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Changes in Oxidative Stress Biomarker and Gene Expression Levels in Workers Exposed to Volatile Organic Compounds

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Abstract: Exposure to volatile organic compounds (VOCs) was known to result in immunologic, respiratory, carcinogenic, reproductive, neurologic, and cardiovascular effects. However, the mechanisms by which VOCs induce these adverse health effects are not well understood. To evaluate the change of oxidative stress biomarker and gene expression levels in workers exposed to VOCs, we obtained urine and blood samples from 21 subjects before and after occupational exposure to VOCs. We measured levels of muconic acid (MuA), hippuric acid (HA), mandelic acid (MaA), and methyl hippuric acid (MHA) as urinary exposure biomarkers for benzene, toluene, ethylbenzene, and xylene (collectively BTEX), and malondialdehyde (MDA) and 8-hydroxydeoxyguanine (8-OHdG) as oxidative stress biomarkers in all subjects. We also evaluated BTEX-mediated RNA expression using cDNA microarray in 14 subjects. HA and MHA levels were higher following occupational exposure to VOCs (p<0.01). In the linear regression analysis, HA ratios of after- and before-exposure were found to be significantly associated with increase of MDA ratios of after- and before-exposure after controlling for age, body mass index, and smoking (β=0.06, p=0.031). Evaluation of the gene expressions by HA showed that 23 gene expressions were found to be significantly associated with HA levels after adjusting for age, body mass index, and smoking (p<0.001). In particular, expressions of *ENO3* and *CDNA FLJ39461 fis* among the 23 genes were significantly associated with the change in MDA level (p<0.05). Our study results suggest that exposure to VOCs, specifically toluene, induces oxidative stress and various gene expression change of which some may be responsible for oxidative stress.

Key words: Volatile organic compounds, Toluene, Oxidative stress, Expression microarray

Introduction

Volatile organic compounds (VOCs) are toxic pollutants ubiquitous in the general environment[1)]. Exposure to them has been known to result in immunologic, respiratory, carcinogenic, reproductive, neurologic, and cardiovascular effects[2, 3)]. VOCs are widely used as constituents of household chemicals such as paints, inks, aerosol sprays and fuels, and threaten health, especially of workers exposed to high concentration of VOCs[4–7)]. Benzene, toluene, ethylbenzene, and xylene (collectively

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BTEX) among VOCs are commonly found in occupational or non-occupational environments. Benzene is a well known carcinogen causally linked to leukaemia[8]. Toluene and xylene was reported to increase cancer incidence in paint industry workers although they were not classified as carcinogens[5, 6]. In addition, reproductive depressions by toluene, ethylbenzene, and xylene were reported in occupationally exposed workers[7]. However, the mechanisms by which these VOCs induce adverse health effects are not well understood despite considerable evidence of their impact on human health.

Previous studies reported that exposure to VOCs induced oxidative stress in animal or cell-line studies[9–11]. Oxidative stress has been involved in the etiology and progression of numerous pathological condition including cancer, aging, and neurodegenerative diseases[5, 12, 13]. Therefore, we hypothesized that BTEX exposure cause oxidative stress by producing highly reactive radicals that might cause progression of numerous pathological conditions in the process of these VOCs metabolism. Furthermore, according to the central dogma, regulation of cellular processes is conducted basically via RNA transcription. For these reasons, in the present study, we evaluated the effect of BTEX on oxidative stress and gene expressions.

In the present study, we measured urinary levels of muconic acid (MuA), hippuric acid (HA), mandelic acid (MaA), and methyl hippuric acid (MHA) as exposure biomarkers for BTEX in order to determine personal exposure to them. We also measured levels of malondialdehyde (MDA) and 8-hydroxydeoxyguanine (8-OHdG) as oxidative stress biomarkers. To explore gene expressions by BTEX exposure, we conducted an investigation of BTEX-mediated RNA expression using cDNA microarray. We also tried to evaluate the relationships between oxidative stress and gene expressions.

Materials and Methods

Study population and sampling

Study subjects were 21 Korean shipbuilding workers who had been occupationally exposed to VOCs. Subjects were 29- to 53-yr-old males. They worked in the department of Painting 1 (n=10), Painting 2 (n=7), or Designing (n=4) during the survey period, and their job types were spray (n=9), touch up (n=5), and coating (n=7). They worked from 9 a.m. to 5 p.m. weekdays. Because they have used various paints during working days, they have been occupationally exposed to a variety of VOCs. We obtained urine and venous blood samples from each subject before (on Sunday evening) and after a week of exposure to VOCs (on Friday evening) at the workplace. All urine samples were placed at –20°C immediately following collection and stored until analysis for the six biomarkers, and blood samples were stored at 4°C until isolating total RNA. We also obtained subject demographics using a structured questionnaire. The study protocol was approved by the Institutional Review Board at Seoul National University Hospital, and each study participant provided written informed consent.

Measurement of VOC exposure level

Study subjects have been checked by periods about how much they are exposed to VOCs in their workplace. To evaluate individual real BTEX exposure levels, we used VOC exposure data obtained from individual badge, the passive diffusion monitor 3500 (3M, USA), wore every 3 h twice for one day between Jan 1, 2005 and Jun 30, 2005. The analytical method (Manual no. 1501, http://www.cdc.gov/niosh/nmam/pdfs/1501.pdf) recommended by the National Institute for Occupational Safety and Health (NIOSH) was used for the measurement of exposed VOC levels.

Measurement of urinary biomarkers

MuA, HA, MaA, and MHA were measured as biomarkers for exposure to benzene, toluene, ethylbenzene, and xylene, respectively, and MDA and 8-OHdG were measured as oxidative stress biomarkers.

To determine MuA levels, 200 μl of urine was mixed with 800 μl of mobile phase (1% acetic acid and methanol, 10:1, v/v) and filtered through a 0.45-μm membrane filter. Absorbance of the filtered solution was measured at 259 nm using HPLC-UV. To determine HA, MaA, and MHA levels, 200 μl of urine was mixed with 2 ml of distilled water and filtered through a 0.45-μm membrane filter. Absorbance of the filtered solution was measured at 225 nm using HPLC-UV. The mobile phase was 0.55% n-tetrabutylammonium bromide / 0.15% KH_2PO_4 and MeOH (60:40, v/v).

MDA levels were determined by measuring thiobarbituric acid reactive substances (TBARS) as follows: 50 μl of urine was mixed with 300 μl of 0.5 M phosphoric acid solution and 150 μl of 23 mM TBA solution (Sigma-Aldrich T-5500, Steinheim, Germany) and heated at 95°C for 1 h. After cooling on ice, the mixture was vortexed with 500 μl of methanol and centrifuged at 5,000 x g. Absorbance of the supernatant was measured at 532 nm using HPLC-UV. The mobile phase was potassium phosphate (0.05 mol/l; pH 6.8) and methanol (58:42, v/v). The urinary levels of 8-OHdG were measured using ELISA kit (JaICA, Fukuroi, Japan). Briefly, 50 μl of 8-OHdG monoclonal antibody and 50 μl of the sample were added to a microtiter plate which had been precoated with 8-OHdG, and the

plate was incubated at 37°C for 1 h. After washing with 250 μl of washing solution, 100 μl of enzyme-labeled secondary antibody was added to the plate and incubated at 37°C for 1 h. After re-washing the plate, substrate solution and phosphoric acid solution were added in turn, and absorbances from the wells were measured at 450 nm using a microtiter plate reader.

For the adjustment of urinary volume, we measured urinary creatinine levels using a HITACHI 7600 instrument (HITACHI, Tokyo, Japan). Briefly, 10 μl of urine and 300 μl of picric acid solution (Wako, Osaka, Japan) were mixed at room temperature for 3 min, and absorbance of the solution was measured at 505 nm. Seventy-five μl of alkaline solution (Wako, Osaka, Japan) was added for 4 min, and absorbance of the solution was measured at 570 nm. The mean of the two values measured at 505 nm and 570 nm was used as the creatinine level.

RNA preparation

One day after collecting venous blood samples, we isolated total RNA from whole blood using TriZol-reagent (GIBCO-Invitrogen, Carlsbad, CA). We purified RNA samples using RNeasy mini kits (Qiagen, Valencia, CA) and assessed yield and quality using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only paired samples with RNA yield >5 μg and 28S/18S ribosomal RNA ratios >1 before and after occupational exposure to VOCs were used in the expression microarray analysis.

Expression microarray

Paired RNA samples taken from each subject before and after occupational exposure to VOCs were processed in a chip to evaluate changes by BTEX exposures. Fluorescence-labeled probes for oligo microarray analysis were prepared using Amino allyl MessageAmp™ aRNA kits (Ambion Inc., Texas). Labeled probes were hybridized to a Phalanx Biotech Human OneArray™ DNA microarray (Phalanx Biotech, www.Phalanxbiotech.com) at 42°C for 16 h. Slides were washed sequentially (once in 2x SSPE/0.1% SDS at 42°C for 10 min, once in 0.1x SSPE/0.1% SDS at 42°C for 10 min, and once in 0.1x SSPE at room temperature for 10 min) and spin dried. DNA chips were scanned using Axon 4000B (Axon Instruments, Union City, CA) and scanned images were analyzed with GenePix 3.0 software (Axon Instruments, Union City, CA) to obtain gene expression ratios. Log-transformed gene expression ratios were normalized by LOWESS regression[14]. Data from the Gene Ontology Consortium (GO;http:/www.geneontology.org/) were used as references to obtain information for biological processes of genes related to 32,050 probes (30,968 human genome/1,082 experimental controls) on the Phalanx Human OneArray™.

Statistical analysis

The levels of BTEX exposure biomarkers before occupational exposure to VOCs were compared with those after occupational exposure to VOCs using the paired *t*-test and Wilcoxon signed-rank test. Changes in oxidative stress biomarker levels related with changes in exposure biomarker levels were evaluated by linear regression analyses after adjusting for age, body mass index, and smoking status. In this analysis, changes in biomarker levels were expressed as ratios: biomarker level after occupational exposure to VOCs / biomarker level before occupational exposure to VOCs. The effects of BTEX exposures on gene expression were evaluated by comparing gene expression ratios with changes in BTEX exposure biomarker levels (expressed as ratio). Oxidative stress-associated gene expressions by BTEX exposures were evaluated using Spearman correlation analysis. SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

Results

VOC exposure level

We measured levels of benzene, toluene, ethylbenzene, and xylene obtained from passive sampler in order to determine common exposure to the volatile organic chemicals in workplace. The mean concentrations of toluene, ethylbenzene, and xylene were 2.16 ppm (range, 1.79–2.55), 6.05 ppm (range, 1.75–23.63) and 9.78 ppm (range, 3.87–31.40), but benzene was not detected (LOD=0.0073 ppm).

Change in levels of VOC exposure biomarkers before and after exposure to VOCs at the workplace

We measured urinary levels of MuA, HA, MaA, and MHA, and compared the levels of these biomarkers before and after a week-long exposure to VOCs at the workplace to evaluate individual exposure to BTEX (Table 1). In the Wilcoxon signed-rank test, significantly elevated urinary concentrations of HA, MaA, and MHA following occupational exposure to VOCs were found (In the Wilcoxon signed-rank test, HA, p=0.001; MaA, p=0.017; and MHA, p<0.001). All three components (*o*-MHA, *p*-MHA, and *m*-MHA) of total MHA showed consistent increase in their levels following occupational exposure to VOCs (In the Wilcoxon signed-rank test, *o*-MHA, p=0.010; *p*-MHA, p=0.035; and *m*-MHA, p<0.001). However, result in paired *t*-test for MaA showed inconsistence with those in Wilcoxon signed-rank test (In paired *t*-test, MaA,

Table 1. Comparison of exposure biomarker levels before and after occupational exposure to VOCs

	Exposure biomarker levels (Mean ± SE)			
Metabolite	Before exposure	After exposure	p-value[a]	p-value[b]
MuA	0.36 ± 0.03	0.38 ± 0.04	0.602	0.743
HA	131.30 ± 25.03	380.45 ± 87.02	0.001	0.008
MaA	20.73 ± 12.44	19.67 ± 4.14	0.017	0.932
MHA	41.15 ± 6.39	114.98 ± 22.07	<0.001	0.005
o-MHA	26.25 ± 2.93	67.66 ± 20.70	0.010	0.064
p-MHA	5.16 ± 1.24	16.83 ± 3.91	0.035	0.011
m-MHA	9.74 ± 3.51	30.48 ± 5.72	<0.001	0.003

The mg/g creatinine was used as concentration unit for MuA, HA, MaA, and MHA. [a]Wilcoxon signed-rank test and [b]paired *t*-test were used for comparison of levels.

Table 2. Changes in oxidative stress biomarker levels related with changes in exposure biomarker levels

	MDA		8-OHdG	
Exposure biomarker	β (SE)	p-value	β (SE)	p-value
HA	0.06 (0.03)	0.031	0.04 (0.05)	0.487
MHA	–0.02 (0.02)	0.451	–0.01 (0.04)	0.829

Ratio values for HA, MHA, MDA, and 8-OHdG were used in linear regression analysis. Regression coefficients and p-values were obtained by linear regression after controlling for age, body mass index, and smoking status.

p=0.932). MuA levels were similar in samples taken before and after occupational exposure to VOCs (In paired *t*-test, p=0.743; and in the Wilcoxon signed-rank test, p=0.602).

Change in levels of oxidative stress biomarkers by exposure to toluene or xylene

Because only HA and MHA showed significant difference between the levels before and after exposure to VOCs at the workplace in both Wilcoxon signed-rank test and paired *t*-test, we evaluated the relations between HA or MHA level and the oxidative stress biomarker levels to assess whether exposure to toluene or xylene induce oxidative stress response. When we conducted linear regression analysis to evaluate changes in oxidative stress biomarker levels related with changes in exposure biomarker levels, we found that HA ratios affected MDA ratios significantly after controlling for age, body mass index, and smoking (β=0.06, p=0.031) (Table 2).

Changes in gene expression by exposure to toluene

We evaluated changes in gene expression after occupational VOC exposure by hybridizing RNA from blood samples to a Human-32K chip. We obtained high-quality of RNA (ribosomal RNA ratio of 28S and 18S >1) from blood samples taken from 14 of the subjects before and after occupational exposure to VOCs, and we used each pair of RNA samples in a single array to evaluate the effects of VOC exposure on gene expression levels. Because only HA showed the relationship with oxidative stress biomarker, we evaluated the gene expressions by HA (Table 3). In the 14 expression microarray analyses, 23 gene (*SSU72, KIN, PAMCI, KIAA1713, UBE2L3, LOC440602, BRP44L, ENO3, CDNA FLJ42306 fis, THOP1, SKP1A, CDNA FLJ39461 fis, NBL1, CPSF1, MGC61571, KLF16, ZNF136, DAZ4, SEC24C, ZNF524, LOC389077, C19orf28*, and *LOC441444*) expressions were found to be associated with HA levels after adjusting for age, body mass index, and smoking when significance level was set to 0.001. In particular, *ENO3* and *CDNA FLJ39461 fis* expressions were found to be significantly associated with the change in MDA level (*ENO3*, ρ=0.55, p=0.041; and *CDNA FLJ39461 fis*, ρ=0.68, p=0.008) (Fig. 1).

Discussion

VOCs are airborne toxins related with various health problems such as headache, nausea, confusion, coma, reproductive depression, respiratory or cardiovascular diseases, and cancer[2–4, 15–17]. However, in spite of their considerable impact on human health, their mechanisms underlying changes in physiological conditions

Table 3. Changes in gene expressions related with changes in HA levels

Gene	Change in gene expression		GenBank_Acc	Biological process or molecular function
	β (SE)	p-value		
SSU72	0.01 (0.00)	<0.001	NM_014188.2	Oxidative stress, cognition, Rb inhibition
KIN	–0.01 (0.00)	<0.001	NM_012311.2	DNA binding, RNA splicing
PAMCI	0.14 (0.03)	<0.001	NM_005447.2	Signal transduction, protein targeting, cytoskeletal organization
KIAA1713	0.18 (0.03)	<0.001	XM_290811.3	Carcinogenesis
UBE2L3	0.01 (0.00)	<0.001	NM_198157.1	Ubiquitin-dependent protein catabolism
LOC440602	0.11 (0.02)	<0.001	XM_498751.1	-
BRP44L	–0.01 (0.00)	<0.001	NM_016098.1	Spinal cord injury and regeneration
ENO3	0.11 (0.01)	<0.001	AW293969	Glycolysis, tumor suppressor
CDNA FLJ42306 fis	–0.02 (0.00)	<0.001	AK124299	-
THOP1	0.03 (0.01)	<0.001	NM_003249.3	Proteolysis and peptidolysis, defence mechanism
SKP1A	–0.02 (0.00)	<0.001	NM_006930.2	Ubiquitin cycle, CYP inhibition, Parkinson's disease, tumor suppressor, development disorder
CDNA FLJ39461 fis	0.03 (0.00)	<0.001	AK096780	-
NBL1	0.03 (0.00)	<0.001	NM_005380.3	Negative regulation of cell cycle, neurogenesis, embryogenesis, homeostasis, carcinogenesis
CPSF1	0.02 (0.00)	<0.001	NM_013291.1	Visual, olfactory, central nervous system
MGC61571	–0.02 (0.00)	<0.001	NM_182523.1	-
KLF16	0.02 (0.00)	<0.001	NM_031918.1	Transcription regulation
ZNF136	0.02 (0.00)	<0.001	NM_003437.2	Negative regulation of transcription from Pol II
DAZ4	0.08 (0.02)	<0.001	NM_020364.2	Spermatogenesis
SEC24C	0.01 (0.00)	<0.001	NM_004922.2	Intracellular protein transport
ZNF524	0.01 (0.00)	<0.001	NM_153219.2	Regulation of transcription
LOC389077	0.02 (0.00)	<0.001	XM_374025.1	-
C19orf28	0.02 (0.00)	<0.001	AF218008	-
LOC441444	–0.01 (0.00)	<0.001	NM_004477.1	-

Ratio values for HA were used in linear regression analysis. Regression coefficients and p-values were obtained by linear regression after controlling for age, body mass index, and smoking status.

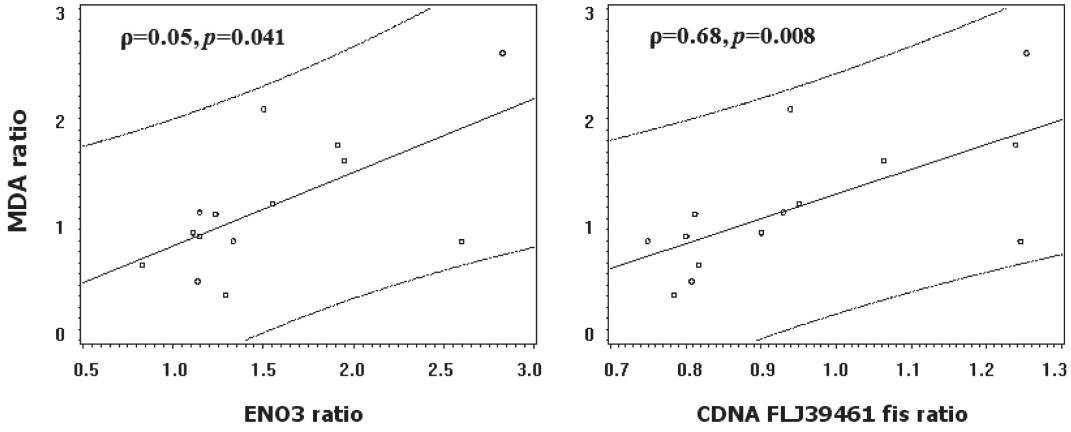

Fig. 1. Correlation between MDA and gene expression ratios.

are largely unknown. Therefore, we tried to evaluate the effect of exposure to VOCs on molecular and biological consequences in this study. We limited the present study to four VOC compounds—benzene, toluene, ethylbenzene, and xylene—to which workers are frequently exposed in the workplace[18–20]. Metabolites of benzene, toluene, ethylbenzene, and xylene are mainly excreted in the urine (as MuA, HA, MaA, and MHA) and level of the metabolites clearly reveal the level of their exposures, which allowed us to quantify their exposures[21].

To evaluate individual's exposures to benzene, tolu-

ene, ethylbenzene, and xylene, we measured urinary levels of MuA, HA, MaA, and MHA before and after occupational exposure to VOCs. In the comparison of the four exposure biomarker levels, the levels of HA and MHA were shown to be increased after occupational exposure to VOCs. However, we did not find significant and consistent difference for the exposure to benzene or ethylbenzene. We measured levels of benzene, toluene, ethylbenzene, and xylene obtained from passive sampler in order to determine common exposure to the volatile organic chemicals in workplace. The mean exposure to toluene, ethylbenzene, and xylene were 2.16 ppm, 6.05 ppm and 9.78 ppm, but benzene was not detected in the workplace. In general, the levels were lower than those of workplace exposure to VOCs in other studies[7, 18–20].

Oxidative stress has been considered as a disease-related change[12, 13]. For this reason, we evaluated the effect of exposure to toluene and xylene on level of the oxidative stress biomarkers. MDA and 8-OHdG used in our study are biomarkers indicating lipid peroxidation and oxidative DNA damage, respectively. In the present study, HA level was found to be significantly associated with MDA level, but not with 8-OHdG level, while MHA level was not found to be significantly associated with the oxidative stress biomarkers. Previous study suggested that oxidative DNA damage was shown in VOC concentration higher than the level of VOC causing lipid peroxidation[10]. No association of HA with 8-OHdG in our study may be due to toluene exposure under threshold for oxidative DNA damage. Another studies suggested that high level of toluene and xylene mixture exerts antagonistic effect on lipid peroxidation[22–24]. In the present study, we did not find any antagonistic effect of toluene and xylene on MDA level because our study subjects were exposed to relatively low levels of toluene and xylene even though they were occupationally exposed to VOCs.

Because only HA was found to be significantly associated with oxidative stress biomarker in our study, we evaluated the changes in gene expressions by change in HA levels and found significant changes of 23 gene expressions by HA. Although biological functions of the 23 genes were largely unknown, many of the 23 genes have been reported to affect carcinogenesis and nervous system such as cognitive function and Parkinson's disease, areas that are known to be affected by chronic VOC exposure[2, 3, 9, 11, 25].

Based on our results that exposure to toluene induces oxidative stress and gene expression changes, we inferred that some of the 23 gene expression changed by HA may be associated with the oxidative stress. In the analysis to see the oxidative stress-associated gene expression changes by HA, we found that expression of *ENO3* and *CDNA FLJ39461 fis* genes was associated with oxidative stress. Therefore, we can postulate that some of the genes regulated by toluene exposure are involved in the oxidative stress pathway. However, because we could not explain all of the gene expression changes by HA with the oxidative stress hypothesis, we need to explore other pathways to regulate genes when exposed to HA in further studies.

Smoking, one of the largest sources for personal and/or indoor toxic compounds, were controlled in the analysis, but other lifestyle factors reported to affect the exposed VOC levels[26–28] were not considered in this study. Since we used the "before" measurements as a reference point and the relevant lifestyle factors are behavior patterns that are unlikely to change in the course of one week, these lifestyle factors most likely did not affect our biomarker levels or gene expression. In fact, we compared cotinine levels before and after occupational exposure to VOCs and confirmed no difference of smoking behavior between them (data was not shown here). However, we cannot exclude the possibility that some of our results may be false positives or negatives because we used small number of samples and did not conduct dye swap in the chip analysis due to insufficient blood sample.

In conclusion, this study suggests that exposure to VOCs, specifically toluene, induces oxidative stress and various gene expression changes of which some may be associated with oxidative stress.

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