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Discovery and biological characterization of 1-(1*H*-indol-3-yl)-9*H*-pyrido[3,4-*b*]-indole as an aryl hydrocarbon receptor activator generated by photoactivation of tryptophan by sunlight

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ABSTRACT

Activation of the aryl hydrocarbon receptor (AHR) by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is required for AHR dependent transcriptional activation and TCDD toxicity. We previously reported that aqueous tryptophan exposed to sunlight through window glass (aTRP) contains multiple photoproducts, including the well characterized 6-formylindolo[3,2-*b*]carbazole (FICZ), capable of activating the AHR and inducing CYP1A and CYP1A-mediated enzyme activities. We report here the isolation from aTRP and chemical characterization and synthesis of 1-(1*H*-indol-3-yl)-9*H*-pyrido[3,4-*b*]indole (IPI), a compound previously identified as a natural product of marine ascidia and now shown to be a TRP photoproduct with AHR-inducing properties. IPI, FICZ and TCDD produced equieffective induction of CYP1A-mediated 7-ethoxyresorufin deethylase (EROD) activity in chick embryo primary hepatocytes and mammalian Hepa1c1c7 cells. EROD induction by IPI was markedly curtailed in AHR-defective c35 cells, supporting the AHR dependence of the IPI response. Although IPI had a higher EC_{50} for EROD induction than FICZ, the much larger amount of IPI than FICZ in aTRP makes IPI a prominent contributor to EROD induction in aTRP. IPI was detected in TRP-containing culture medium under ambient laboratory conditions but not in TRP-free medium, consistent with its production from TRP. Cotreatment of hepatocytes with submaximal EROD-inducing doses of IPI and FICZ or TCDD produced additive increases in EROD without synergistic or inhibitory interactions. IPI and FICZ were readily metabolized by cultured hepatocytes. In addition to increasing *CYP1A4* mRNA and EROD, IPI and FICZ decreased hepatocyte phosphoenolpyruvate carboxykinase mRNA expression and glucose output, biological effects associated with TCDD metabolic dysregulation. The findings underscore a role for sunlight in generating AHR-activating bioactive molecules.

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1. Introduction

The environmental toxin and industrial by-product TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, dioxin) is the most potent and best studied activator of the aryl hydrocarbon receptor (AHR), a ligand activated transcription factor and member of the Per-Arnt-Sim (PAS) protein family [1,2]. Activation of the AHR by TCDD elicits diverse pathological effects, including tumor promotion and metabolic dysregulation leading to a wasting syndrome [1–3].

Ligand activation causes the AHR to migrate from cytosol to the nucleus and form a complex with the aryl hydrocarbon nuclear translocator (ARNT) that can bind dioxin-responsive elements (DREs) in the promoter regions of target genes including xenobiotic-metabolizing cytochrome P450 (CYP1A) enzymes and TCDD-inducible poly [ADP-ribose] polymerase (TiPARP, PARP7) [4]. In addition to environmental toxins like TCDD, some natural products have recently been shown to activate the AHR, among which tryptophan (TRP) derivatives comprise a large group [5]. These include TRP derivatives ingested in the diet or produced by metabolism *in vivo*, such as indolo[3,2-*b*]carbazole (ICZ) [6], and photoproducts formed by exposure of TRP to UV or visible light [7–9], among which 6-formylindolo[3,2-*b*]carbazole (FICZ) is the best characterized

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[10]. The discovery of natural compounds that can bind and activate the AHR has stimulated a search for diverse AHR ligands that may elicit pathologic effects (e.g., tumorigenesis, metabolic dysregulation) or as yet unrecognized physiologic activities of the AHR.

We previously reported that exposure of an aqueous solution of TRP to sunlight passing through window glass (aTRP) produces multiple photoproducts capable of activating the AHR and inducing CYP1A-mediated enzyme activities, including arachidonic acid epoxygenation and 7-ethoxyresorufin deethylation (EROD) [8]. FICZ was identified in one of 14 fractions obtained by separation of aTRP by reverse phase high pressure liquid chromatograph (RP-HPLC), all of which exhibited CYP1A inducing capacity. That prior study showed that photoactivated TRP contains many AHR inducers in addition to FICZ. We report here the identification, chemical characterization and synthesis of a novel photoproduct present in aTRP that induces CYP1A with high efficacy: 1-(1*H*-indol-3-yl)-9*H*-pyrido[3,4-*b*]indole (IPI).

2. Materials and methods

2.1. Materials

Chemicals were from Sigma–Aldrich, (St. Louis, MO), TCI (Portland, OR) or Alfa Aesar (Ward Hill, MA) and were used without further purification. Tissue culture medium and additives were from GIBCO BRL Life Technologies (Gaithersburg, MD). HPLC chemicals were of HPLC or LC/MS grade. Fertilized White Leghorn chicken eggs were obtained from Burr Farm (Hampton, CT), TCDD was from the NCI Chemical Carcinogen Repository, (Kansas City, MO) and FICZ was from Biomol/Enzo Life Sciences (Plymouth Meeting, PA).

2.2. Cell culture, EROD (7-ethoxyresorufin deethylase) and glucose output assays

Chick embryo hepatocytes (CEH) were cultured as previously described [4,8]. Briefly, livers were removed from 15 to 16 day old chick embryos, minced and disaggregated with 0.05% collagenase. Cell suspensions were centrifuged three times (1000 rpm, 4 °C, 7 min). The combined pellets were suspended in standard Ham's medium with 2% FBS (Std. Ham's). Hepatocytes were plated in 96-well microtiter plates (Costar white-walled clear-bottom, Corning Inc.) at 80,000 cells per well in 0.16 ml of medium or in 24-well plates at 0.5×10^6 cells per well in 1 ml of medium for measurement of EROD activity, or in 6-well plates at 3×10^6 cells in 3 ml of medium for measurement of glucose output. Cultured cells were allowed to rest for 48 h before medium change and treatment. All treatments of CEH or Hepa1c1c7 cells were in custom-prepared TRP-free medium (Millipore, Billerica, MA) as previously described [8]. Hepa1c1c7 cells and AHR defective c35 cells were obtained from ATCC (Manassas, VA) and grown in MEMα medium with 10% FBS.

EROD was measured as previously described [8] using a GeminiXS fluorescence plate reader for 96-well plates (Molecular Devices, Sunnyvale, CA) or a Hitachi F-4500 fluorescence spectrophotometer for 24-well plates, (excitation $\lambda = 558$ nm, emission $\lambda = 590$ nm).

Production of glucose by CEH (glucose output, GO) was measured as described [4]. Briefly, after removing treatment medium, hepatocytes were washed with phosphate buffered saline and medium was replaced with 1.5 ml of GO medium (DMEM without glucose and with 2 mM pyruvate and 20 mM lactate). After incubation for 3 h at 37 °C, the medium was collected, centrifuged at 3000 rpm for 10 min to remove cell debris and assayed for glucose using a Glucose (GO) assay kit (GAGO-20; Sigma–Aldrich, Co., St. Louis, MO). The amount of glucose in the medium was calculated with reference to a glucose standard curve, and values were corrected for cellular protein content.

2.3. Photoactivation of TRP

Aqueous solutions of TRP were prepared at a concentration of 1.42 mg/ml of TRP, designated 100X (14.2 μg/ml, 1X, is the physiologic concentration of TRP and the TRP concentration in Std. Ham's medium), and exposed to sunlight passing through window glass for 7 days as described previously [8]. TRP solutions at equivalent concentrations, wrapped in tin foil to prevent exposure to light, were also placed at the window, as controls. Sunlight-exposed TRP is referred to as aTRP (activated tryptophan).

2.4. HPLC resolution of the major AHR inducing component in fraction F7

Reverse phase HPLC separations were performed using a Denali C_{18} monomeric, 100 Å, 5 μm particle size reversed-phase column (4.6 mm × 250 mm; Vydac, Hesperia, CA) and the gradients and flow rates described in Section 3. Absorbance at 254 nm was monitored using a Waters 486 UV detector (Waters Corporation, Milford, MA). The signal was transmitted from the UV detector through a Flo-One Beta detector (Packard Instrument Company, Downers Grove, IL) using the auxiliary hardware option that converts the absorbance signal to "CTS" as the readout. Eluates of interest were collected, dried under N_2, reconstituted in DMSO and water as above and diluted in TRP-free medium for addition to cultured cells to measure EROD induction. The major EROD activity-inducing peak in fraction 7 of 14 original fractions separated on HPLC from a solution of aTRP [8] was isolated as described in Section 3.

2.5. Characterization of F7 by mass spectrometry

The purified F7 was evaporated to dryness and reconstituted in 1 ml of 2% acetonitrile in ddH_2O containing 0.1% formic acid (solvent A), vortexed and then centrifuged at 16,000 *g* for 5 min to remove particulates. Sample supernatants were further diluted 1:9 (vol:vol) with solvent A and were transferred to an autosampler vial to determine the accurate molecular mass and formula of purified F7 by on-line LC–MS analysis. The LC system comprised a Zorbax SB-AQ C_{18} column (2.1 × 100 mm, 1.8 μm particle size, Agilent Technologies, Santa Clara, CA) and a 1200 rapid resolution system containing a binary pump, on-line degasser, thermostat dual 54-well plate autosampler and a thermostat column compartment (Agilent Technologies, Santa Clara, CA). A rapid resolution cartridge (Eclipse XDB-C8, Agilent Technologies) was placed in front of the column to prevent clogging. The LC flow was introduced into an Agilent 6220 accurate mass TOF (time-of-flight) mass spectrometer, equipped with a dual spray electrospray ionization (ESI) source. A separate isocratic pump was set to deliver an internal reference mass solution to the ESI source for continuous mass calibration during sample analysis. LC parameters were set as follows: 5 μl injection volume, 0.4 ml/min mobile phase flow rate, 40 °C column temperature and 8 °C autosampler temperature. The mobile phase consisted of 0.1% formic acid in ddH_2O (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The gradient was: 0–2 min, 2% B; 2–17 min, to 98% B; 17–22 min, 98% B; 22–27 min, to 2% B. Mass spectra were acquired in 4 GHz (high resolution) mode with 1.41 spectra/sec sampled over a mass/charge (*m/z*) range of 50–1000. The TOF capillary voltage was set at 4000 V and the fragmentor at 175 V. The nebulizer pressure was 35 psi, and the N_2 drying gas was maintained at a flow rate of 12 L/min at 250 °C. MS spectra were collected over the range

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