Acute Sarin Exposure Causes Differential Regulation of Choline Acetyltransferase, Acetylcholinesterase, and Acetylcholine Receptors in the Central Nervous System of the Rat

Wasiuddin A. Khan, Anjelika M. Dechkovskaia, Elizabeth A. Herrick, Katherine H. Jones, and Mohamed B. Abou-Donia

Department of Pharmacology and Cancer Biology, Duke University Medical Center, P.O. Box 3813, Durham, North Carolina 27710

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Acute neurotoxic effects of sarin (O-isopropylmethylphosphonofluoridate) in male Sprague-Dawley rats were studied. The animals were treated with intramuscular (im) injections of either 1 × LD₅₀ (100 µg/kg), and sacrificed at 0.5, 1, 3, 6, 15, or 20 h after treatment, or with im injections of either 0.01, 0.1, 0.5, or 1 × LD₅₀ and sacrificed 15 h after treatment. Plasma butyrylcholinesterase (BChE) and brain regional acetylcholinesterase (AChE) were inhibited (45–55%) by 30 min after the LD₅₀ dose. BChE in the plasma and AChE in cortex, brainstem, midbrain, and cerebellum remained inhibited for up to 20 h following a single LD₅₀ treatment. No inhibition in plasma BChE activity was observed 20 h after treatment with doses lower than the LD₅₀ dose. Midbrain and brainstem seem to be most responsive to sarin treatment at lower doses, as these regions showed inhibition (~ 49% and 10%, respectively) in AChE activity following 0.1 × LD₅₀ treatment, after 20 h. Choline acetyltransferase (ChAT) activity was increased in cortex, brainstem, and midbrain 6 h after LD₅₀ treatment, and the elevated enzyme activity persisted up to 20 h after treatment. Cortex ChAT activity was significantly increased following a 0.1 × LD₅₀ dose, whereas brainstem and midbrain did not show any effect at lower doses. Treatment with an LD₅₀ dose caused a biphasic response in cortical nicotinic acetylcholine receptor (nAChR) and muscarinic acetylcholine receptors (mAChR), and muscarinic acetylcholine receptors (m2-mAChR) ligand binding, using [³H]cytisine and [³H]AFDX-384 as ligands for nAChR and mAChR, respectively. Decreases at 1 and 3 h and consistent increases at 6, 15, and 20 h in nAChR and m2-mAChR were observed following a single LD₅₀ dose. The increase in nAChR ligand binding densities was much more pronounced than in mAChR. These results suggest that a single exposure of sarin, ranging from 0.1 to 1 × LD₅₀, modulates the cholinergic pathways differently and thereby causes dysregulation in excitatory neurotransmission.

Key Words: sarin; choline acetyltransferase; acetylcholinesterase; muscarinic acetylcholine receptor; nicotinic acetylcholine receptor; neurotoxicity; Gulf War.

Sarin, O-isopropylmethylphosphonofluoridate, was developed as a warfare nerve agent (Wood, 1951). During the Gulf War, several hundred thousand American soldiers were presumably exposed to a combination of neurotoxic chemicals, and possibly to sarin (Institute of Medicine, 1995). Some of these veterans complained of symptoms that involved defects in, or abnormal regulation of, the central and peripheral cholinergic nervous system (Persian Gulf Veterans Coordination Committee, 1995).

The neurotoxicity of sarin has been evaluated in different rodent and mammalian species, and the acute toxic effects are supposed to be mediated by inhibition of acetylcholinesterase (AChE). The main clinical features associated with acute sarin intoxication are seizure, fasciculation, tremor, and hypothermia (Taylor, 1985). The appearance of these symptoms correlates with the inhibition of AChE, both in the central nervous system (CNS) and peripheral nervous system (PNS) (Gupta et al., 1991). This is followed by excessive accumulation of acetylcholine, leading to hyperactivation of nicotinic and muscarinic acetylcholine receptors.

Excessive accumulation of acetylcholine leads to activation of ligand-gated ion channels, and of nicotinic acetylcholine receptors (nAChR), and muscarinic acetylcholine receptors (mAChR). These receptors activate diverse kinds of cellular responses by distinct signaling mechanisms (Wess, 1996). Indeed, previous studies from our laboratory and others have shown that organophosphate compounds cause differential regulation of nAChR and mAChR (Huff et al., 1994; Katz et al., 1997). In vitro studies by Bakry et al. (1988) suggested that sarin binds to nAChR and modulates its ligand-binding characteristics. A recent study showed a decrease in high-affinity choline uptake by the insecticide chlorpyrifos (O, O-diethyl 3,5,6-trichloropyridenyl phosphorothioate) (Liu and Pope, 1996). The levels of acetylcholine in the CNS can be regulated by different metabolic pathways, e.g., by the inhibition or activation of AChE and choline acetyltransferase and regulation of high-affinity, sodium-dependent choline transporter (Taylor and Brown, 1999). Rats exposed to soman and sarin have been found to have a decrease in high affinity choline uptake in cortex and hippocampus (Whalley and Shih, 1989). From all of these studies, it is apparent that changes in acetylcholine-related metabolism are the key regulators of CNS...
toxicity induced by organophosphate compounds, including sarin.

Several studies in the past have reported consistent inhibition of AChE by sarin; however, its effects on choline acetyltransferase (ChAT) are shown to be variable, with some having no effect (Sivam et al., 1984) and others with inhibition of no consequence (Kobayashi et al., 1986). Yet another study by Brookes and Goldberg (1979), using cultured spinal cord cells, showed activation of ChAT by a closely related compound, diisopropylphosphorofluoridate. Because of the central role of the cholinergic system in the manifestation of toxicity by sarin, we decided to evaluate acute effects of sarin on the interplay between the cholinergic parameters, i.e., AChE, ChAT, nAChR, and mAChR, concurrently, in a single study. Our studies show that sarin caused inhibition of plasma BChE and brain region-specific AChE and persistent activation of ChAT. They also show that sarin caused increased binding of nAChR- and mAChR-specific ligands.

MATERIALS AND METHODS

Chemicals

Butyrylcholine iodide, acetylcholine iodide, acetyl CoA, and choline chloride were obtained from Sigma Chemical Co. (St. Louis, MO). Tetraphenyl boron was from Sigma-Aldrich Chemicals (Costa Mesa, CA). [3H]Acetyl CoA (sp. activity 12 Ci/mmol) was purchased from ICN Chemicals (Costa Mesa, CA). [3H]Cytisine (sp. activity, 15 Ci/mmol) and [3H]AF-DX384[2,3 Dipro (sp. activity 12 Ci/mmol) was purchased from ICN Chemicals (Costa Mesa, CA). [3H]Acetyl CoA (0.25 mM) was obtained from NEN (Boston, MA). Rabbit polyclonal antibodies to choline acetyltransferase were obtained from Chemicon International (Temecula, CA) and also as a kind gift from Dr. L. Hersh, University of Kentucky (Louisville, KY). SDS–PAGE and Western blotting apparti were from Biorad (Richmond, CA). ECL kit was supplied by Amersham Biosciences. All other reagents were of the highest purity available commercially. Sarin (1.90 mg/ml in saline) was obtained from the U.S. Army Medical Research and Materiel Command, Fort Detrick, MD.

Animals

Male Sprague-Dawley rats (200–250g) were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed in the Duke University Medical Center vivarium on a 12-h dark-light cycle. The animals were allowed food and water ad libitum. All the treatments and procedures on the animals were carried out strictly according to the recommended guidelines by the Army and the Duke University Institutional Animal Care and Use Committee.

Treatment of Animals and Tissue Retrieval

Animals were treated with a single intramuscular injection of 100 μg/kg/ml in normal saline into the thigh muscle for the LD50 time-course study (Aboudonia, et al., unpublished observation). For LD90 treatments, a minimum of 10 animals were used, of which 2 to 3 died within 60 min of treatment. The remaining surviving animals were sacrificed for tissue and blood collection, to carry out biochemical estimations. For dose study, sarin was diluted with normal saline to give a final concentration of 0.01, 0.1, 0.5, or 1 × LD90 to each animal. Control animals received an equal volume of vehicle. At the termination of the experiment, the animals were anesthetized with 0.2 ml of ketamine/xylcaine and blood was drawn into a heparinized syringe. Animals were dissected, and the brain was removed and washed thoroughly with ice-cold normal saline to remove blood. Brain regions (cortex, midbrain, cerebellum, and brainstem) were dissected on ice and snap frozen in liquid nitrogen.

Enzyme and Receptor Assays

Cholinesterase determination. AChE in brain regions and BChE in plasma activities were determined according to the method of Ellman et al. (1961), modified for assay in a Molecular Devices UV Max Kinetic Microplate Reader, as previously described (Aboudonia, et al., 1996). In brief, brain regions were weighed and 10% homogenate was prepared in 0.1 M phosphate buffer, pH 8.0, containing 0.5% Triton X-100. The homogenate was centrifuged at 5000 × g for 10 min at 4°C. The supernatant was used as the source of the enzyme. Blood samples were centrifuged at 5000 × g for 10 min to separate plasma. All samples were stored at −70°C until use. All tissue supernatants were diluted 1:10 with PBS containing 10 mM MgCl2, pH 7. Twenty μl of diluted supernatant was used for each assay in a total volume of 200 μl of buffer or 0.2 mM acetylthiocholine iodide. For plasma BChE determination, the plasma was diluted 1:10 in PBS containing 10 mM MgCl2, and assayed as described for brain regions, except that 0.2 mM butrylthiocholine (BSCh) was used as substrate in the presence of 5 × 10−7 M AChE inhibitor, 1,5-bis-(N-allyl-N-dimethyl-4-ammoniumphenyl) pentane-3-one dibromide. The reaction was started by the addition of 0.1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in PBS. The blank contained buffer in the place of substrate, and the enzyme activity was monitored by recording the absorbance at 412 nm. Protein concentration was determined by BCA method according to Smith et al. (1985). The enzyme activities are expressed as μmol substrate hydrolyzed/min/mg protein for brain regions and nmol substrate hydrolyzed/min/mg protein for plasma.

Determination of choline acetyltransferase. Choline acetyltransferase (ChAT) activity in brain regions was determined according to the method of Fonnum (1975). Briefly, the tissues were homogenized in 50 mM phosphate buffer, pH 7.4, containing 0.5% TritonX-100 and centrifuged at 5000 × g for 10 min at 4°C. The supernatant was used as the source of enzyme. The assay was carried out in 50 mM phosphate buffer, pH 7.4, containing 0.2 M sodium chloride, 10 mM EDTA, 100 μM eserine, 5 mM choline chloride, 200 μM acetyl CoA (0.25 μCi [3H]acetyl CoA), in a final volume of 200 μl, for 30 min at 37°C. The reaction was stopped by adding an equal volume of 1.5% tetrphenyl boron in 3-heptanone, vortexed thoroughly, and centrifuged at 5000 × g for 5 min to separate the organic phase. The choline acetylamine level was determined by counting the organic phase. Enzyme activity was expressed as pmol acetylcholine formed/min/mg protein.

SDS–PAGE and Western blotting of ChAT. A suitable aliquot of 5000 × g supernatant containing 25 μg of protein was denatured with sample buffer. Proteins were separated and transferred to PVDF membranes as described by Khan et al. (1994). The membranes were incubated with 5% non-fat dry milk containing 0.5% Surfactin in Tris-buffered saline, pH 7.4, for 1 h at room temperature to block the nonspecific sites. Membranes were incubated with the primary antibody overnight at 4°C at 1:1000 dilution. The membranes were washed with 5% non-fat dry milk containing Surfactin ×3 for 15 min each, following which the incubation with secondary antibody conjugated to horse-radish peroxidase was carried out for 1 h at room temperature. After extensive washing, the reaction was developed by chemiluminescence using an ECL kit supplied by Amersham Biosciences.

Nicotinic acetylcholine receptor (nAChR) binding assay. [3H]Cytisine was used as the specific ligand for binding studies with nAChR according to the method described by Slotkin et al. (1999). The tissue was homogenized by polytron in 50 mM Tris–HCl, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, and 2.5 mM MgCl2. The membranes were sedimented by centrifuging at 40,000 × g for 10 min. The resulting membrane pellet was resuspended in the same buffer, using Teflon pestle glass homogenizer in a volume sufficient to give 1.5 to 2.0 mg/ml protein. An aliquot of membrane preparation containing ~ 200 μg protein was used to carry out the incubation with 1 nM [3H]Cytisine at 4°C for 75 min. Nonspecific binding was carried out in the presence of 1 μM nicotine ditartrate. The labeled membranes were trapped on membrane filters using a rapid vacuum filtration system, and the results are expressed as specific binding (dpm)/mg protein.
Muscarinic acetylcholine receptor (mAChR) binding assay. For the assay of mAChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at 40,000 × g for 10 min, and the membranes were suspended in the same buffer at the protein concentration of 1.5–2.5 mg/ml as described by Huff et al. (1994). Muscarinic receptor in the CNS comprises a family of 5 distinct members (m1–m5). We carried out ligand-binding studies with m2-mAChR, because of its central role in memory and learning, and our previous studies (Huff et al., 1994) have shown that m2-mAChR is selectively regulated by organophosphate. The m2-mAChR binding was carried out by using m2-selective ligand, [3H] AFDX 384 at room temperature for 60 min. Nonspecific binding was carried out in the presence of 2.22 μM atropine. Ligand-bound membranes were trapped on glass filters presoaked with 0.1% polyethyleneimine using rapid vacuum filtration as described for the nAChR assay. The results are expressed as specific binding (dpm)/mg protein.

Statistical analysis. The data were analyzed by Student’s t-test for statistical significance. The graphs were generated on Excel graphics for Macintosh and are presented as mean ± SE.

RESULTS

Clinical Signs

All animals were observed for the development of clinical signs of toxicity. Animals treated with sarin at 1 × LD₅₀ showed severe tremors, seizures, and salivation within 5–15 min of treatment and these signs continued with increasing severity for up to 30 min. Death occurred in some animals that had also exhibited convulsion, possibly because of respiratory paralysis. Animals treated with 0.5 × LD₅₀ developed tremors by 15–30 min after treatment. Treatment with lower doses of sarin did not result in any observable signs of toxicity.

Effect of Sarin on Plasma Cholinesterase Activity

Initially we carried out a time course study to evaluate the inhibitory potential of sarin after a single LD₅₀ dose. Sarin treatment at 1 × LD₅₀ dose resulted in ~ 45% decrease in plasma butyrylcholinesterase activity 30 min after exposure and continued to decrease up to 55–65% by 3 and 15 h (Fig. 1). The maximum inhibitory effect persisted up to 15 h after treatment.

Because exposure to sarin could be to doses lower than the LD₅₀, we wanted to examine the effects of sarin on plasma BChE at 0.01, 0.1, 0.5, and 1 × LD₅₀; we sacrificed the animals 15 h after treatment. The data in Figure 2 show that plasma cholinesterase activity was inhibited significantly only at the 1 × LD₅₀ dose 15 h after treatment (p < 0.04). However, it is likely that a smaller dose may have some inhibitory effect at earlier time periods.

Effect of Sarin on Brain Regional AChE

We studied the effect of sarin on AChE in different regions of brain after a single LD₅₀ dose. The data presented in Figure 3 show the inhibition pattern of AChE in cortex, brainstem, midbrain, and cerebellum. Cortex and midbrain registered a significant decrease in AChE activity 30 min after treatment. Brainstem, midbrain, cortex, and cerebellum all exhibited statistically significant inhibition 3 h post-exposure. Only 13, 20, 16, and 20% activity remained in cortex, brain stem, midbrain, and cerebellum, respectively, after 3 h, and this inhibitory pattern was maintained for up to 15 h, after which there was some recovery in the enzyme activity.

Data on the effect of different doses of sarin, 15 h after
dosing, on brain regional AcChE are presented in Figure 4. Cortex and brain stem AcChE activity registered a decrease only after 0.5 and 1 × LD₅₀. This inhibition was statistically significant only at the 1 × LD₅₀ dose (11% and 34% activity for cortex and brainstem, respectively, remaining after 15 h). Midbrain showed a significant decrease in AcChE activity at 0.1 × LD₅₀ (~60% of control) and continued to decrease at 0.5 and 1 × LD₅₀. Cerebellar AcChE activity was decreased (70%) significantly only at 1 × LD₅₀. At the 0.01 × LD₅₀ dose, midbrain, brainstem, and cerebellum showed consistent increases in AcChE activity. It is apparent from these data that the dose response to sarin is variable in different regions of the rat brain.

**Effect of Sarin on Brain Regional Choline Acetyltransferase Activity (ChAT)**

We carried out a time-course study on the effect of sarin on ChAT activity in different brain regions after a single LD₅₀ exposure. Data presented in Figure 5 show that sarin treatment caused a significant increase in enzyme activity in the cortex at all the post-treatment time points. By 3 h after treatment, there was a significant increase (~138% of control) in cortex activity, and it continued to rise for up to 20 h post-treatment. The brainstem activity showed a biphasic response: a decrease at early time points but a significant increase (~160–170% of control) by 6 to 20 h. These data suggest a differential regulation of ChAT activity in 2 brain regions that exhibit maximum cholinergic innervation. In midbrain, the activity followed a pattern similar to that in the brainstem. The cerebellar activity, even though not a main cholinergic response area, showed a declining trend.

Data from the dose study on ChAT activity are shown in
Figure 6. As with the time course with a single LD$_{50}$ dose study, the cortex area showed a significant increase (181, 157, and 164%) in enzyme activity after 0.1, 0.5, and 1 × LD$_{50}$, respectively. A similar pattern of activation was observed in mid brain.

Effect of Sarin on Nicotinic and m2-Muscarinic Acetylcholine Receptors in the Cortex

Sarin (1 × LD$_{50}$) exposure resulted in a decrease (~ 25%) and an increase (~ 132%) in binding of [³H]cytisine 1 and 6 h after treatment, respectively. Increase in binding density persisted up to 20 h after treatment (Fig. 7). Furthermore, sarin caused an increase in the ligand-binding density at 0.01 of the LD$_{50}$ dose. Because α4β2 is the major receptor type present in the CNS of rats, our results suggest that sarin acutely regulates the nAChR ligand binding (Figs. 7 and 8).

The data presented in Figures 9 and 10 show that sarin treatment resulted in an initial decrease at 1 and 3 h post-treatment and subsequent increase in binding in the m2 mAChR selective ligand 6 h after treatment of the LD$_{50}$ sarin dose. Unlike nAChR, however, the increased binding in mAChR was only modest (10–15%) but persisted for up to 20 h after sarin treatment. Figure 10 shows the effect of various doses of sarin on m2 mAChR. The increase in binding density was observed at 0.01 × LD$_{50}$ dose. These data suggest that sarin exposure causes a persistent increase in receptor binding for nAChR, whereas m2-mAChR shows a different pattern of regulation.

DISCUSSION

The present study demonstrates that a single im dose of sarin, either at LD$_{50}$ or less, induces changes in the CNS characteristic of organophosphate nerve agent toxicity. Besides the major clinical symptoms such as convulsions, fasciculations, tremors, and seizure, sarin treatment resulted in signifi-
cant inhibition in cholinesterase activity both in plasma and brain regions. The acute symptoms observed were a result of excessive cholinergic activity resulting from the accumulation of ACh at the synapse. Our data also provide evidence that activation of ChAT in cortex, brainstem, and midbrain may enhance the availability of acetylcholine above and beyond that afforded by inhibition of AChE by sarin. This could lead to overstimulation of AChR in the CNS.

Several studies have reported inhibition of AChE by sarin (Lim et al., 1983 and references therein). The regulatory role of sarin on biosynthesis and degradation of acetylcholine and its role on the CNS nAChR and mAChR, however, has not been reported in a single study. It is important to evaluate these aspects of the cholinergic pathway, because the interplay between each component of the cholinergic pathway would ultimately affect the neurotoxicity of sarin. The present study provides data on all of these aspects of sarin-induced toxicity.

Inhibition of plasma BChE is the hallmark of the neurotoxicity induced by a large number of organophosphates, including sarin. Our time-course study with \(1 \times \text{LD}_{50}\) sarin dosage shows that sarin treatment resulted in a significant decrease in BChE activity in plasma and AChE inhibition in cortex, brainstem, midbrain, and cerebellum. The dose-response study showed that significant inhibition in BChE activity is observed only at the higher dose of sarin at 15 h of treatment. However, it is likely that lower doses such as 0.01 and 0.1 \(\times \text{LD}_{50}\) may also have an inhibitory effect at earlier time periods, which is subsequently recovered. It is known that sarin-inhibited AChE ages slowly (Clement, 1982), and therefore, the inhibition might have subsided by 15 h, the time of our study. Increase in AChE activity at 0.01 \(\times \text{LD}_{50}\) sarin may be mediated by an indirect mechanism as opposed to the inhibition of AChE by sarin, which is by direct interaction with the enzyme.

Our results also suggest that there are regional differences in the brain severity to inhibition of AChE by lower doses of sarin, with midbrain showing inhibition of AChE activity at 0.1 \(\times \text{LD}_{50}\), whereas in the cortex and brainstem, the inhibition was observed at 0.5 \(\times \text{LD}_{50}\). This differential response could reflect the total enzymatic activity present in each region. Thus, the threshold level of cortex and other brain regions such as striatum AChE may be higher than in the rest of the regions.

ChAT is a specific marker of cholinergic innervation in the CNS, which catalyzes the final step in the biosynthesis of acetylcholine (Wu and Hersh, 1994). Although it is believed that ChAT is not the rate-limiting enzyme in the availability of acetylcholine in the CNS, it can have a modulatory role in the cholinergic system. Indeed, in the past attempts have been made to use selective inhibitors of choline acetyltransferase as possible in vivo protection mechanisms against soman-induced neurotoxicity (Harris et al., 1982; Schoene et al., 1977). Sterling et al. (1988) reported that a quaternary salt of hydroxyethylphosphorylvinyl pyridine provided protection against soman-induced mortality when given 2–3 min prior to soman treatment. Our data on ChAT activation by sarin in cortex and brainstem prove that enhanced enzyme activity may have some consequence, at least in the early period of exposure. Similarly, Brookes and Goldberg (1979), using mouse spinal cord cell culture, found that diisopropylphosphorofluoridate (DFP) exposure caused activation of ChAT. Others found no effect on...
the enzyme activity in response to sarin or DFP exposure (Sivam et al., 1984). The reasons for these differences are not known. Significant inhibition of ChAT activity in the brainstem at early time periods following LD_{50} sarin administration may suggest a direct inhibitory effect.

ChAT activation by sarin in vivo is interesting, because it is known that sarin-inhibited AChE is reactivated faster than other nerve agent-inhibited AChE (Clement, 1991; Clement et al., 1991; Schoene, 1978). Clement (1982) reported that soman-inhibited AChE ages faster than a sarin-inhibited enzyme. Therefore, in view of persistent activation of ChAT, as observed in our studies, it is reasonable to assume that even when AChE inhibition by sarin is not pronounced (because of faster reactivation), the still higher level of acetylcholine could be available at the presynaptic terminals. However, the role of vesicular acetylcholine transporter remains to be evaluated under these conditions.

ChAT activation has been observed under a variety of conditions; mostly related to trophic factors and survival (Cavicchioli et al., 1991; Fusco et al., 1989; Li et al., 1995; Mobley et al., 1985; Wu and Hersh, 1994). However, other modifications such as phosphorylation and proteolysis have also been shown to regulate the enzyme activity (Bruce and Hersh, 1989; Wu et al., 1995). Sarin-induced activation of ChAT activity may involve proteolytic cleavage of the enzyme. An increase in electrical activity has been shown to cause increased proteolytic activity by. Furthermore, it has been shown that cholinergic stimulation causes protease(s) activation that leads to synapse loss in activity-dependent manner (Liu et al., 1994). Therefore, it is likely that increased ChAT activity following sarin exposure may be a consequence of protease-mediated activation of the enzyme. This view needs further studies.

Inhibition of AChE following OP poisoning causes excessive stimulation of CNS AChRs. Under acute exposure conditions, overstimulation leads to seizure and chronic activation may lead to impairment of memory function (Taylor, 1985). A critical role of nAChR and mAChR has been implicated in all these processes (McGehee, 1999; Wess, 1996). Our data suggest that acute sarin exposure significantly increases binding densities of respective ligands for nAChR and m2-mAChR. Earlier in vitro studies (Bakry et al., 1988) reported inhibition in binding of high affinity m2 receptor ligand [3H]CD to mAChR by several organophosphates, including sarin. This effect was found to be selective for mAChR as the inhibition for nAChR was not pronounced. A recent study extended these observations by reporting that not only neuronal type nAChR but also electric ray nAChR, which is very similar to muscular AChR structurally and pharmacologically, binds organophosphates of diverse structures (Katz et al., 1997). Binding of these organophosphate compounds to the nAChR is believed to be at a site distinct from the ligand binding site and this binding induces desensitization of the receptor (Albuquerque et al., 1997; Bakry et al., 1988). Increased binding densities of α4β2-specific nAChR ligand in the cortex in the present study at 0.001 LD_{50} suggest that if the concentration of sarin reaches a high enough level in the cell, it may cause allosteric changes in the receptor conformation, exposing higher ligand binding sites. Whether or not a high ligand binding state of the receptor continues to be active long enough or becomes desensitized remains to be discovered. Alternatively, the number and or function of the receptor increased by sarin can be mediated by second messengers such as c-AMP-dependent or independent mechanisms (Gurantz et al., 1993; Margiotta, 1987) or by changes in intracellular Ca^{2+} concentration. An interesting possibility could also involve upregulation of the mAChR as a consequence of hypothermia induced by sarin (Clement, 1991) because low temperature causes upregulation in surface expression of mAChR (Cooper et al., 1999).

Muscarinic acetylcholine receptors in the CNS are comprised of 5 distinct classes of receptors (m1-m5). These receptors have distinct structural and pharmacological features and show differential cellular localization (Levey et al., 1991). These receptors are coupled to different G-proteins to transduce cellular signaling from the cell surface. m2-mAChR is coupled to Gi protein, leading to inhibition of adenylate cyclase (Hulme et al., 1990). Our laboratory has previously shown that chlorpyrifos oxon binds to m2-mAChR in vitro and inhibits cAMP accumulation (Huff and Abou-Donia, 1995; Huff et al., 1994). Studies by Ward et al. (1993) and Silveira et al. (1990) also have shown that organophosphate compounds selectively regulate m2-mAChR ligand binding. The data in the current study showing increased m2-mAChR-specific ligand binding in cortex following sarin treatment suggest in vivo regulation. Similarly, studies by Chaudhuri et al. (1993) and Liu and Pope (1996) reported an increased m2-mAChR ligand binding in response to chlorpyrifos treatment. It has been previously shown that presynaptic m2-mAChR could regulate acetylcholine release via a feedback inhibitory mechanism (Marchi et al., 1990; Raiteri et al., 1984) and in rat striatal cells, paraaxon inhibited forskolin induced cAMP synthesis, an effect which was blocked by atropine (Jett et al., 1991). These results suggest that selective effects of sarin on m2-mAChR may have modulatory effects on other processes, such as acetylcholine release, second messenger system, etc. that could influence the toxicity of sarin.

In summary, our results suggest that BChE activity in plasma remains inhibited up to 15–20 h following a single LD_{50} dose of sarin, whereas the brain regional AChE shows differential response to sarin treatment. ChAT activity was induced in the cortex followed by midbrain and brainstem. The increased ChAT activation may cause persistent long-term sarin toxicity even after AChE activity has recovered. Furthermore, our results indicate that sarin caused increased nAChR and m2-mAChR binding in the cortex after 6, 15, and 20 h of single-dose LD_{50} treatment. Finally, our data clearly suggest that sarin-induced neurotoxicity has multiple mechanisms. The eventual manifestation of sarin toxicity is primarily a dysregulation of the cholinergic system.
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