Effects of Triethyl Lead on Various Cholinergic Parameters in the Rat Brain In Vitro

Fumio Hoshi, Haruo Kobayashi¹, Akira Yuyama¹ and Naonori Matsusaka¹

Department of Veterinary Internal Medicine, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034, Japan

¹Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Morioka 020, Japan

ABSTRACT—The effects of triethyl lead acetate (triethyl Pb) on the cholinergic system in the brain of the rats were investigated in vitro. Triethyl Pb, at concentrations below 10⁻⁴ M, inhibited the depolarized release of acetylcholine (ACh) from slices of cortex and they synthesis of ACh in such slices, while it potentiated in a dose-dependent manner the non-depolarized release of ACh. In contrast, lead inhibited noncompetitively the high-affinity uptake of choline into synaptosomes with a Kᵢ of 4.03 × 10⁻⁶ M and the activity of choline acetyltransferase with a Kᵢ of 4.07 × 10⁻⁵ M. Triethyl Pb has an inhibitory effect (IC₅₀ = 5 × 10⁻⁵ M) on the binding of [³H]quinuclidinyl benzilate to muscarinic ACh receptors. Triethyl Pb inhibited acetylcholinesterase activity slightly at 5 × 10⁻⁵ and 10⁻⁴ M. It is suggested that ACh transmission, in particular the synthesis of ACh and the release of ACh, is susceptible to organolead neurotoxicity.

Organic lead compounds have been produced in large quantities for use as gasoline anti-knock additives, such as tetraethyl- and tetramethyl lead, but the use of these compounds has declined in recent years. Since organic lead compounds are highly soluble in lipids, they are readily absorbed through the respiratory tract, the gastrointestinal tract and the skin (1). It was reported that the primary product of the metabolism of tetraethyl lead in vivo is the triethyl lead ion, which was found to accumulate in the liver, blood, kidneys and brain (2, 3). The triethyl lead is a potent uncoupler of oxidative phosphorylation (4). The involvement of various neurotransmitter systems, such as the cholinergic, noradrenergic, dopaminergic and GABAergic system has also been proposed to explain the neurotoxicity of lead (5, 6). Several investigators have described the neurobehavioral effects of triethyl lead (7–9) and the neurochemical effects of tri- or tetra-ethyl lead (9, 10).

Bondy and his colleagues (11–13) reported that tri-n-butyl lead inhibited the high-affinity uptake of choline (HACU) and the binding of [³H]quinuclidinyl benzilate (QNB) to muscarinic acetylcholine (ACh) receptors in preparations of membranes from brain tissue in vitro. However, there are few studies in which the cholinotoxicity of organoleads, and in particular that of triethyl lead, have been systematically quantified.

The mechanism by which triethyl lead alters synaptic function is not well understood. Such a mechanism may involve some or all of the following: changes in ACh release into the synaptic cleft and in systems for uptake of choline, effects on hydrolytic and synthetic activities of various enzymes, and altered binding of released ACh to its receptors.
results in the present report suggest the possibility that triethyl lead causes significant changes in the capacity of brain preparations to release ACh to take up choline and to synthesize ACh.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 150 to 250 g were used. They were allowed free access to food and water in a room kept at 23 ± 1°C with a 12-hr light : 12-hr dark cycle.

Triethyl lead acetate (triethyl Pb, Alfa Products, Danvers, U.S.A.) was dissolved in re-distilled water, and a fresh solution was prepared for each experiment.

Experiments with tissue slices

Slices of brain (0.2 mm in thickness) from a rat were prepared with a Mcllwain-type automatic microchopper in a cold room (3 ± 1°C). One hundred milligrams of these slices was suspended in 1 ml of non-depolarizing buffer (14). After addition of different concentrations of triethyl Pb, the suspension of tissue was incubated in an atmosphere of 95%O₂ and 5%CO₂ for 20 min at 0°C, and then it was incubated at 38°C for 30 min in the same atmosphere with shaking or kept at 0°C (to serve as a null control). The suspension was centrifuged at 10,000 × g for 20 min at 0°C. The pellet suspended in 1 ml of depolarizing or non-depolarizing buffer and the supernatant was adjusted to pH 4 with 1 N HCl and immersed in boiling water for 2 min. After adjustment to pH 6.8 with 1 N NaOH, the suspension and supernatant were diluted 10-fold with eserine-free frog Ringer or eserine- and KCl-free frog Ringer when depolarizing buffer was used. The concentrations of ACh in these two samples were determined by bioassay using the rectus abdominis muscles of the frog. The amounts of ACh released and synthesized were calculated by subtraction of the values for each null control (14).

Examination of high-affinity uptake of choline

Cerebral cortex was homogenized in 10 volumes of an ice-cold solution of 0.32 M sucrose in a glass homogenizer with a Teflon pestle, operated at 400 rpm. A crude synaptosomal fraction was obtained by the method of Gray and Whittaker (15), as described by Kobayashi et al. (16). A 0.1-ml aliquot of this crude synaptosomal fraction was added to 0.9 ml of reaction medium (final concentrations: 154 mM NaCl, 6.0 mM KCl, 1.6 mM KH₂PO₄, 1.5 mM MgSO₄, 0.1 mM neostigmine bromide, 1.5 mM CaCl₂, 10 mM glucose, 24.0 mM NaHCO₃, and [³H]choline chloride at 10⁻⁵ or 3 × 10⁻⁴ M), supplemented with 10⁻⁷-10⁻⁴ M triethyl Pb. The radioactive tracer used was [1-methyl-³H]choline chloride (77 Ci/mmole, Radiochemical Center, Amersham). After preincubation at 0°C for 5 min, the reaction mixtures, except for the null control which was kept at 0°C, were incubated at 38°C for 4 min, and then centrifuged at 10,000 × g for 40 min at 0°C. Each resultant pellet was rinsed 5 times with 1 ml of saline (0°C) and then suspended 3 times with 0.3 ml of water. The final suspension of the pellet was put into a vial with 8 ml or scintillation fluid (ACS II, Amersham). The radioactivity was counted in a liquid scintillation counter (LSC-700, Aloka Co., Ltd.).

Assay of choline acetyltransferase (ChAT) activity

A preparation of ChAT was obtained by the method of Fonnum (17). The cerebral cortex of the rat was homogenized twice in 0.01 M potassium-phosphate buffer (pH 6.3, containing 1 mM EDTA) with a Biotron (Biotrona Switzerland, setting 10) for 10 sec. The homogenate was supplemented with 0.01 ml of Triton X-100 and kept at 0°C for 30 min; then it was centrifuged at 10,000 × g for 30 min and the supernatant was used as the preparation of ChAT.

The activity of ChAT was determined by the method of Schrier and Shuster (18), as described by Kobayashi et al. (16). The reaction mixture contained 0.1 ml of reaction solution.
0.02 ml triethyl Pb (0–10⁻⁴ M) and 0.04 ml of the preparation of ChAT, and it was incubated at 0°C for 5 min and then supplemented with 0.04 ml of 0.25 mM [¹⁴C]acetylcoenzyme A (56.6 μCi/mmol, Radiochemical Center, Amersham). After incubation at 38°C for 20 min, the reaction mixture in the test tube was placed in boiling water for 2 min. It was then loaded on to a negative ion-exchange column (Dowex 1 X 8, chloride type, 200–400 mesh) and recovered in a scintillation vial. The test tube was washed twice with 0.3 ml of water, and the washings were also passed through the column and collected in the vial. After addition of 8 ml of ACS II, radioactivity was monitored in a scintillation counter.

**Assay of acetylcholinesterase activity**

The cerebral cortex was homogenized in 10 volumes of 0.1 M phosphate buffer (NaH₂PO₄ plus K₂HPO₄, pH 8) in a Biotron at 0°C. This homogenate was further diluted 20-fold with the buffer. Five minutes after addition of 2.5 ml of phosphate buffer (pH 8) to 0.4 ml of diluted homogenate and 0.1 ml of triethyl Pb in a cuvette at 25°C, the activity of AChE was determined at 25°C over the course of 5 min by the method of Ellman et al. (19) with 0.48 mM acetylthiocholine iodide as the substrate.

**Examination of muscarinic acetylcholine receptors (mAChR)**

The cerebral cortex was homogenized in 10 volumes of an ice-cold solution of 0.32 M sucrose in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 1,000 X g for 10 min at 0°C. The preparation for the assay of binding of [³H]QNB (42.6 Ci/mmol, New England Nuclear, Boston) to mAChR was prepared as previously reported (14). Fifteen microliters of the preparation, 0.955 ml of Tris-HCl (Tris buffer, pH 7.4), and 10 μl of 0 to 10⁻⁴ M triethyl Pb were incubated in the presence or absence of 10⁻⁶ M atropine at 25°C for 30 min; and 0.3, 0.5, 0.8 or 1.0 X 10⁻⁷ M [³H]QNB was then added. The mixture was then incubated at 25°C for 40 min, and the reaction was terminated by filtration through a Whatman GF/B glass fiber filter (20). The filter was washed twice with 5 ml of Tris buffer. After the filters were soaked overnight in 8 ml of ACS II in scintillation vials, radioactivity due to tritium was measured in a scintillation counter.

**Protein determination**

Protein was determined by the method of Lowry et al. (21).

**Statistics**

Data are expressed as means ± S.E. Student's t-test was used for statistical comparisons.

**RESULTS**

The effect of triethyl Pb on the depolarized release and synthesis of ACh in slices of cortex were examined over a range of concentrations from 10⁻⁶ to 10⁻⁴ M. As shown in Fig. 1 (A), triethyl Pb at 10⁻⁵ and 10⁻⁴ M inhibited the release and synthesis of ACh in a dose-dependent manner. The amounts of ACh released in the presence of 10⁻⁵ and 10⁻⁴ M of triethyl Pb corresponded to 70 and 40% of the control value. The amounts of ACh synthesized in the presence of 10⁻⁵ and 10⁻⁴ M of triethyl Pb corresponded to 60 and 23% of the control value.

The synthesis of ACh in slices of cortex and the release of ACh in non-depolarizing buffer that contained triethyl Pb were measured. As shown in Fig. 1 (B), triethyl Pb at 10⁻⁶, 10⁻⁵ and 10⁻⁴ M increased the rate of release of ACh in a dose-dependent manner. The amounts of ACh synthesized in the presence of 10⁻⁵ and 10⁻⁴ M of triethyl Pb corresponded to 60 and 23% of the control value.

The synthesis of ACh in slices of cortex and the activity of AChE in homogenates of rat cortex are shown in Fig. 2 (A). The control activity was 13.2 ± 0.17 μmol acetylthiocholine hydrolyzed/min/g at 25°C. Triethyl Pb inhibited the activity of AChE by about 10 and 20% at concentrations of 5 X 10⁻⁵ M and 10⁻⁴ M.
The effect of triethyl Pb on the activity of ChAT was examined and analyzed by construction of a Dixon plot (22). As shown in Fig. 2 (B), triethyl Pb inhibited the activity noncompetitively with an apparent K_i of 4.07 ± 0.04 × 10^{-5} M. Lead acetate at concentrations of 10^{-6} and 10^{-5} M had no effect on the activity of ChAT in the presence of 500 μM choline, but inhibited the reaction by about 20% at 10^{-4} M (data not shown).

The HACU by synaptosomes was measured in the presence of triethyl Pb. Triethyl Pb at a concentration of 10^{-4} M inhibited the HACU significantly. As shown in Fig. 2 (C), the inhibitory effects of triethyl Pb on HACU were quantified in terms of a K_i of 4.03 ± 0.04 × 10^{-6} M.

In order to clarify the effect on muscarinic receptors, the binding of [3H]QNB was examined in the presence of triethyl Pb. As
shown in Fig. 3, the binding of \[^3H\]QNB was inhibited at each tested concentration of \[^3H\]QNB by triethyl Pb. The mean value of IC\textsubscript{50} (the concentration causing 50\% inhibition) was estimated to be 4.73 ± 0.97 \times 10^{-5} M. When analyzed by construction of a Scatchard plot (Fig. 3, insert), \(K_d\) values were found to increase in the presence of triethyl Pb from 0.29 ± 0.03 nM (control) to 0.60 ± 0.07 nM (10^{-5} Pb) and 1.54 ± 0.13 nM (10^{-4} M Pb) without any significant change in the values of \(B_{max}\).

**DISCUSSION**

Triethyl Pb had significant effects on cholinergic parameters in the cerebral cortex of the rat at concentrations below 10^{-4} M.

The decrease in potassium-induced release of ACh from cerebral slices of cortex in standard calcium containing medium caused by triethyl Pb is inconsistent with results of a previous study (5) which failed to find any effect of lead acetate, an inorganic compound, on the release of ACh from minced cortex of mice in vitro. Some authors have, however, reported inhibitory effects of inorganic lead on the release of ACh from neuromuscular preparations and the superior cervical ganglion in vitro (5, 23). Considerable evidence suggests that lead affects the evoked release of transmitter by acting on nerve terminals to inhibit the influx of Ca\textsuperscript{2+} during depolarization (10, 22, 24). Because of its high solubility in lipids, triethyl Pb may be able to gain access more easily than inorganic Pb to calcium channels, through which Ca\textsuperscript{2+} may be transported when ACh is released, in brain tissue. The release of ACh is linked to the synthesis of ACh (23).

Triethyl Pb inhibited synthesis of ACh in slices of rat cortex in the present experiment. Therefore, the inhibitory effect of triethyl Pb on the release of ACh seems to be due to a direct effect on the mechanism of release and/or an indirect effect on the synthesis of ACh.

A stimulatory effect of triethyl Pb on the spontaneous release of ACh observed in the present experiments is unlikely to be due to a suppression of the synthesis of ACh, since release is linked to synthesis, as described above (23). Bondy et al. (12) reported that tributyl lead stimulated the release of tritium from homogenates of brain that had previous been incubated with \[^3H\]choline. It is generally accepted that lead causes an increase in the spontaneous release of neurotransmitter by interfering with the intracellular regulation of Ca\textsuperscript{2+} independent of Ca\textsuperscript{2+} influx, and this results in increases in levels of Ca\textsuperscript{2+} within the nerve terminal (24, 25).

Suppression of the synthesis of ACh caused by triethyl Pb in the slices of brain incubated in normal or high-potassium medium may be due to suppression of HACU and/or ChAT activity, which are thought to be the main factors that control the synthesis of ACh (26).
Bondy et al. (11) reported that tributyl lead inhibited HACU more effectively than did lead acetate. They speculated that the heavy metal in organometalic compounds is able to dissolve in and diffuse through lipid-rich membranes more easily than the lead supplies as lead acetate. In the present experiment, triethyl Pb inhibited HACU in a noncompetitive fashion. Therefore, inhibition of HACU by triethyl Pb seems to be different from that by hemicholinium-3, which is a specific inhibitor of HACU.

The effects of organic lead on ChAT activity have not been reported. The effects of organic and inorganic lead compounds in vitro on the activity of ChAT have also not been reported to our knowledge. While triethyl Pb inhibited the activity of ChAT in a noncompetitive manner, lead acetate, an inorganic lead salt, did not do so in the present experiments. ChAT contains sulfhydryl (SH) group(s) surrounded by a hydrophobic region within the ChAT molecule (27). Mercury compounds with a high affinity for SH groups have been reported to inhibit the activity of ChAT, a sulfhydryl enzyme (28, 29). Because of their higher solubility in lipids, organic mercury compounds are generally more potent in inhibiting the activity than are inorganic compounds (28). Although lead compounds have a lower affinity for SH groups than do mercury compounds (30), triethyl Pb with its high lipophilicity may be able to bind to the SH group in ChAT and inhibit the activity.

Bondy and Agrawal (13) reported that tri-n-butyl lead acetate inhibited the binding of $[^3H]$QNB to membranes from rat brain in the presence of 1 nM QNB. The inhibition by triethyl Pb in the present experiments, in the presence of 0.3 to 1.0 nM $[^3H]$QNB, was similar to that by tri-n-butyl lead reported by Bondy and Agrawal (13). The mechanisms of the inhibition by organolead are generally accepted to involve interactions with amino acid residues or SH group(s) within the allosteric binding sites of the receptor molecule (13).

There is a lack of information on the effects of organic lead and triethyl Pb on AChE activity in vivo and in vitro. Triethyl Pb at $10^{-4}$ M inhibited AChE activity by about 18% in the present experiments. Since it is generally accepted that more than 50% of the brain ChE is sufficient to hydrolyze the total amount of released ACh in a very short time interval (31), the effect of triethyl Pb on the activity may be negligible in vivo.

There is some evidence that injection of triethyl Pb induces neurochemical and behavioral changes (7–10). In the rat brain, the amount of lead 5 days after a single injection of triethyl Pb, supplied as a dose of 7.9 mg/kg, was 210 μg/g of wet tissue (9), which corresponds approximately to $10^{-5}$ M lead. Therefore, from the results obtained in the present experiment, the concentration of triethyl Pb in the brain of animals exposed to this compound might be sufficient to suppress the release and synthesis of ACh, the high-affinity uptake of choline, the activity of ChAT and the function of mAChR, causing some failure of cholinergic transmission in the brain.

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