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Gene expression in benzene-exposed workers by microarray analysis of peripheral mononuclear blood cells: Induction and silencing of CYP4F3A and regulation of DNA-dependent protein kinase catalytic subunit in DNA double strand break repair

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ABSTRACT

Benzene causes hematotoxicity and leukemia in humans. To analyze benzene-caused aberrant gene expression, we examined differential gene expression by microarray analysis of peripheral mononuclear blood cells from seven workers diagnosed with benzene poisoning and seven matched controls. Twenty-two genes were found up-regulated and 18 down-regulated in benzene patients compared with controls. Here we report the characterization of two benzene-regulated genes. CYP4F3A, which encodes the leukotriene B_4 (LTB_4) ω-hydroxylase, is important for inactivation of LTB_4 in neutrophils. CYP4F3A mRNA was found elevated in all patients; moreover, CYP4F3A mRNA and protein were induced by benzene metabolite phenol in HL-60 and K562 cells as well as ex vivo in human peripheral neutrophils. Silencing of CYP4F3A in HL-60 cells by lentiviral delivery of CYP4F3A-specific siRNA reduced cell survival to 56%, 44%, 22%, 14%, and 3% at 3, 4, 5, 6, and 7 days, respectively; the results suggest that CYP4F3A is a critical positive regulator of HL-60 proliferation. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) regulates non-homologous end joining (NHEJ) in DNA double strand break (DSB) repair. DNA-PKcs mRNA was found consistently increased in the patients and DNA-PKcs mRNA and protein were induced by hydroquinone in HL-60 cells. In a DSB model, hydroquinone induced the formation of γ-H2AX foci, a marker of DSBs, in HL-60 cells. The findings indicate that hydroquinone induces DSBs and induction correlates with elevated levels of DNA-PKcs and NHEJ. Similar results were obtained in K562 cells treated with phenol. Since NHEJ is error-prone, induction of DNA-PKcs and NHEJ may contribute to mutagenesis and leukemia by benzene. To our knowledge, the study demonstrated for the first time that benzene and metabolites induce CYP4F3A and DNA-PKcs both in vivo and in vitro. Induction of the genes may play a role in the pathogenesis of benzene hematotoxicity and serve as biomarkers of benzene exposure.
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1. Introduction

Benzene is an established myelotoxicant and leukemogen in humans [1]. Chronic exposure to benzene leads to bone marrow toxicity that initially manifests as reduction of peripheral blood cell counts but may progress to pancytopenia, aplastic anemia, and myelogenous leukemia [2,3]. Occupational exposure to benzene occurs in industries, such as shoe making, automobile repair, the oil industry, shipping, and chemical manufacturing, whereas environmental sources of benzene include cigarette smoking, gasoline vapor, and automobile exhaust. The possibility that exposure to benzene at or below 1 ppm—the current occupational exposure standard in the United States—may still cause toxicity to hematopoietic cells is an ongoing concern [4].

The mechanism by which benzene induces hematotoxicity and leukemia remains elusive. Benzene is metabolized in the liver to form multiple metabolites that produce the biological effects [5]. CYP2E1 catalyzes the initial and several subsequent oxidation steps required for the formation of phenol, hydroquinone, catechol, muconic acid, and other metabolites. Mice with targeted knockout of *Cyp2e1* have lower levels of benzene metabolites than wild-type and are resistant to benzene hematotoxicity [6]. Other enzymes, such as NAD(P)H: quinone oxidoreductase, microsomal epoxide hydroxylase, glutathione-*S*-transferase, and myeloperoxidase, contribute to benzene metabolism in liver and bone marrow cells to

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modulate benzene myelotoxicity. Benzene metabolites may damage DNA by adduct formation and oxidative stress that involves redox cycling of quinone derivatives [7,8]. At the present, few molecular targets and signaling events that govern benzene hematotoxicity and leukemia have been identified.

The chronic and progressive nature of benzene myelotoxicity and leukemogenesis suggests genomic reprogramming resulting in aberrant gene expression in the mechanism of benzene toxicity. However, few studies have been undertaken to analyze gene expression during benzene myelotoxicity and tumorigenesis. To address this issue, we used cDNA microarray to analyze differential gene expression in peripheral mononuclear blood cells from seven workers diagnosed with benzene poisoning and their matched controls. A number of aberrant gene expressions were found in benzene patients: 22 genes were up-regulated and 18 down-regulated. In this report, we review the characterization of two benzene-regulated genes, CYP4F3A and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). To our knowledge, this study demonstrated for the first time that benzene induces CYP4F3A and DNA-PKcs both in vivo and in vitro via its metabolites phenol and hydroquinone. The findings suggest that induction of the genes plays a role in the pathogenesis of benzene hematotoxicity and serves as biomarkers of benzene exposure and toxicity.

2. Microarray of peripheral mononuclear blood cells from benzene-poisoning patients and matched controls

Seven female workers from a shoe manufacturing factory and a chemical production company in Henen province, China, were diagnosed with benzene poisoning [9]. Major manifestations include various degrees of blood cell count reduction. Diagnosis comprises benzene poisoning (1 case), moderate chronic benzene poisoning (2 cases), medium chronic benzene poisoning (2 cases), severe chronic benzene poisoning (1 case), and aplastic anemia (1 case); diagnosis criteria were described in document GBZ-2002, Department of Health, China at http://www.niohp.net.cn/Contents/Channel_100/2007/1105/12734/content_12734.htm. Concentrations of benzene at which the patients were exposed to at workplace were from 2.87 to 60.27 ppm at the time of diagnosis. Seven local workers who had no history of occupational benzene exposure were chosen as controls and were matched with the patients on age, gender (all female), geographical location, years of working experience, educational level, and history of smoking and alcoholic drinking. Patients and control subjects had no history of exposure to other hematotoxic chemicals or radiation and were not exposed to radiation or chemotherapy within 15 days before the study. Procedures involving human subjects were approved by the institutional review board of Wuhan University Medical Center.

Mononuclear white blood cells were prepared from 10 ml of fresh blood samples. Total RNA was extracted and used for cDNA microarray using a CSC-GE-80 human microarray chip (Shenzhen Chipscreen Biosciences Ltd., Shenzhen, Guandong, China). Each chip contained 384 genes as positive and negative controls to assess interference form nonspecific hybridization, as internal standards to measure repeatability of the results, and as external standards to quantify dose–response relationship and sensitivity of hybridization. A Cy3/Cy5 ratio of larger than 2 or less than 0.5 was considered different between patient and control. The results reveal altered expression of multiple genes, among which 22 genes were up-regulated (Table 1) and 18 down-regulated (Table 2) in all seven patients in comparison with the matched controls. The genes are involved in a spectrum of cellular functions including apoptosis, DNA repair, immune function, and drug metabolism [9–14].

Table 1
Elevated expression of 22 genes in peripheral mononuclear blood cells of benzene patients compared with matched controls.

Gene and function	Gene tag	Fold change (mean)
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cycloxygenase)	PTGS2	17.29
Brain-specific angiogenesis inhibitor 3	BAI3	25.64
Grancalcin	GCL	96.76
Cytochrome P450, subfamily IV polypeptide 3 (leukotriene B4 omega hydroxylase)	CYP4F3	19.02
Leptin receptor overlapping transcript-like 1	MY047	12.36
Human T-cell receptor active alpha-chain mRNA from JM cell line	TRA@	5.94
TRAF and TNF receptor-associated protein	AD022	6.91
Protein kinase C, eta	PRKCH	4.07
RAS guanyl releasing protein 1 (calcium and DAG-regulated)	RASGRP1	4.23
Formyl peptide receptor 1	FPR1	5.27
Transforming growth factor, beta receptor III (beta blycan, 300 kDa)	TGFBR3	3.61
GRO1 oncogene (melanoma growth stimulating activity, alpha)	GRO1	10.34
Sel-1 (support of lin-12, C. elegans)-like	SEL1L	2.99
Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	CSF2RB	4.85
Interferon induced transmembrane protein 1 (9–27)	IFITM1	3.44
Signal transducer and activator of transcription 4	STAT4	3.36
Interferon induced transmembrane protein (1-8D)	IFITM2	4.51
Actin binding LIM protein 1	ABLIM	3.57
Amino acid transporter 2	KIAA1382	3.91
Spectrin, beta, non-erythrocytic 1	SPTBN1	2.72
Hemoglobin, beta	HBB	95.48
Protein kinase, DNA-activated, catalytic polypeptide	PRKDC	2.27

Table 2
Reduced expression of 18 genes in peripheral mononuclear blood cells of benzene patients compared with matched controls.

Gene and function	Gene tag	Fold change (mean)
S100 calcium-binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))	S100A10	−4.10
Integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	ITGB2	−3.42
Transketolase (Wernicke–Korsakoff syndrome)	TKT	−3.87
Vesicle-associated membrane protein 8 (endobrevin)	VAMP8	−5.10
FBJ murine osteosarcoma viral oncogene homolog B	FOSB	−5.44
N-acyl sphingosine amidohydrolase (acid ceramidase)-like	ASAHL	−6.62
CDC37 (cell division cycle 37, S. cerevisiae, homolog)	CDC37	−4.28
Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	SLC25A6	−3.60
Ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky–Bielschowsky disease)	CLN2	−3.07
Actin, alpha 2, smooth muscle, aorta	ACTA2	−3.78
Cystatin C (amyloid angiopathy and cerebral hemorrhage)	CST3	−8.60
Major histocompatibility complex, class II, DM beta	HLA-DMB	−4.83
Aldehyde dehydrogenase 2 family (mitochondrial)	ALDH2	−10.88
Lectin, galactoside-binding, soluble, 2 (galactin 2)	LGALS2	−5.69
Lectin, galactoside-binding, soluble, 1 (galectin 1)	LGALS1	−4.59
Ras homolog gene family, member B	ARHB	−4.03
Kruppel-like factor 4 (gut)	KLF4	−16.46
Activating transcription factor 3	ATF3	−9.60

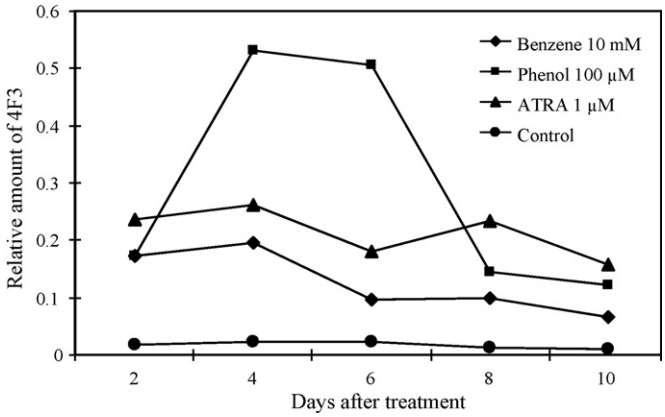

Fig. 1. Induction of CYP4F3 protein. HL-60 cells were treated as indicated for 2, 4, 6, 8, and 10 days. CYP4F3 protein was detected and quantified using immunofluorescent flow cytometry with antibodies against CYP4F3. ATRA at 1 μM was used as a positive control.

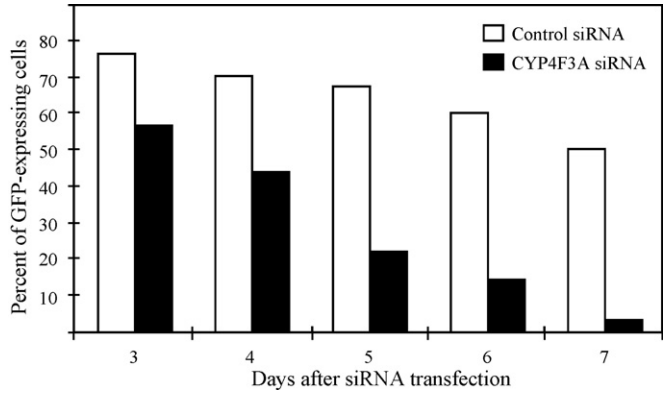

Fig. 2. Expression of CYP4F3A siRNA in HL-60 cells. CYP4F3A siRNA or scrambled siRNA was expressed in HL-60 cells using lentiviral vectors. The cells were cultured for 2, 4, 6, 8, and 10 days. Expression of GFP was used as a marker of siRNA expression. Cells expressing GFP were quantified using fluorescent flow cytometry.

3. Induction and silencing of CYP4F3A

CYP4F3A mRNA was consistently elevated in all seven patients in comparison with matched controls (Table 1) [9]. Induction was also confirmed by real-time PCR. CYP4F3A is known to preferentially express in mononuclear white blood cells and encode leukotriene B_4 (LTB_4) ω-hydroxylase that converts LTB_4 to less active metabolites. LTB_4 is a potent endogenous chemotactic and chemokinetic signaling molecule. Induction of CYP4F3A enhances the inactivation of LTB_4 and reduces the chemotaxis of leukocytes, thereby altering leukocyte functions and dynamics to contribute to the pathogenesis of myelotoxicity in benzene poisoning.

A number of in vitro and ex vivo experiments were performed to analyze the mechanism of CYP4F3A induction. The findings revealed that benzene metabolite phenol effectively induced CYP4F3A mRNA and protein expression in human promyelocytic leukemic HL-60 cells in culture. Fig. 1 shows that the basal expression of CYP4F3 protein in untreated cells was very low throughout the 10-day period. Phenol at 100 μM significantly induced the protein on day 2 after treatment; induction was the highest between days 4 and 6; and induction decreased on days 8 and 10. All-trans retinoic acid (ATRA), a known inducer of CYP4F3A, at 1 μM induced the CYP4F3 protein, but induction was lower than that by phenol on days 4 and 6. Benzene at 10 mM only moderately induced CYP4F3 on day 2 and induction gradually decreased afterwards. Hydroquinone, another major metabolite of benzene, induces extensive apoptosis in the cells [9]. Phenol also induced CYP4F3A in differentiated HL-60 and K562 cells indicating that it is capable of inducing the gene independently of differentiation states of the cells [9]. Finally, human neutrophils were prepared from fresh peripheral blood and were treated with phenol for induction. These ex vivo experiment provided direct evidence demonstrating that phenol induces CYP4F3 in human neutrophils and induction does not require cell differentiation [9].

We examined the function of CYP4F3A by knocking down the gene in HL-60 cells. Human CYP4F3A-specific small interfering RNA (siRNA) was constructed and delivered to HL-60 cells using a lentiviral vector. Scrambled siRNA was used as a negative control. Expression of siRNA was monitored by detection of GFP fluorescence. We found that silencing of CYP4F3A reduced the GFP fluorescent cells to about 56%, 44%, 22%, 14%, and 3% at 3, 4, 5, 6, and 7 days after siRNA transfection, whereas expression of scrambled siRNA only had modest effect on the percentage of cells expressing the GFP protein (Fig. 2). The results indicate that expression of siRNA of CYP4F3A markedly reduced the survival of HL-60 cells. Expression of CYP4F3A siRNA also effectively inhibited HL-60 cell proliferation (data not shown). The findings reveal for the first time that CYP4F3A functions as a positive regulator critical for HL-60 proliferation and survival.

4. Induction of DNA-PKcs, DNA double strand break, and DNA repair

DNA-PKcs encodes the DNA-dependent protein kinase catalytic subunit. DNA-PKcs mRNA was found consistently elevated in benzene-poisoning patients by microarray (Table 1) and real time-PCR [14]. DNA-PK consists of three subunits, the catalytic subunit and the regulatory subunits Ku70 and Ku80 [15]. Ku70 and Ku80 are activated by DNA double strand break (DSB), bind to the break ends, and recruit DNA-PKcs to the break. Localized DNA-PK at the break sites is critical for double strand break repair via a mechanism called non-homologous end joining (NHEJ). Consistent with this notion, cells defective in DNA-PKcs or the Ku proteins through mutation or targeted gene knockout are highly sensitive to ionizing radiation that causes DSB. On the other hand, NHEJ is error-prone; induction of DNA-PK may enhance NHEJ and consequently increases mutations in chromosomal DNA, thereby contributing to genomic instability. Therefore, we further characterized induction of DNA-PKcs in benzene-induced DNA damage.

Hydroquinone is known to be mutagenic and apoptotic in hematopoitic and peripheral blood cells [9]. Hydroquinone may undergo redox cycling resulting in the production of reactive oxygen species (ROS), semiquinone radicals, and other radicals, which can damage DNA and produce DSB [16]. Treatment of HL-60 cells with hydroquinone induced formation of γ-H2AX foci, a marker of DNA double strand break. Induction is both concentration and time-dependent (Fig. 3 and data not shown). The results support the notion that benzene induces DSB via its metabolite hydroquinone and induction of DSB contributes to the apoptotic effect of hydroquinone. We then tested if hydroquinone induces DNA-PKcs in HL-60 cells. The findings revealed that hydroquinone effectively induces DNA-PKcs mRNA and protein expression in the cells and induction correlates with DSB formation (Fig. 4 and data not shown). In separate experiments, phenol was also found to induce DSB and DNA-PKcs expression in K562 cells (data not shown). These in vitro induction studies are in full agreement with the in vivo results in humans. These findings provide mechanistic insights into benzene-induced DNA double strand break and repair, which are important in benzene-induced hematotoxicity and leukemogenesis.

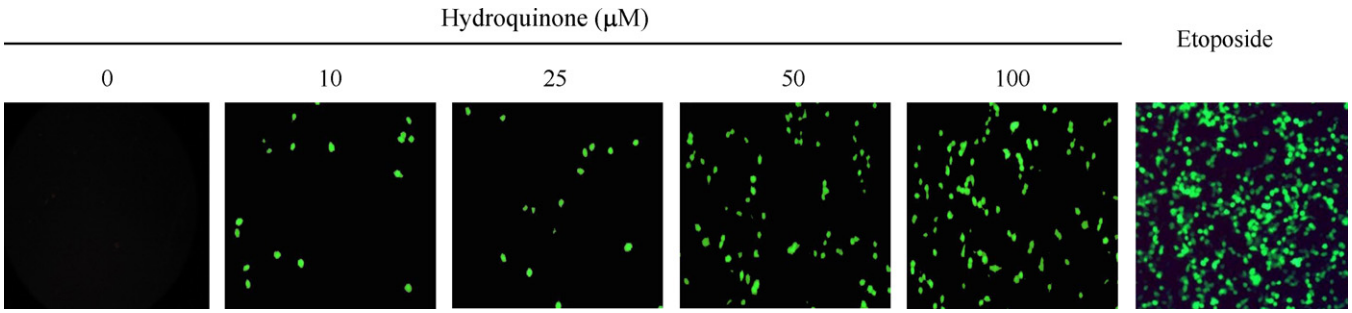

Fig. 3. DNA double strand break by hydroquinone. HL-60 cells were treated with hydroquinone for 24 h at 0, 10, 20, 25, 50, and 100 μM, respectively. Etoposide is used as a positive control of DNA double strand break. Cells were stained with antibodies against phosphorylated γ-H2AX foci and micrographs were taken under a fluorescence microscope with a FITC filter.

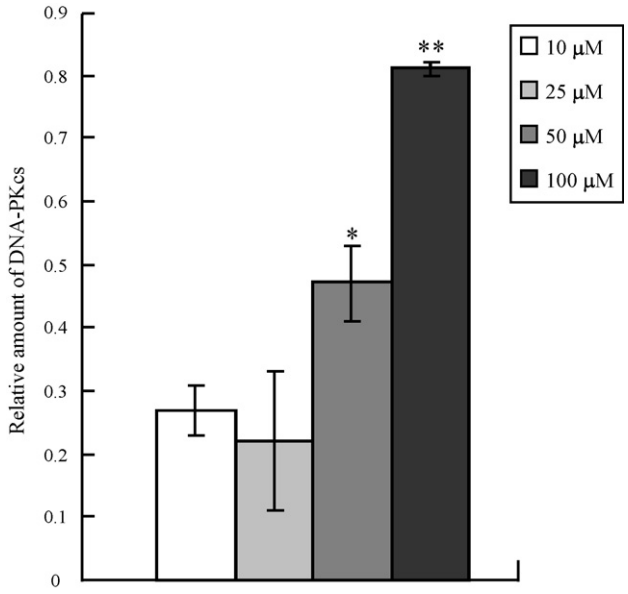

Fig. 4. Induction of DNA-PKcs mRNA. HL-60 cells were treated with hydroquinone for 24 h as indicated. Total RNA was prepared and DNA-PKcs mRNA induction was determined by real-time PCR. Actin mRNA was measured for correction of loading variations. Results represent means and standard deviations from three samples. $^{*}p < 0.05$; $^{**}p < 0.01$.

5. Conclusion

Given the high specificity and chronic and progressive nature of damage to bone marrow cells by benzene, we hypothesized that exposure to benzene elicits large-scale aberrant gene expression in hematopoitic cells that contributes to the development of hematotoxicity and leukemia. Microarray analysis of peripheral mononuclear blood cells from benzene-poisoning patients and their matched controls demonstrated that, indeed, benzene induces differential gene expression that involves a number of cellular functions and pathways, including tumorigenesis, apoptosis, immune function, DNA damage and repair, and metabolism of endogenous and xeno chemicals (Tables 1 and 2). To elucidate the mechanism by which benzene alters gene expression and to identify the function of the genes in relation to benzene toxicity, a number of in vitro and ex vivo model systems were established to replicate induction and function of CYP4F3A and DNA-PKcs.

Our studies show that benzene metabolite phenol induces CYP4F3A in HL-60 and K562 cells as well as ex vivo in human peripheral neutrophils. Furthermore, silencing of CYP4F3A in HL-60 cells inhibits cell proliferation and promotes cell death. Hydroquinone and phenol induce DNA-PKcs in HL-60 cells, and induction correlates with increased DNA double strand break and repair. Together with the results obtained from human subjects, our findings reveal novel mechanistic aspects of benzene hematotoxicity and tumorigenesis. In light of these new findings, we propose that induction of CYP4F3A increases inactivation of LTB_4 and thereby, reduces chemotaxis and function of white blood cells; at the same time, induction of the gene promotes proliferation of the cells; both changes contribute to benzene toxicity in blood cells. On the other hand, hydroquinone and phenol induce DNA double strand break and other lesions via ROS and semiquinone radicals, induce and activate DNA-PK, and increase the error-prone NHEJ, leading to increased genomic instability, cell death, and tumorigenesis. Characterization of other aberrant gene expression in benzene toxicity is currently underway.

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Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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