone concentration, temperature and RH were monitored in the exposure chamber.

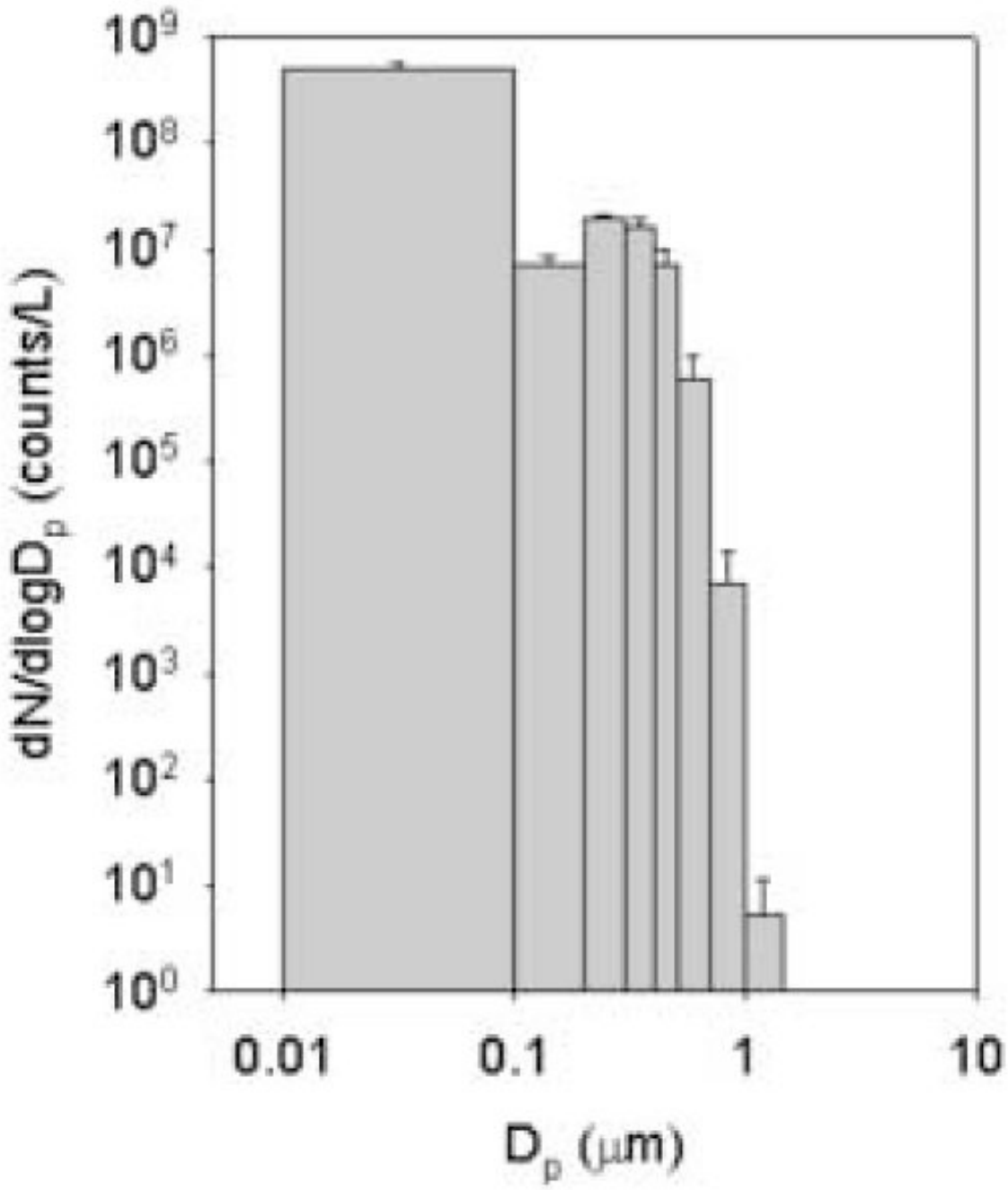

Figure 2.
Particle number size distribution in the exposure chamber. Particle number from 0.1- 2 μm in diameter by LASAIR optical particle counter; particle number from 0.01-0.1 μm in diameter determined by difference between the condensation particle counter (CPC) and LASAIR. Each bar is the mean ± SD (n=13).

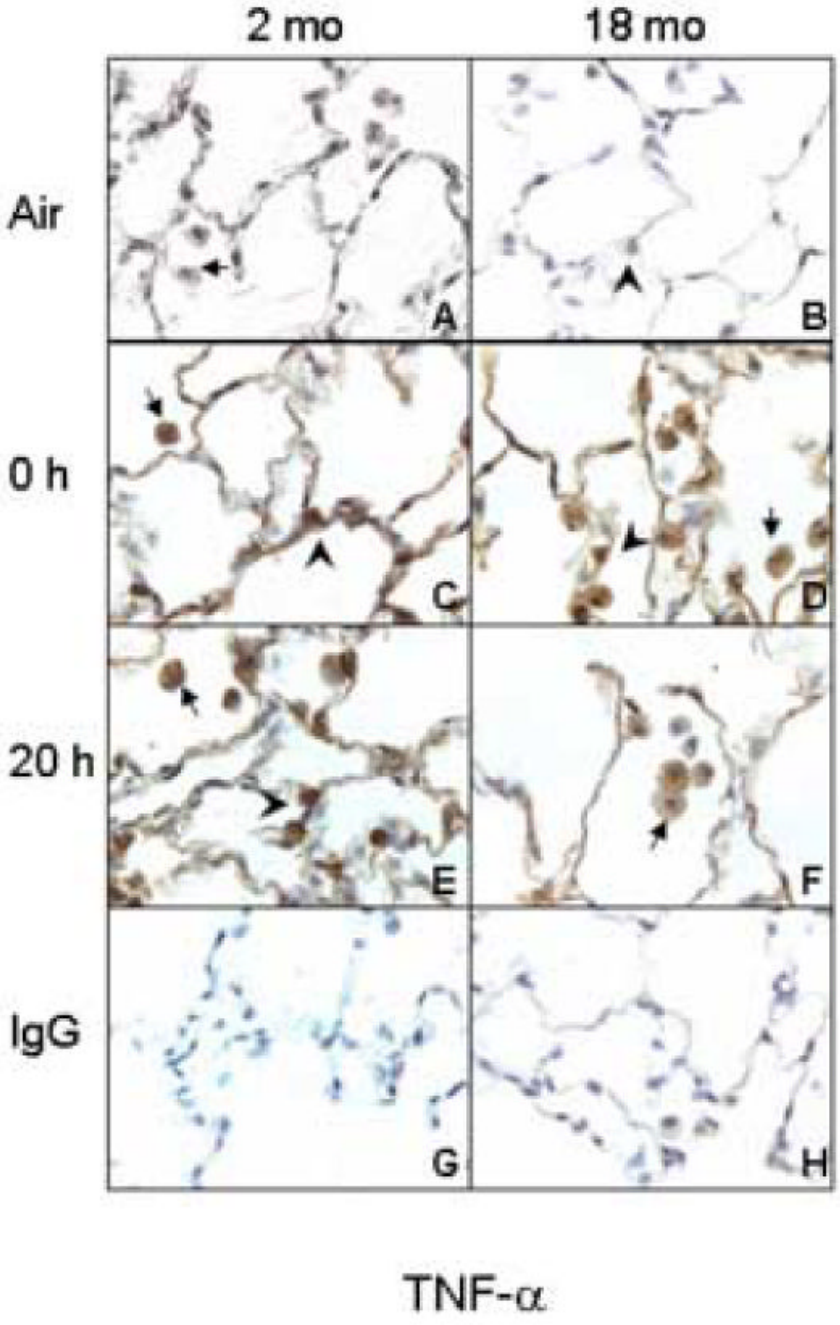

Figure 3.

Effects of inhalation of LOP on TNF-α expression. Lung sections (6 μm) were prepared from younger (2 mo) and older (18 mo) rats immediately (0 h) and 20 h after exposure to air (panels A and B) or LOP (panels C-F). Sections were stained with antibody to TNF-α (panels A-F) or IgG (panels G and H). One of three similar experiments is presented. Brown staining is indicative of TNF-α expression. Arrows, alveolar macrophages; Arrowheads, Type II cells.

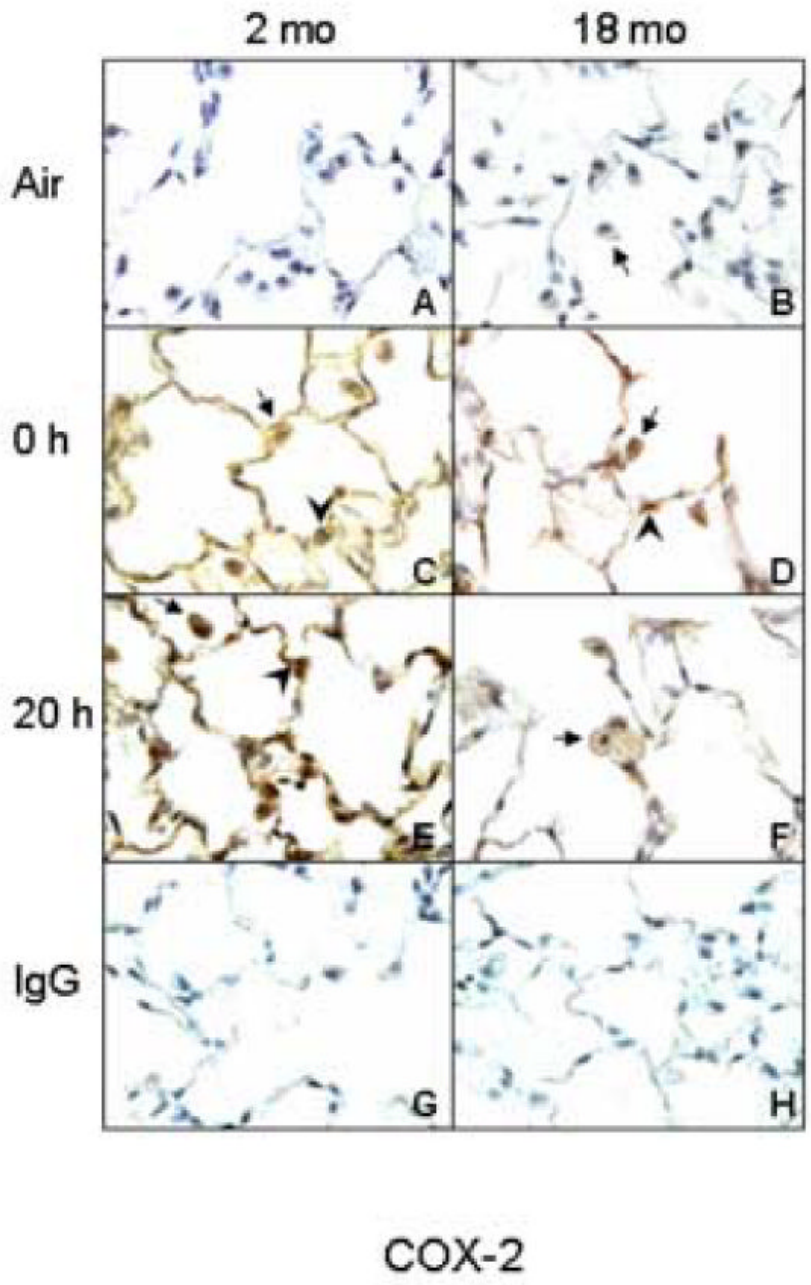

Figure 4.

Effects of inhalation of LOP on COX-2 expression. Lung sections (6 μm) were prepared from younger (2 mo) and older (18 mo) rats immediately (0 h) and 20 h after exposure to air (panels A and B) or LOP (panels C-F). Sections were stained with antibody to COX-2 (panels A-F) or IgG (panels G and H). One of three similar experiments is presented. Brown staining is indicative of COX-2 expression. Arrows, alveolar macrophages; Arrowheads, Type II cells.

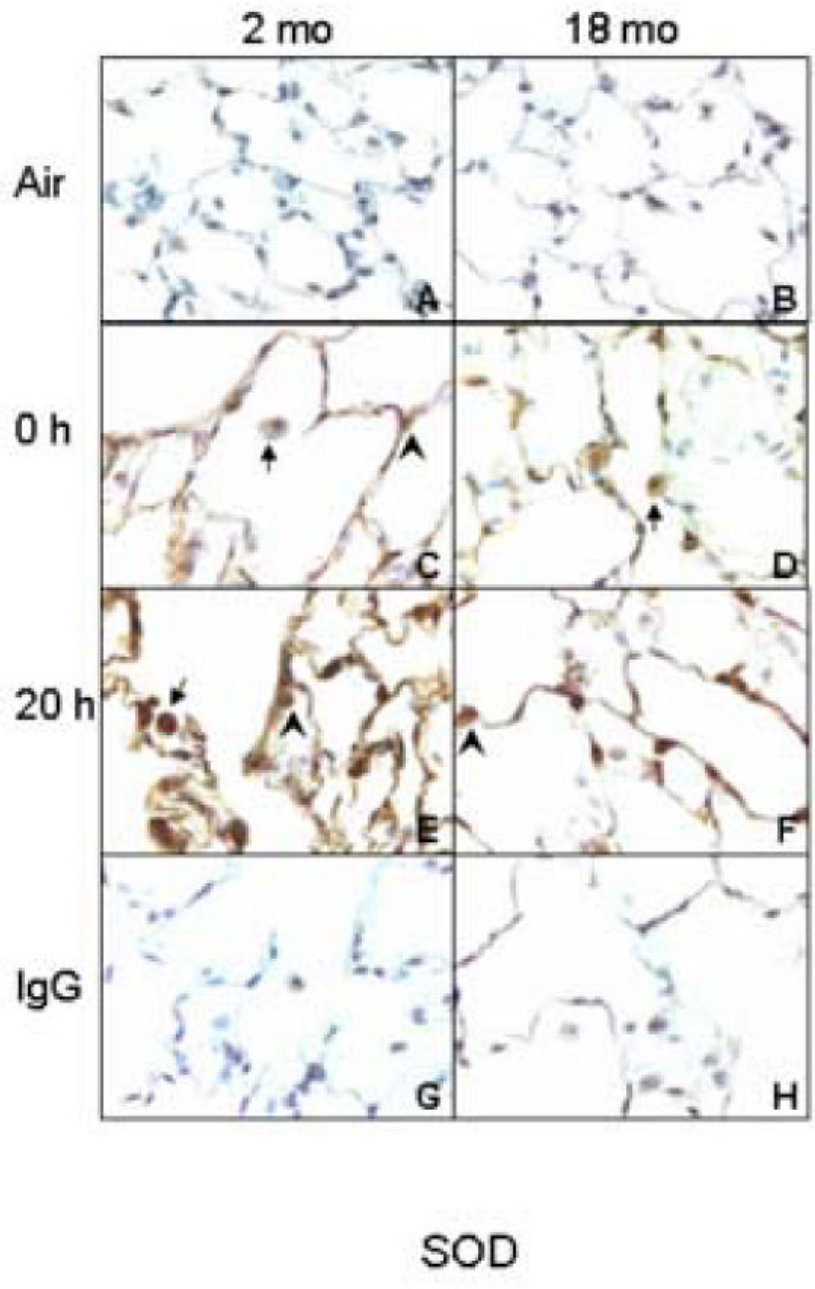

Figure 5.
Effects of inhalation of LOP on Cu/Zn SOD expression. Lung sections (6 μm) were prepared from younger (2 mo) and older (18 mo) rats immediately (0 h) and 20 h after exposure to air (panels A and B) or LOP (panels C-F). Sections were stained with antibody to Cu/Zn SOD (panels A-F) or IgG (panels G and H). One of three similar experiments is presented. Brown staining is indicative of Cu/Zn SOD expression. Arrows, alveolar macrophages; Arrowheads, Type II cells.

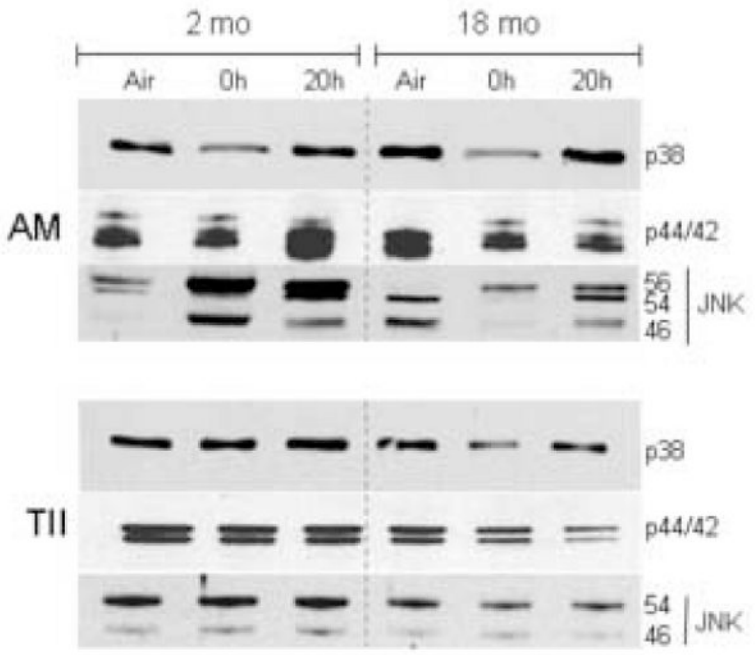

Figure 6.

Effects of inhalation of LOP on MAP kinase expression in alveolar macrophages (AM) and Type II cells (TII). Cells were isolated from younger (2 mo) and older (18 mo) animals immediately (0 h) and 20 h after exposure to air or LOP. Cytoplasmic extracts were prepared and analyzed for p38, p44/42 and JNK MAP kinase expression by western blotting. One representative gel from three separate experiments is shown.

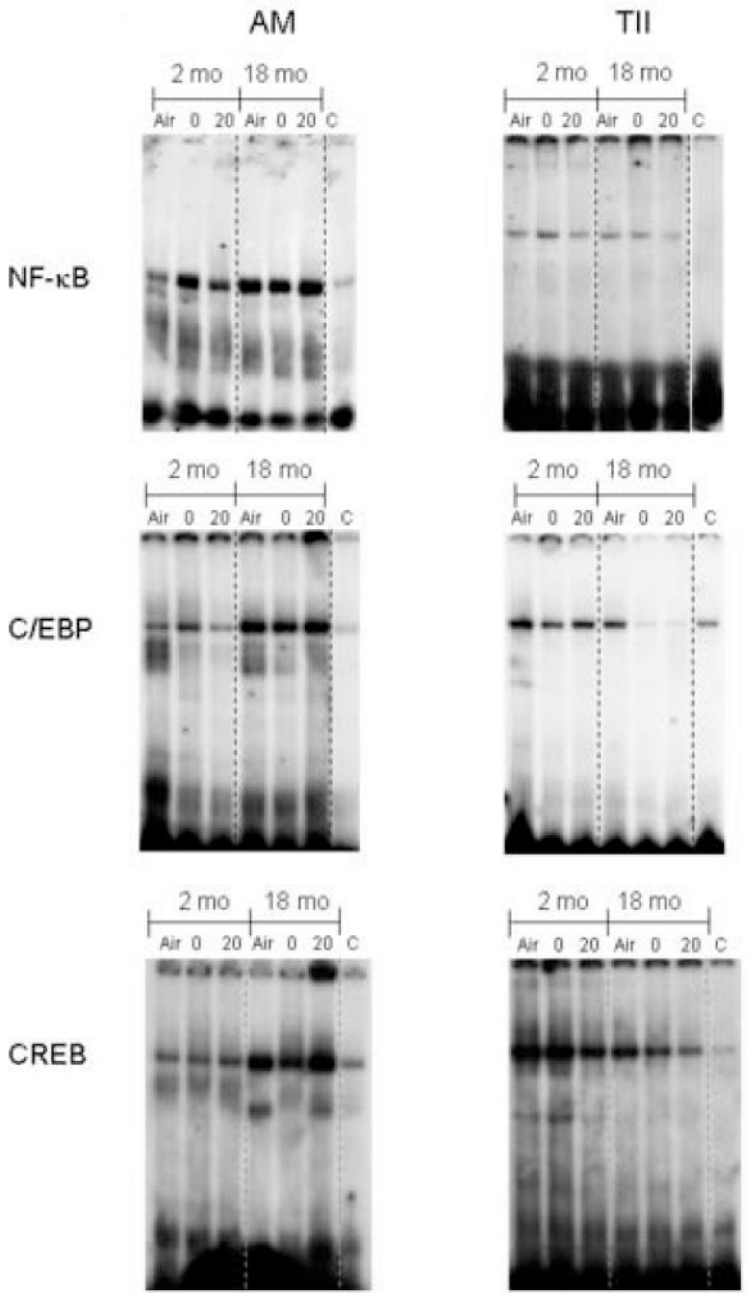

Figure 7.

Effects of inhalation of LOP on NF-κB, C/EBP and CREB nuclear binding activity. Alveolar macrophages (AM) and Type II cells (TII) were isolated from younger (2 mo) and older (18 mo) rats immediately (0) and 20 h (20) after exposure to air or LOP. Nuclear extracts were prepared and analyzed for NF-κB, C/EBP and CREB binding activity by EMSA. For supershift analysis, extracts prepared immediately after exposure of older rats to LOP (18 mo/ 20 h) were pre-incubated with 50-fold excess of unlabeled specific competitor probes (C). One representative of three gels is shown.

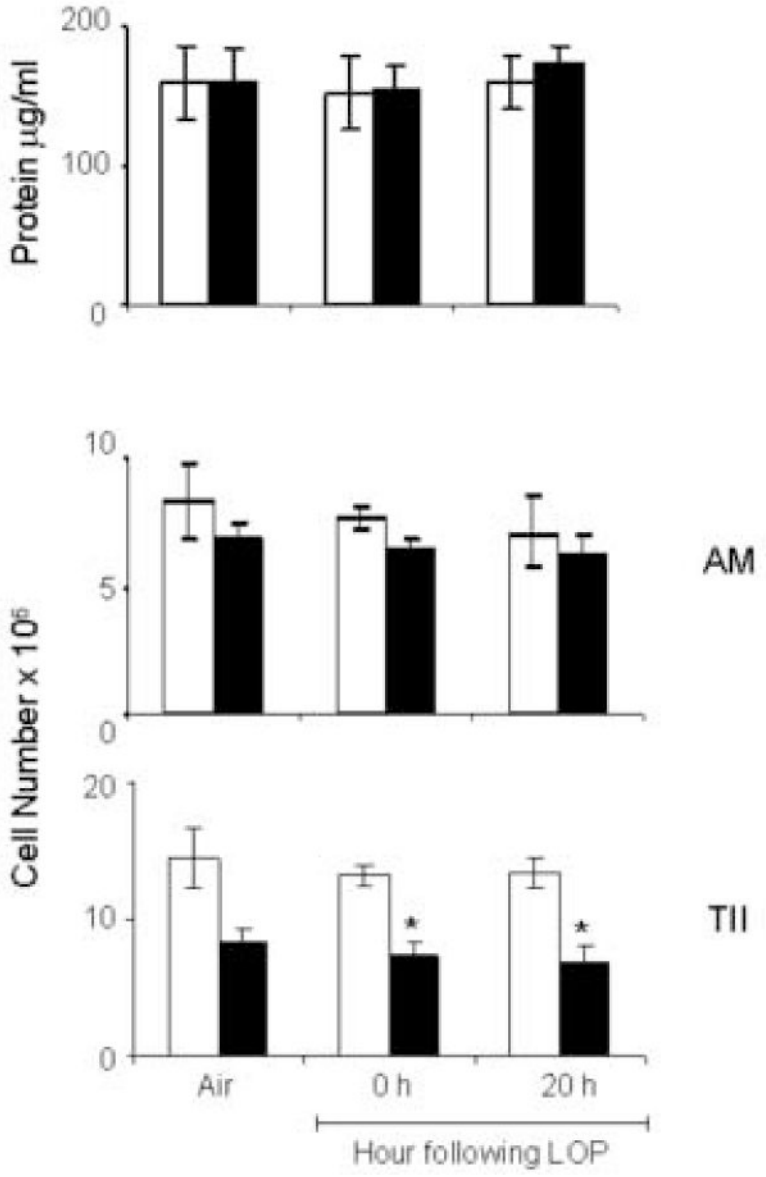

Figure 8.

Effects of LOP on BAL protein and cells. BAL fluid was collected immediately (0 h) or 20 h after exposure of younger (open bars) and older (closed bars) animals to air or LOP. *Upper panel:* Protein in BAL. Each bar is the average ± SEM of triplicate samples from three groups of animals (n=3/group). *Lower panel:* Number of viable alveolar macrophages (AM) and Type II alveolar cells (TII) recovered from the lung. Each bar is the average ± SE (n=3). *Significantly different ($p<0.05$), from younger animals (One way ANOVA).

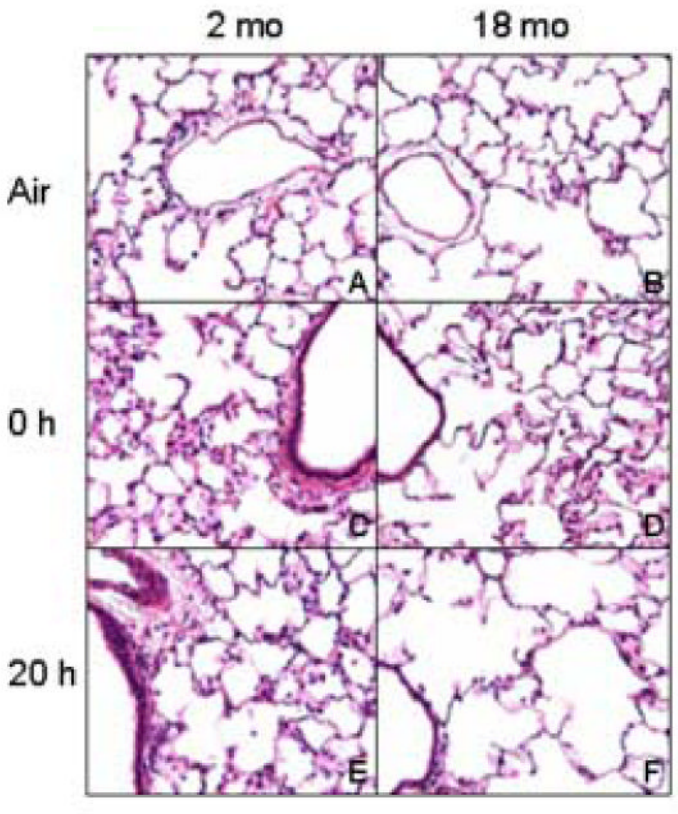

Figure 9.
Effect of LOP on lung histology. Lung sections (6 μm) prepared from younger (2 mo) and older (18 mo) rats immediately (0 h) and 20 h after exposure to air (panels A and B) or LOP (panels C-F) were stained with H & E, Magnification, 40×.