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Evidence for cellular protein covalent binding derived from styrene metabolite

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Abstract

Styrene is one of the most important industrial intermediates consumed in the world. Human exposure to styrene occurs mainly in the reinforced plastics industry, particularly in developing countries. Styrene has been found to be hepatotoxic and pneumotoxic in humans and animals. The biochemical mechanisms of styrene-induced toxicities remain unknown. Albumin and hemoglobin adduction derived from styrene oxide, a major reactive metabolite of styrene, has been reported in blood samples obtained from styrene exposed workers. The objectives of the current study focused on cellular protein covalent binding of styrene metabolite and its correlation with cytotoxicity induced by styrene. We found that radioactivity was bound to cellular proteins obtained from mouse airway trees after incubation with ¹⁴C-styrene. Microsomal incubation studies showed that the observed protein covalent binding required the metabolic activation of styrene. The observed radioactivity binding in protein samples obtained from the cultured airways and microsomal incubations were significantly suppressed by co-incubation with disulfiram, a CYP2E1 inhibitor, although disulfiram apparently did not show a protective effect against the cytotoxicity of styrene. A 2-fold increase in radioactivity bound to cellular proteins was detected in cells stably transfected with CYP2E1 compared to the wild-type cells after ¹⁴C-styrene exposure. With the polyclonal antibody developed in our lab, we detected cellular protein adduction derived from styrene oxide at cysteinyl residues in cells treated with styrene. Competitive immunoblot studies confirmed the modification of cysteine residues by styrene oxide. Cell culture studies showed that the styrene-induced protein modification and cell death increased with the increasing concentration of styrene exposure. In conclusion, we detected cellular protein covalent modification by styrene oxide in microsomal incubations, cultured cells, and mouse airways after exposure to styrene and found a good correlation between styrene-induced cytotoxicity and styrene oxide-derived cellular protein adduction.

Keywords

Styrene; Styrene Oxide; Bioactivation; Protein Adduction

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Introduction

Styrene is one of the top 50 chemicals produced worldwide, and it has been used in a wide range of applications from food containers to automobile parts. Industrial emission and disposal of styrene-based products are the major concern with styrene exposure, and daily exposure to styrene can be attributed to vehicle exhaust, cigarette smoke, and other indoor emissions, such as paints, carpets, and furniture. Styrene shows low to moderate acute toxicity in both laboratory animals and humans [1-2]. The principal pathological findings in rats and guinea pigs exposed to styrene consisted of severe pulmonary irritation, congestion, edema, hemorrhage, and leukocytic infiltration [1]. Ohashi [3,4] and coworkers reported that epithelial changes occurred in the nose and trachea of rats after being exposed to 800 ppm styrene for 4 hour/day for 8 weeks. Pathologic changes in the nasal mucosa were found at 150 ppm styrene exposure [3,4]. In all cases, the morphological damage was more severe in the upper respiratory tract. Cruzan and coworkers also reported respiratory toxicity in mice and rats after chronic inhalation exposure to styrene [5-7]. The acute effects of styrene in humans are consistent with those observed in animal studies. Irritation of the eyes, nose, upper respiratory tract and lung ventilation disorder in humans have been reported at high concentrations of styrene exposure [8,9].

Cytochromes P450 play important roles in styrene metabolism [10]. Styrene is primarily metabolized by P450 isozymes, such as CYP2E1 and CYP2F2, to form a chemically active metabolite, styrene-7,8-oxide (**2**, Scheme 1), which has been suggested to be responsible for styrene toxicity. This electrophile can attack nucleophilic sites on macromolecules, like DNA and proteins, to form adducts. Styrene oxide has been reported to show direct carcinogenic effects in experimental animals. Various DNA adducts have been identified in humans exposed to styrene [11], although the carcinogenesis of styrene in humans is still controversial. Increased chromosomal aberrations were reported in groups of workers with high exposure to styrene [12-14]. However, limited information is available regarding protein adduction derived from styrene oxide. Albumin and hemoglobin are the only two proteins that have been studied for protein modification. Styrene oxide adducts of both proteins have been identified *in vivo* and *in vitro*. A dose-response correlation between styrene or styrene oxide exposure and the protein adduction has been documented [15,16]. Many studies have shown hemoglobin and albumin adducts in occupationally exposed workers [17-20]. Vodicka and co-workers reported that hemoglobin adduct levels correlated with styrene exposure levels [18]. Christakopoulos *et al.* used the Edman assay to quantify the styrene oxide-hemoglobin adducts in a group of reinforced plastics workers exposed to styrene. They found that blood samples from the workers had detectable hemoglobin adducts, with an average styrene-derived protein adduction at 28 pmol/g [21].

The documented blood protein adduction suggested the possibility that there are cellular protein adducts derived from styrene metabolites and encouraged us to investigate the correlation between styrene-derived cellular protein adduction and styrene toxicity. Covalent modification of specific proteins can activate the immune system to cause an autoimmune response [22,23]. It might also disrupt the protein function or cause cell death by disrupting some regulatory pathway [23,24]. Protein covalent binding was originally proposed by Brodie and coworkers as a possible mechanism for bromobenzene induced liver necrosis [23,25]. Since then, protein adduction with xenobiotics or their reactive metabolites has been recognized as a possible mechanism of chemical toxicity [26-29]. Reactive metabolites of acetaminophen have been reported to form adducts with critical proteins in various subcellular fractions, such as selenium-binding protein (cytosol protein) [23,30], and mitochondria proteins carbamyl phosphate synthetase I [31] and aldehyde dehydrogenase [32]. The observed protein adduction has been suggested to associate with acetaminophen-induced hepatotoxicity [33-35]. Association between halothane induced hepatitis and protein

adduction through its reactive intermediate was also established [26]. Various proteins have been identified, such as carboxylesterase [36], calreticulin [37], and CYP2E1 [38,39], as the targets attacked by its reactive metabolite. The volatile solvent trichloroethylene (TRI) was found to be metabolized mainly by CYP2E1 to reactive metabolites TRI oxide, dichloroacetyl chloride and chloral. And the metabolite(s) bind(s) to CYP2E1 itself acting as a mechanism-based inactivator(s) of CYP2E1 [23,40-42]. All these results suggested that protein covalent modifications by reactive metabolites might contribute to the observed toxicity of the xenobiotics. In one of the styrene toxicity studies, Alarie [43] and his coworkers suggested that the sensory irritation of the upper airways by styrene could result from the adduction of styrene metabolites with sulfhydryl groups on the free afferent trigeminal nerve endings located at the surface of the nasal mucosa. Recently, Lanosa and co-workers found a close correlation between metabolic activation of styrene and the sensory irritation response to styrene [44]. Thus, the understanding of the relationship between protein covalent binding and styrene toxicity and further identification of the reactive metabolite-modified proteins are crucial steps to elucidate the mechanisms of styrene-induced cytotoxicity. The objectives of this study were to examine protein covalent binding derived by styrene metabolite, to determine the role of CYP2E1 in styrene-induced protein adduction, to identify the chemical nature of the protein modification, and to verify the correlation between the protein adduction and cytotoxicity of styrene.

Materials and Methods

Chemicals and Instruments

Styrene (99+%), styrene oxide (99+%), disulfiram, *N*-acetylcysteine, Me₂SO, and Lactic Dehydrogenase Cytotoxicity assay kit were obtained from Sigma-Aldrich (St. Louis, MO). ¹⁴C-Styrene [8-¹⁴C] was custom synthesized by American Radiolabeled Chemical, Inc., (St. Louis, MO) with chemical purity of 99+% and radioactive purity of 99+%. Western blots were performed on an Invitrogen Xcell surelock electrophoresis system (Invitrogen, Carlsbad, CA). Structure identification was performed by both a 300-MHz NMR spectrometer (Varian Associates, Palo Alto, CA) and a LC-MS/MS system including Agilent 1100 HPLC pump system interfaced with Sciex API 2000 tandem quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). A reverse phase C18 chromatography column (250 × 4.6 mm) was used for HPLC analysis and purification of synthetic compounds. The transgenic cell line expressing CYP2E1 (h2E1) and the wild-type cell line (cHo1, human B-lymphoblastoid) were obtained from BD-Gentest (Palo Alto, CA). The two cell lines were used earlier for mechanistic studies of styrene toxicity in our laboratory [45]. The activity of CYP2E1 in the wild-type was too low to be detected using 4-nitrophenol as a substrate, while substantial elevation of CYP2E1 activity was observed in the transgenic cells.

Styrene oxide-derived mercapturic acid I (**5**, Scheme 1) (2-(acetylamino)-3-(2-hydroxy-1-phenylethylthio)propanoic acid) and styrene oxide-derived mercapturic acid II (**6**, Scheme 1) (2-(acetylamino)-3-(2-hydroxy-2-phenylethylthio)propanoic acid) were synthesized as reported earlier by our laboratory [46] and their structures were confirmed by both mass spectrometry and NMR. Rabbit antiserum #1043 against styrene oxide cysteinyl protein adducts was developed in our laboratory [46].

Animals

Male CD-1(Crl:CD-1(ICR)BR) mice (20-22 g) were obtained from Charles River Laboratories (Wilmington, MA). They were housed in group cages in environmentally controlled rooms on a 12 hr light:dark cycle. All animals were allowed to adapt for a minimum of one week prior to use, and the animals were on diet before being used in

experiments. The study protocol was approved by the IACUC of Seattle Children's Hospital.

Micro-dissection of airway segments and tissue culture

The micro-dissection of airways has been described in detail by Plopper's group [47] and our laboratory [48]. Briefly, mice were anesthetized with pentobarbital, and the lungs were inflated with low-melting agarose. During dissection, the lungs were immersed in F12-DMEM media and the distal branches were dissected starting from their branch point with the major daughter airways to the most distal terminal bronchioles. The resulting dissected lung airways were gently washed with PBS buffer to remove agarose possibly remaining in the tissues and then placed in serum free F12-DMEM media containing gentamicin (50 µg/mL) for toxicity and protein covalent binding studies.

Cell culture

Parental and h2E1 cells were cultured in RPMI 1640 supplemented with L-glutamine and 10% fetal bovine serum (Hyclone Laboratory, Logan, UT) under 95% air/5% CO₂ at 37°C, according to the protocol our lab published earlier [45].

¹⁴C-Styrene exposure and supernatant preparation

Airway trees freshly dissected from mice were incubated with ¹⁴C-styrene (chemical concentration: 400 or 800 µM; and specific radioactivity: 6.25 Ci/mol). In a separate study, mouse airways were incubated with ¹⁴C-styrene (800 µM) in the presence disulfiram (50 µM). The incubation plates were sealed with Secureseal to prevent possible evaporation of styrene. After 24 hr incubation, the resulting airway segments were homogenized by a tissue homogenizer, and the supernatants were collected after centrifugation at 14,000 rpm for 30 min.

CYP2E1 cells and wild type cells were counted with Trypan Blue staining using a hemocytometer to ensure that a density of 5×10^5 cells/mL was reached. The cells were incubated with ¹⁴C-styrene at a concentration of 100 µM with specific radioactivity of 6.25 Ci/mol in RPMI 1640 medium supplemented with FBS (10%), and the plates were sealed with Secureseal. After 24 hr incubation, the resultant cells were washed twice with ice-cold PBS (pH 7.4) and lysed by sonication. The supernatants were collected after centrifugation at 14,000 rpm for 30 min.

LDH assay and protein assay

LDH assay was performed based on the protocol provided by the manufacturer (In Vitro Toxicology Assay Kit, Sigma-Aldrich, St. Louis, MO). Briefly, 80 µL LDH assay mixture and 40 µL cell culture media were mixed in an individual well of a 96-well microplate. The plate was covered and incubated for 20-30 min at room temperature, followed by the addition of 1 N HCl (12 µL) to terminate the reaction. LDH activity as the biomarker of cytotoxicity was determined by measuring the absorbance at 490 nm. Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL).

Protein covalent binding assay

The supernatants obtained from airway trees and cultured cells were incubated with one unit of DNase and RNase I at 37°C for two hours, followed by dialysis against deionized water (4 hr × 6) using a 3.5KDa MWCO SLIDE-A-LYZER MINI-Dialysis unit system (Pierce, Rockford, IL). The resulting samples were mixed with scintillation cocktail solution, and the radioactivity in each sample was determined by a LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, CA).

Bioactivation of styrene in mouse lung microsomal incubations

Mouse lung microsomal fractions were prepared by differential centrifugation based on the procedure reported by Watt and co-workers [49] and stored in portions at -80 °C until used. The mouse lung microsomes were carefully thawed on ice prior to experiments. ¹⁴C-Styrene (chemical concentration: 1.0 mM; and specific radioactivity: 5.0 Ci/mol) was mixed with the lung microsomes (1.0 mg protein/mL) in phosphate buffer (100 mM, pH 7.4). The reactions were initiated by adding NADPH (2.5 mM). The final volume of the reaction was 0.1 mL. Incubations were carried out at 37°C for 30 min in a water bath. Some incubation mixtures were incorporated with disulfiram at a concentration of 100 μM. Similar reactions were performed with boiled mouse lung microsomes in parallel. After incubation, samples were dialyzed against de-ionized water (1 L, 4 hr × 6) in a 3.5kDa MWCO SLIDE-A-LYZER MINI-Dialysis unit system. Radioactivity in each dialyzed sample was assessed by a scintillation counter.

Styrene toxicity in h2E1 transgenic cells

Before treatment, cells were counted with Trypan Blue staining using a hemocytometer to ensure that a density of 5×10^5 was reached. Styrene was added to 6-well microplates containing h2E1 cells (5×10^5 /well) in RPMI 1640 medium supplemented with FBS (10%), and the final concentrations of styrene were 100, 500, and 1,000 μM, respectively. The resulting plates were sealed with Secureseal. After 24 hr incubation, the cell suspension was centrifuged at 1,000 rpm for 5 min. The resulting cell pellets were collected, washed with PBS buffer twice and suspended in PBS buffer. Cell viability was determined by Trypan Blue staining. The remaining cells were lysed by sonication and centrifuged (14,000 g) at 4°C for 45 min. The resulting supernatants were stored at -80°C until needed.

Western blots

Protein bands were resolved by SDS-polyacrylamide gel electrophoresis using pre-cast 10% Tris-glycine gels (Invitrogen, Carlsbad, CA) and then transferred to an Immobilon-P transfer membrane (Amersham International Plc, England). A protein assay was conducted to ensure that an equal amount of protein was loaded. Blots were then blocked with 5% nonfat milk in TBS-Tween buffer (100 mM Tris-base buffer containing 154 mM NaCl and 0.5% Tween-20, pH 7.4) for 1 hr at room temperature. The blotted membranes were incubated with 1/1000 dilution of rabbit antiserum #1043 in TBS-Tween buffer with 5% nonfat milk in the absence or presence of styrene oxide mercapturic acids I and II (1:1, 50 μM) at 4 °C overnight. The following day, after washing by TBS-Tween buffer for three times, the resulting membranes were incubated with anti-rabbit IgG-horseradish peroxidase solution (1/3,000 in TBS-Tween buffer with 5% nonfat milk) for 1.5 hr at room temperature. After washing, protein bands were detected by chemiluminescence with ECL detection kit (Amersham International Plc, England).

Statistical analyses

All data are given as means ± SE, and statistical differences among treatment groups were analyzed by one-way analysis of variance and Duncan's multiple range test using SAS statistical software package version 6.12 (SAS Institute, Cary, NC).

Results

Styrene-induced cytotoxicity and protein covalent binding in mouse airways

Mouse airway injury induced by styrene was evaluated by monitoring LDH released to media from the airway tissues after incubation with styrene. Figure 1A shows the changes in LDH activity in the media. At a concentration of 400 μM, styrene did not induce significant

cytotoxicity compared with vehicle-treated control. However, LDH activity in the media markedly increased when styrene concentration reached 800 μM . Protein adduction was monitored by examining the radioactivity binding in cellular proteins recovered from the dissected airway tissues after incubation with ^{14}C -styrene in serum free F12-DMEM media for 24 hr (the same time for the *in vitro* toxicity study). Radioactivity was detected in the recovered cellular protein samples after exhaustive dialysis, and the radioactivity bound to cellular proteins doubled when styrene concentration for the tissue exposure increased from 400 μM to 800 μM (Figure 1B). The effect of disulfiram on styrene-induced protein adduction and cytotoxicity was determined by using the same tissue model. Disulfiram is a known CYP2E1 inhibitor and CYP2E1 has been reported to be responsible for the formation of styrene oxide. The inhibitor was incorporated in the tissue incubations to probe the role of styrene oxide in protein adduction induced by styrene. As shown in Figure 1B, the co-incubation of disulfiram (50 μM) dramatically suppressed protein covalent binding induced by styrene. However, disulfiram did not show a protective effect against styrene-induced cytotoxicity in the cultured tissues. Then we evaluated the cytotoxicity of disulfiram itself and found that disulfiram at the same concentration caused the elevation of LDH activity in the media at a similar level as that for the exposure to a mixture of styrene (800 μM) and disulfiram (50 μM), as shown in Figure 1A.

Metabolism dependency of styrene-induced protein covalent binding

To investigate whether the observed styrene-induced protein covalent binding depends on metabolism, we incubated ^{14}C -styrene with native or boiled mouse lung microsomes in the presence or absence of NADPH, a coenzyme of cytochromes P450. All biochemical reactions involved by cytochromes P450 require NADPH. The presence of NADPH in the incubations of the radioactive styrene with native mouse lung microsomes caused an over 2-fold increase in radioactivity bound to the microsomal proteins compared to the group lacking NADPH (Figure 2). In addition, the presence of disulfiram (100 μM) substantially suppressed the radioactivity binding in microsomal proteins induced by styrene (Figure 2). As expected, the radioactivity bound to proteins remained the same in the incubations of the radioactive styrene with boiled mouse lung microsomes no matter in the presence or absence of NADPH.

CYP2E1 dependency of cellular protein covalent binding induced by styrene

CYP2E1 is one of cytochromes P450 reported to participate in the bioactivation of styrene. To test the role of CYP2E1 in styrene-induced protein adduction, we examined the radioactivity bound to cellular proteins obtained from the wild-type and h2E1 cells after exposure to ^{14}C -styrene at the same chemical and radioactive concentration. An approximate 2-fold elevation of radioactivity was observed in cellular proteins harvested from h2E1 cells relative to that obtained from the wild-type (Figure 3).

Styrene-induced cytotoxicity and protein covalent binding in h2E1 cells

To further study the association between styrene cytotoxicity and styrene induced cellular protein adduction, we examined styrene-induced cytotoxicity and protein covalent binding in h2E1 cells. The cells were exposed to styrene at concentrations of 100, 500, or 1000 μM , respectively. After 24 hr incubation, half of the cells was harvested for cell viability test by Trypan Blue staining, and the other half was collected for the determination of protein covalent binding. Figure 4 shows the cell viability and protein adduction in h2E1 cells after exposure to styrene at the indicated concentrations. With the increasing concentration of styrene, the cell viability decreased. At a concentration of 1 mM, less than 30% of the cells survived compared with the vehicle-treated cells.

Protein adduction was monitored by immunoblot using the polyclonal antibodies we developed earlier [46]. The antibodies were raised to specifically recognize styrene oxide cysteinyl protein adducts **3** and **4** (Scheme 1). This allows us to detect the cellular protein covalent binding derived from styrene oxide. The protein samples obtained from styrene-exposed cells were resolved by SDS-PAGE, followed by blotting to an Immobilon-P transfer membrane. The blotted membrane was incubated with the polyclonal antibodies (antiserum #1043) and consecutively incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase. The resulting antibody-exposed membrane was treated with ECL chemiluminescence kit. The results of the immunoblots are shown in Figure 4. Several chemiluminescent bands with molecular weight from 40 kDa to 80 kDa were detected, and the band density increased with the increasing concentration of styrene exposure. Protein bands with the highest chemiluminescence were detected in the protein samples recovered from h2E1 cells treated with 1000 μ M of styrene. As expected, no chemiluminescent bands were observed in the lane loaded with proteins obtained from the cells after exposure to vehicle.

To confirm the observed protein binding resulted from the modification of cysteine residues by styrene oxide, competitive immuno-blots were conducted using styrene oxide mercapturic acids I and II as competitors. The mercapturates are mimics of styrene oxide-derived cysteinyl protein adducts **3** and **4** (Scheme 1). Our previous work has shown that the mercapturic acids competed binding to the primary antibody with bovine albumin adduct derived from styrene-oxide, indicating that the mercapturates and the protein adduct share similar structural identity. Co-incubation of the mercapturic acids with the primary antibody allows us to probe the topological structure of an antigen recognized by an antibody. Protein samples obtained from h2E1 cells treated with 1000 μ M styrene were loaded onto 10% Tris-glycine gel. After separation and transfer, the blotted membrane was cut into two pieces and incubated with the primary antibodies in the absence or presence of styrene oxide-derived mercapturic acids I and II (1:1, 50 μ M), respectively. As expected, at the selected concentration, the presence of the mercapturic acids I and II completely inhibited the immunostaining of the protein adducts (Figure 5).

Discussion

The formation of styrene oxide mediated by cytochromes P450 has been suggested to be the critical step to initiate the cytotoxicity induced by styrene. Styrene oxide is a known electrophilic species reactive to nucleophiles. With the same rationale, we reasoned that styrene oxide formed *in situ* in biological systems shows intrinsic chemical reactivity to biomolecules, such as proteins and nucleic acids. Styrene oxide-induced DNA damage has been well studied. It has been reported that styrene oxide primarily reacts with DNA at N-7 of guanine as well as nitrogens of other nucleic bases [11]. Although the adduction of albumin and hemoglobin by styrene oxide has been well documented, whether the reactive metabolite of styrene modifies cellular proteins is unknown. We hypothesized that styrene oxide generated *in situ* reacts with cellular proteins to form protein adducts and the protein adduction contributes to cytotoxicity of styrene.

To explore whether the observed protein covalent binding involves styrene metabolism, native or boiled mouse lung microsomes were incubated with 14 C-styrene in the presence or absence of NADPH. The microsomal incubation study showed that both NADPH and active (native) microsomes are required for the bioactivation of styrene and the consequent protein covalent binding (Figure 2). This indicates that the protein adduction involves metabolic activation. In addition, the observed suppression of styrene-derived microsomal protein adduction (Figure 2) and cellular protein adduction in cultured mouse lung airways by

disulfiram further provided evidence for the important role of metabolic activation in protein adduction induced by styrene.

The notable radioactivity bound to the boiled microsomes may result from non-covalent binding, possibly due to the high lipophilicity of styrene. Similar levels of radioactivity resulting from non-covalent binding in the boiled microsomal proteins were observed no matter whether NADPH was present or not in the incubation system. In the absence of NADPH, similar levels of radioactivity bound to the microsomal proteins were observed in both native and boiled microsomes incubated with ^{14}C -styrene. However, the presence of NADPH in the incubation of the radioactive styrene with native microsomes caused substantial increase of radioactivity bound to microsomal proteins, and extensive dialysis was unable to reduce the radioactivity to the level of those observed in the incubations without the presence of NADPH. This may explain that microsomal proteins are adducted through covalent modification by NADPH-dependent products (metabolites) formed *in situ* (the nature of protein-metabolite reactions is discussed separately). Additionally, disulfiram was found to suppress the styrene-induced protein adduction in both cultured mouse airways and mouse lung microsomal incubations. This provides a strong evidence for the contribution of styrene-induced protein covalent binding in the observed radioactivity bound to proteins.

Three approaches were tested to minimize non-covalent protein binding, they were organic solvents washing, excessive dialysis against 1% SDS in water and dialysis against water only. We had difficulty to re-constitute protein pellets either in organic solvents or in aqueous solution after washing with organic solvents. Dialysis with water was chosen for removal of non-covalent binding radioactivity, due to the compromising between protein handling and low radioactivity remained as background. We do not exclude that some non-covalent binding contributed to the radioactivity observed in the protein samples. However, we believe that such levels of non-covalent binding unlikely affect the assessment of protein covalent modification by reactive metabolites of styrene.

CYP2E1 is one of the major enzymes responsible for styrene bioactivation. We observed that CYP2E1 inhibitor disulfiram suppressed the radioactivity binding in proteins obtained from cultured mouse airways and microsomal incubations after exposure to ^{14}C -styrene (Figures 1B and 2). However, disulfiram did not appear to decrease the susceptibility of the cultured tissues to the cytotoxicity of styrene (Figure 1A). Additionally, we noticed that disulfiram at the same concentration as for the covalent binding study showed significant cytotoxicity in cultured mouse airways. We anticipate that the intrinsic toxicity of disulfiram mainly attributed to the cytotoxicity observed in cultured mouse airways after exposure to the mixture of styrene and disulfiram.

In our previous studies, we found that CYP2E1 transgenic cells were more susceptible to styrene toxicity than the wild-type cells [45]. In the current study, we examined styrene-induced protein adduction in CYP2E1 and the wild-type cell lines. As expected, much more radioactivity was bound to cellular proteins in CYP2E1 cells than that in the wild-type cells (Figure 3) after exposure to ^{14}C -styrene. This indicates that CYP2E1 not only participates in styrene-induced cytotoxicity but is also involved in styrene-induced protein covalent modification. The data support the possible correlation between protein adduction and the cytotoxicity induced by styrene.

Styrene oxide is considered as the primary metabolite responsible for styrene toxicity. Chemically, the electrophilic species possesses high reactivity towards nucleophilic amino acids, and cysteine residues were found to be the major amino acid residues modified by styrene oxide [50,51]. To investigate the association between styrene cytotoxicity and

styrene-induced cellular protein covalent binding, we evaluated the cytotoxicity of styrene and monitored cellular protein adduction in h2E1 cells after exposure to various concentrations of styrene. As expected, a concentration-dependency of cytotoxicity was observed. Protein adduction was assessed by immunoblots, using the polyclonal antibodies we developed previously. The antibodies have been proved to selectively recognize styrene oxide cysteine adducts, and the recognition selectivity was probed by competitive ELISA and competitive immunoblots [46]. As shown in Figure 4, several chemiluminescent bands were detected in the lanes loaded with protein samples obtained from h2E1 cells after exposure to styrene, particularly at concentration of 1000 μ M. The chemiluminescent intensity of the bands increased with the increasing concentration of styrene exposure. A good correlation between styrene cytotoxicity and styrene induced protein modification is indicated by the evidence that the cell viability after exposure to various concentrations of styrene is inversely proportional to the cellular protein covalent binding induced by styrene.

The radioactivity tracing studies allowing us to quantify the protein adduction were unfortunately unable to tell the chemical nature of the protein adduction with the reactive metabolite(s) of styrene. The polyclonal antibody we developed was raised specifically against styrene oxide cysteinyl protein adducts **3** and **4** (Scheme 1) by rational design of appropriate haptens and immunogens [46]. Competitive immunoblot was performed to probe whether the protein adduction resulted from the conjugation of styrene oxide with cysteine residues. The presence of mercapturic acids I and II completely prevented the antibodies from binding to the protein adducts (Figure 5). The diminishing of antibody binding occurred due to the affinity of the antibodies to the mercapturic acids present in the incubation system. Not only does it indicate that the protein modification attributed to the conjugation of styrene oxide with cysteine residues, but also it tells us that the antibody binding resulted from immunochemical recognition and not from non-specific binding. Recently, Linhart and co-workers reported urinary aromatic mercapturic acids in mice given styrene, indicating the formation of arene oxides as metabolic intermediates [52]. We may not exclude possible involvement of the reported arene oxides in protein adduction.

In conclusion, we provided good evidence for the bioactivation of styrene to styrene oxide *in situ* and its consequent modification of cellular proteins and for the involvement of CYP2E1 in the metabolic activation of styrene. Our results suggest a good correlation between styrene-induced cytotoxicity and styrene oxide-derived cellular protein modification. The protein modification might trigger the consequent toxicity. However, the identities of the adducted proteins remain unknown, and the identification of the proteins modified by styrene oxide is being undertaken.

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Abbreviations

PBST	200 mM phosphate-buffered saline solution containing 0.02% Tween-20 at pH 7.4
TBS-Tween	100 mM tris-base buffer containing 154 mM NaCl and 0.5% Tween-20 at pH 7.4
TRI	trichloroethylene

Figure 1. Styrene-induced cytotoxicity and cellular protein covalent binding in dissected mouse airway trees

Airway trees freshly dissected from CD-1 mouse lungs were incubated with ^{14}C -styrene (chemical concentration: 400 or 800 μM ; and specific radioactivity: 6.25 Ci/mol), disulfiram (50 μM) or ^{14}C -styrene/disulfiram (800 μM /50 μM) in serum free F12-DMEM media at 37°C for 24 hr. Cytotoxicity was evaluated by monitoring the LDH activity (A) in media. Protein covalent binding was determined by measuring the radioactivity (B) bound to proteins recovered from the mouse airways. Lowercase letters denote statistical difference: (a,b) = $P < 0.05$; (a,c) = $P < 0.01$.

Figure 2. Metabolism-dependency of styrene-induced protein adduction

^{14}C -Styrene (chemical concentration: 1.0 mM; and specific radioactivity: 5.0 Ci/mol) in DMSO was mixed with mouse lung microsomes (native or boiled, 1.0 mg protein/mL) in phosphate buffer (100 mM, pH 7.4) with or without disulfiram (100 μM). The reactions were initiated by adding NADPH (final concentration: 2.5 mM). Incubations were carried out at 37°C for 30 min, followed by excessive dialysis. Protein adduction was assessed by determining the radioactivity bound to the microsomal proteins.

Figure 3. CYP2E1-dependency of styrene-induced protein covalent modification

Both wild type (WT) and h2E1 cells were treated with ^{14}C -styrene at concentration of 100 μM with specific radioactivity of 6.25 Ci/mol for 24 hr, and the cells were then harvested after intensive wash. The supernatants from the cell lysate were treated with DNase and RNase, followed by extensive dialysis. Protein adduction was assessed by measuring the radioactivity remaining in the supernatants. Data represent the values of triplicates (means $\pm$ SEM). Lowercase letters denote statistical difference: (a,b) = $P < 0.05$.

Figure 4. Immunochemical detection of styrene oxide modified cellular proteins in h2E1 cells h2E1 cells were incubated with vehicle or styrene at the indicated concentrations for 24 hr. Cell viability was monitored by Trypan Blue staining. Cellular protein adduction was determined by immunoblots, using antiserum #1043 as primary antibodies. Each lane was loaded with 25 μg of proteins.

Figure 5. Competitive immunostaining of cellular proteins in h2E1 cells treated with styrene h2E1 cells were incubated with vehicle or 1,000 μM styrene for 24 hr. Proteins in the supernatants were resolved by SDS-PAGE and blotted. The membrane blotted with the cellular proteins was incubated with antiserum #1043 in the presence or absence of styrene oxide mercapturic acids I and II (50 μM) as competitors. Each lane was loaded with 25 μg of proteins.

Scheme 1.