Effect of styrene oxide and diethyl maleate on expression of cytochrome P450 family 1 and glutathione store in mouse liver

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Abstract

Purpose: To determine the effect of the glutathione (GSH) suppressors styrene oxide (SO) and diethyl maleate (DEM) on the hepatic expression of cytochrome P450 family 1 (Cyp1) isoforms that are related to carcinogenesis including Cyp1a1, Cyp1a2, and Cyp1b1.

Methods: Seven-week-old ICR mice were intraperitoneally injected with SO (150 and 300 mg/kg/day), DEM (175 and 350 mg/kg/day), or N-acetylcysteine (NAC; 300 and 600 mg/kg/day) for 7, 14, or 28 days. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, hepatic Cyp1 expression, total glutathione, reduced glutathione (GSH), and oxidized glutathione (GSSG) were determined.

Results: ALT and AST levels were markedly increased by SO and DEM while GSH/GSSG ratio was decreased by SO in all treatments (p < 0.05), while high dose (350 mg/kg/day) DEM significantly suppressed GSH/GSSG ratio at 28 days (p < 0.05). The expressions of Cyp1a1, Cyp1a2, and Cyp1b1 were induced by SO and DEM, corresponding with induction of ethoxy/methoxy-resorufin O-dealkylase activities.

Conclusion: The Cyp1 family metabolizes procarcinogens to carcinogenic DNA adducts; exposure to the industrial solvents, SO and DEM, raises the risk of carcinogenesis via GSH depletion coupled with Cyp1 induction.

Keywords: GSH, Carcinogenesis, Diethyl maleate, N-acetylcysteine, Cytochrome P450 family 1, Hepatotoxicity

INTRODUCTION

A wide array of xenobiotics can cause cellular damage and affect cellular protective systems depending on their concentrations and the period of exposure. Glutathione (GSH) is a stably controlled antioxidant that is present both intracellulary and extracellulary [1]. Some chemicals such as diethyl maleate (DEM), carbon tetrachloride, acetaminophen, and chloroform are metabolized to mercapturic acid derivatives, which are then detoxified by GSH
and glutathione-S-transferase leading to GSH depletion in tissue stores [2].

Extensive research has shown a relationship between imbalance in GSH stores and cancer or disease. Chloroform-induced hepatotoxicity progresses from GSH depletion to GSH deficiency, which subsequently results in cell lysis [3]. Moreover, GSH depletion has been shown to potentiate the toxicity of the anti-cancer drugs cisplatin and melphalan, and to affect dopamine toxicity in the brain [4]. Therefore, GSH depletion can cause damage to multiple cell types and organs.

Cytochrome P450 (CYP) is a super family of hepatic enzymes responsible for the metabolism of drugs and other xenobiotics into water-soluble compounds for excretion. Therefore, the study of CYP expression has been developed and used to predict and explain the toxicology of drugs and xenobiotics [5]. Disruption of the oxidative protective system, such as depletion of GSH stores, affects the metabolism of xenobiotics. Although the toxic effects of GSH depletion have been reported, the impact of GSH depletion on the expression of CYP1 remains unclear. Therefore, we investigated the effect of GSH depletion on the expression of the carcinogenesis-associated CYP 1 isoforms, Cyp1a1, Cyp1a2, and Cyp1b, in mouse livers.

EXPERIMENTAL

Chemicals

Diethyl maleate (DEM), styrene oxide (SO), N-acetylcysteine (NAC), reduced glutathione (GSH), and oxidized glutathione (GSSG) were from Sigma-Aldrich Chemical (Steinheim, Germany). Taq-polymerase, reverse transcriptase, ribonuclease inhibitor, dNTP mixture, and other reagents for quantitative reverse transcription PCR (RT-qPCR) were products of Applied Biosystems (Branchburg, NJ, USA) and Toyobo Co. Ltd. (Osaka, Japan).

Animal studies

Male seven-week-old ICR mice were provided by the National Laboratory Animal Center of Thailand. All animal experiments were performed in the Animal Unit of Faculty of Pharmaceutical Sciences, Khon Kaen University. All protocols were approved by the Animal Ethics Committee for Use and Care, Khon Kaen University (approval no. AEKKU 93/2555), and the study was conducted in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals [6]. Mice (n = 4 - 6 per group) were intraperitoneally (i.p.) injected daily with 150 or 300 mg/kg/day of styrene oxide (SO), 175 or 350 mg/kg/day of diethyl maleate (DEM), or 300 or 600 mg/kg/day of N-acetylcysteine (NAC) for 7, 14, and 28 days. Twenty-four hours after the last treatment, the mice were anaesthetized with pentobarbital sodium at a dose of 100 mg/kg. The abdominal tissue was removed. Whole blood was collected from inferior vena cava using an IV catheter and a heparin syringe. The whole blood (0.5-0.8 mL) was centrifuged at 825 × g for 10 min at 4° C to obtain plasma (80-100 µL). The liver was immediately removed for further studies.

Determination of plasma alanine- and aspartate-aminotransferase (ALT and AST)

Plasma ALT and AST levels were determined using the α-ketoglutarate reaction with some modifications [7]. The final reaction mix of plasma with α-ketoglutarate and either L-alanine for ALT, or α-ketoglutarate and L-aspartate was incubated for AST, at 37 °C for 30 min. Then, 2,4-dinitrophenylhydrazine and sodium hydroxide were added. The UV-absorbance (505 nm) was measured. The amount of ALT or AST in international units (IU/L) was calculated from a pyruvate standard curve.

Determination of glutathione profiles

Liver homogenates were prepared in cold sulfosalicylic acid as previously described [7]. The supernatants were analyzed for GSH or GSSG content using 5,5′-dithiobis (2-nitrobenzoic acid) thiol formation. For GSSG assay, the sample supernatant was treated with 4-vinylpyridine before performing the assay. Thiol formation was measured at a wavelength of 405 nm every 1 min until the kinetic curve was saturated (5 - 10 min). The slope (ΔAbs505/Δtime) of GSH or GSSG content was calculated and compared to the slope of GSH or GSSG standard curve.

Determination of the expression of hepatic CYP1 mRNA

Hepatic total RNA was extracted with acid guanidinium thiocyanate-phenol-chloroform, then reverse-transcribed to cDNA. The mRNA expression of Cyp1 mRNA was quantified using RT-qPCR. The levels of Cyp1a1 (Mm00487218_m1), Cyp1a2 (Mm00487224_m1), and Cyp1b1 (Mm00487229_m1) mRNA were determined using Taqman® Gene Expression assay (Applied Biosystems, Branchburg, NJ). Fold difference of mRNA expression was normalized to a reference
gene (\textit{Gapdh}, forward primer: CCTCGTCCCGTA
GACAAAAATG and reverse primer: TGAAGG
GGTCGTTGATGGC) for each sample and
calculated using $\Delta C_{t}$ method.

Assay of hepatic microsomal CYP1 activity

Microsomal fractions were prepared by ultra-
centrifugation of the homogenate-liver
supernatants at 104,000 $\times$ g at 4 °C for 1 h. The
concentration of microsomal protein was
determined using the Bradford assay, with BSA
as standard [8].

7-Ethoxyresorufin O-deethylase (EROD) and 7-
methoxyresorufin O-demethylase (MROD)
activities represent CYP1 activity. A reaction
mixture containing microsomal protein, and
ethoxyresorufin (for EROD) or methoxyresorufin
(for MROD) substrates in Tris-buffer (pH 7.8)
were incubated at 37 °C. After adding NADPH,
resorufin formation was kinetically measured at
excitation and emission wavelength of 520/590
nm [8].

Statistical analysis

The data are presented as mean±S.D. and were
analyzed using one-way ANOVA followed by
Tukey post hoc test (IBM SPSS statistics version
23, Armonk, NY). $p < 0.05$ was considered
statistically significant.

RESULTS

Effect of styrene oxide (SO) and diethyl
maleate (DEM) on hepatic toxicity and
glutathione profiles

Mean mouse weight was not significantly
changed by any treatments (data was not
shown).

Table 1 shows plasma ALT and AST levels. After
treatment for 7 days, SO and DEM (at the higher
concentrations) significantly increased ALT, while
AST was unchanged. After prolonged treatment
for 14 days, SO markedly increased both ALT
and AST levels, while DEM elevated only ALT. In
contrast, NAC suppressed ALT levels compared
to control. For long term treatment (28 days),
neither DEM nor NAC changed ALT or AST
levels, while both enzyme levels were increased
by SO. These results reveal that SO and DEM
caused hepatotoxicity, while NAC exerted
hepato-protective effect after 14-days of
treatment by reducing ALT levels.

Table 2 shows hepatic GSH profiles. After 7 days
of treatment, total GSH and GSSG contents were
raised by SO and DEM-350, which decreased
the GSH/GSSG ratio in the SO treated mice. After
prolonged treatment for 14 days, SO-150

| Table 1: Plasma ALT and AST levels |
|-------------------------------|
| Group | ALT* (UI/L) | AST** (UI/L) |
|-------|-------------|-------------|
| Before treatment | | |
| Control | 19.64±2.73 | 23.13±2.81 |
| SO-150 | 24.83±3.01 | 22.11±2.59 |
| SO-300 | 29.20±5.52* | 21.25±4.38 |
| DEM-175 | 31.17±3.86* | 23.85±3.80 |
| DEM-350 | 20.40±7.66 | 23.24±3.31 |
| NAC-300 | 33.47±3.60* | 22.11±2.06 |
| NAC-600 | 24.81±4.49 | 19.84±1.10 |

| 7-day treatment | | |
| Control | 23.07±3.16 | 25.06±3.90 |
| SO-150 | 33.34±4.87* | 30.26±3.01* |
| SO-300 | 32.66±6.04* | 29.17±3.14* |
| DEM-175 | 28.58±3.43* | 25.57±6.25 |
| DEM-350 | 34.01±3.79* | 26.15±5.26 |
| NAC-300 | 17.26±3.87* | 23.37±6.13 |
| NAC-600 | 18.27±3.70* | 23.08±2.63 |

| 14-day treatment | | |
| Control | 25.14±3.22 | 21.95±4.39 |
| SO-150 | 30.63±4.55* | 26.33±2.35* |
| SO-300 | 39.04±5.13* | 27.89±4.18* |
| DEM-175 | 27.80±4.80 | 24.69±4.49 |
| DEM-350 | 27.96±2.52 | 22.10±2.01 |
| NAC-300 | 27.49±1.15 | 22.60±4.78 |
| NAC-600 | 26.46±1.69 | 22.85±4.03 |

*Alanine aminotransferase, **aspartate aminotransferase; *$p < 0.05$, vs control at the same
period, based on one-way ANOVA with Tukey post hoc test

Effect of styrene oxide (SO) and diethyl
maleate (DEM) on expression of Cyp1 mRNA

At treatment for 7 days (Figure 1), SO (300
mg/kg/day) significantly induced expression of
\textit{Cyp1a1} (Figure 1 A), \textit{Cyp1a2} (Figure 1 B), and
\textit{Cyp1b1} (Figure 1 C) mRNAs. Both doses of
Table 2: Hepatic glutathione profiles

| Group            | Total GSH (nmols/mg) | GSH* (nmols/mg) | GSSG** (nmols/mg) | Ratio of GSH/GSSG |
|------------------|----------------------|----------------|------------------|------------------|
| **7-day treatment** |                      |                |                  |                  |
| Control          | 57.09±0.69           | 22.78±0.80     | 34.30±0.23       | 0.66±0.03        |
| SO-150           | 73.33±0.20*          | 26.12±0.84     | 47.21±0.64*      | 0.55±0.03*       |
| SO-300           | 69.13±0.79*          | 24.59±2.07     | 44.54±1.32*      | 0.55±0.06*       |
| DEM-175          | 53.87±1.49           | 19.32±1.97     | 34.56±0.95       | 0.56±0.07        |
| DEM-350          | 66.41±1.23*          | 24.71±1.86     | 41.94±0.69*      | 0.58±0.05        |
| NAC-300          | 58.15±2.44           | 21.69±2.73     | 36.55±0.43       | 0.59±0.08        |
| NAC-600          | 56.79±2.03           | 21.61±1.68     | 35.18±0.40       | 0.61±0.04        |
| **14-day treatment** |                    |                |                  |                  |
| Control          | 59.34±1.64           | 22.41±1.31     | 36.93±0.51       | 0.61±0.02        |
| SO-150           | 62.64±1.79           | 21.69±0.55     | 40.95±1.61*      | 0.53±0.02*       |
| SO-300           | 50.67±0.70*          | 16.84±1.10*    | 33.83±1.39       | 0.50±0.05*       |
| DEM-175          | 66.13±1.08*          | 23.90±1.30     | 42.23±0.51*      | 0.57±0.03        |
| DEM-350          | 84.05±0.70*          | 31.91±0.52*    | 52.14±0.66*      | 0.61±0.01        |
| NAC-300          | 58.30±1.77           | 22.44±1.28     | 35.87±0.57       | 0.63±0.03        |
| NAC-600          | 50.22±1.04*          | 19.44±1.21*    | 30.78±1.13*      | 0.63±0.06        |
| **28-day treatments** |                  |                |                  |                  |
| Control          | 54.31±1.05           | 12.12±0.39     | 42.19±0.72       | 0.29±0.01        |
| SO-150           | 54.40±0.91*          | 10.45±1.32     | 43.97±1.17       | 0.24±0.03        |
| SO-300           | 50.86±1.10           | 8.37±1.60*     | 42.51±1.61*      | 0.20±0.05*       |
| DEM-175          | 54.67±0.98           | 10.82±1.69     | 43.85±1.43       | 0.25±0.05        |
| DEM-350          | 40.47±0.37*          | 7.56±1.06*     | 32.90±0.70*      | 0.23±0.04*       |
| NAC-300          | 49.70±0.76           | 10.67±0.89     | 39.03±0.83       | 0.27±0.03        |
| NAC-600          | 38.07±0.43*          | 8.19±1.04*     | 29.89±0.62*      | 0.27±0.04        |

*reduced glutathione, **oxidized glutathione; *p < 0.05, vs control at the same duration, based on one-way ANOVA with Tukey post-hoc test.

DEM markedly elevated expression of all Cyp1 mRNAs, while NAC did not modify expression of any isoform. The activities of EROD (Figure 1 D) and MROD (Figure 1 E) were increased by SO and DEM, corresponding to the increased expression of Cyp1 mRNAs. For the long-term treatment of 28 days, SO and DEM significantly induced expressions of Cyp1a1 (Figure 3 A), Cyp1a2 (Figure 3 B), and Cyp1b1 (Figure 3 C) mRNAs, which corresponded with increased EROD (Figure 3 D) and MROD (Figure 3 E) activities. NAC did not change either the expression of any Cyp1 isoforms or the EROD and MROD activities.

**DISCUSSION**

Glutathione is the major non- enzymatic protective factor against oxidative stress in the liver [1]. Reduced glutathione (GSH) scavenges reactive oxygen species (ROS) and is transformed to GSSG by glutathione peroxidase, which is returned to GSH by glutathione reductase [1]. Suppression of glutathione activity has been shown to be involved in several abnormal conditions such as increased lipid peroxidation, pneumo-toxicity, liver steatosis, and Parkinson's disease.

Figure 1: Effect of SO and DEM on the expression of Cyp1 after 7 day-treatment. Results are expressed as mean ± SD of expression of Cyp1a1 (A), Cyp1a2 (B), Cyp1b1 (C) mRNAs, EROD activity (D), and MROD activity (E). *p < 0.05, vs control based on one-way ANOVA with Tukey post-hoc test.
disease. Moreover, GSH is crucial for detoxification of toxicants and carcinogens [9].

Styrene has been used for plastic and resin production since 1940 [10]. Styrene vapor can be found in the air around manufacturing areas and can be a contaminant in drinking water. It is also found in cigarette smoke, coal tar, coal gas, and petroleum products produced by the organic molecule cracking process [10]. Styrene has been found in polystyrene micro-plastic products and is classified by the IARC as probably mutagenic due to its metabolite; styrene oxide (SO). The current study showed that SO suppressed the GSH profile in mouse livers, which agrees with a previous report that showed SO reduced GSH and GSSG levels in mouse lung and liver [11] and depleted GSH level in rat brain [12]. Diethyl maleate (DEM) is an environmental contaminant found as a pesticide metabolite and a component of plastic wrapper. DEM has also been shown to suppress GSH levels in rat brain and HepG2 cells [13]. N-Acetyl-L-cysteine (NAC) is a well-known GSH precursor. Consumption of NAC (100-300 mg/kg/day, i.p. for 42 days) has been shown to exhibit antioxidant properties and reduce cholestasis-associated complications in rats [14]. Therefore, these three compounds were chosen as GSH modifiers in this study.

The CYP1 family consists of three isoforms, i.e. CYP1A1, CYP1A2, and CYP1B1. CYP1A1 and CYP1A2 are found mostly in the liver while CYP1B1 is found in extrahepatic organs, e.g. lungs, lymphocytes, mammary glands, and placenta [15]. The major function of the CYP1 family is the metabolism of exogenous compounds such as polycyclic aromatic hydrocarbons, which are broadly distributed environmental contaminants that cause carcinogenic, teratogenic, and immune toxicities in mammals [15]. In addition, the CYP1 family is implicated in the carcinogenic process. For example, CYP1A1 and CYP1B1 induction by cigarette smoke was shown to increase the risk of oral squamous carcinoma [16], and excessive CYP1A2 activity has been related to a higher risk of lung adenocarcinoma in Chinese women [17].

In this study, SO and DEM increased plasma levels ALT and AST, which are biomarkers for liver injury in humans and rodents. The GSH/GSSG ratio represents oxidative stress status and a decrease in this ratio indicates GSH depletion. Both SO and DEM decreased the GSH/GSSG ratio, while NAC did not. The lungs of rats exposed to styrene (750 ppm) for 4 weeks exhibited increases in malondialdehyde (MDA) levels, with associated decreases in catalase, superoxide dismutase, and GSH activities [18] and DEM has been shown to increase ROS levels in breast cancer [19]. The CYP1 inducer, benzo[a]pyrene induced EROD and glutathione-S-transferase activities in HT-29 colorectal cells [20]. The current study is the first that connects...
SO- and DEM-mediated up-regulation of Cyp1 expression with GSH depletion in mouse livers. Previously, polychlorinated diphenyl sulfides have been shown to induce CYP1A1 via the aryl hydrocarbon receptor (AhR), causing overproduction of ROS in human HepG2 cells [21]. In this model, SO and DEM might cause excessive levels of ROS in the mouse livers leading to GSH depletion and Cyp1 up-regulation. Thus, the mechanism of SO and DEM associated ROS production and the AhR-mediated pathway for Cyp1 activation should be examined further.

CONCLUSION
Consumption of SO and DEM causes liver injury in mice with elevated ALT and AST levels, imbalance of GSH profile, and induction of Cyp1 mRNA. Exposure to these compounds increases the risk of carcinogenesis via GSH depletion and Cyp1 activation.

DECLARATIONS
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Conflict of interest
No conflict of interest is associated with this work

Contribution of authors
The authors declare that this study was done by the authors named in this article and that all liabilities pertaining to claims relating to the content of this article will be borne by them. WC designed the conceptual framework, carried out the experiments and statistical analysis, and drafted the manuscript. KJ supervised the experimental work, verified the data, proof read and revised the manuscript. The manuscript was comprehensively read and approved for publication by all authors.

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