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Effects of the organochlorine pesticide methoxychlor on dopamine metabolites and transporters in the mouse brain

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ABSTRACT

Pesticide exposure has been suggested as a risk factor in developing Parkinson's disease (PD). While the molecular mechanism underlying this association is not clear, several studies have demonstrated a role for mitochondrial dysfunction and oxidative damage in PD. Although data on specific pesticides associated with PD are often lacking, several lines of evidence point to the potential involvement of the organochlorine class of pesticides. Previously, we have found that the organochlorine pesticide methoxychlor (mxc) causes mitochondrial dysfunction and oxidative stress in isolated mitochondria. Here, we sought to determine whether mxc-induced mitochondrial dysfunction results in oxidative damage and dysfunction of the dopamine system. Adult female CD1 mice were dosed with either vehicle (sesame oil) or mxc (16, 32, or 64 mg/kg/day) for 20 consecutive days. Following treatment, we observed a dose-related increase in protein carbonyl levels in non-synaptic mitochondria, indicating oxidative modification of mitochondrial proteins which may lead to mitochondrial dysfunction. Mxc exposure also caused a dose-related decrease in striatal levels of dopamine (16–31%), which were accompanied by decreased levels of the dopamine transporter (DAT; 35–48%) and the vesicular monoamine transporter 2 (VMAT2; 21–44%). Because mitochondrial dysfunction, oxidative damage, and decreased levels of DAT and VMAT2 are found in PD patients, our data suggest that mxc should be investigated as a possible candidate involved in the association of pesticides with increased risk for PD, particularly in highly exposed populations.

1. Introduction

Parkinson's disease (PD) is a progressive neurological disorder characterized by abnormal posture, bradykinesia, rigidity, akinesia and resting tremor. Although the vast majority of affected patients have no clear causative factors, both genetic and environmental influences have been implicated (Warner and Schapira, 2003; Kamel and Hoppin, 2004; BenMoyal-Segal and Soreq, 2006; Brown et al., 2006). Several epidemiological studies (Priyardarshi et al., 2000; Ascherio et al., 2006; Frigerio et al., 2006; Kamel et al., 2007) have shown an association between pesticide exposure and increased risk of developing PD. Although the mechanism behind this association is unclear, oxidative stress and brain mitochondrial dysfunction, which has been implicated in neurodegenerative diseases, particularly PD (for reviews see Fiskum et al., 2003; Lin and Beal, 2006), has been proposed as a possible mechanism for the association of pesticides with PD.

In support of a role for mitochondrial dysfunction in PD, inhibition of complex I of the electron transport chain (ETC) has been determined to be the mechanism of action of 1-Methyl-4-phenylpyridinium (MPP^+) (Nicklas et al., 1985; Richardson et al., 2007), the active metabolite of 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) which has been demonstrated to induce parkinsonism (Langston et al., 1983). Evidence linking pesticides and complex I in PD was provided following the observation that rats chronically exposed to the complex I inhibitor rotenone reproduces the nigrostriatal neurodegeneration associated with PD (Betarbet et al., 2000). In the rotenone model, oxidative damage resulting from complex I inhibition has been demonstrated to be the key mechanism responsible for dopaminergic degeneration by rotenone

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Abbreviations: DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; mxc, methoxychlor, 1,1,1-trichloro-2,2-bis(p-methoxyphenyl)ethane; HVA, homovanillic acid; VMAT2, vesicular monoamine transporter; 5HT, serotonin; 5HIAA, 5-hydroxyindoleacetic acid; GFAP, glial fibrillary acidic protein; TH, tyrosine hydroxylase.

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both *in vivo* and *in vitro* (Sherer et al., 2002, 2003). Taken in concert, these data suggest that other pesticides that disrupt proper mitochondrial function may damage the dopamine system and increase vulnerability to PD.

In an earlier study, we demonstrated that the organochlorine pesticide 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane (methoxychlor, mxc) inhibited complex I of the ETC of isolated non-synaptosomal rat brain mitochondria *in vitro* and following repeated *in vivo* mxc exposure (Schuh et al., 2005). Based on our previous findings, and the association of complex I dysfunction in PD, we sought to test the hypothesis that mxc-induced mitochondrial dysfunction leads to oxidative damage and dysfunction of the dopamine system. Increased protein carbonylation, loss of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) and changes in dopamine transporter levels were observed suggesting that mxc may act as an environmental neurotoxicant capable of promoting neurodegeneration.

2. Materials and methods

2.1. Chemicals and reagents

1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane (methoxychlor, mxc) was purchased from ChemService (Westchester, PA) in a powdered form and was 99% pure. All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Animals

Female CD1 (Charles River Laboratories, Charles River, CA) mice (25 g, 39 days old) were housed five animals per cage at the University of Maryland School of Medicine Central Animal Facility and provided food and water ad libitum. Animals were subjected to 12-h light-dark cycles. Mice were weighed daily to assess the amount of mxc to give them, then dosed via intraperitoneal injection with 16, 32, or 64 mg/kg/day mxc, or sesame oil (vehicle) for 20 continuous days. The mice were sacrificed when in estrus to minimize variability due to hormonal fluctuations within 24 h after the final mxc treatment. The gender, dosing regimen and route of administration was selected based on our previous studies (Schuh et al., 2005; Gupta et al., 2006) and had been determined to cause no overt toxicity as demonstrated by a lack of tremor or lethargy and no significant weight loss in the mxc-treated animals. The University of Maryland School of Medicine Institutional Animal Use and Care Committee approved all procedures involving animal care, euthanasia and tissue collection.

2.3. Striatal isolation

Following decapitation, striata were dissected from the mouse brains on ice, and then the left striata were weighed and immediately frozen in liquid nitrogen until high performance liquid chromatography could be performed. The right striata from the same mouse brains were homogenized in ice-cold mannitol-sucrose (MS) buffer pH 7.4 (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mg/ml fatty acid free BSA) plus protease inhibitor cocktail (Calbiochem, San Diego, CA). Right striatal homogenates were centrifuged at 2000 × *g* for 5 min, then, the supernatants were further centrifuged at 30,000 × *g* for 30 min. The final pellet was resuspended in homogenization buffer then frozen and stored at −80 °C until Western blots could be performed. Protein concentrations were determined by the method described by Lowry et al. (1951) and ranged from 8 to 17 mg/ml.

2.4. Mitochondrial isolation

The fresh mouse forebrain tissues were processed to isolate non-synaptosomal mitochondria using the percoll isolation method as previously described (Sims, 1990). Briefly, the tissue was placed in ice-cold MS buffer pH 7.4 (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mg/ml fatty acid free BSA and 1 mM EGTA). The brain was homogenized and then centrifuged twice at 1317 × *g* for 3 min. Following a further 10 min centrifugation at 21,074 × *g*, the pellet was resuspended in 15% percoll (Amersham Biosciences, Piscataway, NJ) and then layered on a discontinuous percoll gradient and centrifuged at 29,718 × *g* for 8 min. The mitochondrial fraction was centrifuged at 16,599 × *g* for 10 min, the pellet resuspended and then spun at 6668 × *g* for 10 min. The mitochondrial pellet was resuspended in the above buffer but without BSA or EGTA. Protein concentrations were determined by the method described by Lowry et al. (1951) and ranged from 8 to 17 mg/ml.

2.5. Protein carbonyl determination

Isolated mitochondria were lysed in buffer containing 50 mM potassium phosphate (pH 7.8), 1 mM EDTA, 0.1% Triton X-100 (2:1 volume ratio of mitochondria:lysis buffer), then subjected to two freeze/thaw cycles. Protein concentrations were re-measured as described above. Mitochondrial lysates (15 μg) were assessed for oxidative modification using an OxyBlot kit (Chemicon, Temecula, CA) that recognizes carbonylated proteins. Carbonylation was determined according to the manufacturer's protocol.

2.6. High performance liquid chromatography (HPLC)

Supernatants from mouse left striata were analyzed for DA and its metabolites DOPAC and HVA, and serotonin (5HT) and its metabolite 5-hydroxyindoleacetic acid (5HIAA) as performed previously (Hatcher et al., 2007).

2.7. Western blot procedure

Proteins (15 μg) as determined by the Lowry method (1951) from mouse right striatal lysates were resolved using sodium dodedcyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% precast NuPage gels (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride membrane. Immunoblotting for Dopamine transporter (DAT), Vesicular monoamine transporter 2 (VMAT2), Tyrosine hydroxylase (TH) (Chemicon), and Glial fibrillary acidic protein (GFAP) (Sigma) was performed as previously described (Richardson and Miller, 2004; Richardson et al., 2008). Briefly, nonspecific sites were blocked in 7.5% nonfat dry milk in Tris–buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris and 0.1% Tween 20, pH 7.4). Membranes were then incubated overnight in DAT monoclonal antibody and detected using a goat anti-rat horseradish peroxidase secondary antibody and enhanced chemiluminescence. The luminescence signal was captured on an Alpha Innotech Fluorochem Imager and stored as a digital image. Membranes were stripped for 15 min at room temperature with Pierce stripping buffer (Rockford, IL) and sequentially reprobed with polyclonal antibodies against VMAT2, TH and GFAP. β-actin (Sigma) blots were used to ensure equal protein loading.

2.8. Statistical analysis

Data are expressed as means ± SE and the comparisons between experimental groups were made using SPSS statistical software (SPSS, Inc., Chicago, IL) using one-way analysis of variance (ANOVA). Holm–Sidak test was used for post hoc analysis. Statistical significance was assumed at $p < 0.05$.