Toxicity of Styrene Vapor in Hepatocyte Monolayers at Low Oxygen Tensions

Anita K. Costa* and James R. Trudell*

Hepatocyte monolayer cultures were exposed to 6000 ppm styrene vapor at 20%, 2%, or 1% O₂ and assayed for signs of cell damage immediately following the 2-hr exposure and then 24 hr later. Oxygen concentrations were used that were previously shown to maximize lipid peroxidation and to predispose hepatocyte monolayers to chemical injury. The use of two time points allowed assessment of acute injury as well as injury that requires several hours to manifest itself. The uptake of styrene into the buffer in the culture dishes was measured by gas chromatography and was found to be 0.49, 0.68, and 0.74 mM at 15, 60, and 120 min, respectively. However, as measured by release of aspartate aminotransferase and inclusion of trypan blue, no toxicity was evident at either time point, irrespective of the oxygen concentration. This study shows that despite the weakening of hepatocyte defense mechanisms by hypoxia, styrene is not acutely toxic to these cells. Furthermore, if any damage to DNA, RNA, or the capability for protein synthesis occurs during exposure to styrene, it is insufficient to cause lysis within 24 hr.

Introduction

Over 3 million tons of liquid styrene monomer are used annually in the manufacture of polystyrene plastics; styrene therefore poses a potential occupational hazard for many workers. The maximum allowable concentration (MAC) for styrene in the United States is 100 ppm with the threshold limit value (TLV) and time weighted average (TWA) set at 50 ppm (1). It has been established that the biotransformation of styrene monomer is a prerequisite for hepatic injury [for reviews on the metabolism, see (2,3)]. The reactive metabolite responsible is proposed to be the epoxide of styrene, styrene oxide, resulting from the oxidation of styrene by the hepatic mixed-function oxidases. However, conflicting reports cast doubt on whether the toxic or mutagenic species is the epoxide (4–7), and other reactive intermediates have been proposed and are being actively sought (7). Over the years there have been many studies with varying results concerning the toxicity, mutagenicity, or carcinogenicity of this compound, but the general theme emerging from these reports is that damage is only observed after multiple exposures (1,8). There has been one report of acute liver injury in young rats following a single high IP dose of styrene (875 mg/kg) (9). In this study, using 35% of the LD₉₀ of styrene, the addition of inducers and inhibitors of the mixed-function oxidases had no effect on the extent of injury, in contrast to other studies using lower styrene concentrations where inhibitors and inducers played a significant role (7,10). The in vivo damage observed in the former study would be consistent with styrene causing direct solvent injury (11) or alterations in hepatic blood flow (12). Halothane, which is a CNS depressant, causes alterations in blood flow to the liver and results in oxygen concentrations in the centrilobular area of the liver lobule dropping from 20 to 40 μM, under conditions of normal blood flow to below μM (12,13). Therefore, it is possible that styrene, which is also a CNS depressant above 50 ppm (1), could also cause similar alterations.

This paper addresses the role that oxygen may play in acute hepato cellular injury by styrene in monolayers of primary hepatocytes: Do low prevailing oxygen concentrations influence styrene toxicity? Styrene metabolism is known to deplete liver glutathione (GSH) and, in turn, cause lipid peroxidation (14), a scenario that theoretically should result in potentiation of the toxicity of the compound as the oxygen concentration drops because lipid peroxidation proceeds maximally at 0.2% O₂ (15,16). We investigated the toxicity of 6000 ppm styrene in hepatocyte monolayer cultures at 20%, 2%, and 1% O₂, and assayed for signs of cell damage immediately following a 2-hr exposure and 24 hr later.

*Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305-5117.
Address reprint requests to J. R. Trudell, Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305-5117.
Methods

Hepatocyte Isolation

Hepatocytes were prepared from male Fischer 344 rats (180 to 240 g) by in situ perfusion of the liver with collagenase (17,18). The isolated and washed hepatocytes were suspended in 100 mL of a medium (complete M-199) containing GIBCO M-199 with Earle's salts, pH 7.3, 100 U penicillin/mL, 100 µg streptomycin/mL, 4.5 µg gentamicin/mL, 1 µg transferrin/mL, 0.25 mM ascorbic acid, 4.12 µM folic acid, 1.24 mM pyruvate, 8 U insulin/mL, 100 µM 5-aminolevulinic acid, 0.25% bovine serum albumin, and 10% heat-inactivated fetal calf serum (GIBCO). The 5-aminolevulinic acid has been shown to maintain total cytochrome P-450 content at in vivo levels (19). Aliquots of 1 mL containing approximately 3 × 10⁶ cells were added to collagen-coated 60 mm Lux Permanox Contur (Flow Laboritories) culture dishes, and allowed to attach for 120 min at 37°C in 5% CO₂/95% air. These culture dishes were preincubated at 37°C for 3 hr in complete M-199 with 20% fetal calf serum and 1 µM dexamethasone to form a matrix, which facilitates attachment, prior to addition of cell suspension aliquots. The high oxygen permeability of the Permanox dishes permits rapid equilibration of the cells and medium with any desired gaseous environment.

The hepatocyte monolayers were then washed twice after the 120-min attachment period and covered with 2 mL complete M-199. Cells at this point were more than 95% viable as assayed 5 to 15 min after addition of trypan blue, by observing five fields of approximately 200 cells at 200× magnification with a Nikon Diaphot inverted microscope. Cytochrome P-450 content at isolation and 24 hr after plating were ascertained, as described previously (18), and corresponded closely to in vivo levels. Previous studies in this laboratory involving identically prepared hepatocytes demonstrated that the metabolic activity of these cells was unchanged after 3.5 hr of 2% O₂ hypoxia (20).

Experimental Protocol

The styrene exposure experiments were initiated 18 hr following the isolation and plating of the hepatocytes. Prior to each styrene or control exposure, the monolayers were washed twice with buffer and covered with 2 mL complete M-199 medium and then checked for confluency with an inverted microscope. Cells were then removed from a standard incubator (5% CO₂/95% air at 37°C) and placed in a Billups-Rothenberg (Del Mar, CA) modular incubator chamber for exposure to styrene and/or selected hypoxic gas mixtures. The incubator chamber was altered to permit the gas mixture to flow in from the top of the chamber onto the surface of a 100-mm Petri dish filled with sterile H₂O that was placed on the top shelf. The resulting turbulence caused a uniform gas flow over five culture dishes placed on each of the two lower shelves. The outflow port at the incubator chamber was extended upward to the surface with tubing, and the entire sealed incubator chamber was immersed in a 37°C water bath.

The cells were exposed to stable concentrations of styrene over the 2-hr exposure period at the various oxygen tensions. The uptake and distribution of styrene vapor into fluids and tissue compartments has been thoroughly studied (21,22).

In order to measure the actual concentration of styrene to which the hepatocytes were exposed, the Billups-Rothenberg modular incubator was modified as follows: Four holes were drilled in the top such that each hole allowed withdrawal of samples from two culture dishes. The holes were plugged with vacuum wax except during sampling. After 15, 30, 45, 60, and 120 min of exposure of Lux Permanox culture dishes containing Earle's buffer to saturate styrene vapor from a copper kettle vaporizer (Foregger, Roslyn Heights, NY), 0.5-mL samples (in duplicate) were withdrawn and added to 0.5 mL carbon disulfide in a 1-mL reaction vial that was then sealed with foil. The sample was mixed vigorously on a vortex mixer for 1 min, the phases were allowed to separate, and two 1-µL aliquots were analyzed by gas chromatography (n = 4 for each time point).

Extraction of styrene into carbon disulfide and analysis by gas chromatography has been previously described (1). Gas chromatography was performed on a Varian 2100 on a 30 × 0.53 mm (i.d.) DX-624 column (J & W Scientific, Folsom, CA) with a helium flow of 8 mL/min. The column, injector, and detector temperatures were 90, 130, and 130°C, respectively. Styrene was dissolved in carbon disulfide to prepare a 1-mM reference sample. Styrene vapor directly from the copper kettle vaporizer was bubbled for 30 min into 10 mL of Earle's buffer, pH 7.4, maintained at 37°C in order to determine the highest styrene concentration that could be achieved in a culture dish at full equilibrium. Earle's buffer, rather than complete M-199, was used in these control experiments in order to prevent the formation of an emulsion during extraction that is caused by serum proteins.

The gas mixtures of either 1, 2, or 20% O₂/5% CO₂/balance N₂ were made with mass flow controllers (Porter Instrument Co., Inc., Hatfield, PA) at 1 L/min. An oxygen monitor (OHMEDA 5100, B.O.C. Health Care, Madison, WI), calibrated against room air and various O₂/N₂ mixtures, was used to confirm the desired O₂ concentrations. The concentration of styrene (6000 ppm) was the result of the entire flow going through the copper kettle vaporizer at 20°C (1). The gas mixture or gas/styrene mixture was passed through a glass frit humidifier that humidified and warmed it to 37°C before entry into the exposure chamber. All groups of dishes remained in the incubator chamber for a 2-hr exposure with lids off. Experiments in the different groups were performed
sequentially throughout the day, with the controls done before the chamber was exposed to styrene. Immediately following each experimental exposure, supernatants were removed from 5 of the 10 identical monolayers in each group. The supernatants were centrifuged to separate dead cells and debris, and the resulting supernatants were analyzed for aspartate aminotransferase (AST) release using Sigma Kit No. 505 (Sigma Chemical Co., St. Louis, MO). A supplementary assessment of cell death was obtained by measuring trypan blue exclusion of the cells following removal of the supernatant. The remaining five dishes were incubated in 5% CO₂/95% air at 37°C for 24 hr. At the end of this 24-hr period, the supernatants from these dishes were tested for AST release and trypan blue exclusion as described above.

Data Analysis

Experiments were performed on two separate hepatocyte preparations (total n = 10 for each point) to average dish-to-dish and rat-to-rat variations. The AST release for each dish was determined as described in the Sigma Kit No. 505; it was compared to complete AST release caused by lysis with 0.2% Triton X-100 and expressed as a percent. It should be noted that 80% cell lysis, as measured by AST release, corresponded to 100% cell death as monitored by their inability to exclude trypan blue. Multiple regression was performed with Statworks software (Cricket Software Inc., Malvern, PA) on an Apple MacIntosh II computer.

Results

A preliminary study in this laboratory using 1000 ppm (10-fold MAC) for 2 hr at 1%, 2%, and 20% O₂ resulted in no significant cell damage above that found with control monolayers as monitored by the release of AST and by the ability of the monolayers to exclude trypan blue (data not shown). To maximize the possibility of measuring styrene toxicity, a study was performed with saturated styrene vapor (6000 ppm) at oxygen concentrations of 1%, 2%, and 20% for 2 hr. The styrene concentration was measured by gas chromatography in the buffer contained in Lux Permanox dishes during actual incubation conditions at 15, 30, 45, 60, and 120 min. The uptake of styrene is shown in Figure 1. It is seen that at 15 min the concentration is 65% of the final value of 0.74 ± 0.02 mM at 120 min. Bubbling the styrene vapor from the copper kettle vaporizer at 20°C directly into Earle’s buffer maintained at 37°C for 30 min produced a final styrene concentration of 0.73 ± 0.02 mM. Saturating Earle’s buffer with liquid styrene at 37°C produced a concentration of 3.5 mM that agrees well with the 2.5 mM in water saturated at 20°C as previously described (1). The concentrations measured in culture dishes at 60 and 120 min were not statistically different (p < 0.05) from each other, or from buffer saturated directly for 30 min, by students unpaired t-test.

No hepatocellular damage was observed immediately following the exposure at any of the oxygen concentrations (Fig. 2). Since the adverse effects of phenomena such as depletion of GSH, accumulation...
of lipid peroxides, or the inhibition of protein synthesis may take time to develop, as has been found with halothane (18) and 1, 2-dibromoethane exposure (23), half of the monolayers from each group were incubated for another 24 hr at 20% O\textsubscript{2}. Cell damage was ascertained as above and no increase in cell death was observed at the 24-hr time point.

Multiple regression was performed on the data. Styrene and oxygen had no significant effect on cell death, while time exhibited a weak correlation with increasing cell death at 24 hr. However, the adjusted coefficient of determination (R\textsuperscript{2}) was only 0.145, indicating that none of the three variables posed a significant factor in cell death.

**Discussion**

The subject of styrene toxicity was thoroughly reviewed in 1983 by the National Institute for Occupational Safety and Health (NIOSH) in a document titled “Criteria for Recommended Standard, Occupational Exposure to Styrene” (1). Although this review presented clear evidence for toxicity to several other organ systems, it concluded that research to clarify the role of styrene on liver status is needed. Previous studies in animals (7-10) have shown that it is very unlikely that hepatotoxicity would result from acute exposure to styrene at the threshold limit value of 50 ppm set by NIOSH (1). Therefore, it was appropriate to investigate possible hepatotoxicity resulting from the very much higher styrene concentrations that could occur during industrial accidents or spills of liquid styrene. However, exposure of animals to high concentrations of styrene is complicated by a variety of CNS effects (1); among these is abnormal pulmonary function. Because hypoxia alone has been shown to cause hepatotoxicity (12,18), it may be impossible to separate the effect of styrene metabolism alone in an in vivo model. In the present study, exposure to high concentrations of styrene was investigated in an in vitro system where both oxygen and styrene concentrations could be defined.

The hepatic metabolism of styrene proceeds with the formation of the reactive common epoxide intermediate, styrene oxide (2,3,10), the half-life of which depends on the amount of epoxide hydrolase present (7). Styrene metabolism is known to result in the depletion of GSH and, in turn, cause lipid peroxidation (14). In analogy to other xenobiotics that cause depletion of GSH and lipid peroxidation, styrene theoretically should be hepatotoxic. Indeed, in vivo experiments have shown that it can be under certain conditions (7–9).

Our laboratory has had a particular interest in the interrelationship between cellular hypoxia and metabolism of xenobiotics (18,19,23). Oxygen tensions in the pericentral region of the liver can range from 4% under normoxia to 1% or lower under severe hypoxia (19). Hypoxia has been shown to lessen the ability of hepatocytes to withstand damage from the calcium ionophore A23187 (24) or t-butyl hydroperoxide (25,26) while at the same time increasing lipid peroxidation (16). In addition, we have used the stability of monolayers of hepatocytes in culture (17) to show that some components of metabolic damage to hepatocytes require several hours to be observable (18,27).

The present study was designed to measure possible toxicity to hepatocytes at a high concentration of styrene and also at low but physiologically reasonable oxygen concentrations where we (18,24,25,27) and others (16,26) have observed increased hepatotoxicity. Because it is possible that metabolites of styrene cause damage to DNA, RNA, or other components of protein synthesis that may result in a delayed onset of toxicity, the viability of the hepatocytes was also measured 24 hrs after exposure. However, a saturated vapor of styrene at concentrations 60-fold higher than the occupational MAC at oxygen concentrations ranging from 1 to 20% for 2 hr was not toxic immediately or after an additional 24 hr of normoxic incubation.

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