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# Gene expression and phosphoprotein profile of certain key neuronal signaling proteins following soman intoxication

G. RamaRao*, B.K. Bhattacharya, Subodh Kumar, C.K. Waghmare

*Defence Research and Development Establishment, Jhansi Road, Gwalior, M.P., India*

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ABSTRACT

Nerve agents irreversibly inhibit acetylcholinesterase (AChE), leading to cholinergic crisis and death at acute exposure levels. The complexity, delayed onset, and persistent nature of nerve agent induced CNS effects need to be elucidated to block their multiple effects. In the present study gene expression and phosphoprotein profile of certain key neuronal proteins were studied after soman exposure. Quantitative real time PCR analysis of c-Fos, Bax, CREB and caspase 3 genes in the hippocampus, cortex and cerebellum showed that only c-Fos and Bax mRNA expression was increased significantly. Western blot analysis also confirmed the induction of c-Fos at early time points both at 0.5 and 1.0 $LD_{50}$ dose of soman exposure. Acute soman exposure caused perturbations in the phosphorylation status of ERK, JNK, p38 MAPK, CREB, c-Jun and NF-κB in all the three brain regions. The primary target for soman toxicity, AChE was inhibited in blood and brain up to 90%. Therapeutic treatment comprising of HI-6, atropine and diazepam has completely protected animals from death and reactivated soman inhibited AChE up to 40% in the plasma and RBC. This therapeutic regime also reduced soman induced Bax expression to near control levels, but could not reverse the soman induced changes in c-Fos expression and phosphorylation levels completely. Results suggest that exposure to soman caused persistent changes in these key brain proteins, which could lead to the development of complex neurotoxic effects and there is an urgent need for development of better drugs to stop multiple effects of nerve agents poisoning.



## 1. Introduction

Chemical warfare agents were designed to kill or incapacitate enemy forces, disrupt military operations, and deny terrain to the adversary. Nerve agents bind to the active site of the AChE, thus preventing it from hydrolyzing acetylcholine resulting in the accumulation of acetylcholine and subsequent cholinergic crisis. The acute lethal effects of the nerve agents are generally due to respiratory failure caused by a combination of effects at both central and peripheral levels and are further complicated by copious secretions, muscle fasciculations, and convulsions (Taylor, 2001). These agents are highly lipophylic and readily penetrate the CNS and cause several neuropathological effects. Due to ubiquitous nature of cholinergic receptors and synaptic networks, exposure to organophosphorus (OP) compounds has wide spread consequences on several target organs. Among OP nerve agents, soman is one of the most studied, being one of the most dangerous and difficult to counteract. When it does not kill rapidly, soman produces epileptic seizures and related brain damage, which, if not treated immediately, may resist current therapies. The potential for exposure to nerve agents in a real world situation is likely to occur as a result of military operations, a terrorist incident, or accidental exposure, including demilitarization of weaponized material. Although it was originally thought that these agents act solely through inhibition of cholinesterase and consequent cholinergic hyper stimulation, it is now evident that there are multiple mechanisms that contribute to neuronal abnormalities (Duysen et al., 2001; Richards et al., 1999). There are numerous studies in both humans and animals showing that survivors of high level OP exposure can experience subtle but significant long term neurological effects that are detectable months or even years following the acute exposure (Damodaran, 2009; Miyaki et al., 2005; Kassa et al., 2001). In the event of nerve agent poisoning, immediate treatment with an anticholinergic drug, such as atropine sulphate, will antagonize the effects of excess acetylcholine at muscarinic receptor sites, and an oxime, such as pyridine-2-aldoxime methylchloride (2-PAM), is used to reactivate inhibited AChE (Moore et al., 1995; Taylor, 2001). This regimen, however, does not prevent nerve agent induced

* Corresponding author at: Biochemistry Division, Defence Research and Development Establishment, Jhansi Road, Gwalior, M.P., India. Tel.: +91 751 2341859; fax: +91 751 2341148.
*E-mail address:* ramugolime@gmail.com (G. RamaRao).

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irreversible brain damage that can result in long-term deficits in cognitive function and behavior (McDonough and Shih, 1997; Shih et al., 2003). The biological changes that lead to persistent impairments in cognition and sensorimotor function after nerve agent exposure are poorly understood.

Multiple intracellular signaling pathways that converge on MAPKs exist in all eukaryotic cells and play pivotal roles in a wide range of cellular functions. Activation of a receptor by neurotransmitters or any other stimuli leads to posttranslational modification (i.e. phosphorylation) of constitutively expressed transcription factors such as CREB, which then rapidly activate de novo transcription and translation of inducible transcription factors (ITFs) such as Fos and Jun (within minutes to hours). The transient expression of these ITFs then regulates the more persistent and delayed expression of ITF target genes. For example, it has been shown that pharmacological activation of central cholinergic receptors with the muscarinic agonist pilocarpine will induce transcription of c-Fos mRNA and detectable translation to Fos protein in both hippocampal and neocortical neurons within 30 min of the drug being injected intraperitoneally (Dragunow and Preston, 1995). In the nervous system, many proteins including neurotransmitter receptors, ion channels, cytoskeletal proteins, synaptic vesicle associated proteins, enzymes, and various regulatory proteins have been shown to be regulated by phosphorylation. It is generally recognized that phosphorylation/dephosphorylation of target proteins is involved in many cellular functions ranging from cell proliferation, differentiation, apoptosis, or degeneration by toxic chemicals (Gupta and Abou-Donia, 2001).

The possibility of further terrorist attacks with nerve agents and the escalating use of OP insecticides underscore the urgent need to develop effective and safe antidotes against OP poisoning. Recent gene expression studies during nerve agent exposure demonstrated several pathways in neurons including cholinergic, purinergic, NMDA-glutamatergic, GABAergic, catecholaminergic, serotogenic, calcium, and MAP kinase signaling along with molecules related to neurodegeneration, learning and memory, dementia/ataxia, mitochondrial dysfunction and apoptosis were altered significantly (Damodaran et al., 2006; Dillman et al., 2009; Bansal et al., 2009; Pachiappan et al., 2009). While most of these studies focused on changes at the mRNA levels, it would be interesting to carry out studies on changes at protein levels in different brain regions at time points spanning several days or weeks after toxicant exposure. These studies will help in developing a mechanistic view on the possible mode of initiation, modification, persistence or disappearance of distinct molecular changes that may cause cellular/tissue injury, leading to clinically and behaviourally observable effects and for the development of protective drugs with better central nervous system efficacy than currently available drugs such as atropine and diazepam.

## 2. Materials and methods

### 2.1. Chemicals

Soman and HI-6 were obtained from Process Technology Development Division of DRDE, Gwalior. Purity of soman was greater than 98%, as verified by gas chromatography. Mouse monoclonal antibodies for anti c-Fos (clone 2G9C3, Calbiochem) and anti-β actin (clone, Ac-15), atropine sulphate and all other chemicals were obtained from Sigma Chemicals Co. (St. Louis, U.S.A.), unless otherwise mentioned. Luminal and HRP substrate for chemiluminescence detection was purchased from Millipore Corporation (Bangalore, India). HRP conjugated anti mouse IgG was obtained from Dako Denmark A/S, DK2600 (Glostrup, Denmark).

### 2.2. In vivo exposure to soman

Wistar rats (100–120 g, 8–10 week old) were used in the present study. Animals were housed in polycarbonate cages with rice husk as bedding and maintained in controlled temperature and humidity on a standard 12 h light/dark cycle with free access to food and water. All animal experiments were approved by the Animal Care and Use Committee. Soman was dissolved in saline and administered to rats at 105 μg/kg ($1 \times LD_{50}$) through subcutaneous (s.c.) route and sacrificed at 15 min, 30 min, 2.5 h, 1, 3, 7, 14 and 30 days ($n = 4$ per each time point). 55 μg/kg ($0.5 \times LD_{50}$, s.c.) of soman was injected to 3 groups of rats and sacrificed at 15, 30 and 150 min time points ($n = 4$ per each time point). Vehicle (saline) control animals ($n = 4$ per each time point at 1, 7, 14 and 30 days time points) received an equivalent volume of 0.9% sodium chloride. In another set of experiments, animals were pretreated with the oxime HI-6 (50 mg/kg, i.m), 30 min prior to challenge with soman (210 μg/kg, s.c). One minute after soman exposure, animals were treated with atropine sulphate (10 mg/kg, i.m) followed by diazepam (5 mg/kg, i.m) on the onset of symptoms and sacrificed at 30 min, 2.5 h, 1, and 7 day ($n = 4$ per each time point) time intervals. Control animals of this group were injected with antidotes comprising of HI-6, (50 mg/kg, i.m), 30 min prior to challenge with saline instead of soman ($2 \times LD_{50}$). One minute later animals were treated with atropine sulphate (10 mg/kg, i.m) followed by diazepam (5 mg/kg, i.m) ($n = 4$; per each time point at 1 and 7 day). Rats were sacrificed by decapitation and brains were processed for further analysis. Samples were stored at −80 °C until use.

### 2.3. Assay of AChE activity (EC 3.1.1.7.)

AChE activity was assayed according to the method of Ellman et al. (1961), using acetylthiocholine iodide as the substrate. The enzyme activity was calculated as nmole substrate hydrolyzed $min^{-1}$ $ml^{-1}$ for plasma and nmole substrate hydrolyzed $min^{-1}$ $mg^{-1}$ protein for RBC and brain. Protein was estimated by Bradford method (1976).

### 2.4. RNA isolation

Total RNA was extracted from 50 mg of different regions of rat brain using RNeasy kit (Qiagen, Germany) following manufacturer's protocol. The purity and quantity of total RNA was determined by measuring absorbance at A260/A280 ratios and then A260, respectively, using a UV-Spectrophotometer (BioTek, U.S.A). RNA having high purity ratio ranging from 1.9 to 2.1 was used for further real time PCR studies.

### 2.5. Real-time RT-PCR

The quantitative real-time RT-PCR was carried out for the selected genes using gene specific primers from Quantitect primer assay Kit (Qiagen, Germany). QuantiFast one step RT-PCR kit was used for real time PCR and RNA polymerase-II (RP-II) was used as an endogenous reference gene. Briefly, the reaction mixture consisted of 12.5 μl of 2× QuantiFast SYBR Green RT-PCR Master Mix, 2.5 μl 10× Quantitect primer mix, 0.25 μl of Quantifast RT Mix, 100 ng (2 μl) of template RNA and 8.75 μl nuclease free water in 25 μl reaction volume. The Roche Light cycler-480 system was used to monitor the SYBR Green signal at the end of each extension period for 40 cycles. The thermal profile consisted of 10 min of reverse transcription at 50 °C for one cycle and 5 min of polymerase activation at 95 °C, followed by 40 cycles of PCR at 95 °C for 10 s, 60 °C for 30 s for combined annealing/extension. The relative quantification levels in expression were determined using the 2nd derivative maximum analysis with the determination of the crossing points for each transcript. Crossing point values for each gene were normalized to the respective crossing point values for the reference gene RP-II. Data are presented as normalized ratios of genes along with standard error using Roche Applied Science E-Method (Tellmann and Olivier, 2006).

### 2.6. Immunoblotting

Hippocampus, cerebellum and piriform cortex tissues were homogenized in buffer containing 10 mM Tris, pH 7.6, 0.5 M sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and a protease inhibitor mixture (1 mM PMSF, 2 μg/ml aprotinin, leupeptin, and pepstatin A). The crude nuclear fraction was isolated by centrifugation at $4000 \times g$ for 5 min at 4 °C. The nuclear pellet was resuspended in a lysis buffer containing 20 mM Tris (pH 7.6), 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3 M NaCl, 0.5 mM dithiothreitol, and 0.5 mM PMSF. Nuclear proteins were derived from the supernatant following centrifugation at $12{,}000 \times g$ for 20 min at 4 °C. After measuring protein concentrations using Bradford method (1976), equal amounts of protein (40 μg) was diluted in 2× Laemmli's buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred on to PVDF membranes and blocked with 5% non-fat dried milk dissolved in PBS (pH 7.5). Immunoblotting was carried out with anti-c-Fos antibody at 1:1000 dilution for overnight at 4 °C. PVDF membranes were washed thrice in PBS containing 0.05% Tween 20 and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody at 1:3000 dilution for 1 h at room temperature. Bands were developed by chemiluminescence detection using Luminol substrate. Immunoreactive bands of proteins were quantified as optical density (OD) by using Bio-Rad Quantity one software.