Long term neurotoxicity of styrene. A quantitative study of glial fibrillary acidic protein (GFA) and S-100

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ABSTRACT Little information exists about the possible neurotoxicity of styrene. The present study was designed to explore whether long term inhalation exposure (three months) to styrene (90 and 320 ppm) could induce long lasting astroglial alterations in Sprague Dawley rats, traceable four months after exposure ceased. Styrene exposure at 320 ppm induced such alterations as shown by raised concentrations of the glial cell marker, glial fibrillary acidic protein (GFA) in the sensory motor cortex and in the hippocampus. GFA is the structural protein of the astroglial filaments and formation of these filaments has been shown after damage to the central nervous system from any cause. It is concluded that exposure to styrene at moderate exposure levels induces regional, long lasting astroglial reactions that serve as an indicator of solvent induced brain damage.

Styrene, a common industrial solvent, is highly lipid soluble and the adverse effects associated with acute exposure primarily concern the nervous system and are related to its depressant properties.^{1,2} In the occupational setting chronic exposure has been reported to induce an increased prevalence of peripheral neuropathies, abnormal EEGs, and an impaired central nervous system function as judged by neurophysiological tests.³⁻⁷

The neurochemical and structural basis for styrene induced brain dysfunction has not been elucidated. Several animal studies have been concerned with the acute effects of the solvent but few long term exposure experiments have been undertaken. It has been shown that styrene interferes with the dopamine system of the brain⁸⁻¹⁰ and in a recent report the ototoxic effect of the solvent was established.¹¹ To our knowledge, however, no studies have shown structural changes in the brain, such as neuronal death or other alterations of the cellular composition, as a consequence of long term exposure to styrene.

The present paper is concerned with the irreversible effects of long term exposure to styrene on the cellular composition of rat brain as studied by the use of two astroglial markers, the S-100 protein and glial fibrillary acidic protein (GFA). The aim of the study was to show astroglial reactivity as a sign of a preceding brain injury, as has been shown after long term exposure to several other organic solvents.¹²⁻¹⁷

Materials and methods

MATERIALS

Barbital, sodium barbital, sodium azide, sodium chloride, perhydrol (H₂O₂), tris, EDTA, and 2-mercaptoethanol were of analytical grade (Merck, Germany). Agarose HSA (Litex, Denmark), 96 well flat bottom microtest plates (NUNC, Denmark), horseradish peroxidase conjugated pig anti-rabbit IgG (Dako, Denmark), bovine albumin, and o-phenylenediamine (Sigma Chemical Co, USA) were used. Styrene, analytical grade, was obtained from Merck (Germany).

ANIMALS

Thirty two male Sprague Dawley rats were used. The initial weight of the rats was 220 g. The animals were housed in transparent plastic cages (45 × 24 × 15 cm, two rats in each). Commercial laboratory rat food (Astra/Ewos, Sweden) and water were freely available. Sawdust was used as bedding. Environmental temperature was kept to 22°C. Light supplementing daylight was automatically controlled for 12 hours daylight, with 30 minutes twilight at dawn and in the evening.

EXPOSURE

During the experimental runs, exposure was continuous. Styrene was vaporised by injection into a temperature controlled glass vaporiser (60°C), mixed with a small volume of air (20 l/h), and subsequently

diluted with clean air (1000 l/h) to produce the desired concentration. The concentration in the inhalation chambers was monitored with a Miran 1A spectrophotometer fitted with a multipath gas cell. All air was filtered to remove oil and particles larger than 0.3 μm . The long term stability of the system permitted the solvent concentration to be held within 10% of the predetermined level. Interruptions occurred only one to two hours a week for changing water, bedding material, and food.

Two experiments were performed. In one the rats were exposed to 90 ppm and in the other to 320 ppm styrene. The two exposure groups were exposed sequentially, each with its own contemporaneous control group. Exposed and control animals were housed in identical chambers throughout the experiment. The duration of each exposure period was three months. After the exposure all animals were removed from the inhalation chambers and kept free from exposure for four months.

PREPARATION OF TISSUE

At the end of the solvent free period the animals were decapitated, and the brains removed and dissected on ice. Cerebral cortices were divided by transverse cuts into three equal parts, frontal (frontal cerebral cortex), middle (sensory motor cerebral cortex), and dorsal (occipital cerebral cortex). The cerebellar hemispheres were divided at the position of the sulci intercrurales into anterior and posterior parts. The anterior (lobuli I-V) and posterior (lobuli VI-X) cerebellar vermis and the brainstem and hippocampus were also dissected. Tissue samples were weighed and then quickly frozen on dry ice and stored in tight fitting boxes at -80°C until they were homogenised. Barbital buffer (25 mM, pH 8.60, with 2.5 mM EDTA and 1.0 mM 2-mercaptoethanol) was used for homogenisation at 1:20 (wt/vol). The homogenates were then centrifuged at 25 000 g for 60 minutes.

A normal rat brain supernatant was prepared from the brain of a non-exposed rat by homogenisation in barbital buffer at 1:10 (wt/vol) and centrifugation at 25 000 g for 60 minutes.

ANALYTICAL PROCEDURE

Protein concentrations in the homogenates were determined according to the method of Lowry *et al*¹⁸; bovine serum albumin was used as a standard.

The S-100 concentrations in the supernatants were determined by rocket immunoelectrophoresis,¹⁹ using bovine S-100 as the reference standard.

GFA concentrations in the supernatants were determined by a sandwich ELISA. A first layer, guinea pig anti-GFA diluted 1:5000 in incubation buffer (0.25 mM barbital buffer, pH 8.60, containing 2.5 mM EDTA, 1.0 mM 2-mercaptoethanol, and 0.1% sodium

azide) was adsorbed to the wells of the microtest plates by incubation overnight at $+4^{\circ}\text{C}$. The plates were then washed in 0.15 M NaCl, containing 0.05% Tween 20 and incubated with 3% bovine albumin for one hour at room temperature and they were then washed again. Incubation buffer containing 0.5% bovine albumin and 0.25 M NaCl together with samples of the supernatants (5 μl) or dilution series of the normal rat supernatant were then added to the wells and incubated for three hours at room temperature. After washing, rabbit anti-GFA diluted 1:4000 in incubation buffer was added and similarly incubated for four hours. After washing, peroxidase conjugated pig anti-rabbit IgG, diluted 1:200 in incubation buffer was added to the wells and allowed to incubate overnight at $+4^{\circ}\text{C}$. The plates were washed and the enzyme substrate was added: o-phenylenediamine, 0.5 mg/ml in 50 mM citrate buffer, pH 5.0, containing 0.01% H_2O_2 . The reaction was stopped with 1.0 M H_2SO_4 and the absorbance was measured at 490 nm in an automatic ELISA reader (Dynatech, UK). The arbitrary units used refer to the amount of GFA in 1 μl of normal rat brain supernatant.

ANTIBODIES

The S-100 and GFA antisera were prepared as described.^{14,20} The specificity of the sera have previously been shown.^{14,21}

STATISTICAL EVALUATION

Fisher's non-parametric permutation test was used to test the differences observed between the exposed and control groups.²² The correlations between the different exposure levels and the GFA concentrations (GFA/ww) were analysed within the hippocampus and the sensory motor cortex respectively, using the Spearman rank correlation test.²²

Results

Body and brain weights No animal died during the exposure or the solvent free periods. There were no significant differences in body weight between the exposed animals and the controls, either at the end of the exposure or after the solvent free period. Brain weights did not differ between control and exposed animals after exposure to 90 or 320 ppm. The weights of the dissected brain regions did not differ significantly between exposed and control animals.

S-100 concentrations per wet weight in the different brain regions studied were not significantly changed after exposure to styrene at 90 or 320 ppm compared with controls (fig 1), nor were any changes observed when the S-100 concentrations were calculated per protein.

GFA concentrations per wet weight were sig-

Fig 1 S-100 protein per wet weight (ng/mg) in nine brain regions after exposure to styrene at 320 ppm. F: frontal cerebral cortex; S-M: sensory motor cerebral cortex; O: occipital cerebral cortex; HIP: hippocampus; HA: anterior cerebellar hemispheres; HP: posterior cerebellar hemispheres; VA: anterior cerebellar vermis; VP: posterior cerebellar vermis; BS: brainstem. Black columns: control values. Striped columns: values of exposed animals. SEM indicated.

nificantly increased in the sensory motor cortex and in the hippocampus after exposure to 320 ppm (fig 2). These changes were also significant when the GFA concentrations were calculated per protein (fig 3). No significant changes were observed after exposure at 90 ppm. The correlations between the different exposure levels and the GFA concentrations per wet weight were tested in the hippocampus (fig 4) and in the sensory motor cortex, giving significant Spearman rank correlation coefficients (ρ) of 0.67 and 0.42 respectively ($p < 0.01$ and $p < 0.05$ respectively).

Discussion

The possibility that chronic mental disorders may be

Fig 2 GFA protein per wet weight (AU/mg) in nine brain regions after exposure to styrene at 320 ppm. * $p < 0.05$; ** $p < 0.01$. (See fig 1 for details.)

Fig 3 GFA protein per protein (AU/mg) in nine brain regions after exposure to styrene at 320 ppm. * $p < 0.05$; ** $p < 0.01$. (See fig 1 for details.)

induced by long term exposure to organic solvents is recognised by several reviewers^{23,24}; case histories and epidemiological studies support this concept. Experimental studies have also shown a connection between long term exposure to organic solvents and permanent brain damage.¹²⁻¹⁷ In this paper we have shown that astroglial alterations may be induced by long term exposure to styrene.

In the central nervous system the astroglial cells are of prime interest in the study of the chemically injured brain because these cells are known to react in a characteristic manner in response to different types of brain injury. Progressive reactions of the astroglial cells (hypertrophy or proliferation, or both) and the appearance of astroglial fibrils and the formation of a

Fig 4 GFA protein per wet weight (AU/mg) in hippocampus after exposure to styrene at 90 ($n = 8$) and 320 ($n = 8$) ppm and in control groups ($n = 16$). Linear regression curve is drawn. Spearman rank correlation coefficient is 0.64 ($p < 0.01$) between exposure levels and GFA concentrations.

glial scar is known as astrogliosis. These progressive reactions may follow damage to the central nervous system from any cause, such as nerve cell degeneration, mechanical tissue destruction, irradiation, and infectious disease.²⁵ Thus the demonstration of progressive astroglial reactions represents clear evidence of an abnormality in the central nervous system.²⁵

To estimate progressive astroglial reactions we have developed methods to quantify two astroglial proteins, S-100 and GFA, in the central nervous system tissue. Of these proteins, S-100 is found in the cytoplasm of both protoplasmic and fibrillary astrocytes and is consequently a marker for all astroglial cells. As GFA is the main component of the astroglial filaments, however, it is principally found in the fibrillary astrocytes.^{26 27}

In the present study our main finding was a raised concentration of GFA protein in the sensory motor cortex and in the hippocampus after exposure to styrene at 320 ppm; S-100 protein concentrations in these regions remained unchanged. These observations were equally significant when calculated per wet weight or per protein, thus excluding the possibility that the changes are due to changed wet/dry weight ratios of the tissue. At the 90 ppm exposure level no statistically significant changes were observed. Nevertheless, the significant correlations between GFA concentrations and exposure levels in the hippocampus and the sensory motor cortex indicate a dose response effect in these regions.

The formation of astroglial filaments has previously been shown in several animal models of damage to the central nervous system. For example, stab wounds in the cerebral cortex induced progressive changes in astroglial cells and increased GFA content in the damaged zone.²⁸ Spinal cord transection,²⁹ cryogenic lesions of the brain,³⁰ and viral and experimental allergic encephalomyelitis^{31 32} are also known to induce the formation of GFA positive filaments in the damaged tissue.

Thus the increased GFA concentrations observed in this study are compatible with an astrogliosis in the hippocampus and in the sensory motor cerebral cortex after exposure to styrene. The lack of concomitant changes in S-100 concentrations probably reflects the differential distribution of S-100 and GFA in the two astroglial cell subpopulations. Protoplasmic astrocytes are known to have the capacity to be transformed into fibrillary astrocytes as a reaction to pathological states of the brain.²⁵ Thus astrogliosis may occur by a shift from protoplasmic to fibrillary astrocytes in response to styrene exposure.

Although the 320 ppm exposure concentration used in this experiment is relatively high, similar levels have been reported in working environments.⁷ Thus, with the hazards of extrapolating animal data for risk assessment of solvent toxicity in man, this paper

presents evidence for a styrene induced injury in the rat brain that may be connected with the brain dysfunction observed among workers exposed to styrene.⁷ Furthermore, the astroglial alterations observed in this study should be considered as permanent. They appear after three months of continuous exposure followed by a four month postexposure solvent free period. This solvent free period was included in the experiment to allow time for neuronal regeneration and the restoration of transient astroglial cell reactions.^{20 33 34} In man persisting brain dysfunction has been shown both by behavioural methods and by regional cerebral blood flow measurements after exposure to organic solvents.^{35 36}

The observations made in this study correlate well with previous reports from our group. In a similar experimental model exposure to xylene resulted in increased GFA levels in the frontal cerebral cortex and in the cerebellar vermis.¹⁷ Exposure to dichloromethane, ethanol, 1,1,1-trichloroethane, trichloroethylene, or perchloroethylene resulted in astroglial alterations mainly in the frontal and sensory motor cortex but also in the hippocampus and in the cerebellar vermis.¹²⁻¹⁶ We have no explanation for these regional patterns, but it is known from studies of man that frontal and frontotemporal regions are affected by chronic exposure to ethanol and other organic solvents and that the hippocampus is also affected by ethanol.³⁶⁻³⁹ In the present study the regional distribution observed might be in accordance with the increased occurrence of fast activity in EEG recordings found in central and percentral areas of the brain in workers chronically exposed to styrene.⁶

The mechanism behind the neurotoxicity of styrene is unclear. Styrene is highly lipid soluble, and high concentrations of the solvent are likely to be reached in the central nervous system during exposure.⁴⁰ Hence, interference with neuronal membrane function may be an important element in the development of styrene induced brain damage. In addition to the lipid solubility of the compound, biotransformation of styrene to toxic metabolic intermediates, especially styrene epoxide,⁴¹ may explain its neurotoxicity. The enzymatic oxidation is catalysed by the mixed function mono-oxygenase system, which is mainly found in the liver but is also present to some extent in the brain.⁴² Thus autoxidative injury of the central nervous system is one possibility.⁴³

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