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Alterations in the nigrostriatal dopamine system after acute systemic PhIP exposure

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ABSTRACT

Heterocyclic amines (HCAs) are primarily formed during cooking of meat at high temperature. HCAs have been extensively studied as mutagens and possible carcinogens. Emerging data suggest that HCAs are neurotoxic and may be relevant to Parkinson's disease (PD) etiology. However, the majority of HCAs have not been evaluated for *in vivo* neurotoxicity. Here, we investigated acute *in vivo* neurotoxicity of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP). PhIP is the most prevalent genotoxin in many types of meats. Adult, male Sprague-Dawley rats were subjected to acute, systemic PhIP at doses and time-points that have been extensively utilized in cancer studies (100 and 200 mg/kg for 8, 24 h) and evaluated for changes in dopaminergic, serotoninergic, GABAergic, and glutamatergic neurotransmission. PhIP exposure resulted in decreased striatal dopamine metabolite levels and dopamine turnover in the absence of changes to vesicular monoamine transporter 2 levels; other neurotransmitter systems were unaffected. Quantification of intracellular nitrotyrosine revealed higher levels of oxidative damage in dopaminergic neurons in the substantia nigra after PhIP exposure, while other neuronal populations were less sensitive. These changes occurred in the absence of an overt lesion to the nigrostriatal dopamine system. Collectively, our study suggests that acute PhIP treatment *in vivo* targets the nigrostriatal dopaminergic system and that PhIP should be further examined in chronic, low-dose studies for PD relevance.

1. Introduction

Dietary heterocyclic amines (HCAs) are primarily produced in meat during high-temperature cooking through the Maillard reaction between amino acids, sugar and creatine, or pyrolysis of amino acids such as tryptophan (Matsumoto et al., 1981; Skog et al., 1998). Many HCAs are mutagenic (Felton et al., 1984). Potential links between HCAs and cancer have been extensively studied for the last three decades, where HCAs have been found to cause DNA damage in tissues such as the mammary gland and liver (Dietrich et al., 2011; Dobbernack et al., 2011) and induce oxidative stress (Li et al., 2013); resulting in tumor formation in several organ systems (Fujita et al., 1999; Ohgaki et al., 1987; Sugimura et al., 2004). Far less attention has been devoted to potential neurotoxic effects. However, early and recently emerging data do suggest that many HCAs should be evaluated as neurotoxins. β-carbolines, an HCA subclass, have been examined as possible Parkinson's disease (PD) relevant neurotoxins due to structural similarity to known dopaminergic neurotoxicants (Matsubara et al., 1998). Levels of the β-carboline 1-methyl-9H-pyrido[3,4-b]indole (harmane) are elevated in blood and cerebrospinal fluid of patients with PD and essential tremor (Kuhn et al., 1996; Louis et al., 2011; Louis et al., 2013; Louis et al., 2014). In essential tremor, harmane accumulates in the brain compared to control showing that it crosses the blood-brain barrier (BBB) (Louis et al., 2013). With respect to aminoimidazoaazarenes (AIAs), another HCA subclass, early studies showed that 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) inhibit monoamine oxidase (MAO) activity, resulting in increased dopamine and decreased metabolite levels in striatum (Ichinose et al., 1988; Kojima et al., 1990; Maruyama et al., 1994). While two of these studies used PC12 h rat pheochromocytoma cells and human brain synaptosomes, the third one performed unilateral infusion into the rat striatum. Although they provided valuable insight on MAO activity effects, the route of administration was not relevant to human health, and Trps are found in meat at very low levels compared to other HCAs; perhaps explaining why follow-up studies were not published (Skog et al., 1997). Taken together, these findings suggest that *in vivo* evaluation of HCA neurotoxicity should be a priority.

Of dietary HCAs, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) has been extensively studied, because PhIP is the most

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abundant AIA isolated from the crust of cooked meat, where levels may reach ~15 micrograms/kg uncooked meat (~75% of genotoxic material) (Felton et al., 1986a; Felton et al., 1986b). While PhIP has been extensively studied as a mutagen, effects on the nervous system have not yet been substantially evaluated. Recently, our group showed that PhIP is selectively toxic to dopaminergic neurons in primary rat midbrain cultures (Griggs et al., 2014). In that study, PhIP and its bioactive phase I metabolite, *N*-OH-PhIP induced loss of dopaminergic neurons and neurite retraction, whereas nondopaminergic neurons were spared. Further, we showed that PhIP induced oxidative damage in dopaminergic neurons, and intervention of this damage with anti-oxidants alleviated toxicity. Selective dopaminergic neurotoxicity and oxidative damage suggest potential relevance to PD, because these features are hallmarks of the disease (Cannon and Greenamyre, 2011). Given the devastating neurological symptoms of the disease, and that the causes of most cases are unknown, the identification of new etiological factors is critical. The prevalence of PhIP in the diet, the findings that it crosses the BBB, and selective toxicity in a primary culture system suggest that PhIP should be evaluated *in vivo* for effects on the nigrostriatal dopamine system, the primary neuropathological target in PD and PD models (Cannon and Greenamyre, 2010; Enokizono et al., 2008; Teunissen et al., 2010). To address whether PhIP might selectively target the nigrostriatal dopamine system, we evaluated effects on several neurotransmitter systems and oxidative damage after acute, systemic PhIP exposure. The goals of these first *in vivo* PhIP neurotoxicity studies were to use established doses from the genotoxicity literature to assess acute effects, and to determine whether time and cost intensive, chronic, long-term *in vivo* studies are justified and needed in the future to test PD relevance.

2. Materials and Methods

2.1. Animals

Wild type male Sprague Dawley rats (6–7 weeks old rats at 225–250 g) were purchased from Envigo (Indianapolis, IN). Rats were housed in a temperature-controlled facility with 12 h light/dark cycle, and allowed to acclimate for at least 48 h before treatments. During the entire experiment, animals received food and water *ad libitum*. All animal studies were approved by the Purdue Animal Care and Use Committee.

2.2. Dose rationale and PhIP treatment

To the best of our knowledge, the neurotoxic effects of PhIP have not been studied in mammalian systems. As the most relevant route of exposure, we chose to administer PhIP by oral gavage. The doses utilized in our study were chosen from extensive published cancer bioassays, where *in vivo* genotoxicity has been extensively studied as an adverse outcome. An extensive review of the literature (more than 70 studies examined; condensed citation list reported here) found that in rodents, the typical dose of PhIP administered via gavage ranged from 5 to 200 mg/kg body weight (bw) daily, while the frequency might vary from a single dose to every other day for 10 weeks (Hikosaka et al., 2004; Inaguma et al., 2003; Khan et al., 2013; Naito et al., 2004). To examine acute toxicity of PhIP, we chose two doses: 100 and 200 mg/kg bw. Animals were sacrificed either 8 or 24 h following the single oral gavage. These doses were chosen from the numerous reported regimens, because similar doses and/or time points have been shown to induce adduct formation, histopathological changes in gastric mucosa and oxidative stress in the stomach, and increase DNA adduct formation in prostate and colon (Inaguma et al., 2003; Li et al., 2013; Metry et al., 2009). It is worth noting that the doses used there are 2000–40,000 times higher than the average human consumption (5–50 ng/kg daily). However, humans consume numerous HCAs (~30 known, likely many unknown) vs. PhIP alone, suggesting that considering dietary intake of a single HCA underestimates total HCA consumption (Augustsson et al., 1997; Roemer et al., 2016). Further, our previous studies found that of the two primary metabolic pathways, the *N*-hydroxylation product is far more neurotoxic than ring-hydroxylation (Griggs et al., 2014). *N*-hydroxylation is at least 13-fold less efficient in rodents vs. humans (rodent CYP1A2 converts far more PhIP to 4′-OH-PhIP than to the genotoxic and neurotoxic *N*-hydroxylated metabolite, *N*-OH-PhIP), suggesting that far higher doses may be required in rodents vs. humans to produce neurotoxic metabolites (Cheung et al., 2005; Griggs et al., 2014; Turesky et al., 1998). Lastly, PhIP food content can vary by > 500-fold due to differences in meat types, cooking times, and temperatures (Augustsson et al., 1997; Byrne et al., 1998; Keating and Bogen, 2004; Knize et al., 1994; Layton et al., 1995; Zimmerli et al., 2001). Thus, rodents may require far higher doses of this single representative HCA to elicit neurotoxicity. Taken together, our doses in these initial acute neurotoxicity studies are higher than that which humans are exposed to, but are based on an extensive literature from cancer studies and justified by key differences between rodents and humans.

PhIP (TRC, A617000) was dissolved in corn oil (Sigma, C8237) at final concentration of 15 or 30 mg/ml for doses of 100 or 200 mg/kg bw, respectively. Suspensions were sonicated in an ultrasound water bath (Branson, 1800) at 37 °C until a homogenous solution was formed with no precipitates. For oral gavage, 18-gauge feeding tubes at 3” in length (Instech, FTP-18-75) attached to 3 ml syringes (BD Biosciences, 309657) were used. PhIP was administered only once, and animals were sacrificed after either 8 or 24 h. Treatment groups were as following: (1) Vehicle-treated (8 h and 24 h pooled), (2) 100 mg PhIP/kg bw for 8 h,(3) 200 mg PhIP/kg bw for 8 h, (4) 100 mg PhIP/kg bw for 24 h, (5) 200 mg PhIP/kg bw for 24 h (n = 10 per treatment).

2.3. Tissue collection and preparation

After 8 or 24 h following a single oral gavage, animals were placed in a medium size decapicone bag (BrainTree Sci, DC2000) and euthanized by decapitation. The brains were quickly removed from the skull and placed in pre-chilled PBS for 3 min. After brains were cut into half sagittally with the help of a sagittal brain matrix (BASi, RBM-4000S), one hemisphere was fixed in pre-chilled, 4% paraformaldehyde for histological examination. The other hemisphere was placed in a coronal brain matrix (BASi, RBM-4000C), a 2 mm section of striatum was extracted, and flash frozen in liquid nitrogen for neurochemistry.

2.4. High performance liquid chromatography

Neurochemical analysis was performed as described in our previous studies (O'Neal et al., 2014; Wang et al., 2014; Wirbisky et al., 2015) using a high-performance liquid chromatography (HPLC) system that consisted of a Dionex Ultimate 3000 Model ISO-31000BM pump, a model WPS-3000TBSL autosampler, Coulochem III electrochemical detector and an ESA Coulochem data station (ThermoScientific, Waltham, MA). Samples were separated on a Waters XBridge reverse phase C18 column (150 × 3.0 mm, 3.5 µm particle size) (Waters Corp, Milford, MA). For monoamine separation, the mobile phase was: 80 mM NaH_2PO_4, 10% methanol, 2 mM octanesulfonic acid, 0.025 mM ethylenediaminetetraacetic acid and 0.2 mM trimethylamine, at pH 2.4. Monoamines were detected by analytical cell set at E1 = –150 mV and E2 = +350 mV. For the quantification of GABA and glutamate, the mobile phase was: 0.1 M Na_2HPO_4, 22% methanol and 4% acetonitrile, at pH 6.75. Quantification of amino acid neurotransmitters requires derivatization. The samples were mixed with a derivatization agent (containing 0.2 M o-phthalaldehyde (OPA), 0.05% 2-mercaptoethanol, 10% methanol in OPA diluent) prior to separation. Neurotransmitters in the sample was detected by analytical cell set at E1 = −150 mV and E2 = +550 mV. Levels of neurotransmitters tested here were calculated using area under the curve by comparison with a standard curve.