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Protection of DFP-Induced Oxidative Damage and Neurodegeneration by Antioxidants and NMDA Receptor Antagonist

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Abstract

Prophylactic agents acutely administered in response to anticholinesterases intoxication can prevent toxic symptoms, including fasciculations, seizures, convulsions and death. However, anticholinesterases also have long-term unknown pathophysiological effects, making rational prophylaxis/treatment problematic. Increasing evidence suggests that in addition to excessive cholinergic stimulation, organophosphate compounds such as diisopropylphosphorofluoridate (DFP) induce activation of glutamatergic neurons, generation of reactive oxygen (ROS) and nitrogen species (RNS), leading to neurodegeneration. The present study investigated multiple affectors of DFP exposure critical to cerebral oxidative damage and whether antioxidants and NMDA receptor antagonist memantine provide neuroprotection by preventing DFP-induced biochemical and morphometric changes in rat brain. Rats treated acutely with DFP (1.25 mg/kg, s.c.) developed onset of toxicity signs within 7-15 min that progressed to maximal severity of seizures and fasciculations within 60 min. At this time point, DFP caused significant ($p<0.01$) increases in biomarkers of ROS (F_2-isoprostanes, F_2-IsoPs; and F_4-neuroprostanes, F_4-NeuroPs), RNS (citrulline), and declines in high-energy phosphates (HEP) in rat cerebrum. At the same time, quantitative morphometric analysis of pyramidal neurons of the hippocampal CA1 region revealed significant ($p<0.01$) reductions in dendritic lengths and spine density. When rats were pretreated with the antioxidants *N-tert*-butyl-α-phenylnitrone (PBN, 200 mg/kg, i.p.), or vitamin E (100 mg/kg, i.p./day for 3 days), or memantine (18 mg/kg, i.p.), significant attenuations in DFP-induced increases in F_2-IsoPs, F_4-NeuroPs, citrulline, and depletion of HEP were noted. Furthermore, attenuation in oxidative damage following antioxidants or memantine pretreatment was accompanied by rescue from dendritic degeneration of pyramidal neurons in the CA1 hippocampal area. These findings closely associated DFP-induced lipid peroxidation with dendritic degeneration of pyramidal neurons in the CA1 hippocampal area and point to possible interventions to limit oxidative injury and dendritic degeneration induced by anticholinesterase neurotoxicity.

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Introduction

Pesticide residues are now among the most ubiquitous synthetic chemicals in our environment, as they are detectable in the tissues of humans, animals, aquatic and wildlife worldwide. Of the wide variety of pesticides available, organophosphate (OP) and carbamate (CM) insecticides are the most commonly used and encountered in accidental, suicidal, and occupational poisonings. Presently, more than 100 different OPs are used as insecticides worldwide (Kwong, 2002; Gupta, 2006). The widespread use and easy accessibility to these compounds result in a significant number of intoxications and several hundred thousand fatalities annually (Gunnell and Eddleston, 2003). Other derivatives of phosphoric or phosphonic acid, such as chemical warfare nerve agents, are considered to be the most toxic compounds among all chemical weapons (Watson et al., 2006, 2009). Therefore, OP compounds (industrial chemicals or weapons of mass destruction) pose a potential threat to civilians as well as military personnel.

Pharmacologically, OPs and CMs are acetylcholinesterase (AChE) inhibitors and their acute symptoms are attributed to accumulation of acetylcholine (ACh), thus exhibiting the signs of cholinergic hyperactivity. Depending upon the degree of AChE inhibition, the severity of poisoning can vary from mild (mild dyspnea, blurred vision and glandular hypersecretion) to severe (severe dyspnea, skeletal muscle fasciculations, convulsions and unconsciousness) cases, and eventually death ensues from respiratory failure (Goldfrank et al., 1982; Weinbroum, 2005).

However, anticholinesterases have long-term pathophysiological effects that are not yet well characterized, making rational prophylaxis and treatment for these effects problematic. Long-term neurological impairments following anticholinesterase exposure including: (a) an intermediate syndrome (IMS) affecting muscles, which can occur within 24 to 96 hours following recovery from severe acute affects (De Bleecker, 2006); (b) a delayed peripheral polyneuropathy associated with some anticholinesterases, that usually occurs within weeks following an acute exposure (Lotti, 1992: Lotti and Moretto, 2005); and (c) subtle, long-term neurological effects which may last months or even years (Behan, 1996; Jamal, 1997). Anticholinesterase initiation of adverse health effects is also associated with potential involvement of glial cells in the neurotoxicity of OPs (Aschner, 2000). Neuronal injury caused by seizures is accompanied by an inflammatory reaction involving gliosis, and induction of inflammatory mediators including prostaglandins, cytokines, cell adhesion proteins and matrix metalloproteinases (Jorgensen et al., 1993; Vezzani et al., 2002; Jourquin et al., 2003; Lehtimaki et al., 2003; Borges et al., 2003). Animals exposed to soman at doses producing convulsions exhibit a rapid increase in active astrocytes and the accumulation of glial fibrillatory acidic protein (GFAP) (Zimmer et al., 1997). In addition to oxidative damage and interference with adenylyl cyclase cell signaling, chlorpyrifos inhibits DNA synthesis to a greater extent in glioma cell lines (C6 cells) than neuronal cell lines (PC12) (Garcia et al., 2001). These effects are independent of cholinergic receptors as the cholinergic antagonist fails to block chlorpyrifos-induced inhibition of DNA synthesis. The findings are also consistent with exposures to another OP compound, diazinon, suggesting that anticholinesterase compounds target glial cells by additional mechanisms of cholinergic toxicity (Walker and Nidiry, 2002).

Involvement of non-cholinergic mechanisms in OP toxicity is also supported by evidence suggesting that anticholinesterases induce activation of glutamatergic neurons. For example, soman-induced seizures increased extracellular glutamate in the pyriform cortex (Wade et al., 1987) and cornu ammonis (CA) region of the hippocampus (Lallement et al., 1992) followed by activation of *N*-methyl-D-aspartate (NMDA) receptors in the CA1 region. Overstimulation of glutamate receptors causes synaptic and cellular degeneration in the hippocampus (Siman

et al., 1989; Bahr et al., 2002, Munirathinam and Bahr, 2004). Excitotoxicity in hippocampal neurons is also associated with enhanced vulnerability to other types of neuropathogenesis (Bahr et al., 1994). Moreover, glutamate stimulates ACh release (Anderson et al., 1994), further contributing to excitatory stimulation and prolongation of the seizures, and thus like a brushfire, it propagates excitotoxic neurodegeneration in vulnerable brain regions. Microdialysis studies revealed an immediate increase in extracellular glutamate concentrations in the septum, pyriform cortex, hippocampal regions and amygdala following soman-triggered seizures (Lallement et al., 1991a, b; Wade et al., 1987). Furthermore, blockage of specific glutamate receptors reduces neuropathogenic responses, including nerve agents' toxicity (Sheardown et al., 1990; Sparenborg et al., 1992). In addition to the activation of NMDA receptors, glutamate release also leads to massive Ca^{2+} fluxes into the post-synaptic cells, generation of reactive oxygen (ROS) and nitrogen species (RNS), ensuing in neurodegeneration. Elevation of cytosolic free Ca^{2+} leads to derangement of many intracellular processes that normally regulate Ca^{2+} sequestration and energy metabolism (Siesjo, 1988). Modulations of Ca^{2+}, glutamate and NMDA receptors also induce some other biochemical mechanisms such as oxidative stress which further compromise cell viability.

There are many methods to quantify oxidative damage to tissues, but in the CNS no method distinguishes oxidative damage between neurons and glia. This is potentially a serious limitation because glia outnumbers neurons with a further increase in this ratio in neurodegenerative diseases. We have shown previously that free radical damage to the brain can be sensitively and accurately quantified by measuring chemically stable oxidative damage products of arachidonic acid (AA) and docosahexaenoic acid (DHA); F_2-IsoPs and neuroprostanes (F_4-NeuroPs), respectively (Morrow et al., 1990; Milatovic and Aschner, 2009). AA is relatively evenly distributed in brain with similar concentrations in gray matter and white matter, and within glia and neurons. Thus, F_2-IsoPs quantification is a reflection of oxidative damage to the brain in general and F_4-NeuroPs in particular. Unlike AA, DHA is highly concentrated in neuronal membranes to the exclusion of other cell types. Moreover, F_4-NeuroPs are by far the most abundant products of this pathway in the brain (Reich et al., 2000; 2001). Thus, quantification of F_4-NeuroPs provides a highly selective quantitative window for neuronal oxidative damage *in vivo*.

In this study, we have used diisopropylphosphorofluoridate (DFP) as a model compound for OP insecticides or nerve agents, and investigated non-cholinergic mediated activities in rat cerebrum. Novel biomarkers of neuronal oxidative (F_4-NeuroPs) and nitrosative (citrulline) damage and Neurolucida-assisted neuronal tracings were employed to explore the mechanisms involved in OP-induced neurotoxicity. Different pharmacological tactics were utilized to attenuate oxidative/nitrosative damage induced by anticholinesterase exposure and investigate extent to which such attenuation is accompanied by protection of dendritic damage in the CA1 sector of hippocampal neurons.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing about 200 g (7-8 weeks old), purchased from Harlan Laboratories (Indianapolis, IN, USA), were used in this investigation. They were housed five per cage in a room with controlled conditions: temperature 21±1 °C, humidity 50±10 %, and 12-h/12-h light/dark cycle. Animals had free access to pelleted food and tap water. Rats were acclimatized to these conditions for 7-10 days before being used. During the treatment, rats were placed in individual cages. The animal facility is approved by the Institutional Animal Care and Use Committee (IACUC), and is under the supervision of a veterinarian. All experiments were conducted in accordance with the guidelines from the National Institutes of Health (USA), with adequate measures taken to minimize any discomfort to the rats.

Drugs and Chemicals

Diisopropylphosphorofluoridate (DFP), vitamin E (α-tocopherol, 97%), *N-tert*-butyl-α-phenylnitrone (PBN), acetylthiocholine iodide, adenosine 5′-triphosphate (ATP), phosphocreatine (PCr) and L-citrulline were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO., USA). Memantine HC1 (MEM) was purchased from Panorama Research, Inc (Mountain View, CA). All other chemicals used were of highest purity and were purchased from Cayman Chemical Company (Ann Arbor, MI), VWR (West Chester, PA) or Fisher Scientific (Fair Lawn, NJ, USA).

Experimental Design and Animal Treatment

In time-course experiments, the levels of F_2-isoprostanes (F_2-IsoPs) and F_4-neuroprostanes (F_4-NeuroPs) were assayed in brain at 30 min, 1 h, 2 h or 6 h post-DFP (1.25 mg/kg, s.c.) injection in rats. The rationale for selecting the DFP dose was based on our previous reports (Gupta et al., 2001a, b; Zivin et al., 1999; Milatovic et al., 2006) showing that 1.25 mg/kg of this anticholinesterase provide sings of maximal severity without causing the fatality. Following DFP administration, rats showed the signs of onset within 7-15 min and signs of maximal severity within 30 to 60 min that lasted for about 2 to 3 h. Rats in the control group received normal saline (1 ml/kg, sc). In protection experiments, pretreatment with memantine HCl (MEM, 18 mg/kg, i.p.) or *N-tert*-butyl-α-phenylnitrone (PBN, 200 mg/kg, i.p.) was given as a single injection 60 min (time of maximum distribution of MEM, Wesemann et al., 1983) or 30 min before DFP, respectively. Supplemental vitamin E (100 mg α-tocopherol/kg body wt., i.p.) was given daily for 3 days (48 hours, 24 hours, and 30 min before DFP exposure). To test protective effects of these drugs, rats were sacrificed 1 hour after the last treatment.

Biochemical assays

Quantitation of F_2-IsoPs and F_4- NeuroPs as a marker of ROS—At a predetermined time, rats were sacrificed and brains were rapidly harvested, the cerebral hemispheres flash frozen in liquid nitrogen, and stored at −80 °C until analysis. F_2-IsoPs and F_4-NeuroPs were determined with a stable isotope dilution method using a gas chromatograph coupled with a mass spectrometer (negative ion chemical ionization and selective ion mode) as previously described (Morrow et al., 1990; Milatovic et al., 2003; Milatovic and Aschner, 2009).

Quantitation of nitric oxide (NO) as a marker of RNS—The levels of citrulline (the co-product of NO synthesis) were determined by the reversed-phase HPLC method of Bagetta et al. (1995) with minor modifications (Gupta et al., 2001b; Gupta et al., 2007). Tissues were homogenized in a aliquot of 0.4 M perchloric acid using a Brinkman homogenizer with a PT-10 probe, followed by sonication for 10 s with a Biosonic Cell Disruptor equipped with a microprobe. The homogenates were allowed to extract for 30 min at ice-cold temperature, followed by centrifugation (10,000 rpm for 20 min at 4°C) using a Sorvall centrifuge (RC 26 plus). The supernatants were aspirated and neutralized to pH 7 with 1 M KOH before being centrifuged again to remove fine precipitate ($KClO_4$). The supernatants were derivatized with OPA and assayed for citrulline concentrations using an HPLC system coupled with a fluorescence detector (excitation at 334 nm and emission at 440 nm). The data are expressed as nmol citrulline/g wet tissue weight.

Measurement of ATP and Phosphocreatine—Levels of ATP and phosphocreatine (PCr) were measured in the perchloric acid extracts (as described above for citrulline), using an HPLC method (Gupta et al., 2001a; 2007). The Waters HPLC system was coupled with a UV detector (Model 2487), which allowed simultaneous determination of ATP and PCr at a wavelength 206 nm.

Quantitative morphology of pyramidal neurons

Quantitative neuronal analysis was conducted on a tissue stained with Golgi impregnation that was uniform throughout the section. Length of dendrites and spine density counts of pyramidal neurons were evaluated in Golgi impregnated 50 microns thick hippocampal sections from paraffin-embedded blocks prepared according to the manufacturer's specifications (FD Rapid GolgiStain Kit). Six or more pyramidal neurons with no breaks in staining along the dendrites from the CA1 area of the hippocampus were selected and spines counted according to the published methods (Leuner et al., 2003; Milatovic et al., 2003). Tracing and counting were performed with a Neurolucida system at 100× under oil immersion (MicroBrightField, VT). Dendritic systems were also quantified to a centrifugal nomenclature by Sholl-method (Scholl, 1953) where spine density and length of dendrites arising from the soma are in the first- (50 μm), second- (50 μm -100 μm) and third-order segments (100 μm -150 μm) from the center of the soma.

Statistical analysis

The data presented are means ± SEM of 4-6 rats in each group. Statistical significance between groups was determined by analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test with statistical significance set at $p<0.05$. All analyses were carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA, USA).

Results

A single injection of DFP with an acute dose of 1.25 mg/kg, s.c. produced toxic signs in rats, including salivation, tremors, wet dog shakes, fasciculations with rearing and rolling over within 15- 20 min. Signs of maximal intensity, such as severe muscle fasciculations, seizures, and convulsions developed within 30 min and lasted for about 2 – 3 h before tapering off. By 24 h, animals were free of toxic signs. DFP- induced signs were of typical hypercholinergic preponderance involving both the central and peripheral nervous systems as confirmed by markedly depressed AChE activity. Following DFP exposure, brain AChE activity was reduced to 10.12 ± 1.12 %, 10.05 ± 1.96 %, 14.30 ± 1.41 % and 16.51 ± 1.27% compared to control (569.12 ± 26.80 μmol/hr/g of tissue) at 30 min, 1 h, 2h and 6h, respectively.

Time-course analysis of cerebral biomarkers of oxidative damage revealed significant increases in F_2-IsoPs (142%) and F_4-NeuroPs (225%) as early as 30 min after a single acute exposure to DFP (1.25 mg/kg, sc). Both F_2-IsoPs and F_4-NeuroPs showed transient increase reaching maximum levels at 1h with a return to basal levels by 6 h (Fig. 1). The selective increase in F_4-NeuroPs indicates that neurons are specifically targeted by this mechanism (Fig. 1b). Thus, a one-time challenge with DFP produced a transient increase in both F_2-IsoPs and F_4-NeuroPs, which are sensitive and specific *in vivo* biomarkers of oxidative damage to AA and DHA, respectively.

In an attempt to discern additional mediators of oxidative damage in the rat model of DFP-induced neurotoxicity, the levels of citrulline and high- energy phosphates (HEPs: ATP and PCr) were determined in the cerebral hemispheres of rats 1 h after DFP exposure. NOS catalyzes the conversion of arginine to NO and citrulline, and quantification of citrulline is widely employed as an *in vivo* marker of NOS/NO activity. While citrulline levels were increased > 3-fold compared to control (139.2 ± 8.4 nmol/g tissue), significant depletion of ATP and PCr (47% and 38 % compared to control values (2.23 ± 0.05 and 8.90 ± 0.33 μmol/g tissue, respectively) was observed in brain of rats 1 h post-DFP injection. Alterations in HEPs are considered as indicators of mitochondrial dysfunction that increases neuronal vulnerability to injury.

We next investigated if neuronal oxidative/nitrosative damage and depletion of energy metabolites is accompanied by altered integrity of dendritic system. Data revealed that high intensity of seizures and peak of oxidative damage to neuronal membranes at 1 h coincided with dendritic degeneration of pyramidal neurons in the CA1 hippocampal area. Representative images of Golgi impregnated hippocampal sections with their traced pyramidal neurons from control and DFP-exposed animal are presented in Figure 2. Images of neurons with Neurolucida-assisted morphometry show that DFP-induced brain hyperactivity targeted the dendritic system with profound dendrite regression of hippocampal neurons. Dendritic morphology of randomly selected pyramidal neurons from CA1 hippocampal area from control and DFP-exposed animals was also evaluated by the Sholl method of concentric circles (Fig. 3). Sholl analysis represents a quantitative method for morphometric neuronal studies with consecutive-circles (50, 51-100, 101-150 μm from the center of soma) that analysis specifies dendritic geometry. Results of the present investigation demonstrate that DFP treatment caused a significant decrease in total dendrite length in the proximal (0-50 μm) and intermediate (51-100 μm) Sholl compartment of CA1 pyramidal neurons (Fig. 3a). Results also revealed that DFP treatment induced a significant decrease in spine density (number of spines per 100 μm of dendrites) in all three (proximal, intermediate and distal) Sholl compartments of CA1 pyramidal neurons (Fig. 3b).

The last part of this investigation determined the effectiveness of two antioxidants (vitamin E and PBN) and NMDA receptor antagonist memantine to suppress DFP-induced oxidative/nitrosative damage and neurodegeneration. Vitamin E, PBN and memantine treatment in non-DFP exposed rats did not alter basal levels of F_2-IsoPs, F_4-NeuroPs, citrulline, ATP and PCr in brain. But when given as pretreatment E, PBN or memantine completely suppressed DFP-induced alterations in markers of neuronal oxidative damage (Fig. 4), nitrosative damage (Fig. 5), and depletion of HEPs (Fig. 6). Vitamin E, PBN or memantine pretreatment also completely protected against DFP- induced declines in dendrite length (Fig. 7a) and dendritic spine density (Fig. 7b) when measured 1 h after DFP exposure.

Discussion

Previous reports have highlighted the involvement of non-cholinergic mechanisms involved in OP induced toxicity (McDonough and Shih, 1997; De Groot et al., 2001). Several lines of evidence have suggested that excessive cholinergic stimulation following anticholinestrase exposure is associated with activation of glutamatergic neurons, NMDA receptors, Ca^{2+} fluxes into the post-synaptic cells and generation of ROS/RNS, ensuing in neurodegeneration. The present study explores the mechanisms associated with OP-induced neurotoxicity by probing their effects on oxidative/nitrosative stress and associated dendritic degeneration. Findings revealed that DFP induced reversible oxidative damage to cerebral neuronal membranes, alterations in NOS/NO and impairment of mitochondrial function as evidenced by significant depletion of ATP and PCr. Furthermore, data demonstrated that DFP-induced increases in biomarkers of global free radical damage (F_2-IsoPs) and the selective peroxidation biomarkers of neuronal membranes (F_4-NeuroPs) were accompanied by dendritic degeneration of pyramidal neurons in the CA1 hippocampal area. Importantly, results demonstrated that both neuronal oxidative damage and dendritic degeneration of CA1 pyramidal neurons induced by DFP were completely suppressed by the antioxidants (vitamin E or PBN) or the NMDA receptor antagonist (memantine).

The present results corroborate the findings strongly suggesting that the non-cholinergic system (s) is recruited at an early stage of the OP poisoning (Lallement et al., 1991a, b; Wade et al., 1987). Excessive amounts of glutamate are associated with intense transient influx of Ca^{2+}, leading to mitochondrial structural and/or functional impairments characterized by activation of the permeability transition pores in the inner mitochondrial membrane, cytochrome c release,

depletion of ATP and simultaneous formation of ROS (Heinemann et al., 2002; Cadenas and Davies, 2000; Patel, 2002; Nicholls and Ward, 2000; Nicholls et al., 2003). In addition, increase in cytoplasmic Ca^{2+} triggers intracellular cascades through stimulation of enzymes, including proteases, phospholipase A_2, and NOS, which also lead to increased levels of ROS and oxidative stress (Lafon-Cazal et al., 1993; Farooqui et al., 2001). The present data demonstrated that DFP - induced reversible oxidative damage to cerebral neuronal membranes accompanied with depletion of HEP. Oxidative injury was quantified by measuring F_2-IsoPs and lipid peroxidation of AA by free radicals. In a recent multi-investigator study, i.e., Biomarkers of Oxidative Stress Study (BOSS), sponsored by the National Institutes of Health, it was suggested that the quantification of F_2-IsoPs represents the most accurate method to assess oxidative stress status *in vivo* (Kadiiska et al., 2005). Furthermore, quantification of F_4-NeuroPs (oxidative damage to DHA – highly concentrated in neuronal membranes) provided unique insight into oxidative damage occurring in neurons. Both biomarkers of oxidative damage showed transient increase that reached maximum at the time of the most intensive seizure activity (i.e., 1 h after DFP) and returned toward basal levels by 6 h (Fig. 1). Activation of nNOS and generation of NO, as evidenced by elevation of citrulline at 1 h post-DFP exposure (Fig. 5), may be stimulated by elevation of intracellular Ca^{2+}. Increased NO in the presence of superoxide anion radical (O_2^-) generates the peroxynitrite radical ($OONO^-$), a powerful oxidant exhibiting a wide array of tissue damaging effects ranging from lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation and nitration to inhibition of mitochondrial respiration (Montine et al., 2002; Milatovic et al., 2002; Virag et al., 2003). NO is involved in glutamate receptor-mediated neurotoxicity by decreasing intracellular ATP levels. There are two possible mechanisms responsible for energy depletion caused by NO in neuronal cells. One is the prolonged activation of poly-(ADP ribose) polymerase (Zhang et al., 1994), leading to depletion of ATP. The other mechanism is the inhibition of the mitochondrial complexes, leading to diminished ATP production. NO impairs mitochondrial/cellular respiration and other functions by inhibiting the activities of several key enzymes, particularly cytochrome c oxidase, and thereby causing ATP depletion (Yang and Dettbarn, 1998; Milatovic et al., 2001; Gupta et al., 2001a, b; Dettbarn et al., 2001). NO was also reported to inhibit complexes II and III (Bolanos et al., 1994), as well as complex IV (Lizasoain et al., 1996) in neuron-derived mitochondria and neuronal energy production in cultured hippocampal neurons (Brorson et al., 1999), leading to rapid ATP depletion. Results of the present study confirmed that an increase in the levels of cerebral citrulline (Fig. 5) was accompanied by depletion of ATP and PCr (Fig. 6) in rats 1 h after DFP administration.

Excitotoxic levels of glutamate with influx of Ca^{2+} leading to mitochondrial dysfunction are also involved in anticholinesterase-induced synaptic architectural changes (Munirathinam and Bahr, 2004). Although seizures can induce neuronal death, they may also have “nonlethal” pathophysiological effects on neuronal structure and function. Dendritic spines represent the structural sites of contact for the majority of excitatory, glutamatergic synaptic inputs onto neurons and are strongly implicated in mechanisms of synaptic plasticity and learning (Rao and Craig, 1997; O'Brien et al., 1998). NMDA and other glutamate receptor subtypes are clustered in dendritic spines (Yuste and Denk, 1995; Rao and Craig, 1997; O'Brien et al., 1998) suggesting that receptor localization at synapses might be critical to excitotoxicity. Findings of the present study with the Sholl method of concentric circles confirmed an early neuronal damage and showed that anticholinesterase-induced brain hyperactivity targeted the dendritic system with profound dendrite regression of hippocampal neurons. The data also demonstrated that DFP-induced oxidative damage to neuronal membranes is associated with degeneration of the pyramidal dendritic system in the CA1 hippocampal area.

An additional goal of this study was to determine whether suppression of lipid peroxidation prevents neurodegeneration of pyramidal neurons in the CA1 hippocampal area in the model of DFP-induced neurotoxicity. Therefore, efficacy of the antioxidants (vitamin E and PBN)

was evaluated. Antioxidants play an important role in preventing many human diseases, including but not limited to cancer, atherosclerosis, stroke, rheumatoid arthritis and neurodegeneration (Fang et al., 2002). Vitamin E has been recognized as one of the most potent and important antioxidants. It acts as a chain- breaking antioxidant and radical scavenger and thus protects cells from peroxidation of PUFA in phospholipids and consequent membrane degeneration (Topinka et al., 1989; VanAcker et al., 1993). Decreased level of vitamin E in response to hyperoxia or treatment with a convulsant (Mori et al., 2004; Onodera et al., 2003; Rauca et al., 2004) suggested that it is consumed to prevent oxidative damage. In addition, vitamin E maintains oxidative phosphorylation in mitochondria, and accelerates restitution of high-energy metabolites (Punz et al., 1998; Kotegawa et al., 1993; Milatovic et al., 2005b).

The synthetic spin trapping agent, *N*-*tert*-butyl-α-phenylnitrone (PBN) is also an efficient scavenger of free radicals. PBN is widely used to trap ROS in a variety of physical, chemical and biological conditions. PBN concentrates in the mitochondria, where it reacts with ROS and forms stable adducts, and thereby maintains normal levels of energy metabolites. The protective effects of PBN have been described in experimental models of brain ischemia/reperfusion (Phillis and Clough-Helfman, 1990; Carney and Floyd,1991; Gido et al., 1997; Fetcher et al., 1997), excitotoxicity (Cheng and Sun,1994; Lancelot et al., 1997; Milatovic et al., 2002; Zaja-Milatovic et al., 2008), inhibition of NOS induction (Krishna et al., 1996; Miyajima and Kotake, 1997) and in different models of seizures (He et al., 1997; Thomas et al., 1997). Thus, these agents have been proven to rescue neurons in multiple experimental injury models. Results of the present study confirmed protective effects of these antioxidants and showed that both agents fully suppressed DFP-induced increases in cerebral and neuronal markers of oxidative damage, F_2-IsoPs and F_4-neuroPs, respectively (Fig. 4). In addition to attenuating DFP-induced increased production of citrulline and depletion of ATP and PCr (Fig. 5 and 6), both antioxidants fully protected hippocampal CA1 pyramidal neurons from dendritic degeneration (Fig. 7). Vitamin E pretreatment did not prevent DFP- seizure severity, indicating that its protective effect is most likely mediated by scavenging ROS, thus preventing lipid peroxidation and consequent neuronal damage. However, PBN pretreatment 1 h prior to DFP exposure substantially decreased the intensity of induced seizures. Corroborating with our previous studies, this effect appears to reflect the protective interaction of PBN with AChE, sufficient to protect a critical fraction of AChE against phosphorylation by DFP (Zivin et al., 1999; Milatovic et al., 2000a, b). Despite the protective interaction of PBN with AChE and NOS, the suppression of lipid peroxidation and the parallel reduction in neuronal damage following the increase in vitamin E and PBN, strongly supports oxidative stress mechanisms as causal mediators of DFP-induced seizures and neurodegeneration.

Since excitotoxicity induced neuronal damage in the model of anticholinesterase- seizures is associated with excessive release of glutamate, we tested if the NMDA receptor antagonist, memantine, could afford protection against deleterious effects of DFP. Memantine is an uncompetitive NMDA receptor antagonist, clinically used for the treatment of Alzheimer's disease, Parkinson's disease and spasticity (Ozsuer et al., 2005; Lipton, 2005). Memantine exerts various pharmacological effects by multiple mechanisms: (1) blockage of nicotinic acetylcholine receptor-ion channel complex (Masuo et al., 1986), (2) prevention of neural hyperexcitability (McLean et al., 1992), (3) reduced high-frequency repetitive activation of peripheral nerves (Wesemann et al., 1983) and (4) protection of AChE activity from inhibition by OP and CM insecticides, and OP nerve agents and prototype compound DFP (Gupta and Kadel, 1990; Gupta and Dettbarn, 1992; McLean et al., 1992; Gupta and Dekundy, 2005). Since memantine is able to prevent the pathogenic Ca^{2+} influx caused by continuous activation by low-level glutamate, it is expected that memantine would also suppress formation ROS/RNS, depletion of high-energy phosphates, and mitochondrial/neuronal damage. Indeed, pretreatment with memantine (18 mg/kg, s.c.) suppressed DFP-induced lipid peroxidation (Fig.

4), increased citrulline production (Fig. 5) and reduced HEP levels (Fig. 6). Memantine, when administered alone, did not induce any alteration in neuronal morphometry; however, when administered as a pretreatment, it provided protection against DFP-induced morphometric changes in hippocampal neurons. Memantine completely suppressed both the reduction in dendrite length and spine density of pyramidal neurons in the CA1 hippocampal area of DFP exposed rats (Figs. 7). These effects are consistent with our recent studies on memantine's efficacy in reversing carbofuran's effects on lipid peroxidation, and alteration in citrulline and HEP levels in muscles and brain (Milatovic et al., 2005a; Gupta et al., 2007). Taken together, memantine can reduce free radical generation and lipid peroxidation, prevent HEPs depletion and attenuate the morphological injury, thus providing further support for the role of ROS and RNS in anticholinesterase-induced neurotoxicity.

In conclusion, data of the present investigation suggest that oxidative and nitrosative stress and alterations in energy metabolism are early responses to DFP exposure. These data closely associated DFP-induced lipid peroxidation with dendritic degeneration of pyramidal neurons in the CA1 hippocampal area. This study also investigated different pathways to attenuate biomarkers of oxidative damage associated with anticholinesterase exposure and the extent to which such attenuation is accompanied by rescue from neurodegeneration. Specifically, vitamin E, PBN and memantine efficiently suppressed oxidative injury. Future studies should investigate not only the prophylactic, but also therapeutic effects of these neuroprotectants. Successful identification of safe and effective neuroprotectants that suppress non-cholinergic activities associated with anticholinesterase exposure will provide new pharmacological modalities to protect and treat both the acute and delayed effects of anticholinesterase agent.

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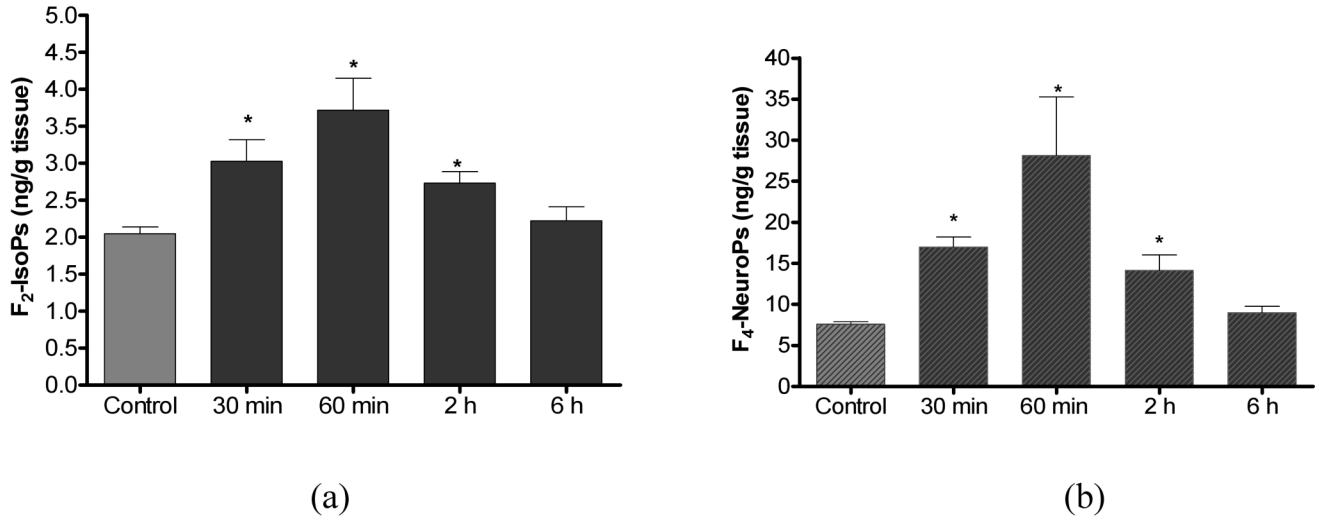


Figure 1.

Cerebral concentrations of F_2-IsoPs (a) and F_4-NeuroPs (b) following DFP (1.25 mg/kg, s.c.) exposure in rats. Values represent mean±SEM (n=4-6). *One-way ANOVA had $p<0.001$ with Bonferroni's multiple comparison tests showed significant difference ($p<0.05$) for vehicle injected control vs. DFP treatment.

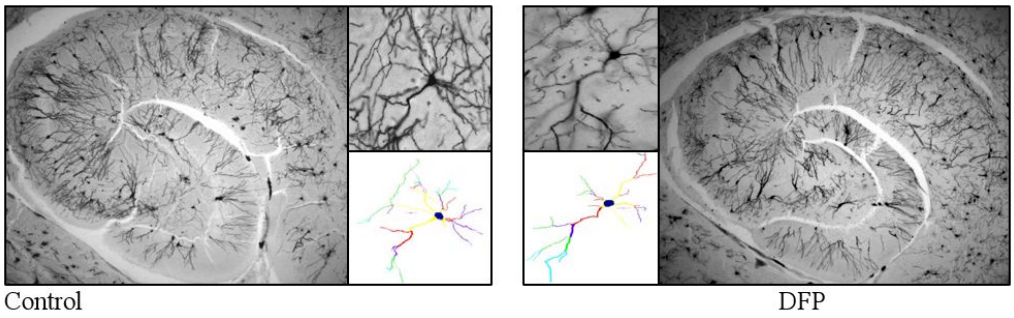
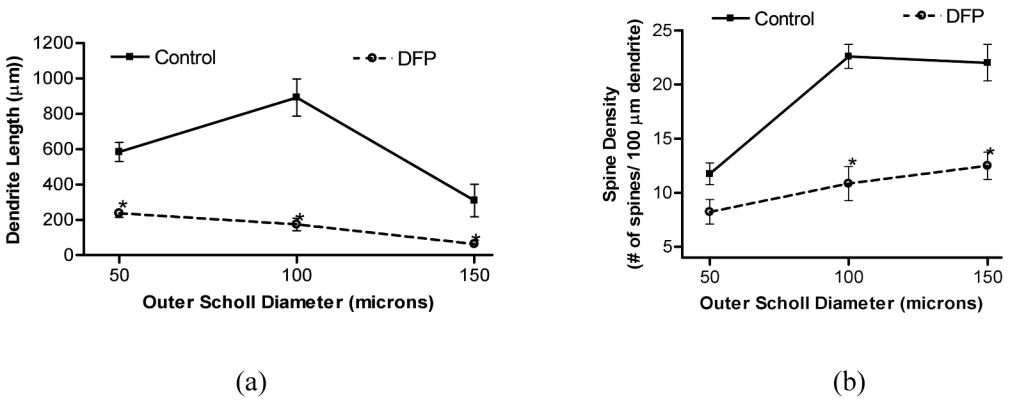


Figure 2.
Photomicrographs of rat hippocampi (2.5×) with pyramidal neurons (10×) from CA1 hippocampal area of rat brains 1 h after saline (control) and DFP (1.25 mg/kg, s.c) injections. Treatment with DFP induced degeneration of hippocampal dendritic system, decrease in total length of dendrite and spine density of hippocampal pyramidal neurons. Tracing and counting were done using a Neurolucida system at 100 × under oil immersion (MicroBrightField, VT). Colors indicate the degree of dendritic branching (yellow=1°, red=2°, purple=3°, green=4°, turquoise=5°, gray=6°).



(a) (b)

Figure 3.

Dendritic length (a) and spine density (b) in each Sholl compartment of pyramidal neurons from CA1 hippocampal area of rats following DFP (1.25 mg/kg, s.c.) exposure. Brains from rats exposed to DFP were collected 1 h post injections ($n \geq 4$). *One-way ANOVA had $p < 0.001$ with Bonferroni's multiple comparison tests showed significant difference for Sholl compartment ($p < 0.05$) from control vs. DFP treatment.

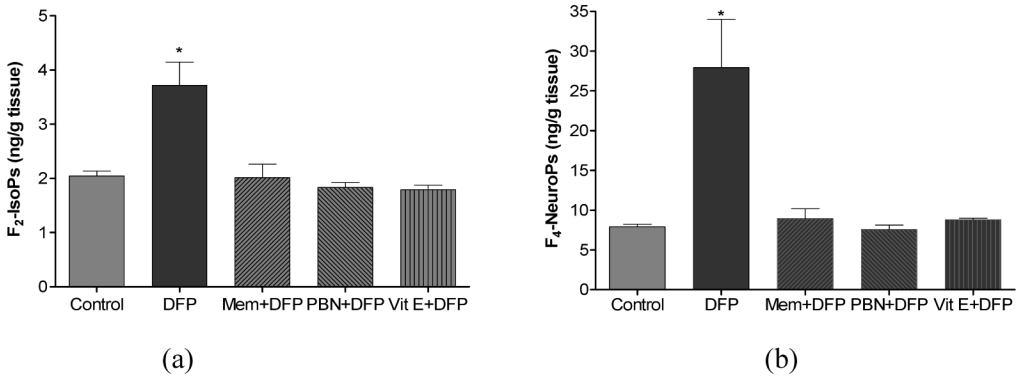


Figure 4.

Cerebral F_2-IsoPs (a) and F_4-NeuroPs (b) concentrations following saline (control) or DFP (1.25 mg/kg, s.c.) exposed rats with or without pretreatment with memantine (MEM, 18 mg/kg, s.c.), *N-tert*-butyl-α-phenylnitrone (PBN, 200 mg/kg, i.p.) or Vitamin E (Vit E, 100 mg/kg, i.p./day for 3 days). Brains from rats exposed to DFP were collected 1 hr post injections. Values of F_2-IsoPs and F_4-NeuroPs represent mean±SEM (n=4-6). *One way ANOVA had $p<0.001$ with Bonferroni's multiple comparison tests significant ($p<0.05$) for DFP vs. control, Mem+DFP, PBN+DFP or Vit E+DFP treatment.

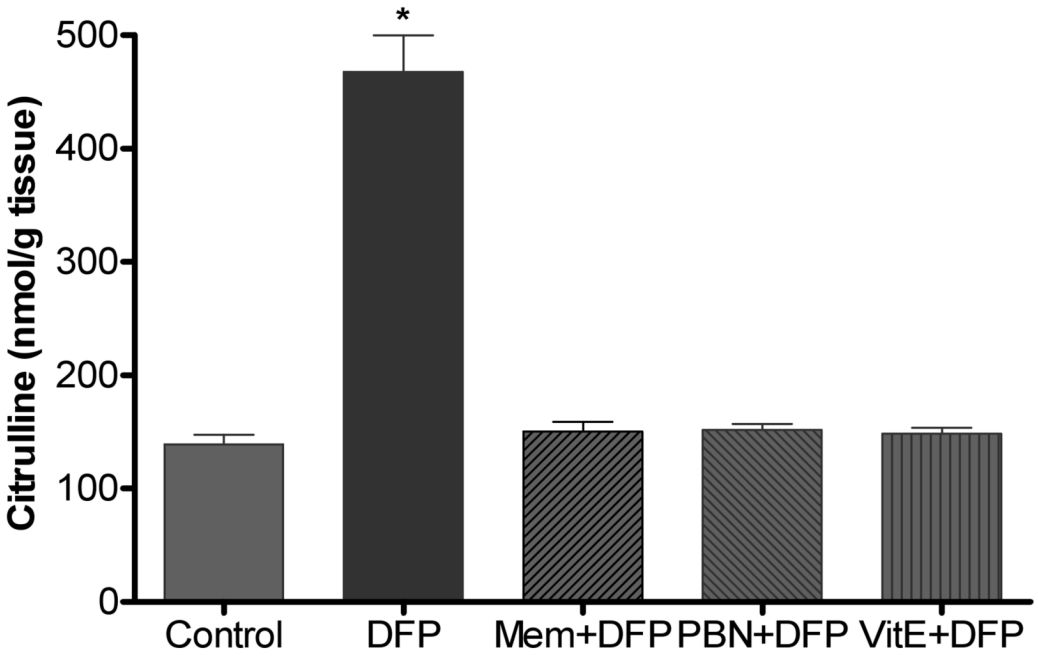
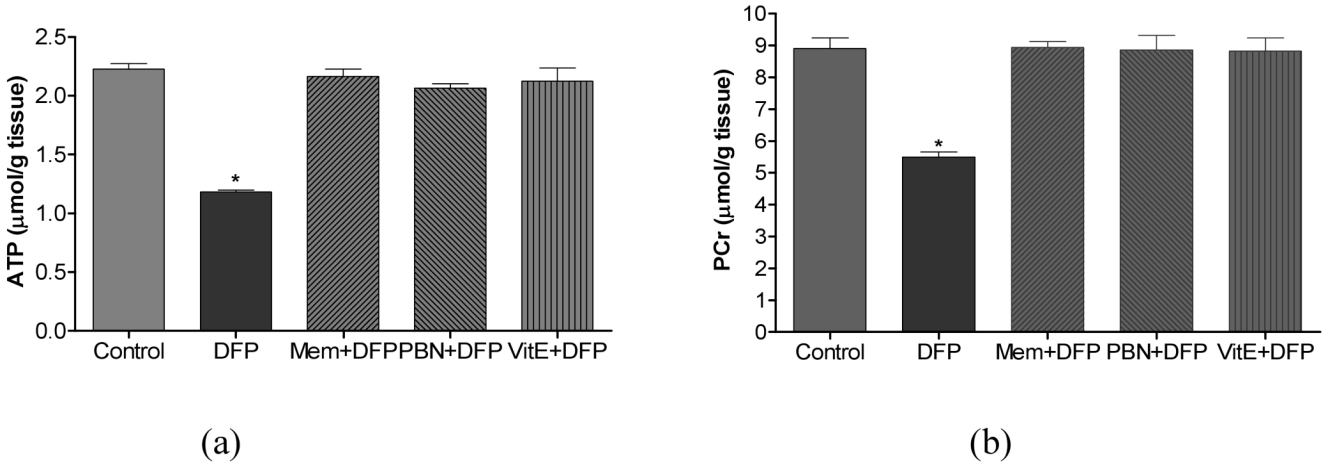


Figure 5.

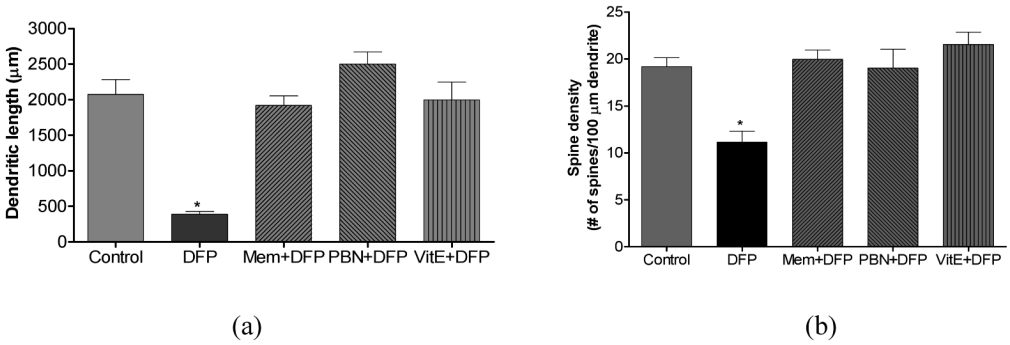
Cerebral citrulline concentrations following DFP exposure with or without pretreatment with memantine (MEM, 18 mg/kg, s.c.), alpha-phenyl-N-tert-butylnitrone (PBN, 200 mg/kg, i.p.) or Vitamin E (Vit E, 100 mg/kg, i.p./day for 3 days). Brains from rats exposed to DFP were collected 1 hr post injections. Values of citrulline represent mean±SEM (n=4-6). *One way ANOVA had $p<0.001$ with Bonferroni's multiple comparison tests significant ($p<0.01$) for DFP vs. control, Mem+DFP, PBN+DFP or Vit E+DFP treatment.



(a) (b)

Figure 6.

Cerebral high-energy phosphates concentartion, ATP (a) and PCr (b) following DFP exposure with or without pretreatment with memantine (Mem, 18 mg/kg, s.c.), alpha-phenyl-N-tert-butylnitrone (PBN, 200 mg/kg, i.p.) or Vitamine E (Vit E, 100 mg/kg, i.p./day for 3 days). Brains from rats exposed to DFP were collected 1 hr post injections. Values of ATp and PCr represent mean±SEM (n=4-6). *One way ANOVA had $p<0.001$ with Bonferroni's multiple comparison tests significant ($p<0.01$) for DFP vs. control, Mem+DFP, PBN+DFP or Vit E +DFP treatment.



(a) (b)

Figire 7.

Dendritic length (a) and spine density (b) of pyramidal neurons from CA1 hippocampal area of rats following DFP exposure with or without pretreatment with memantine (Mem, 18 mg/kg, s.c.), alpha-phenyl-N-tert-butylnitrone (PBN, 200 mg/kg, i.p.) or Vitamine E (Vit E, 100 mg/kg, i.p./day for 3 days). Brains from rats exposed to DFP were collected 1 hr post injections. Values of dendritic length and spine density represent mean±SEM ($n \geq 12$ neurons from 4-6 animals). *One way ANOVA had $p<0.001$ with Bonferroni's multiple comparison tests significant ($p<0.01$) for DFP vs. control, Mem+DFP, PBN+DFP or Vit E+DFP treatment.