



Expression of multidrug resistance 1 and multidrug resistance-related protein 1 in C57BL/6 mice treated with benzene

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ABSTRACT. ATP-binding cassette super family (ABC) proteins are considered key to oncology and pharmacology studies. We examined the effect of benzene on ABC pump protein levels in C57BL/6 mouse bone marrow mononuclear cells. After a 2-week gavage (200 mg/kg, 5 days per week), the number of peripheral leukocytes, lymphocytes and basophils dropped significantly; there was also a significant decrease in MDR1 and MRP1 gene expression. A significant reduction in expression of P-gp was found; however, there was no significant decrease in the expression of MRP1 and NF- κ B p65. We conclude that regulation of membrane efflux transport protein could be a factor in benzene hematotoxicity.

Key words: Benzene; Bone marrow mononuclear cells; MDR1; P-glycoprotein

INTRODUCTION

Benzene is a well-known environmental pollutant found in automobile exhaust, crude oil, gasoline, cigarette smoke, and chemical production (Chiu et al., 2011; Lovreglio et al., 2011; Morrens et al., 2012). Exposure to benzene results in an increased risk of hematotoxicity, including aplastic anemia, myelodysplastic syndrome, acute myelogenous leukemia, and lymphoma (Liu et al., 2011; Jiao et al., 2012; Schnatter et al., 2012; Vlaanderen et al., 2012). Benzene, once absorbed, is metabolized into a variety of intermediate compounds, including catechol, hydroquinone, p-benzoquinone and muconaldehyde, which are reported to be the most potent metabolites in producing toxic effects in hematopoietic cells and to persist in the bone marrow for a long period even termination of benzene exposure (Kerzic et al., 2010; Linhart et al., 2011; Zolghadr et al., 2012).

There is some, albeit limited, evidence that expression of genes encoding ATP-binding cassette (ABC) super family proteins, including multidrug resistance protein 1 (ABCB1B)/ATP-binding cassette, sub-family B (MDR/TAP), member 1B and multidrug-associated protein 1 (ABCC1)/ATP-binding cassette, sub-family C (CFTR/MRP), member 1, may be associated with protecting hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs) from xenobiotics. Lentivirus-mediated MDR1 overexpression in human CD34⁺ cells may increase the radiation tolerance of the hematopoietic system (Maier et al., 2008). Transduction of the MDR1 gene into hematopoietic stem cells suggests a bone marrow-protective effect with no serious side-effects observed (Takahashi et al., 2007). The high expression of ABCC1 in hematopoietic cells also reduces antineoplastic drugs-induced leukopenia and mortality (Omori et al., 1999; D'Hondt et al., 2001).

In our previous study, subjects exposed to benzene with *ABCB1* 3435 mutation genotype (T/T) had the significantly lower white blood cells (WBC) than those carrying wild type (C/C) and heterozygous (C/T) (Huang et al., 2011a,b). As a result of this observation, a greater emphasis has been put on the ABC super family proteins with respect to the hematotoxicity of benzene. However, despite the observed polymorphism in *ABCB1* in peripheral blood cell counts of benzene-exposed workers, relatively little is yet known about the influence of benzene on the ABC transport proteins of bone marrow hematopoietic cells. In order to address this, we purified bone marrow mononuclear cells (BMNCs) and investigated the expression level of *Abcb1b* and *Abcc1* in BMNCs in benzene-treated mice. Besides the mRNA and protein expression, the mouse peripheral blood cell counts, and NF- κ B p65 level were also analyzed. Our results demonstrate that the down-regulation of the ABCB1B (P-gp) protein may be involved in benzene toxicity.

MATERIAL AND METHODS

Animals

Benzene was purchased from Sinopharm Chemical Reagent and was >99.9% pure. Male C57BL/6 mice (7-8 weeks old, weighing 22-26 g), obtained from Shanghai Institutes for Biological Sciences Animal Center, CAS, were housed in stainless steel wire cages, and maintained at 19°-22°C with a 12:12-h light-dark cycle. They were first accustomed to the cages for 1 week prior to treatment. This study was carried out in strict accordance with the recommen-

dations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Jinshan Hospital, Fudan University.

Benzene exposure

Benzene was prepared in corn oil. Mice received this by oral gavage, at a dose of 200 mg/kg body weight, once daily, 5 days per week, for 2 weeks. This protocol was shown to cause hypoplasia of mouse bone marrow in our pre-experiment. Control mice were gavaged with just corn oil during the same period. The mice were sacrificed 4 h after the last gavage. Eight mice per group were used for each experiment.

Peripheral blood cell counts

After anesthesia, mice were opened and 0.5 mL blood was withdrawn from the heart with a 22-gauge needle. The blood was immediately placed into EDTA-K₂-coated tubes and thoroughly mixed. Peripheral blood counts were analyzed by XE-2100 analyzer (Sysmex, Japan), including counts of total WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, erythrocytes, platelets, and the total hemoglobin, the hematocrit, the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), and the mean corpuscular hemoglobin concentration (MCHC).

BMNCs

Immediately after sacrifice, the femurs were surgically removed. Total bone marrow cells were obtained by flushing the cavity of femurs with phosphate-buffered saline (PBS). BMNCs were isolated using Percoll (Sinopharm Chemical Reagent). Briefly, 60% Percoll was added to the centrifuge tube and bone marrow liquids were carefully loaded onto the surface. Then, the tube was centrifuged at 2000 rpm for 20 min at room temperature. After discarding the upper layer, the opaque interface containing the BMNC layer was transferred to a 10-mL tube and washed twice with PBS. After the final wash, the BMNC extracts were stored at -20°C until further study.

RT-PCR

Total RNA was extracted from the harvested BMNCs using TRIzol reagent (Invitrogen) according to the manufacturer protocol. Total RNA was reverse transcribed using a Reverse Transcription System (Promega) according to the manufacturer protocol. Gene expression of *Abcb1b* and *Abcc1* was evaluated by real-time PCR. The *Gapdh* gene was used as an endogenous reference for normalizing target gene mRNA. All primers were designed using PRIMER 5 (<http://www.uea.ac.uk/~e130/Primer5.htm>) and BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primer sequences are shown in Table 1. Amplification was detected by SYBR Green I on an ABI 7500 (Applied Biosystems). cDNA of each sample was diluted 1:30 in RNase-free water. Thermo cycling was carried out in a final volume of 10 µL containing 5 µL SYBR Green mix, 1 µL of each forward and reverse primer and 3 µL cDNA. The thermal cy-

clinging conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 s, 60°C for 1 min. Two replicates were performed for each sample. Normalized Δ CT values were obtained by subtracting the *Gapdh* CT from the CT of the gene of interest.

Table 1. Sequence of primers used for real-time PCR.

Target gene	GenBank No.	Product length (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
MDR1	NM_011075.2	157	CCCATCATTGCAATAGCAGG	GTTCAAACTTCTGCTCCTGA
MRP1	NM_008576.3	256	GCAGAACTTTCAGCCAGTGAACAA	ACAATACCAGCCGGGTCAAGAG
GAPDH	NM_008084.2	439	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC

Western blotting

All protein samples were separated by 8-15% SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h. The blots were stained overnight at 4°C with primary antibodies [including ABCB1B (1:500, ab3366; Abcam), ABCC1 (1:200, sc-13960; Santa Cruz Biotechnology, Inc.), NF- κ B p65 (1:1000; Santa Cruz Biotechnology, Inc.)], and then treated with HRP-conjugated goat antimouse IgG (1:5000) or HRP-conjugated antirabbit goat IgG (1:5000) for 1 h at room temperature. The signal was detected with an ECL Western blot detection kit (ZhongShan Co., Beijing, China). After normalization by the corresponding GAPDH expression, protein expression level was determined by densitometry scans and measured with the Quantity One 1-D Analysis Software (Bio-Rad, USA).

Statistical analysis

Data are reported as means \pm SEM and were analyzed by the 2-tailed independent sample Student *t*-test using SPSS 16.0 (IBM, USA). A difference in mean values with a value of $P < 0.05$ was considered to be significant.

RESULTS

Blood parameters

In C57BL/6 mice, 2-week benzene treatments resulted in considerable decreases in the number of peripheral leukocytes ($P < 0.01$), lymphocytes ($P < 0.01$) and basophils ($P < 0.05$). The marked decrease in the number of lymphocytes was attributed to the significant decrease in the number of leukocytes. The decrease in the number of lymphocytes was 11.58% relative to the control, whereas that of neutrophils was 27.27% relative to the control (Table 2). In contrast, no significant decrease was observed in the erythrocytes, hemoglobin, MCV, MCH, MCHC, or platelets in the mice.

MDR1 and MRP1 mRNA levels

Figure 1 shows the effects of benzene on *Abcb1b* and *Abcc1* mRNA levels, as determined by RT-qPCR in C57BL/6 mice BMNCs. After 2 weeks of benzene exposure the

significant decreases of *Abcb1b* and *Abcc1* gene expression ($P < 0.01$, $P < 0.05$, respectively) were demonstrated.

Table 2. Peripheral blood cell counts of C57BL/6 mice.

	Controls (N = 8)	Benzene-treated mice (N = 8)
Leukocyte	7.28 (1.07)	0.70 (0.17)**
Neutrophil	0.11 (0.05)	0.03 (0.01)
Lymphocyte	4.75 (1.10)	0.55 (0.12)**
Monocyte	0.19 (0.10)	0.01 (0.01)
Eosinophil	0.00 (0.00)	0.00 (0.00)
Basophil	0.60 (0.15)	0.11 (0.04)*
Erythrocyte	8.20 (0.45)	7.54 (0.30)
Hemoglobin	122.86 (6.71)	112.13 (3.25)
Hematocrit	0.44 (0.02)	0.41 (0.03)
MCV	54.30 (0.92)	53.59 (1.28)
MCH	14.94 (0.24)	14.91 (0.50)
MCHC	275.71 (4.41)	279.75 (8.63)
Platelet	1006.43 (66.44)	967.38 (71.11)

Values are reported as mean (SE). MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration. * $P < 0.05$. ** $P < 0.01$.

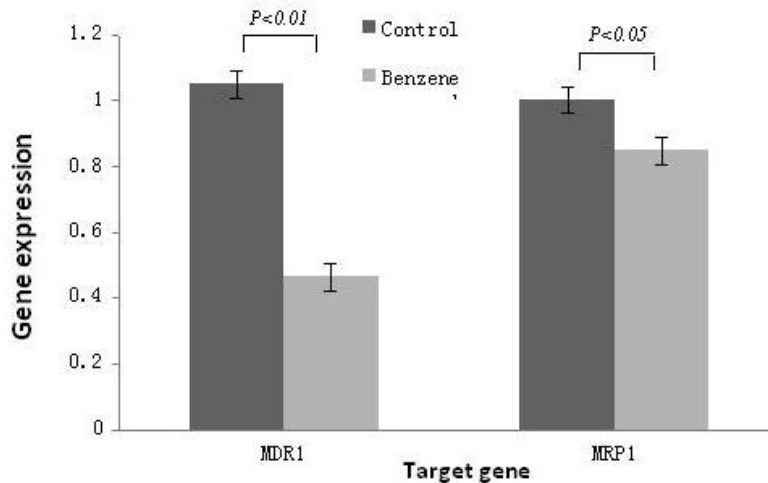


Figure 1. MDR1 and MRP1 gene expression in bone marrow mononuclear cells after benzene treatment. Expression was assessed by real-time RT-PCR. Bars represent means \pm SE of normalized Δ CT values by subtracting the GAPDH CT from the gene of interest CT. MDR1 gene expression in benzene-treated groups was 0.46 ± 0.06 , compared to 1.05 ± 0.03 in controls ($P < 0.01$). MRP1 gene expression in benzene-treated groups was 0.85 ± 0.04 , compared to 1.00 ± 0.05 in controls ($P < 0.05$) by the two-tailed independent sample Student *t*-test.

ABCB1B, ABCC1 and NF- κ B p65 expression

Our results showed that 2-week benzene gavage caused a significant reduction in the expression of ABCB1B when compared to the group treated only with corn oil. In contrast, no significant decrease was observed in expression of ABCC1 or NF- κ B p65, as confirmed by Figures 2 and 3.

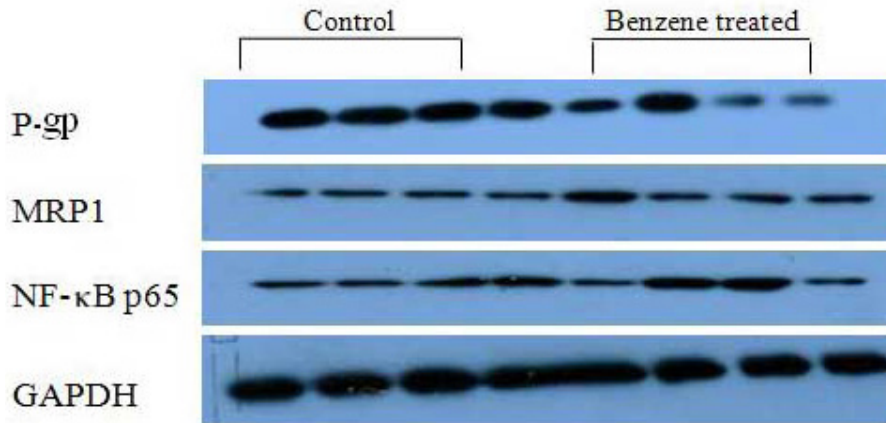


Figure 2. Effects of benzene treatments on the expression of P-gp, MRP1 and NF- κ B p65. C57BL/6 mice (N = 8) were gavaged once daily with corn oil only (control) or 200 mg/kg benzene for 2 weeks. P-gp, MRP1 and NF- κ B p65 levels of mice bone marrow mononuclear cells were measured with Western blotting. GAPDH was used as control.

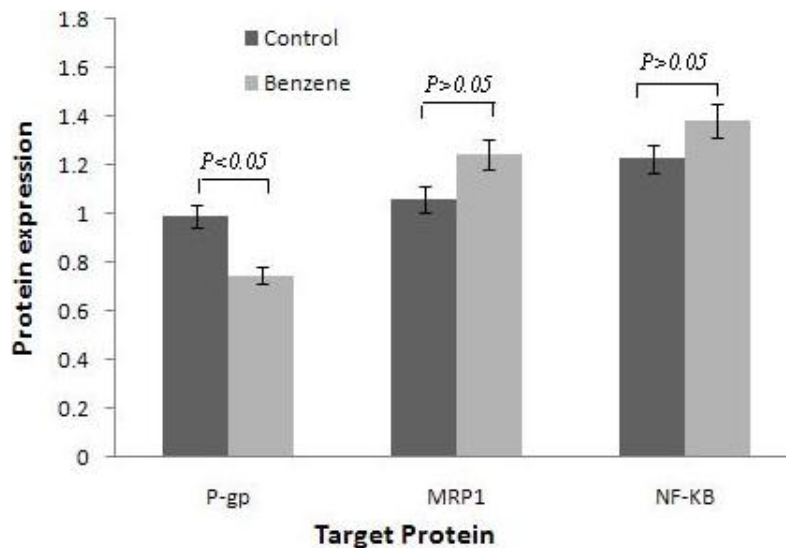


Figure 3. Bars represent means \pm SE. P-gp protein expression in benzene-treated groups was 0.74 ± 0.06 , compared to 0.99 ± 0.01 in controls ($P < 0.05$). There was no difference in MRP1 protein expression, NF- κ B p65 protein expression between controls and benzene-treated groups ($P > 0.05$) by the two-tailed independent sample Student *t*-test.

DISCUSSION

Proteins ABCB1B and ABCC1 were first identified three decades ago as drug export pumping intracellular xenobiotics out of cells, the function and expression of which were induced after clinical drug application over time, leading to multidrug resistance. They have now been described in many normal tissues such as the lung, gut, liver, kidney, testis, placenta,

hematopoietic cells, and blood-cerebrospinal fluid barrier (Huls et al., 2009; Gazzin et al., 2011). Despite their similarities in efflux pump profiles, ABCB1B and ABCC1 differ markedly in their substrate selectivity and tissue disposition. ABCB1B predominantly transports neutral or mildly cationic molecules, and is present in many hematopoietic cell types, including CD34⁺ stem cells (Licht et al., 1994). Conversely, ABCC1 is capable of transporting many lipophilic anions, including conjugates of glutathione, glucuronic acid, and sulfate, and has been shown to be present in human bone marrow cells (Legrand et al., 1996).

In this research, we have demonstrated that a 2-week benzene gavage (200 mg/kg, once daily, 5 days per week, for 2 weeks) results in considerable decreases in the number of peripheral blood cell counts, including leukocytes and some leukocyte phenotypes, such as lymphocytes and basophils (Table 2). Furthermore, this protocol exhibits an inhibitory effect on *Abcb1b* mRNA expression and ABCB1B protein expression in mononuclear cells purified from C57BL/6 mice bone marrow, along with the hematotoxicity of benzene. Conversely, the expression of protein ABCC1 does not change significantly although its mRNA expression exhibits apparent down-regulation (Figures 1 and 2). The major tissue affected by benzene toxicity is bone marrow, in which the BMNC highly enriched with HPCs and HSCs are targets of benzene intermediate compounds (Irons and Stillman, 1996). These results are in accordance with previous studies conducted in benzene-exposed workers, whose decreased WBC counts correlated with *ABCB1* C3435T (Huang et al., 2011a,b). Presumably, *ABCB1* inhibition can thus be considered to deteriorate hematotoxic reactions caused by the increased accumulation of activated benzene intermediate compounds in the bone marrow. Except for other well-known mechanisms, such as lipid peroxidation, apoptosis, DNA damage, and stem cell microenvironment disturbance, the downregulation of membrane efflux transport protein may be involved in benzene hematotoxicity. Benzene intermediate compounds are likely to be the substrates of ABCB1B but not ABCC1.

Previous studies have indicated that numerous signals have been identified to be involved in the activation of *Abcb1b* expression. The transcription factor NF- κ B has been shown to activate *Abcb1b* independently. A binding site (CAAT) of NF- κ B has been verified in the *Abcb1b* promoter and has been demonstrated to participate in the transcriptional activation of *Abcb1b* (Ogretmen and Safa, 1999; Wang et al., 2007). In the current study, we also investigated the level of NF- κ B p65. The 2-week benzene gavage (200 mg/kg, once daily, 5 days per week, for 2 weeks) does not give rise to an obvious change of NF- κ B p65 expression with the reduction in blood cell counts and ABCB1B inhibition. One possible explanation is that the BMNC are not actually 100% hematopoietic cells, in which some impurities could disturb the NF- κ B p65 expression (Pösel et al., 2012). Another potential explanation is that the phosphorylation of the p65 subunit of NF- κ B might genuinely be required for its transcriptional activation (Nazar et al., 2012). NF- κ B p65 cannot be the active ingredient itself.

From another point of view, other signal pathways may play an important role in the regulation of *Abcb1b* gene expression. Increased transcription activity of c-Jun by the JNK activation contributes to the down-regulation of *Abcb1b* and suppression of ABCB1B expression (Takano et al., 2010). Wild-type p53 is widely known to suppress the expression of *Abcb1b* through interactions with basal transcription factors, such as TATA-binding protein (Zastawny et al., 1993). Celecoxib inhibits *Abcb1b* expression through COX-2-dependent mechanism, thus it is suggested that COX-2 take part in the up-regulation of *Abcb1b* expression (Roy et al., 2010).

The present study has provided some valuable clues for revealing some aspects of benzene toxicity in relation to drug export pumping proteins in BMNCs. Further experiments are still required to determine the nature of ATP-binding cassette super family proteins in purified hematopoietic progenitor/stem cells. Applying *Abcb1b*-retroviral transduction techniques to upregulate ABCB1B in bone marrow stem cells and observing the effect on the protection to benzene toxicity could be valuable.

Conflicts of interest

The authors declare no conflict of interest.

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