Original Article

Effect of exposure to volatile organic compounds (VOCs) on airway inflammatory response in mice

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ABSTRACT — Volatile organic compounds (VOCs) are the main substances causing multiple chemical sensitivity reactions in human. The effects of single VOCs exposure on airway inflammatory responses in mice lung have been reported. Previous studies have demonstrated the role of reactive oxygen species (ROS) in lung inflammation induced by single VOCs inhalation. However, effects of VOCs exposure on NO signaling and neurological signaling pathways in airway remain less clear. We exposed male Kunming mice to filtered air (0) and four types of VOCs mixture (formaldehyde, benzene, toluene, and xylene) treated air. Group 1 is 1.0, 1.1, 2.0 and 2.0 mg/m³, group 2 is 3.0, 3.3, 6.0 and 6.0 mg/m³, group 3 is 5.0, 5.5, 10.0 and 10.0 mg/m³, group 4 is 10.0, 11.0, 20.0 and 20.0 mg/m³, which respectively corresponded to 10, 30, 50 and 100 times of indoor air quality standard in China 2 hr per day, 5 days per week, for 2 weeks in the whole body exposure chamber. One day following VOCs exposure, we collected lung, bronchoalveolar lavage fluid (BALF) from each mouse and examined oxidative stress markers, cellular infiltration and production of cytokines, neurotrophin and substance P. We found that VOCs exposure influenced significantly NOS activity, GSH, or IL-6 concentration. The number of total cells, macrophages and eosinophils increased significantly in group 4. In addition, the production of interferon-gamma (IFN- γ) and substance P were significantly decreased. In contrast, neurotrophin-3 production in BALF was significantly increased in group 3 and 4. Our findings suggest that NO signaling pathways may induce airway inflammatory in short term VOCs exposure mice and the airway inflammatory response may be modulated by neurological signaling.

Key words: VOCs, Short-term, NO signaling, Airway inflammation, Neurotrophin, Mice

INTRODUCTION

Sick building syndrome (SBS) is the suite of adverse health effects caused by serious indoor air quality problems in houses, offices, and other work places. The syndrome is generally characterized by mucosal irritation and nonspecific hypersensitivity (Li *et al.*, 2007). Moreover, this symptom has been reported in humans suffering from multiple chemical sensitivity (MCS) following exposure to volatile organic compounds (VOCs), such as toluene and formaldehyde (FA) (Takafuji and Nakagawa, 2000; Fujimaki *et al.*, 2007).

Recent studies showed that the exposure of FA and toluene inhalation induce airway inflammation and neurotrophin production in hippocampus of mice (Fujimaki *et al.*, 2004a, 2004b and 2007; Jung *et al.*, 2007; Win-Shwe

et al., 2010). Moreover, studies have demonstrated that the numbers of corticotrophin releasing hormone-immunoreactive neurons (CRH-ir) in the hypothalamus were increased in mice exposed long term to low-level FA and toluene (Sari *et al.*, 2004; Kesuma *et al.*, 2006). Benzene and xylene also could irritate the airway, and cause dizziness in humans (WHO, 1997; ATSDR, 2007). Animal studies show neurologic effects from inhalation exposure to benzene and xylene (WHO, 1997; ATSDR, 2007). These results show that VOCs inhalation can modulate not only inflammatory responses in lung but also neurological responses in brain.

The mechanisms whereby VOCs inhalation exerts its effects on allergic lung inflammation are not completely understood. In recent reports, FA inhalation increased oxidative burst and nitration of proteins in the lung, sup-

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porting the presumed involvement of oxidative stress on FA-induced lung inflammation (Lino-dos-Santos-Franco *et al.*, 2010). FA exposure induces airway inflammation by increasing eosinophil infiltrations through the regulation of reactive oxygen species production (Jung *et al.*, 2007). However, effects of VOCs exposure on NO signaling and neurological signaling pathways in airway have been given little attention. In the airway, the three types of NOS, eNOS (NOS III), nNOS (NOS I), and iNOS (NOS II) have been identified. Increased exhaled NO levels have been seen in some environmental lung diseases, such as asthma (Ricciardolo *et al.*, 2004). Exhaled NO levels were significantly elevated in children living in homes with average FA levels ≥ 50 ppb (Franklin *et al.*, 2000). Moreover, employees with respiratory symptoms had markedly higher exhaled NO values (Roponen *et al.*, 2001). These results suggested that VOCs could increase the activity of NOS. Recently, it has been found that channeling of NO into S-nitrosothiols (SNO) plays an important role in NO signaling.

Neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) play an important role in the development of airway inflammation and airway hyperresponsiveness (Braun *et al.*, 2000; Fujimaki *et al.*, 2004a) and affect the production of tachykinins such as substance P (Vedder *et al.*, 1993; Malcangio *et al.*, 1997). It has been reported that the expression of neurotrophins and their receptors in peripheral lung cells of mice and some signal trafficking occurs through neurotrophins in peripheral lungs (Hikawa *et al.*, 2002). In addition, low-level toluene exposure act on the HPA axis as stressor and CRH is the possible candidate to modulate airway inflammatory responses in lung (Fujimaki *et al.*, 2007).

FA, benzene, toluene and xylene among volatile organic compounds usually occur in indoor air from the use of common household products and cigarette smoke. Up to now, NO signaling and neurological signaling pathways have effects on airway inflammation, hypersensitivity reaction and compensatory mechanism following VOCs mixture exposure which remain unknown. It is surmised that homeostatic mechanisms involving the nervous and immune systems may compensate toxic chemical induced respiratory changes to maintain a relatively constant internal environment. In addition, Kunming mice are the most widely used outbred colony in China. Some mechanisms of formaldehyde-induced asthma in Kunming mice have been studied (Lu *et al.*, 2005; Yan *et al.*, 2005; Cao *et al.*, 2007). Therefore, the aim of the present study was to investigate the effect of VOCs exposure on airway inflammatory responses via NO signaling and the role of neuro-

trophic factor in the modulation of pulmonary inflammation in Kunming mice. Oxidative stress was also assessed in the present study since the activation of NOS may generate reactive oxygen species (ROS) and reactive nitrogen species (RNS).

MATERIALS AND METHODS

Animals

5 weeks' old male (18-20 g) Kunming mice were purchased from Experimental Animal Care Center of Dalian Medical University. The mice were maintained under the following laboratory conditions of temperature: 22 ± 2 , humidity: $50 \pm 10\%$ and 12 hr/12 hr: light and dark cycle. Distilled water and sterilized food for mice were available *ad libitum*. At the end of 1 week of acclimation, the mice were 6 weeks old on their first day of VOCs mixture exposure.

VOCs inhalation

Benzene, toluene, and xylene (BTX) and FA were purchased from the Sigma Co. (St. Louis, MO, USA). Four VOCs mixture exposure groups and an unexposed control group were used in this study. The concentrations of four VOCs mixture exposure groups corresponded to respectively 10, 30, 50 and 100 times of indoor air quality standard in China (http://kjs.mep.gov.cn/hjbhzbz/bzwb/dqhjbh/dqhjzlbz/200303/t20030301_67375.htm). Animals were exposed for 2 weeks at 2 hr/day (from 14:00-16:00) and 5 day/week in an inhalation exposure chamber. 10 mice were exposed in each of the five experiments ($n = 50$ mice). During the study period except VOCs exposure, food and water were provided to the animals.

Exposure conditions and analysis of chamber VOCs concentrations

The cylindrical experimental chamber (100 l) was made from glass and consisted of two compartments: the animal exposed chamber and the VOCs injection chamber, respectively. The two compartments were connected via a circular perforated baffle palate with ventilator fan to diffuse VOCs into the animal exposed chamber. A hole (5 cm diameter) was located in the VOCs injection chamber for VOCs injection. The other two holes (5 cm diameter) were located in the animal exposed chamber to determine the concentrations of FA and BTX. FA and BTX gas were generated in 100 ml injector using BTX high performance liquid chromatographic (HPLC) purity and FA analytical reagent by 50°C oven and were injected into the chambers in turn to achieve the desired gas concentrations. In order to keep VOCs concentrations in expo-

sure chamber relatively stable, VOCs concentrations in exposure chamber were determined every 10-20 min and VOCs mixture were supplemented in time. The air in the exposure chamber was conditioned to $22 \pm 2^\circ\text{C}$ and $50 \pm 5\%$ humidity. Environmental chemical components, such as BTX and FA were determined in breeding room, and no significant chemical components were detected in breeding room (detection limit $0.05 \mu\text{g}/\text{m}^3$).

An Interscan 4160 digital electrochemical analyzer (4160-19.99m, Interscan, Chatsworth, CA, USA) was used to measure the concentrations of gaseous FA. To determine the BTX concentrations, air samples in the chamber were collected, Gas Chromatograph (GC) (GC-2010, Shimadzu, Kyoto, Japan) were used to determine BTX levels. GC conditions are as follows, detector: FID; column: a HP-5 column ($30 \text{ m} \times 0.53 \text{ mm} \times 1.5 \mu\text{m}$); column (oven) temperature: from 75°C to 105°C ; detector temperature: 320°C ; gas flow rate: $1 \text{ ml}/\text{min}$; injection sample volume: 2 ml . Before the formal test, we detected volatile organic compounds concentrations in the exposure chamber, found the dynamic change trend of VOCs after timely supplement corresponding gas under the same condition with formal test during 2 hr exposure period (Support information Fig.1). The mean VOCs concentrations were achieved over the 2-week exposure period (Table 1).

Collection of samples and cell counting in bronchoalveolar lavage fluid (BALF)

One day following the final VOCs inhalation for 2 weeks, the mice were sacrificed under pentobarbital anesthesia, BALF was collected from each mouse ($n = 5$). The lung for measure of oxidative stress markers and histological analysis was collected from each group of mice ($n = 5$). BALF was collected by cannulating the trachea and lavaging the lung four times with 1 ml of sterile saline as described previously (Fujimaki *et al.*, 1997). The recovered fluids about 3.5 ml were pooled.

BALF was centrifuged and the resulting BAL cell pellet was diluted. Aliquots were analyzed by hemocytometer and trypan blue dye exclusion for viable cell count, and differential inflammatory cells were counted by light microscopy after Wright-Giemsa-staining. The supernatant from BALF which was centrifuged and the other lungs were stored at -80°C .

Measurement of lung oxidative stress markers

ROS generation was measured using the method described by other researchers (Wang and Joseph, 1999). Just before the exposure to HCHO or H_2O_2 , Cells (5×10^5 cells/sample) isolated from the lung tissues were incubated with culture medium containing $20 \mu\text{mol}/\text{l}$ DCFH-DA, for 30 min at 37°C and $5\% \text{ CO}_2$. DCFH-DA was then removed, and cells were washed with PBS and exposed to HCHO or H_2O_2 as described above. After exposure, fluorescence of each well was read immediately using a Spectra Max Gemini XPS microplate reader (RF-5301PC, Shimadzu, Japan) with an excitation filter set at 485 nm and an emission filter set at 530 nm . Data was expressed as the fluorescence intensity.

GSH, MDA, and T-AOC were measured by using commercially available kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The mice's lungs were washed in normal saline and homogenate 10% prepared in $1.15\% \text{ w/v}$ of potassium chloride. The homogenate was centrifuged in $7,000 \text{ g}$ for 10 min at 4°C and supernatant were used for measurement of oxidative stress by estimation of reduced glutathione (GSH) and determination of malondialdehyde (MDA) as well as total anti-oxidation capacity (T-AOC).

Reduced GSH content was determined according to the protocol (Sedlak and Lindsay, 1968). GSH reacts with 5, 5'-dithiobis-2-nitrobenzoic acid, and the absorbance spectra of the product have a maximum absorbance at 410 nm . The results were expressed as $\mu\text{mol}/\text{g}$ protein. Lung homogenate MDA levels were expressed as nmol MDA

Table 1. VOCs concentrations during exposure experiments

Group	Formaldehyde (mg/m^3)	Benzene (mg/m^3)	Toluene (mg/m^3)	Xylene (mg/m^3)
Control	-	-	-	-
1	0.94 ± 0.08	1.05 ± 0.11	1.97 ± 0.19	2.04 ± 0.22
2	3.11 ± 0.17	3.23 ± 0.38	5.98 ± 0.49	6.10 ± 0.55
3	5.14 ± 0.63	5.88 ± 0.51	9.77 ± 1.12	10.14 ± 1.08
4	9.66 ± 0.93	10.87 ± 1.42	19.11 ± 2.28	21.25 ± 2.49

Concentration of each exposure chamber was determined every 10-20 min. Number of determinations: 10; Data was shown as mean $\pm$ S.D.

per mg protein. Degree of lipid peroxidation in lung tissue homogenates was determined in terms of thiobarbituric acid reactive substances (TBARSs) formation with maximal absorbance at 532 nm by following the protocol (Esterbauer and Cheesman, 1990). T-AOC reflects the overall cellular endogenous antioxidative capability including both enzymatic and non-enzymatic antioxidants. All these antioxidants can reduce Fe^{3+} to Fe^{2+} , the latter can form colored and stable chelates combining with phenanthroline. T-AOC was measured by the method of ferric reducing-antioxidant power assay (Benzie and Strain, 1996) and detected at 520 nm with the spectrophotometer.

Total NOS (TNOS) activity and inducible NOS (iNOS) activity were determined using an NOS activity assay kit (Nanjing Jiancheng Bioengineering Institute). In brief, Lung tissue was minced and homogenized in lysis buffer (Tris 20 mmol/l, NaCl 50 mmol/l, NaF 50 mmol/l, $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ 5 mmol/l, $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ 25 mmol/l, DTT 1 mmol/l, Na_3VO_4 2 mmol/l, and 1% protease inhibitor cocktail, pH 7.4) with a tissue homogenizer. The homogenate was centrifuged (12,000 g at 4°C for 10 min), the supernatant were used for measurement of TNOS and iNOS following the manufacturer's instructions. Supernatant were added to the reaction buffer containing L-arginine, NADPH, calcium (not present in iNOS assay buffer), calmodulin, tetrahydrobiopterin, nitroblue tetrazolium (NBT), and phenazine methosulfate (PMS). Formazan, the reaction product of NBT/PMS with NADPH in the presence of NO, was quantified spectrophotometrically at 530 nm. One NOS enzymatic unit was defined as $1 \text{ nmol} \cdot \text{NO}^{-1} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. The NOS activities were expressed as U/mg prot.

NO concentration assay in the lung was performed according to kit protocols (Nanjing Jiancheng Bioengineering Institute Jiangsu, China). The OD value was determined by a spectrophotometer (U-3010, Hitachi, Tokyo, Japan). Results of NO were read with OD value at 550 nm. The result was calculated using the following formula:

$$\text{NO } (\mu\text{mol/l}) = (\text{A sample} - \text{A blank}) / (\text{A standard} - \text{A blank}) \times 20 (\mu\text{mol/l})$$

The final level of NO in liver homogenates was calculated and expressed as nmol/ mg lung protein.

Assay of cytokines, neurotrophins and substance P

The amount of various chemical mediators in each sample supernatant from BALF was measured by using ELISA kit for IL-6, IL-4, interferon (IFN)- γ , neurotrophin (NT)-3 and substance P (R&D system, Minneapolis, MN,

USA). Absorbance of ELISA plates at wavelengths of 450 and 550 nm was measured with a microplate reader (BioRad, Model 550). Determinations were made in duplicates for every sample using standard curves according to the manufacturer's specifications.

Histopathological analysis

The lung tissues of male Kunming mice that had been exposed to one of 4 concentrations of VOCs mixture for 2 hr/day, 5 days/week, for 2 weeks, and control mice were removed, and fixed in 10% neutral buffered formalin overnight at 4°C. The tissues were dehydrated through increasing concentrations of ethyl alcohol (70%, 80%, 90%, and 100%), cleared in xylene, infiltrated in paraffin and paraplast by an automatic tissue processor (Shandon Co., San Luis Obispo, CA, USA), and embedded in paraffin wax with an embedding machine (Leica Co., Wiesbaden, Germany). Three-micron sections of the lung tissues were cut from the paraffin blocks and stained (hematoxylin and eosin). All tissue sections were examined qualitatively by an experienced pathologist, and in a study blind fashion.

Statistical analysis

Data analyses were performed using SPSS software (Ver13.0; SPSS). Data were expressed as means $\pm$ S.D. One-way analysis of variance (ANOVA) was carried out to compare the differences of means among multi-group data. Dunnett's test was carried out when each group of experimental data was compared with control data.

RESULTS

Coefficient of lung to body weight

With increasing dosages, the coefficients of the lung to body weight and the net increase of body weight were gradually decreased, no significant differences were found in the coefficients of the lung to body weight of five groups, whereas, the net increase of body weight of group 3, 4 were significantly lower than that of the control ($p < 0.05$ or $p < 0.01$), indicating that higher doses of VOCs might cause the mouse damages (Table 2).

Effects of exposure to VOCs on oxidative stress markers of lung

The significant increases of ROS and MDA contents were observed in the lungs in group 3 and 4 mice ($p < 0.01$ or $p < 0.05$). Further, ROS in group 1 and 2 were higher than control ($p < 0.05$ or $p < 0.01$) (Table 3). In group 1 mice, T-AOC and GSH were not significantly different from those of the control. However, in the oth-

Airway inflammatory response by VOCs exposure

Table 2. Increase of net weight and coefficients of lung of Kunming mice exposure to VOCs

Index	Group				
	control	1	2	3	4
Net increase of BW (g)	12.80 ± 1.10	10.06 ± 2.26	10.02 ± 1.82	8.79 ± 1.79*	3.17 ± 0.41**
Lung/BW (mg/g)	0.82 ± 0.12	0.76 ± 0.07	0.80 ± 0.10	0.77 ± 0.16	0.73 ± 0.12

Values were means ± S.D. $n = 5$ in each group; * $p < 0.05$ ** $p < 0.01$ compared with control. BW represents body weight.

Table 3. Effects of ROS and oxidative stress markers in lung in mice exposure to VOCs

Group	GSH ($\mu\text{mol/g prot}$)	T-AOC (U/mg prot)	ROS (Fluorescence intensity)	MDA (nmol/mg prot)
Control	24.73 ± 2.31	2.71 ± 0.19	12.83 ± 0.15	4.06 ± 0.38
1	21.81 ± 3.41	2.40 ± 0.27	18.51 ± 0.66*	4.54 ± 0.30
2	21.02 ± 3.62	2.15 ± 0.18*	23.15 ± 1.05**	4.94 ± 0.34
3	17.59 ± 1.34*	1.46 ± 0.40*	34.91 ± 3.06**	4.99 ± 0.26*
4	17.63 ± 0.84*	1.99 ± 0.27*	88.25 ± 5.88**	5.01 ± 0.23*

Values were means ± S.D. $n = 5$ in each group; * $p < 0.05$ ** $p < 0.01$ compared with control.

Table 4. Effect of VOCs mixture on the number of inflammatory cells in BAL cells

Group	BAL cells ($\times 10^4$)	Macrophages	Neutrophils	Lymphocytes	Eosinophils
control	2.47 ± 0.14	2.40 ± 0.04	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
1	2.48 ± 0.18	2.41 ± 0.18	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00
2	2.25 ± 0.26	2.17 ± 0.24	0.02 ± 0.00	0.04 ± 0.01	0.02 ± 0.01
3	2.48 ± 0.15	2.39 ± 0.14	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
4	3.72 ± 0.38**	2.92 ± 0.17*	0.03 ± 0.01	0.80 ± 0.17*	0.08 ± 0.02*

Data are reported as mean ± S.D. ($n = 5$), * $p < 0.05$, ** $p < 0.01$ compared with control.

er exposure groups, T-AOC and GSH were significantly lower than control ($p < 0.01$ or $p < 0.05$).

The activities of TNOS and iNOS concentration of the mouse lung are shown Fig. 1. TNOS and iNOS activities were significantly higher than control in the lung of mice exposure to VOCs except group 1 ($p < 0.05$) (Fig. 1a). NO in the whole lung homogenates would reflect NO produced for neurotransmission, and NO produced by inflammatory responses that were initiated by VOCs in group 3, 4 ($p < 0.05$) (Fig. 1b).

Effects of exposure to VOCs on the inflammatory responses in lung of the mice

The effects of exposure to VOCs on airway inflammatory responses in the mice were examined. After exposure for 2 weeks, the total number of inflammatory cells in BALF was significantly increased in group 4 mice as

compared to that in the control mice (Table 4). Differential cell count analysis revealed that the number of macrophages, lymphocytes and eosinophils were significantly increased in group 4 mice, while the count of neutrophils did not show any significant change as compared to those of control mice. However, the number of inflammatory cells in BALF from other VOCs- exposed group mice returned to control level (Table 4).

However, there was no significant change in the levels of IL-4 in the specimens obtained from the mice exposed for 2 weeks. A significant suppression of the level of IFN- γ in the BALF in group 3, 4 mice was observed, while IL-6 in BALF of mice except group 1 was significantly increased than control (Fig. 2).

Fig. 1. TNOS and iNOS in lung from Kunming male mice treated VOCs. Data are reported as mean $\pm$ S.D. ($n = 5$), * $p < 0.05$, ** $p < 0.01$ compared with control. (a) NOS; (b) NO.

Effects of low-level exposure to VOCs on the production of NT-3 and substance P in BALF of the mice

To examine the effects of inhalation of VOCs on the release of neurotrophins, we measured the production of NT-3. After exposure for 2 weeks, a significant increase in the production of NT-3 was observed in the BALF of VOCs-exposed group 3, 4 mice (Fig. 3a). While no significant change in the production of NT-3 in BALF was observed in other VOCs-exposed group mice. The content of neuropeptide substance P in BALF from group

Fig. 2. Effect of cytokines in BALF from mice exposed VOCs. Data are reported as mean $\pm$ S.D. ($n = 5$), * $p < 0.05$ compared with control.

3, 4 mice (440.29 ± 48.93 ng/l; 451.46 ± 45.63 ng/l) ($p < 0.05$) exposed to VOCs significantly reduced compared with control (510.40 ± 13.94 ng/l) (Fig. 3b).

Histological observation in lung of mice exposed to VOCs

Histological examination of the respiratory epithelium from the lung in mice exposed to VOCs mixture for 2 weeks was performed under a light microscope. The epithelium of trachea and bronchus consisted of pseudostratified ciliated epithelial cells, basal cells and goblet cells. The terminal bronchial epithelium consisted mainly of Clara cells. The bronchiolus and alveolar epithelium did not undergo any changes in VOCs-exposed mice compared with control mice (Support information Fig. 2). In the airway, there was no infiltration of inflammatory cells including mast cells within the epithelium in both VOCs exposed and control mice. In conclusion of pathological study, there were no effects by VOCs inhalation in epithelium tissues of the lung.

DISCUSSION

In the present study, we focused on the induction of airway inflammatory responses to short-term VOCs exposure. It is of interest whether or not sensitization with short-term VOCs inhalation induces immunological abnormalities. Our study could help to clarify factors responsible for increased frequency of symptoms of immunological abnormalities regarding SBS and MCS.

Airway inflammatory response by VOCs exposure

Fig. 3. Effect of VOCs inhalation for 2 weeks on the levels of NT-3 and substance P in the BALF of mice ($n = 5$). * $p < 0.05$ compared with control. (a) NT-3; (b) Substance P.

Our results demonstrate that oxidative stress responses occurred in lung, the production of IL-6 and NT-3 were significantly increased in BALF in higher VOCs mice, while the IFN- γ and substance P level were significantly decreased in mice exposed to higher dose VOCs, histological examination of lung did not occurred significantly. Our data have revealed that the inflammation appears to be rather low.

Previous work has demonstrated that single VOCs is a lung irritant and may cause inflammation (Fujimaki *et*

al., 2004a, 2007; Jung *et al.*, 2007), but studies of VOCs exposure on NO signaling pathways in airway have been lacking. In this study, we demonstrated that VOCs exposure could induce NOS in mouse lungs. NO is synthesized from L-Arginine by three kinds of NO synthase (NOS). In the airway, the three types of NOS, eNOS (NOS III), nNOS (NOS I), and iNOS (NOS II) have been identified. Elevated NOS activity and NOx concentrations in the airway are normally regarded as key inflammatory events in environmental lung diseases (Ricciardolo *et al.*, 2004). iNOS play an important role in host defence and immunological reaction and it is thought to be mediator in vasodilatation, edema, cytotoxicity and other cytokine dependent processes. In inflammatory disease, NO is increased by an upregulation of the inducible form of NOS (iNOS) (Barnes, 1995). In the airway, NOS may produce NO and GSNO. NO may further generate NO₂, which may deplete GSH, and result in airway inflammation, characterized by typical cytokine profiles, e.g., IL-6 up-regulation, as well as bronchoconstriction (Fig. 4). GSNO may convey the bioactivity of NO, and lead to bronchodilation. However, the activity of GSNO could be dismissed by the decomposition of GSNOR, the only GSNO decomposing enzyme (Que *et al.*, 2005) (Fig. 4).

In the present study, TNOS and iNOS activity was significantly increased in group 2, 3, 4 (Fig. 1a), which suggested that inflammation was induced by VOCs exposure. This was further confirmed by the eosinophil count, inflammatory cells that are the major cells to elicit oxidative stress in the airway (Table 4). While GSH concentration in mice lung homogenates was significantly decreased, IL-6 measurement also suggested that VOCs exposure induce inflammatory responses in this study. VOCs exposure to lead to inflammation is consistent with a recent report (Lino-dos-Santos-Franco *et al.*, 2011). In rat model, direct signs of inflammation were seen after exposure to FA as examined by IL-6 secretion, NO, NOS and total and differential cell counts, suggesting that NOS was induced. Our data were also consistent with previous work using toluene diisocyanate exposure, which showed that pulmonary inflammatory responses induced by TDI exposure are associated with increases in inducible NO production (Huffman *et al.*, 1997).

Besides its role as a signaling molecule, NO may also be cytotoxic since it is a small reactive molecule. Furthermore, NO can interact with ROS to form other RNS. ROS, NO, and ROS may damage DNA, lipids, proteins, and carbohydrates in environmental lung diseases (Fig. 4) (Weinberger *et al.*, 2001; Ricciardolo *et al.*, 2004; Masri, 2010; Zhao *et al.*, 2009). In diseases such as asthma, ele-

itor of substance P like immunoreactivity in rat isolated spinal cord (Malcangio *et al.*, 1997). In our study, we found that NT-3 was significantly increased in group 3, 4 mice. NT-3 may suppress airway inflammatory responses via inhibition of substance P production in short-term VOCs exposure. Our results were also consistent with previous work using toluene exposure (Fujimaki *et al.*, 2007). On the other hand, the increase of the secreted CRH in the hypothalamus in turn, might suppress the release of substance P in the lung, which needs to be further validated.

Our study showed that exposure to VOCs mixture in group 3 and 4 mice suppressed the production of IFN- γ in the BAL fluid. IL-4 is known to promote airway eosinophilia by suppressing the production of IFN- γ (Cohn *et al.*, 2001), while IL-4 was not significantly increased in our experiment. A decrease in the production of IL-2 has been reported in mice given single VOCs (Gulec *et al.*, 2006). IL-2 stimulated the synthesis of IFN- γ in peripheral leukocytes (Cousens *et al.*, 1995), and induced expression and secretion of IFN- γ in murine peritoneal macrophages (Puddu *et al.*, 2005). Thus, it appeared that the suppression of IFN- γ production in the BALF in mice exposed to VOCs mixture might be due to a decrease of IL-2 production. IFN- γ has been suggested as the key cytokine in the T-helper type 1 immune response required for adequate control of pulmonary *Mycobacterium tuberculosis* infection (Schluger and Rom, 1998). Our data showing the suppressed IFN- γ production in mice exposed to VOCs mixture was consistent with previous studies showing a toluene-mediated decreased in IFN- γ level in BALF of mice as well as toluene/n-hexane mediated impairment of hamster's resistance against mycobacterial infection (Fujimaki *et al.*, 2007; Palermo-Neto *et al.*, 2001).

In conclusion, short-term VOCs exposure might induce low airway inflammation which may be induced by NO signaling pathways, and oxidative stress was seen post-exposure. However, the airway inflammatory responses are also suppressed in our present study. Our data suggest that NT-3 mediated protective mechanism in the lung is activated in short-term VOCs exposed mice (Fig. 4).

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