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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 ^{14}C-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 was 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 ^{14}C-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.

1. 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 et al. [3,4] reported that epithelial changes occurred in the nose and trachea of rats after being exposed to 800 ppm styrene for 4 h/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 et al. 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 has 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

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.

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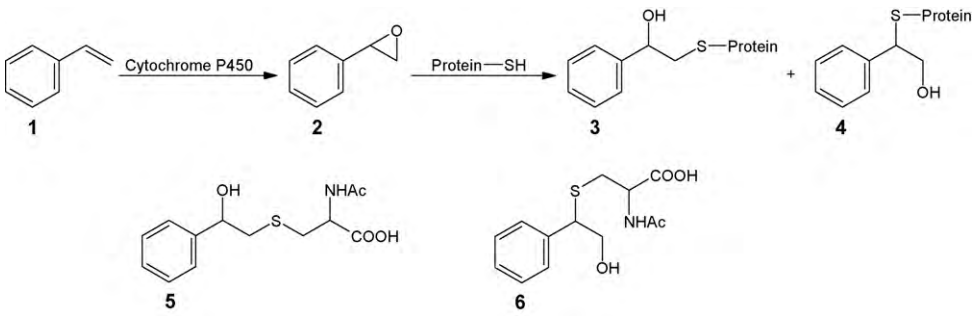

Scheme 1.

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 et al. 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 co-workers 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] 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 et al. 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.

2. Materials and methods

2.1. Chemicals and instruments

Styrene (99+%), styrene oxide (99+%), disulfiram, *N*-acetylcysteine, Me_2SO, and Lactic Dehydrogenase Cytotoxicity assay kit were obtained from Sigma–Aldrich (St. Louis, MO). ^{14}C-Styrene [8-^{14}C] 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 mm × 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