Biochemical and Biophysical Research Communications **255,** 70–74 (1999)
Article ID bbrc.1998.9935, available online at http://www.idealibrary.com on IDEAL®

Styrene and Styrene Oxide Affect the Transport of Dopamine in Purified Rat Striatal Synaptic Vesicles

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Received December 3, 1998

Animal and human studies suggest a dopamine-mediated effect of styrene neurotoxicity. To date, mechanisms of cerebral membrane transport of neurotransmitter amines in the presence of styrene in relation to its neurotoxicity have not been addressed properly. So, the present study has examined to test the hypothesis that dopaminergic malfunction in vesicular transport is a critical component in styrene-induced neurotoxicity in rats. Both styrene and its intermediate reactive metabolite, styrene oxide antagonized the *in vitro* striatal binding of [^{3}H] tyramine, a putative marker of the vesicular transporter for dopamine. Both styrene and styrene oxide potently inhibited the uptake of [^{3}H] dopamine in purified synaptic vesicles prepared from rat brain striata, in a dose-related manner, with inhibitory constants (K_i) 2.5 and 2.2 μM respectively. However, neither styrene nor styrene oxide significantly increased the basal efflux of [^{3}H] dopamine that has been preloaded into striatal vesicles *in vitro*. On the other hand, both styrene and styrene oxide have failed to significantly inhibit the uptake of either [^{3}H] norepinephrine, or [^{3}H] serotonin into striatal synaptic vesicles. It is concluded that both styrene and styrene oxide are capable of producing impairments in dopaminergic transport in purified striatal synaptic vesicles, an effect which may be a critical component in styrene-induced neurotoxicity.

Symptoms of neurological disorders involving both the central and peripheral nervous system have been reported in workers exposed to styrene monomer during manufacturing reinforced plastic products containing polystyrene (1–3). Styrene is also reported to leach out from the finished plastic materials. As such, migration of styrene monomer into food material stored in polystyrene plastic containers have caused great concern due to their toxic properties (4). Various approaches including assessments of prevalence of central nervous system (CNS) symptoms (3, 5, 6), psychomotor reaction tests (7–9) and electroencephalography (5, 10) have been studied. A wide variety of barely detectable to severe adverse acute neurologic effects of occupational styrene exposure have been reported involving decreased nerve conduction velocities and electroencephalographic, functional and psychiatric impairments (11–13). Toxicity of the CNS due to occupational exposures to styrene in excess of 100 ppm in air has been well established, although the effects at lower concentrations of styrene in the workplace are also possible (14). Most neurologic effects have been observed at levels of about 100 ppm of styrene, although memory and other neurobehavioral dysfunctions were observed at levels 10-30 ppm and above (15).

However, the exact mechanism of styrene-induced neurotoxicity is still unknown. Although animal and human studies suggest a dopamine mediated effect of styrene-induced neurotoxicity, the results reported to date were not consistent. Thus, in one study, dose-dependent depletions in striatal and tubero-infundibular dopamine (DA) concomitant with increases in homovanillic acid contents in the same areas due to short-term very high exposure to styrene have been reported, such data are found to be inconsistent with the observed inhibition of tyrosine hydroxylase (15). On the other hand, increased levels of serotonin (5-HT) and norepinephrine (NE) as well as reduced activity of monoamine oxidase have been observed in the whole brain of rats treated orally with styrene, but without any effect on DA levels (16). Dopamine depletion has been observed in the brains of styrene-treated rabbits (17, 18) and an increase in DA receptor binding in rat brain striatum, possibly as a compensatory reaction to DA depletion, has also been reported (19). Dose-related increases in serum prolactin and thyroid hormone concentrations among workers exposed to styrene have been attributed to a toxic mechanism involving reduction of tuberinfundibular DA content (15, 20). Color vision loss (an early appearance) due to styrene (21) has been suggested to be due to an interference of

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TABLE 1

The Effects of Styrene, Styrene Oxide on [^{3}H] Tyramine Binding to Rat Striatal Membranes and on the Uptake of [^{3}H] Dopamine, [^{3}H] Norepinephrine and [^{3}H] Serotonin in Rat Striatal Synaptic Vesicles

	Inhibition of			
Treatment	[^{3}H] Tyramine binding, Ki (μM)	[^{3}H] Dopamine uptake, Ki (μM)	[^{3}H] Norepinephrine uptake, Ki (μM)	[^{3}H] Serotonin uptake, Ki (μM)
Styrene	2.4 ± 0.1	4.8 ± 0.4	127 ± 10	106 ± 9
Styrene oxide	2.05 ± 0.1	3.9 ± 0.4	106 ± 9	118 ± 12
Styrene glycol	128 ± 10.6	156 ± 13	185 ± 16	212 ± 18

Note. In binding studies, triplicate aliquots (~100 μg protein) of rat striatal membranes were incubated for 10 min at 37°C with 5 nM [^{3}H] tyramine in the absence or presence of seven different concentrations of competing chemicals. A K_D value of 20 nM was used for calculation of K_i coefficients (see Methods). In uptake studies, duplicate aliquots of rat striatal vesicular proteins (10-12 μg) were incubated for 5 min at 37°C with 500 nM of [^{3}H] dopamine, or [^{3}H] norepinephrine, or [^{3}H] serotonin, with or without five different concentrations of competitives. A K_M value of 450 nM was used for calculation of K_i coefficients. The results are averages ± SE of four experiments.

styrene on dopaminergic mechanism of retinal cells, as suggested for other neurotoxic effects (22).

To date, mechanisms of cerebral membrane transport of neurotransmitter monoamines in the presence of styrene in relation to its neurotoxicity have not been addressed properly. Therefore, in this paper, studies on the effects of styrene and its important metabolite styrene oxide on transport mechanisms of neurotransmitter amines into striatal synaptic vesicles from rat brain have been presented, as a first step in elucidating the mechanism of styrene neurotoxicity.

MATERIALS AND METHODS

[^{3}H] Tyramine binding to rat striatal membranes. The assay for [^{3}H] tyramine binding to striatal homogenates from adult male Sprague-Dawley rats (210-260g) was performed following the method of Vaccari (23). The striata were quickly dissected over ice and homogenized in 10 volumes of ice-cold 50 mM tris-HCl buffer (pH 7.4) containing 5 mM KCl and 120 mM NaCl. Homogenates were then diluted to 31 ml with buffer and centrifuged at 48,000 g for 10 min. The resulting pellets were vortexed in 20 ml of buffer, incubated for 10 min at 37°C, followed by further dilution to 31 ml and centrifugation at 48,000 g for 10 min. After an additional resuspension of the pellets and centrifugation, the final pellets were homogenized 1:60 (w/v) with cold buffer supplemented with 10 μM pargyline and 50 μM ascorbic acid (incubation buffer). Aliquots of membrane (0.1 ml/100 μg protein) were immediately distributed to polypropylene tubes and stored at 4°C until analysed. Appropriate aliquots of incubation buffer for a final volume of 1 ml were then added, followed by 10 μM dopamine (for measuring nonspecific binding), or the competing drugs, in a volume of 0.1 ml. Compounds dissolved in dimethyl sulfoxide were then added in a volume of 10 μl. [^{3}H] Tyramine (5 nM) was finally added (0.1 ml). In drug-competition experiments, 8 concentrations of each drug were used in duplicate. After the incubation at 37°C for 10 min, samples were placed over ice and diluted with 3 ml of ice-cold 0.9% NaCl. Immediately after incubation, they were filtered on glass fiber Whatman GF/B filters and the filters were then washed two times with 3 ml of cold saline. The binding parameters (K_i) for competition study were calculated, based on a weighed, iterative nonlinear curve-fitting program (24) which is largely based on the routine and equation described by Rodbard (25). For calculation of the K_i values, the K_D of the labeled ligand for the binding site was needed, using a rearrangement of the Cheng and Prusoff (26) equation (24).

Preparation of rat striatal synaptic vesicles. Purified striatal synaptic vesicles from rat brain were obtained according to Erickson *et al.* (27). Briefly, freshly dissected tissues were homogenized in 0.32 M sucrose with a Teflon homogenizer at 800 rpm. The homogenate was then centrifuged at 2000 g for 10 min and the resulting supernatant removed and centrifuged at 10,000 g for 30 min to obtain a crude synaptosomal fraction. The synaptosome-containing pellet was resuspended by a gentle swirling in 2 ml of 0.32 M sucrose and then was subjected to an osmotic shock by addition of distilled water and homogenized with 5 up- and down-strokes. Osmolarity was restored by addition of 0.25 M 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) and neutral potassium tartrate buffer (pH 6.8). The suspension was centrifuged at 55,000 g for 60 min, the pellet was discarded, and the supernatant, treated with 1 mM $MgSO_4$, was further centrifuged at 100,000 g for 50 min. The final pellet so obtained (synpatic vesicles) was then immediately resuspended gently in the assay buffer before use. The purity of such preparations was verified by electron microscopy (28).

Vesicular uptake of [^{3}H] dopamine, [^{3}H] serotonin and [^{3}H] norepinephrine. The standard uptake medium (pH 7.4) contained the following:25 mM Hepes, 2 mM ATP-$MgSO_4$, 100 mM potassium tartrate, 50 μM EGTA, 100 μM EDTA. After a 5-min preincubation at 37°C, duplicate aliquots of the synaptic vesicle fraction containing 10-15 μg protein were incubated in the presence of 5 μM pargyline in 100 μl of the medium at 37°C for 5 min with 400 nM [^{3}H] dopamine, or [^{3}H] serotonin or [^{3}H] norepinephrine in the absence or presence of styrene, styrene oxide, or styrene glycol (0-100 μM) dissolved in dimethyl sulfoxide (Table 1). Control samples contained an equal volume (2 μl) of dimethyl sulfoxide. The reaction was stopped by filtering the samples through glass-fiber GF/F filters followed by washing them three times with 4 ml-aliquots of ice-cold buffer containing 2 mM $MgSO_4$. Nonspecific uptake of [^{3}H] monoamine was defined as that occurring at 4°C and was subtracted from the total uptake obtained at each chemical concentration to yield specific uptake for [^{3}H] monoamine.

Vesicular loss of [^{3}H] dopamine. Triplicate aliquots of the vesicle fraction containing ~10 μg protein were incubated as above with 1 μM [^{3}H] dopamine for 5 or 8 min and thereafter they were filtered and the radioactivity was counted (5 min- and 8-min controls). To determine the efflux of [^{3}H] dopamine induced by test compounds, tetrabenazine was added directly to vesicle suspensions at 5 min of incubation, in the absence, or followed by test compounds such as

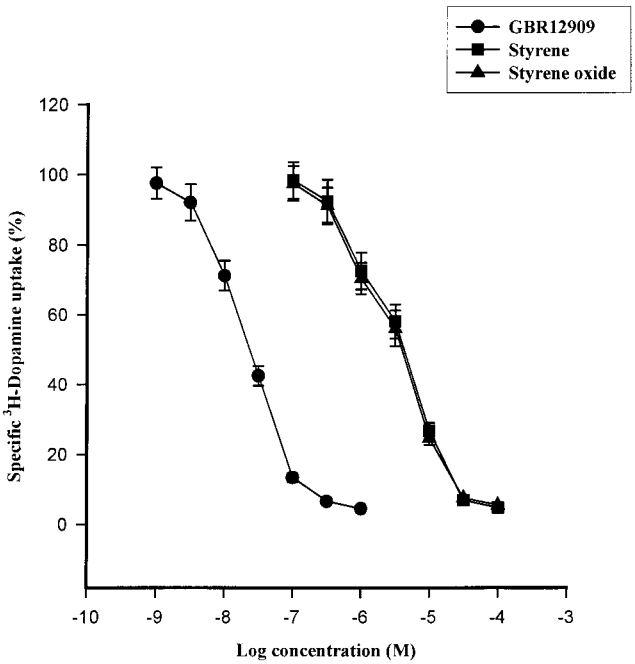

FIG. 1. Effects of styrene and styrene oxide on the specific uptake of [^{3}H] dopamine in rat striatal synaptic vesicles. Each point represents the mean ± SE for 4 determinations of each concentration of each compound. The prototype dopamine uptake inhibitor GBR 12909 was about 145-fold more potent than styrene and styrene oxide, whereas both styrene and styrene oxide seem to have almost equal potency. For details, see Materials and Methods.

styrene, styrene oxide and tyramine, 100 μM each, or saponin at 6 min. In the absence of tetrabenazine, test compounds were added at 6 min of incubation. All samples but 5-min controls were filtered at 8 min. Results were expressed as the absolute [^{3}H] dopamine content (pmol/mg protein) measured after the end of incubations and as the residual content in vesicles in percentage of the tyramine value.

Statistics. The data are reported as means ± SE for 4-5 determinations of each concentration of each compound. Specific uptake for the monoamines and other chemicals was analyzed by nonlinear regression using the sigmoid algorithm of the Inplot program (Graphpad Inc., San Francisco, CA). Such analysis provided IC_{50}, slope and R^2 (goodness-of-fit) values for each curve. The level of significance was set at $P < 0.05$.

RESULTS

[^{3}H] Tyramine binding. Both styrene and styrene oxide inhibited the specific binding of [^{3}H] tyramine to rat striatal membranes fairly potently and in a noncompetitive manner (data not shown) with K_i value of 2.2 μM and 2.5 μM respectively. Both were approximately equipotent.

Vesicular uptake of [^{3}H] monoamines. Both styrene and styrene oxide potently inhibited the uptake of [^{3}H] dopamine in purified synaptic vesicles prepared from rat striata, in a dose-related manner (Fig. 1). Styrene at 1 mM blocked > 80% of the specific uptake of dopamine, whereas styrene oxide (0.5 mM) inhibited over 90% of the specific uptake of dopamine. On the other hand, styrene glycol was found to be much less potent in such inhibition of dopamine uptake. Styrene and styrene oxide inhibited norepinephrine uptake much less potently than they inhibited dopamine uptake (Fig. 2). Nonlinear regression analysis yielded IC50 values of 4.5 ± 0.3 and 2.2 ± 0.4 respectively for the inhibition of [^{3}H] DA uptake by styrene and styrene oxide. The IC50 of styrene for norepinephrine uptake was 25.6 ± 1.2 μM for styrene, and 18.2 ± 0.8 μM for styrene oxide. Both styrene and styrene oxide exhibited low potency toward inhibition of [^{3}H] 5-HT uptake (i.e. IC50s > 100 μM (Fig. 3). Styrene and styrene oxide inhibited no more than 25 and 33% of the specific uptake of 5-HT, respectively at the highest concentration (100 μM) tested.

Vesicular efflux of [^{3}H] dopamine. Results in Table 2 have been presented both as absolute vesicular contents (pmol/mg protein) of [^{3}H] dopamine and release from vesicles as a percentage of tyramine measured after 8 min of incubation. All post hoc comparisons were made between groups and 8-min controls. [^{3}H] Dopamine-loaded striatal vesicles displayed no significant loss of radioactivity upon increasing the incubation time from 5 to 8 min at 37°C (Table 2), consistent with the reported very long half-life (73 min) for this process (29). The addition of 1 mM styrene to the vesicular mixture at 6 min of incubation followed by

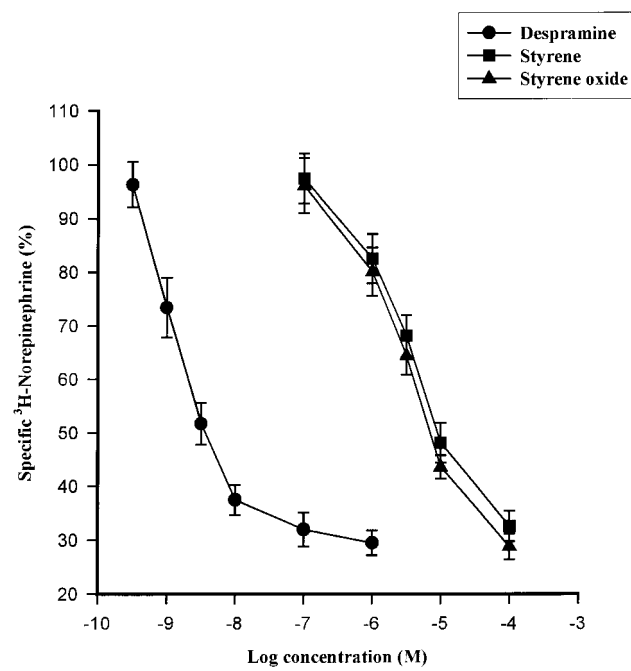

FIG. 2. Effects of styrene and styrene oxide on the specific uptake of [^{3}H] norepinephrine in rat striatal synaptic vesicles. Each point represents the mean ± SE for 4 determinations of each concentration of each compound. For details, see Materials and Methods.

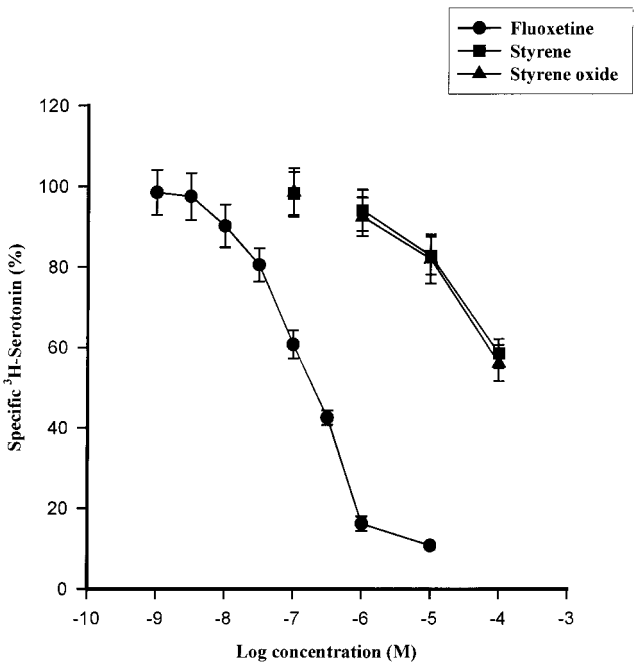

FIG. 3. Effects of styrene and styrene oxide on the specific uptake of [^{3}H] serotonin in rat striatal synaptic vesicles. Each point represents the mean ± SE for 4 determinations of each concentration of each compound. For details, see Materials and Methods.

filtration at 8 min produced only a 30% deficit in the amount of residual [^{3}H] dopamine, compared to 8-min controls. Furthermore, the same concentration of styrene oxide provoked a little higher vesicular loss of [^{3}H] dopamine. The above concentrations of styrene were chosen as roughly corresponding to those putatively present in the brain (30), assuming a uniform distribution of *in vivo* dopamine-releasing doses. Doses of styrene used in this study were in the range of those tested for effects on monoaminergic transmission.

Saturation of the vesicular transporter for monoamines with the potent, selective ligand tetrabenazine (0.5 μM) did not affect the vesicular content of [^{3}H] dopamine, compared to 8-min control (Table 2). Furthermore the addition of styrene, and styrene oxide in the presence of 0.5 μM tetrabenazine, did not further reduce the content of [^{3}H] dopamine left in the vesicles, compared to tetrabenazine control (Table 2). This suggests an absence of an extra-carrier, membrane-permeabilizing component in styrene/styrene oxide-induced dopamine loss. Furthermore, it is seen that there was a nonsignificant release of dopamine from striatal synaptic vesicles due to styrene and styrene oxide (Table 2).

DISCUSSION

The present results have shown that styrene/styrene oxide antagonized the *in vitro* striatal binding of [^{3}H] tyramine, a putative marker of the vesicular transporter for dopamine (23), and the vesicular uptake of [^{3}H] dopamine. However, neither styrene nor styrene oxide significantly increased the basal efflux of [^{3}H] DA that had been preloaded into striatal vesicles *in vitro.* Thus, the present results support the concept that a major component of dopamine release depended upon a direct interaction of styrene/styrene oxide with the synaptic vesicles. This may indicate a striatal synaptic vesicles-mediated role in styrene neurotoxicity. Based on the previous information regarding dopamine impairment in relation to the neurotoxicity of styrene, the present *in vitro* inhibitory effect of dopamine-release from striatal vesicles, its interaction with the [^{3}H] tyramine-labeled carrier, and its inhibitory activity on the vesicular uptake of [^{3}H] dopamine, taken as a whole, suggest consistent changes in storage and re-

TABLE 2

Differential Effects of Styrene and Its Metabolite Styrene Oxide on the Vesicular Release of [^{3}H] Dopamine

Treatment	[^{3}H] dopamine residual in vesicles (pmol/mg protein)	[^{3}H] dopamine release (% control) 8 min	Stimulation (% tyramine)
Control, 5 min	53.7 ± 5.4		
Control, 8 min	51.8 ± 5.2		
Tetrabenazine, 0.5 μM	53.6 ± 4.9		
Tetrabenazine, 5 μM	25.5 ± 3.2*		
Tyramine, 100 μM	34.6 ± 3.85	33.2 ± 2.4	100
Styrene, 100 μM	38.1 ± 3.0	26.4 ± 1.8	80.0
Styrene oxide, 100 μM	36.8 ± 4.65	29.0 ± 1.7	87.1
Tetrabenazine, 0.5 μM + Styrene, 100 μM	34.4 ± 3.9	33.6 ± 2.2	101
Tetrabenazine, 0.5 μM + Styrene oxide, 100 μM	34.8 ± 3.95	32.8 ± 1.9	98.8

Note. Duplicate aliquots (8-10 μg protein) of rat striatal vesicles were incubated with 1 μM [^{3}H] dopamine for 8 min at 37°C (controls); thereafter they were filtered. The test compounds were added at 6 min of incubation. All treatment samples were filtered at 8 min. Values are averages ± SE of five experiments. * Significantly different from the controls, $p < 0.05$.

lease functions for dopamine in acute styrene intoxication.

Neurotransmitter metabolite concentrations are thought to represent functional transmitter release and degradation in conjunction with intraneuronal deamination of newly synthesized transmitter (31). Thus, the altered regulation of dopamine content and its metabolites in response to styrene is likely due to styrene-induced changes in vesicular storage or release of transmitter. In fact, our previous *in vivo* study has demonstrated decreased synaptosomal release of dopamine (unpublished results). Furthermore, the decreased DOPAC and HVA contents observed in our previous *in vivo* study (32) are consistent with this latter finding. Thus, the neurotoxic action of styrene on dopaminergic neurons is primarily presynaptic in nature and related to impaired regulation of DA synthesis and decreased DA release (manuscript submitted).

Styrene oxide, an intermediate metabolite of styrene, is known to react with GSH to form GSH conjugates (2). Whether the cellular uptake of styrene oxide is postulated to occurr via a ligand exchange shuttle, shuttling – SH bound styrene oxide via sequential membrane – SH groups has not been verified in this study. However, such mechanism does not appear to apply to uptake of styrene oxide by striatal synaptic vesicles, since such uptake was not diminished by alkylation of membrane –SH groups by NEM (results not shown).

Based on our present study it may be concluded that the neurotoxicity of styrene is due in large measure to its action as indirect acting dopamine antagonist. It is suggested that future studies should be extended to examine brain regional differences (if any) in the uptake, metabolism and release of dopamine by synaptic vesicles due to exposure to styrene and its reactive metabolite styrene oxide and hence, to find out any possible determinants of the regional specific dopaminergic transport impairments due to styrene.

ACKNOWLEDGMENTS

This research was supported in parts by Institut de recherche en santé et sécurité du travail, Québec and CAFIR, Université de Montréal. The author sincerely thanks Mr. Anwar Malick and Mrs. Sanae Yamaguchi for expert technical assistance and Mrs. Chantal Bélisle for expert secretarial assistance.

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