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BRAIN RESEARCH

Research Report

Nerve agent exposure elicits site-specific changes in protein phosphorylation in mouse brain

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ABSTRACT

Organophosphorus (OP) compounds cause toxic symptoms, including convulsions, coma, and death, as the result of irreversible inhibition of acetylcholinesterase (AChE). The development of effective treatments to block these effects and attenuate long-term cognitive and motor disabilities that result from OP intoxication is hampered by a limited understanding of the CNS pathways responsible for these actions. We employed a candidate method (called CNSProfile™) to identify changes in the phosphorylation state of key neuronal phosphoproteins evoked by the OP compound, diisopropyl fluorophosphate (DFP). Focused microwave fixation was used to preserve the phosphorylation state of phosphoproteins in brains of DFP-treated mice; hippocampus and striatum were analyzed by immunoblotting with a panel of phospho-specific antibodies. DFP exposure elicited comparable effects on phosphorylation of brain phosphoproteins in both C57BL/6 and FVB mice. DFP treatment significantly altered phosphorylation at regulatory residues on glutamate receptors, including Serine897 (S897) of the NR1 NMDA receptor. NR1 phosphorylation was bi-directionally regulated after DFP in striatum versus hippocampus. NR1 phosphorylation was reduced in striatum, but elevated in hippocampus, compared with controls. DARPP-32 phosphorylation in striatum was selectively increased at the Cdk5 kinase substrate, Threonine75 (T75). Phencynonate hydrochloride, a muscarinic cholinergic antagonist, prevented seizure-like behaviors and the observed changes in phosphorylation induced by DFP. The data reveal region-specific effects of nerve agent exposure on intracellular signaling pathways that correlate with seizure-like behavior and which are

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Abbreviations: OP, organophosphorus; PCH, phencynonate hydrochloride; AChE, acetylcholinesterase; DFP, diisopropyl fluorophosphate; Cdk5, cyclin-dependent kinase-5

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reversed by the muscarinic receptor blockade. This approach identifies specific targets for nerve agents, including substrates for Cdk5 kinase, which may be the basis for new anti-convulsant therapies.

1. Introduction

Organophosphorus (OP) cholinesterase inhibitors, including sarin, soman, and the insecticide diisopropylfluorophosphate (DFP), irreversibly inhibit the primary metabolic enzyme acetylcholinesterase (AChE), blocking the breakdown of the neurotransmitter acetylcholine (ACh). The resulting overactivity of cholinergic neurotransmission at peripheral nerve endings and throughout the central nervous system (CNS), leads to toxic symptoms, including convulsions, coma, and death (Taylor, 1985). Further, individuals who survive the acute phase of high-level nerve agent exposure (e.g., survivors of the Tokyo sarin attack) often experience persistent impairments in complex behaviors, including cognition (Suzuki et al., 1997; Nozaki et al., 1995; Miyaki et al., 2005). Individuals sustaining sub-chronic or low-level exposure to OP agents (e.g., Persian Gulf War veterans; agricultural workers) also display persistent learning and memory impairments (Rohlman et al., 2007; Golomb, 2008). Experimental animals exposed to sub-lethal doses of soman nerve gas display spatial learning and sensorimotor processing deficits that can be measured months after exposure (Shih et al., 2003).

The biological changes that lead to persistent impairments in cognition and sensorimotor function after nerve agent exposure are poorly understood. Nerve agents evoke massive and sustained elevations in brain levels of ACh (Lallement et al., 1992), which activate muscarinic-type and nicotinic-type cholinergic receptors throughout the brain, and ultimately flood intracellular signaling responses and open voltage-gated ion channels (Felder, 1995; Hogg et al., 2003). The resultant long-term deficit of cholinergic neurotransmission is one likely contributor to persistent cognitive deficits. Loss or dysregulation of ACh-containing neurons in the basal forebrain, which includes cholinergic inputs to the hippocampus, would impact spatial memory function. Degeneration of cholinergic neurons in the hippocampus and cortex is associated with the loss of cognitive function in Alzheimer's disease (Mesulam, 2004). Additionally, cholinergic interneurons within the striatum contribute to complex forms of learning that require behavioral flexibility (e.g., reversal learning) (Ragozzino et al., 2009). However, simple loss of cholinergic neurons does not entirely explain the persistent effects of nerve agent exposure. Non-cholinergic neurotransmitter systems are also impacted by nerve agent exposure and may contribute to continuing cognitive and motor dysfunction. The initial phase of cholinergic hyperactivity following nerve agent exposure in animals predictably leads to delayed phases of hyperactive glutamatergic and GABAergic neurotransmission that are independent of continued cholinergic receptor activation (Lallement et al., 1991; Lallement et al., 1992; Shih et al., 2003). It is hypothesized that this sequential activation of cholinergic neurotransmission, followed by non-cholinergic neurotransmission persisting for hours after the initial exposure, results in cognitive and motor deficits in animals (Shih et al., 2003; Phillippens et al., 1992). In support of this hypothesis, actions of nerve agents in the CNS rapidly become non-responsive to reversible cholinergic receptor blocker treatments such as atropine (Shih and McDonough, 1997). Therefore, effective treatments that can prevent or reverse the effect of OP intoxication after the initial cholinergic activation phase are needed to attenuate persistent cognitive and motor disabilities. However, development of these agents has been hampered by our limited understanding of the non-cholinergic substrates for OP compounds.

Since multiple neurotransmitter systems are recruited by nerve agent exposure, points of signal integration might be exploited to develop effective treatments able to prevent the long-term consequences of nerve gas exposure. For example, the second messenger pathways that regulate glutamatergic neurotransmission via protein phosphorylation are potential signal transduction targets for the persistent effects of nerve agents on cognition and motor performance. The present study is designed to determine whether nerve agents and other compounds that elevate brain cholinergic neurotransmission leave a distinct neuronal imprint in the form of the phosphorylation of critical signaling molecules. For this purpose we have analyzed the effects of DFP on protein phosphorylation in the mouse brain using CNSProfile, a candidate method that characterizes the intracellular actions of drugs by focusing upon representative phosphorylation sites/phosphoproteins that are involved in the regulation of neuronal excitability. These studies provide insight into the molecular mechanisms underlying the biological effects of nerve agents.

2. Results

2.1. *DFP treatment of female C57BL/6 mice elicits robust seizure-like behavior*

Administration of DFP to female C57BL/6 mice consistently generally induced robust seizure-like behaviors within minutes following intraperitoneal (i.p.) injection, often leading to death. The effects of DFP were dose-dependent over a narrow dose range; a 2 mg/kg dose of DFP did not elicit seizure-like behavior, whereas 3 mg/kg and 4 mg/kg doses did elicit seizure-like behavior within 5 min post-injection. The mean seizure scores for animals in each DFP treatment group are shown in Fig. 1.

2.1.1. *DFP exposure elevates DARPP-32 phosphorylation selectively at the Cdk5 substrate, T75*

DFP treatment significantly increased the state of phosphorylation of DARPP-32 in striatum of female C57BL/6 mice. The increase was detected as a selective increase at T75, a residue