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Alteration of dopamine uptake into rat striatal vesicles and synaptosomes caused by an *in vitro* exposure to atrazine and some of its metabolites

Muhammad M. Hossain, Nikolay M. Filipov*

Center for Environmental Health Sciences, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, MS 39762-6100, USA

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ABSTRACT

Studies have shown that both *in vivo* and *in vitro* exposure to the herbicide atrazine (ATR) results in dopaminergic neurotoxicity manifested by decreased striatal dopamine (DA) levels. However, the mechanism behind this reduction is largely unknown. A decrease in striatal DA could be due to ATR exposure affecting vesicular and/or synaptosomal uptake resulting in disrupted vesicular storage and/or cellular uptake of DA. Hence, we investigated the effects of *in vitro* ATR exposure on DA uptake into isolated rat striatal synaptosomes and synaptic vesicles. In addition to ATR, effects of its major mammalian metabolites, didealkyl atrazine (DACT), desethyl atrazine (DE) and desiopropyl atrazine (DIP) were investigated. ATR (1–250 μM) inhibited DA uptake into synaptic vesicles in a dose-dependent manner. Of the three ATR metabolites tested, DACT did not affect vesicular DA uptake. DE and DIP, on the other hand, significantly decreased vesicular DA uptake with the effect of 100 μM DE/DIP being similar to the effect of the same concentration of ATR. Kinetic analysis of vesicular DA uptake indicated that ATR significantly decreased the V_{max} while the K_m value was not affected. Contrary to the inhibitory effects on vesicular DA uptake, synaptosomal DA uptake was marginally (6–13%) increased by ATR and DE, but not by DACT and DIP, at concentrations of ≤100 μM. As a result, ATR, DIP and DE increased the synaptosomal/vesicular (DAT/VMAT-2) uptake ratio. Collectively, results from this study suggest that ATR and two of its metabolites, DIP and DE, but not its major mammalian metabolite, DACT, decrease striatal DA levels, at least in part, by increasing cytosolic DA, which is prone to oxidative breakdown.

1. Introduction

Genetics is not a major contributory factor to Parkinson's disease (PD) when the onset of symptoms occurs after the age of 50 (Tanner et al., 1999). Increasing evidence indicates that the environment plays an important role in the etiology of basal ganglia pathologies, such as PD. Living in a rural area, farming, drinking well water, and occupational exposure to agricultural chemicals are all suspected PD risk factors (Gorell et al., 1998; Tanner et al., 1999; Priyadarshi et al., 2001; Di Monte et al., 2002; Brown et al., 2006). Among the risk factors for PD, pesticide exposure has received particular attention and it has been associated with increased incidence of PD in agricultural workers in rural environments (Semchuk et al., 1992; Schapira, 1999; Priyadarshi et al., 2000; Brown et al., 2006). For example, increased incidence of PD associated with high use of agricultural pesticides was observed in rural California (Ritz and Yu, 2000), while in the state of Washington, a trend towards increased PD incidence in pesticide applicators and crop farmers was observed (Firestone et al., 2005). Furthermore, case control studies suggest that the risk of PD is increased when the duration of exposure to pesticides exceeds a particular threshold (Seidler et al., 1996; Gorell et al., 1998). Specific pesticides that have been associated with PD-like symptomology (i.e., dopaminergic toxicity) are paraquat (Fredriksson et al., 1993; McCormack et al., 2002), maneb (Morato et al., 1989; Takahashi et al., 1989), and rotenone (Alam and Schmidt, 2002; Gao et al., 2003). In the case of paraquat and maneb, greater dopaminergic toxicity was observed in animals exposed to both of these pesticides (Thiruchelvam et al., 2000). Increased levels of organochlorine pesticides, such as dieldrin, have also been detected in the brains of PD patients (Fleming et al., 1994) and dieldrin has been demonstrated to be toxic to the basal ganglia in animal models (Richardson et al., 2006).

Atrazine (ATR) is an extensively used herbicide throughout most of the world; about 76.5 million pounds are used per year throughout the U.S. to control variety of weeds in agricultural crops, as well as on golf courses and residential lawns where its use has increased (EPA, 2003). ATR is moderately volatile and water soluble, and tends to persist in ground and surface water, which is a main reason for long-term, low environmental exposures. Occupational exposure to higher levels of ATR is of particular concern as levels of ATR indica-

* Corresponding author. Tel.: +1 662 325 1208; fax: +1 662 325 1031.
E-mail address: filipov@cvm.msstate.edu (N.M. Filipov).

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tive of substantial exposure are detected in the saliva and urine of pesticide applicators and farmers during spraying (Hines et al., 2006). A recent study reported that not only ATR applicators but also their families are at risk of high level of exposure to this pesticide (Curwin et al., 2007). They found considerably higher amounts of urinary ATR and its metabolites in applicators and their families living on the farm.

The metabolism of ATR has been studied in different species (Bakke et al., 1972; Ikonen et al., 1988; Catenacci et al., 1990; McMullin et al., 2003; Ross and Filipov, 2006). Major ATR metabolites reported in mammals and humans are desethyl atrazine (DE), desiopropyl atrazine (DIP), and didealkyl atrazine (DACT; Bakke et al., 1972; Erickson et al., 1979; Catenacci et al., 1993; McMullin et al., 2003; Ross and Filipov, 2006). DACT is the primary urinary metabolite detected in mice (Ross and Filipov, 2006) and rats (McMullin et al., 2003). While DE and DIP were two major ATR metabolites found in urine of occupationally exposed humans in earlier studies (Ikonen et al., 1988; Catenacci et al., 1990), very recent evidence, using modern analytical technology, indicates that DACT is the most frequently detected human metabolite of ATR (Barr et al., 2007).

Recently, ATR has been identified as a potential basal ganglia toxicant. Thus, two separate studies found that ATR exposure decreased striatal dopamine (DA) levels and caused a loss of tyrosine hydroxylase (TH)-positive dopaminergic neurons in both the substantia nigra parts compacta (SNpc) and the ventral tegmental area (VTA) in rats (Rodriguez et al., 2005) and mice (Coban and Filipov, 2007). In addition, our laboratory (Filipov et al., 2007) found that *in vitro* exposure to ATR decreased the tissue levels of DA in striatal slices while media levels of DA and its turnover [(DOPAC + HVA)/DA] ratio were increased. Earlier studies with PC12 cells demonstrated that *in vitro* exposure to ATR ($\geq$12.5 μM) significantly decreased intracellular DA in a concentration-dependent manner (Das et al., 2000). In another study, this group (Das et al., 2001) reported that the ATR metabolites, DE, DIP, and DACT, affected PC12 cells DA homeostasis in a manner different from the effects of ATR, i.e., they increased intracellular DA. While experimental evidence for the dopaminergic toxicity of ATR is increasing, at this time, there is no epidemiological data linking ATR exposure and PD.

Dopamine is converted to 3,4-dihydroxyphenylacetic acid (DOPAC) intraneuronally and to homovanillic acid (HVA) extraneuronally in the synaptic cleft (Cooper et al., 2003). Upon its release, DA undergoes rapid reuptake to terminate its action and maintain DA homeostasis. Reuptake is accomplished in two ways: synaptosomal uptake through the dopamine transporter (DAT), which transports DA from the extracellular space into the cytosol, and vesicular uptake by vesicular monoamine transporter-2 (VMAT-2), which stores DA into synaptic vesicles (Cooper et al., 2003). Perturbation of either of these two uptake mechanisms will result in altered DA homeostasis. For example, the importance of both DAT and VMAT-2 was demonstrated in DAT-knockout mice (Giros et al., 1996) and VMAT-2-knockout mice (Takahashi et al., 1989; Miller et al., 1999; Fon et al., 1997; Wang et al., 1997). Thus, compared to normal mice, extracellular DA remained 300 times longer in DAT-knockouts (Giros et al., 1996), and reduced vesicular storage was observed in VMAT-2-knockout mice (Miller et al., 1999).

Based on our previous findings with striatal slices (Filipov et al., 2007) and on the mechanisms of the maintenance of DA homeostasis, we hypothesized that decreased tissue DA could be caused by direct effects of ATR on vesicular and/or synaptosomal uptake resulting in disruption of vesicular DA storage and/or cellular uptake. Therefore, to test this hypothesis, the present study investigated the effects of *in vitro* ATR exposure on DA uptake into rat striatal synaptosomes and vesicles. In addition, we investigated the effects of ATR's major metabolites, DACT, DE, and DIP on vesicular and synaptosomal uptake of DA.

2. Materials and methods

2.1. Animals

Adult Sprague-Dawley rats (2–4 months old, 260–300 g, Harlan, Madison, WI) were used as source of striatal synaptosomes and vesicles in these studies. Animals were housed in a climate controlled room (23 ± 0.5 °C and humidity at 55 ± 5%) under a 12-h light/dark cycle (lights on at 06:00 h) with free access to 18% protein rodent diet (Harlan) and water. All procedures were in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23) and were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of Mississippi State University.

2.2. Chemicals

Atrazine, 2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine (ATR; lot # 301-49A, purity: 98%), desethyl atrazine (DE; lot # 266-60A, purity: 99.5%), desiopropyl atrazine (DIP; lot # 282-112A, purity: 98%) and didealkyl atrazine (DACT; lot # 285-100A purity: 96%) were purchased from ChemService (West Chester, PA, USA). MicroScint^{TM}-20 cocktail, Top Count .NXT^{TM}, Filtermate 196 and plate sealer 496 were purchased from Packard Bioscience Company (Meriden, CT, USA). HEPES was purchased from (Acros, NJ, USA) and Whatman unifilter-24^{TM} GF/B and [^{3}H] dopamine hydrochloride ([^{3}H] DA; 1 mCi) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Polyethylenimine, dopamine hydrochloride (DA), pargyline, adenosine 5-triphosphate disodium (ATP), and reserpine were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.3. Exposure solutions

Stock solutions (50 mM) of ATR, DE and DIP were prepared in absolute ethanol (EtOH) and DACT and reserpine were prepared in dimethyl sulfoxide (DMSO); ATR, DE and DIP completely dissolved in EtOH, whereas DACT and reserpine were not soluble in EtOH but dissolved completely in DMSO. Exposure solutions were prepared with incubation buffer (described below). Since 250 μM of ATR was used in a single vesicular/synaptosomal study and 100 μM was the highest concentration used for ATR/DE/DIP and DACT in all other studies, control, vesicles, and synaptosomes were exposed to EtOH and DMSO vehicles in volume present in 100 μM ATR/DE/DIP and DACT, respectively. In pilot experiments, we determined that EtOH vehicle equivalent to 500 μM ATR did not cause any alterations in synaptosomal and vesicular uptake.

2.4. Preparation of synaptic vesicles

Striatal vesicles were prepared according to (Roz and Rehavi, 2003) with minor modifications. Briefly, rats were euthanized with CO_2, the striata were quickly removed, weighed and homogenized in 10 volumes of ice-cold physiological sucrose solution (0.32 M sucrose, 10 mM Tris–HCl; pH 7.4) using a glass–Teflon pestle homogenizer (RZR1, Heidolph, Germany). Homogenates were centrifuged (1500 × *g* for 10 min at 2 °C) and the supernatants were briefly stored at 4 °C. The pellets were re-suspended in the same volume of ice-cold homogenizing buffer and centrifuged at 1500 × *g* for 10 min at 2 °C. The two supernatants were combined and centrifuged at 20,000 × *g* for 20 min at 2 °C. The resulting supernatant was discarded and pellets were disrupted by osmotic shock via homogenization in 10 volumes of 5 mM Tris–HCl buffer using a glass–Teflon homogenizer followed by incubation on ice for 45 min. Each homogenate was centrifuged at 20,000 × *g* for 20 min and the supernatant was further centrifuged at 62,000 × *g* for 40 min. The resulting pellet (synaptic vesicles) was re-suspended in 0.32 M sucrose containing 5 mM Tris–HCl (pH 7.4) and stored at 2 mg protein/ml at −80 °C until use. Protein concentration was measured by the method of Bradford (1976).

2.5. Assay for vesicular uptake of DA

Vesicular uptake of DA was determined according to Roz and Rehavi (2003) with slight modifications. Two sets of experiments were performed: studies with [^{3}H] DA present in the incubation medium and kinetic studies where in addition to [^{3}H] DA, a concentration-range of cold DA was also present. The total reaction incubation volume was 300 μl. Frozen synaptic vesicles were thawed and diluted to a concentration of 15 μg protein/100 μl with assay buffer (100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM potassium phosphate buffer; pH 7.4). In the presence of 1 mM Na_2ATP (final concentration), aliquots of 200 μl vesicles (30 μg protein) were incubated at 30 °C for 15 min with either vehicles (EtOH or DMSO), ATR (0.1–250 μM), its metabolites (DE, DACT, or DIP at 1 and 100 μM), or with reserpine (1 μM), as a positive control. This was followed by an incubation with cold DA (0.1–100 nM, final concentration, kinetic experiments only) and [^{3}H] DA (6.6 nM, final concentration) for 3 min.