DOPAMINE IS AN ARYL HYDROCARBON RECEPTOR AGONIST

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Running Title: Dopamine is an AhR agonist

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Keywords:
Carbidopa, L-DOPA, selective AhR modulator, pancreatic cancer, colon cancer, glioblastoma

Conflict of Interest: The authors declare that there are no competing interests associated with the manuscript.
ABSTRACT

Tryptophan metabolites exhibit aryl hydrocarbon receptor (AhR) agonist activity and recent studies show that the phenylalanine metabolite serotonin and carbidopa, a drug used in treating Parkinson’s disease, activated the AhR. In this study, we identified the neuroactive hormone dopamine as an inducer of drug metabolizing enzymes CYP1A1, CYP1B1, and UGT1A1 in colon and glioblastoma cells and similar results were observed for carbidopa. In contrast, carbidopa but not dopamine exhibited AhR activity in BxPC3 pancreatic cancer cells whereas minimal activity was observed for both compounds in Panc1 pancreatic cancer cells. In contrast to a previous report, the induction responses and cytotoxicity of carbidopa was observed only at high concentrations (100 µM) in BxPC3 cells. Our results show that similar to serotonin and several tryptophan metabolites, dopamine is also an AhR-active compound.
INTRODUCTION

Gut microbiota plays an important role in human health and disease, and this is due, in part, to microbial metabolites and their direct influence on the intestine and also distal organs [1-3]. Metabolite formation can be influenced by multiple factors including the diet, and there is evidence that microbial metabolism of diets enriched in fiber generate short chain fatty acids which contribute to intestinal resilience [4-6]. Tryptophan is one of the nine dietary-derived essential amino acids and microbial degradation of tryptophan generates multiple indole-derived compounds that also contribute to intestinal health [7-11]. There is evidence that dietary tryptophan and its metabolites enhance intestinal immunity and inhibit inflammation, and this has been linked, in part, to AhR-active tryptophan metabolites and their AhR-dependent activities [10,12-14]. In addition, dietary tryptophan and AhR-active metabolites of tryptophan such as indole-3-propionic acid and indole-3-aldehyde also exhibit anti-inflammatory activity in the central nervous system (CNS) of mouse models of multiple sclerosis and decreased serum levels of tryptophan metabolites are observed in multiple sclerosis patients [15,16].

Microbial degradation of tryptophan utilizes multiple pathways to produce an array of metabolites in the intestine as well as the CNS where tryptophan is converted (via tryptophan hydroxylase) into serotonin which has also been identified as an AhR active compound [17]. Indoleamine 2,3-dioxygenase in the host catalyzes metabolism of tryptophan into kynurenine and kynurenic acid which in turn are metabolized into both neurotoxic (e.g. quinolinic acid) and neuroprotective (e.g. kynurenic acid) metabolites which are also AhR ligands [18,19]. Carbidopa, a drug used for treatment of Parkinson’s disease to enhance neuronal levels of L-DOPA, is also an AhR ligand. Carbidopa exhibits AhR activity in multiple cancer cell lines and was shown to inhibit BxPC3 pancreatic cancer cell growth [20].

In this study, we demonstrate that L-DOPA exhibits minimal to non-detectable AhR activity despite structural similarities to carbidopa. We have re-examined the effects of all 3 structurally similar 3,4-dihydroxyphenyl derivatives including carbidopa, L-DOPA, and dopamine. We show that dopamine also exhibited AhR activity similar to carbidopa; however, these effects are cell context and response specific. Thus, the neuroactive hormone dopamine derived from the amino acid phenylalanine is an AhR active compound.
Materials and Methods

Cell Culture

Panc1, BxPC3, Caco-2 human colon cancer cells and human glioblastoma cell lines U87-MG were obtained from American Type Culture Collection (ATCC, Manassas, VA) and the patient-derived 15-037 cells, 14-015, and 14-104 glioblastoma cells have previously been described [21,22]. Panc1, SW480, and HCT116 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) with nutrient supplemented with 10% fetal bovine serum (FBS) (Gibco, Dublin, Ireland). BxPC3 cells were maintained in RPMI 1640 medium with 10% FBS (Gibco). Caco-2 cells were maintained in DMEM with nutrient mixture supplemented with 20% FBS and 10 m/L MEM non-essential amino acid solution (Gibco). Patient-derived xenografts from human gliomas cell lines were incubated at 37°C in the presence of 5% CO₂. Dopamine, L-DOPA, and carbidopa were purchased from Sigma-Aldrich (St. Louis, MO). The 15-037-AhRKO cells were generated by CRISPR/Cas9 as described (22). A humanAhR CRISPR/Cas9 guide RNA (AGACCGACTTAATACAGAGT) in a pSpCas9 BB-2A-GFP PX458 vector was purchased from GenScript (Piscataway, NJ). Individual clones were isolated and two of these exhibited the targeted frameshift mutations in exon 2 and the resulting AhR cells did not express the AhR and were TCDD non-responsive (22).

Western blot analysis

Cells (3×10⁵ cells/well) were plated in 6-well plates and grown for 24 hr. Cells were treated with different concentrations of the compounds for 24 hr. Whole-cell lysates from different cell lines were analyzed by Western blot analysis as described previously (22). Aliquots of cellular proteins were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). The membrane was allowed to react with CYP1A1, CYP1B1 (Santa cruz biotechnology, Santa cruz, CA) and AhR(Enzo Life Sciences, Farmingdale, NY) antibodies, and detection of specific proteins was carried out by enhanced chemiluminescence. Loading differences were normalized by using GAPDH antibody.

Chromatin immunoprecipitation (ChIP) Assay

The ChIP assay was performed using ChIP-IT Express Magnetic Chromatin Immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. Caco-2 cells were incubated for 24 hr and then treated with
TCDD, Dopamine and Carbidopa for 2 hr prior to cell harvest. Cells were analyzed by ChIP Assay as described previously (21, 22). DNA was extracted from the immunoprecipitate and PCR amplified using CYP1A1 (forward 5'-CCCGTGACCTCAGGGCT-3', reverse 5'-TTGCACCCACTGGAACGC-3') primer. PCR products were resolved on a 2% agarose gel in the presence of ethidium bromide.

**Quantitative Real-Time Reverse Transcriptase PCR**

Total RNA was extracted using RNA isolation kit from cells according to the manufacturer’s protocol. cDNA synthesis was performed from the total RNA of cells using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Real-Time PCR was carried out in triplicate using Bio-Rad SYSR Universal premix for 1 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min in the Bio-Rad iCycler (MyiQ™2) real-time PCR System. The expression of genes was analyzed with the comparative CT method and normalized to expression levels of TATA-binding protein (TBP). The sequences of the primers used for real-time PCR were as follows: CYP1A1 sense 5'-GAC CAC AAC CAC CAA GAA C-3', antisense 5'-AGC GAA GAA TAG GGA TGA AG-3'; UGT1A1 sense 5'-GAA TCA ACT GCC TTC ACC AAA AT-3', antisense 5'-AGA GAA AAC CAC AAT TCC ATG TTC T-3'; TBP sense 5'-GAT CAG AAC AGC CTG CC-3', antisense 5'-TTC TGA ATA GGC TGT GGG GT-3'.

**Cell Viability Assay**

Cells (1×10⁵) were plated in 12-well plates for 24 hr and then treated with different concentrations of the compounds in media containing 2.5% FBS. Cells were then trypsinized and counted after 24 hr using a Coulter Z1 cell counter (Sykesville, MD). The 100 µM concentration of dopamine, DOPA and carbidopa were the highest concentrations that exhibited a ≤ 15% decrease in cell viability.

**DNA Binding assay**

DRE binding of AhR was measured using an Episeeker DNA-protein binding assay kit (Abcam) according to the manufacturer’s protocol. A biotinylated 25-bp double-stranded oligonucleotides of DRE promoter region (wild-type; 5-GATCTGGCTTTTCTCAGCAACTCCG-3 and mutant-type; 5-GATCTGGCTTTTCTGTACATCATCACTCCG-3) containing AhR binding consensus sequence was used as a capture
probe, and 25 bp double-stranded unlabeled oligonucleotide containing the identical consensus sequences was used as a competitor. Whole cell extracts from Caco-2 cells were used in this experiment.

**Clonogenic Assay**

Clonogenic assay was performed as described recently [23,24]. The cells were seeded at a low density (500 cells/well) and allowed to attach to the substratum. Cultures were then treated with different concentrations of carbidopa (0, 1, 5, 10, 50, and 100 μM), and the colonies were allowed to grow for 10-14 days. Carbidopa was added to cells every 3 days. At the end of incubation period, the colonies were fixed and stained with crystal violet.

**RESULTS**

Several AhR-active microbial degradation products of tryptophan [25-27] and dietary AhR active compounds [28,29] induce CYP1A1 in Caco2 cells which are highly responsive to structurally-diverse AhR ligands. Results in Figure 1A show that carbidopa induced CYP1A1 in Caco2 cells; this is consistent with previous studies on carbidopa in other cell lines (20). We also observed that the neurotransmitter dopamine also induced CYP1A1 in Caco2 cells and the induction responses observed for carbidopa, dopamine, and TCDD (positive control) were inhibited after co-treatment with the AhR antagonist CH223191. Both dopamine and carbidopa induced CYP1B1 (Fig. 1B) and UGT1A1 (Fig. 1C) in Caco2 cells and the magnitude of this response was greater than observed for TCDD. These induced responses (CYP1B1, UGT1A1) were also inhibited after co-treatment with the AhR antagonist CH223191, thus confirming that these were AhR-mediated responses.

A previous report (20) indicated that DOPA was inactive as an AhR agonist in HepG2 cells whereas our results (Supplemental Figure S1A) showed that 50-100 μM DOPA induced CYP1A1 and UGT1A1 in Caco2 cells; however, the magnitude of these responses was low compared to dopamine and carbidopa. Since carbidopa and dopamine have important neuronal functions, we also examined their AhR activity in U87 glioblastoma cell lines. Carbidopa, dopamine, and TCDD induced CYP1A1 (Fig. 1A), CYP1B1 (Fig. 1B), and UGT1A1 (Fig. 1C) in U87 cells and these responses were also inhibited after co-treatment with CH223191. The fold induction of CYP1A1, CYP1B1, and UGT1A1 were significantly lower in U87 cells compared to Caco2 cells, and this was particularly evident for the low (but significant) induction of CYP1A1 and CYP1B1 observed for dopamine in U87 cells. Nevertheless, these results show, for the first time, that dopamine is an AhR-active ligand. We also compared the
Recent studies in our laboratories characterized the Ah-responsiveness of a series of patient-derived glioblastoma cell lines which expressed variable AhR levels and observed that the AhR inhibited cell growth and invasion [22]. Figure 2 summarizes the Ah-responsiveness of patient-derived 15-037 wild-type cells and 15-037 (AhRKO) cells in which the AhR has been silenced by CRISPR/Cas9 [22]. TCDD and carbidopa significantly induced CYP1A1 (Fig. 2A) and CYP1B1 (Fig. 2B) whereas only minimal induction was observed for dopamine. In contrast, dopamine but not TCDD or carbidopa, induced UGT1A1 in 15-037 glioblastoma cells (Fig. 2C) illustrating the gene-specific induction responses by these compounds. No induction responses were observed in 15-037 (AhRKO) cells. We further investigated induction of CYP1A1 (Fig. 3A), CYP1B1 (Fig. 3B), and UGT1A1 (Fig. 3C) by dopamine and carbidopa in two additional (Ah-responsive 14-104 and Ah-non-responsive 14-015) patient-derived glioblastoma cell lines [22]. Carbidopa and TCDD induced CYP1A1 and CYP1B1, whereas dopamine only induced CYP1B1 (<2-fold) in 14-104 cells whereas UGT1A1 was not inducible by these compounds in 14-104 cells. Higher concentrations of dopamine resulted in lower levels of CYP1A1 and CYP1B1. The 14-015 cells express low AhR levels and inducibility of CYP1A1 and UGT1A1 by TCDD, dopamine, and carbidopa were not observed (Fig. 3A-C). CYP1B1, on the other hand, was induced by all 3 compounds suggesting that this response was AhR-independent.

We also analyzed effects of TCDD, dopamine and carbidopa on expression of the AhR, CYP1A1 and CYP1B1 in selected cell lines (Caco-2, 14-104s and U87MG) and showed that the AhR protein is expressed in these cells and TCDD decreases expression of the receptor (Figure 4A). Induction of CYP1A1 and CYP1B1 protein was both ligand and cell context dependent; CYP1A1 was induced by TCDD in Caco2 cells and detectable induction was also observed for dopamine and carbidopa. In contrast, CYP1A1 protein was not induced in 14-104s and U87 MG cells. CYP1B1 protein was induced by all three compounds in U87MG but not in Caco2 or 14-104s cells. A chromatin immunoprecipitation assay (Figure 4B) was used to show that after treatment of Caco2 cells with TCDD, dopamine and carbidopa there was an increase in AhR binding to the DRE region of the CYP1A1 promoter and pol II binding was also increased and these results correlated with the CYP1A1 mRNA and protein induction studies.
We used an Episeeker DNA-protein binding kit to determine interactions between TCDD and dopamine with an oligonucleotide containing a dioxin responsive element (DRE) and a second oligonucleotide in which the DRE is mutated (negative control). The free DNA probe contains the DRE and competitively decreases binding to the oligonucleotides (Fig. 4C). The results show that 2 and 10 nM TCDD and 25 and 100 µM dopamine bind the wild-type but not the mutant oligonucleotides. In addition, the “free DNA” probe containing the DRE competitively decreased binding to the wild-type (DRE) oligonucleotide. These results show that like TCDD, dopamine binds a consensus DRE which is consistent with the AhR agonist activity observed for this compound.

Previous studies showed that carbidopa was also an AhR agonist in pancreatic cancer cells and inhibited BxPC3 cell and tumor growth in a xenograft model (20). Results illustrated in Figures 5A and 5B confirm that TCDD and carbidopa induced CYP1A1 and CYP1B1 in BxPC3 cells whereas only minimal induction of UGT1A1 (Fig. 5C) was observed. In contrast, dopamine and DOPA were inactive in BxPC3 cells and in the highly invasive Panc1 cells only TCDD slightly induced (< 3-fold) drug metabolizing enzyme gene expression. A key observation reported by Ogura and co-workers was that carbidopa decreased growth of BxPC3 cells (20). We therefore initially examined effects of carbidopa, dopamine, and DOPA in Panc1 (Fig. 6A) and BxPC3 (Fig. 6B) cells using a cell viability/counting assay. None of these compounds at concentrations up to 100 µM affected growth of BxPC3 and Panc1 cells and the results for TCDD were comparable to previous reports on the activity of this compound in Panc1 cells [30]. We also observed no cytotoxic effects of dopamine and related compounds in Caco2, SW480, and HCT116 colon cancer cell lines (Suppl. Fig. 1B). Similar results were observed in 15-037 patient-derived glioblastoma cells (data not shown). Previous studies showed that 1 µM carbidopa significantly inhibited cell viability in a clonogenic assay in BxPC3 cells (20); however, our results showed that significant inhibition was observed only at the 100 µM concentration (Fig. 6C). In contrast, treatment of the more aggressive Panc1 cells with 1–100 µM carbidopa did not affect cell viability (Fig. 6D). Thus, our results show that like carbidopa, dopamine is also an AhR ligand; however, the cytotoxicity of carbidopa in BxPC3 cells was considerably lower than previously described (20) and cytotoxic effects of carbidopa were not observed in Panc1 cells.

DISCUSSION

The AhR was first discovered as the intracellular protein that mediated the biochemical and toxic responses of TCDD and related halogenated aromatics [31]. Subsequent studies have identified structurally diverse chemicals

(Figs. 1A and 4A).
as AhR ligands and they include other industrial compounds, pharmaceuticals, phytochemicals including flavonoids, alkaloids, other polyhydroxy compounds, and other phytochemicals found in plant extracts, microbial metabolites, and endogenous biochemicals [32-35]. In contrast to TCDD, many of these compounds are health-promoting and this is consistent with the identification of multiple functions of the AhR in maintaining cellular homeostasis and in many diseases including cancer [36-38]. There is considerable evidence that many AhR ligands act as tissue type-specific AhR agonists and antagonists and are selective AhR modulators (SAhRMs) [39-43]. For example, the first AhR antagonist developed in this laboratory [6-methyl-1,3,8-trichlorodibenzofuran (MCDF)] antagonized TCDD-induced CYP1A1 induction and some TCDD-induced responses but acted as an AhR agonist (like TCDD) and an inhibitor of estrogen signaling [39,44].

Several studies show that tryptophan and tyrosine metabolites such as tryptamine, indole-3-aldehyde, several indole acids, serotonin, and carbidopa (an L-DOPA derivative) exhibit AhR agonist activity [20,25-27,12]. Moreover, carbidopa not only induced CYP1A1 in some cancer cell lines but this compound also inhibited BxPC3 and Capan-2 cell proliferation and tumor growth in an athymic nude mouse xenograft model (20). Research in our laboratories has used Caco2 cells as a sensitive model for screening phytochemicals and tryptophan metabolites as inducers of drug metabolizing enzyme gene expression. We confirmed that like TCDD, carbidopa induced CYP1A1, CYP1B1, and UGT1A1 in Caco2 cells and similar results were also observed for dopamine in this cell line (Fig. 1) whereas only minimal (but significant) induction was observed for L-DOPA (Supplemental Fig. S1). These responses were inhibited by the AhR antagonists CH223191 and induction responses were minimal in CRISPR/Cas9 generated 15-037-AhRKO cells (Fig. 2) and in 14-015 GBM cells (Fig. 3) which exhibit minimal AhR-responsiveness (22). We also observed variable responses for TCDD, carbidopa and dopamine among the colon and GBM cells with Caco2 cells being the most responsive (Fig. 1-3). In Caco2 cells, TCDD dopamine and carbidopa induced CYP1A1 and enhanced DNA Binding in a ChIP and modified gel shift assay (Fig. 4).

Subsequent studies in established and patient-derived glioblastoma cells and pancreatic cancer cells demonstrate tumor-type and gene-specific induction responses for dopamine and carbidopa. Both compounds induced drug metabolizing enzyme gene expression in Caco2 cells and to a lesser extent in glioblastoma cells; whereas, only carbidopa was AhR-active in BxPC3 cells and both compounds were inactive in Panc1 cells. These differences in Ah-responsiveness between BxPC3 and Panc1 cells are not surprising since ligand-induced nuclear
uptake of the AhR is observed in BxPC3 (20) but not Panc1 cells (30). Despite the differences in Ah-responsiveness (e.g. CYP1A1 induction) of carbidopa and dopamine in BxPC3 cells, neither compound affected proliferation of BxPC3 or Panc1 cells (Fig. 6A and 6B) or colon cancer cells (Suppl. Fig. S1B). We further confirmed that carbidopa inhibited cell viability in a clonogenic assay in BxPC3 cells (Fig. 6C) but this was observed only after treatment with 100 µM carbidopa; whereas, 1 µM carbidopa was previously reported to be inhibitory in this same cell line (20). In contrast, inhibition of cell viability by carbidopa in the more aggressive Panc1 cells was not observed (Fig. 6D).

Results of this study demonstrate that dopamine is an AhR agonist and activation of Ah-responsive genes by dopamine and carbidopa is gene- and cell-context specific which is consistent with their activity as SAhRMs. The cytotoxicity of dopamine and carbidopa is relatively low in pancreatic and colon cancer cells and they are unlikely to affect tumorigenicity at current physiological (dopamine) levels or pharmacological (carbidopa) doses. We also observed that norepinephrine did not exhibit AhR activity (data not shown) and are currently investigating other catecholamines and drugs used for treating Parkinson’s disease for their activity as AhR ligands.

**Abbreviations**

AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; DOPA, 3,4-dihydroxy-L-phenylalanine; DRE, dioxin responsive element; FBS, fetal bovine serum; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; PCR, polymerase chain reaction; SAhRM, selective AhR modulator; UGT, UDP glucuronosyl transferase.

**Author Contributions**

Hyejin Park, Un-ho Jin and Keshav Karki carried out most of the experiments; Sharon K. Michelhaugh and Sandeep Mittal developed and validated the glioblastoma cell lines; Arul Jayaraman, Robert Chapkin, Clint Allred, and Stephen Safe contributed to the study design and manuscript preparation; Stephen Safe wrote the manuscript, collected the data, and initiated the study.

**Funding:** The National Institutes of Health (R01-AT010282, R01-CA202697, R35-CA197707, and P30-ES029607), the Syd Kyle Chair, Allen Endowed Chair in Nutrition & Chronic Disease Prevention, Ray Nesbitt Endowed Chair, and Texas Agrilife is gratefully acknowledged.
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FIGURE CAPTIONS

Figure 1. Dopamine and carbidopa as AhR ligands in Caco2 and U87 cells. Cells were treated with DMSO (control) 10 nM TCDD, 10–100 µM dopamine, and carbidopa alone in or in combination with 10 µM CH233191, and induction of CYP1A1 (A), CYP1B1 (B), and UGT1A1 (C) was determined by real-time PCR as outlined in the Methods. Results are expressed as means ± SD for at least 3 replicates for each treatment group and significant (p<0.05) induction (*) or inhibition by CH223191 (**) is indicated.

Figure 2. Dopamine and carbidopa as AhR ligands in 15-037 wild-type and AhRKO cells. Cells were treated with DMSO, 10 nM TCDD, 10–100 µM dopamine and carbidopa, and induction of CYP1A1 (A), CYP1B1 (B) and UGT1A1 (C) was determined by real-time PCR as outlined in the Methods. Results are expressed as means ± SD for at least 3 determinations per treatment group and significant (p<0.05) induction as indicated (*).

Figure 3. Dopamine and carbidopa as AhR ligands in patient-derived 14-014 and 14-015 glioblastoma cells. Cells were treated with DMSO, 10 nM TCDD, 10–100 µM dopamine and carbidopa and induction of CYP1A1 (A), CYP1B1 (B) and UGT1A1 (C) were determined by real-time PCR as outlined in the Methods. Results are expressed as means ± SD for at least 3 determinations for each treatment group and significant (p<0.05) induction is indicated (*).

Figure 4. Induction and binding assay. A. Caco2 cells were treated with DMSO, TCDD, dopamine and carbidopa for 24 hr and whole cell lysates were analyzed by western blots as outlined in the Methods. B. Using as similar procedure treatment related interactions of the AhR and pol II with the DRE region of the CYP1A1 promoter were determined in a ChIP assay. C. DRE binding of dopamine wild-type and mutant oligonucleotides derived from the human AhR promoter were incubated with whole cell lysates from Caco2 cells treated with two different doses of TCDD (2 and 10 nM) and dopamine (25 and 100 µM). Binding was determined in a colorimetric Episeeker DNA-protein assay as outlined in the Methods. Results are expressed as means ± SD for at least 3 determinations for each treatment group.
Figure 5. Dopamine, carbidopa, and L-DOPA as AhR ligands in BxPC3 and Panc1 cells. Cells were treated with DMSO, 10 nM TCDD, 10-100 µM dopamine, carbidopa, and L-DOPA. Induction of CYP1A1 (A), CYP1B1 (B) and UGT1A1 (C) was determined by real-time PCR as outlined in the Methods. Results are expressed as means ± SD for at least 3 determinations for each treatment group and significant (p<0.05) induction is indicated (*).

Figure 6. Cytotoxicity of dopamine, carbidopa, and L-DOPA in Panc1 (A) and BxPC3 (B) cells treated with DMSO, 10 nM TCDD, and 10–100 µM dopamine, carbidopa and L-DOPA for 24 h. Cell viability were determined by cell counting as outlined in the Methods. Cell viability was also determined in BxPC3 (C) and Panc1 (D) cells in a clonogenic assay as described (20) and as outlined in the Methods. Results are expressed as a means ± SD for at least 3 determinations for each treatment group and significant (p<0.05) inhibition is indicated (*).
