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Evidence for New Light-Independent Pathways for Generation of the Endogenous Aryl Hydrocarbon Receptor Agonist FICZ

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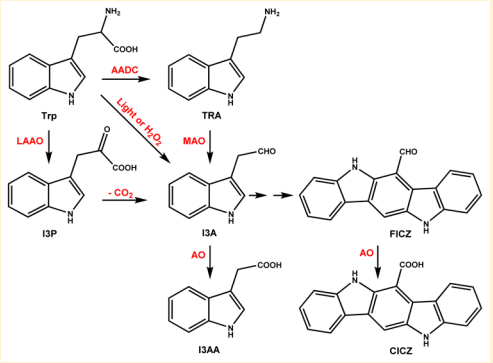
Supporting Information

ABSTRACT: Activation of the aryl hydrocarbon receptor (AhR), a conserved transcription factor best known as a target for highly toxic halogenated substances such as dioxin, under normal xenobiotic-free conditions is of considerable scientific interest. We have demonstrated previously that a photoproduct of tryptophan, 6-formylindolo[3,2-*b*]carbazole (FICZ), fulfills the criteria for an endogenous ligand for this receptor and proposed that this compound is the enigmatic mediator of the physiological functions of AhR. Here, we describe novel light-independent pathways by which FICZ can be formed. The oxidant H_2O_2 was shown to convert tryptophan to FICZ on its own in the absence of light. The enzymatic deamination of tryptamine yielded indole-3-acetaldehyde (I3A), which then rearranged to FICZ and its oxidation product, indolo[3,2-*b*]carbazole-6-carboxylic acid (CICZ). Indole-3-pyruvate (I3P) also produced I3A, FICZ, and CICZ. *Malassezia* yeast species, which constitute a part of the normal skin microbiota, produce a number of AhR activators from tryptophan. We identified both FICZ and CICZ among those products. Formation of FICZ from tryptophan or I3P produces a complex mixture of indole derivatives, some of which are CYP1A1 inhibitors. These can hinder the cellular clearance of FICZ and thereby increase its power as an AhR agonist. We present a general molecular mechanism involving dehydrogenations and oxidative coupling for the formation of FICZ in which I3A is the important precursor. In conclusion, our results suggest that FICZ is likely to be formed systemically.



INTRODUCTION

The aryl hydrocarbon receptor (AhR), a ubiquitous ligand-activated transcription factor belonging to the bHLH-PAS family of proteins, was shown 40 years ago to bind dioxins and other polycyclic aromatic compounds.[1] We propose that, rather than acting as a cytosolic sensor of exogenous small molecules and regulator of their biotransformation and detoxification, the major role of AhR and the enzymes belonging to the cytochrome P450 1 family (CYP1A1/1A2/1B1) is to help maintain cellular homeostasis in response to endogenous high-affinity ligands. AhR is already known to be involved in the expansion of progenitor cells and to play key roles in connection with development, immunity, and cancer (reviewed in refs 2−6). Accordingly, persistent activation of this receptor and disturbance of autoregulatory feedback control by its endogenous ligand probably explains the toxicity of the dioxins, which is unmatched by any other man-made substance.

To date, extensive efforts to identify endogenous ligands for AhR have revealed only a few candidates.[7] In 1987, we demonstrated that certain photo-oxidized derivatives of tryptophan (Trp) bind to the AhR with very high affinity,[8] and we have since described the properties of one such derivate, 6-formylindolo[3,2-*b*]carbazole (FICZ), and shown that FICZ is present *in vivo*.[9−15] Indeed, FICZ binds to the AhR with the highest affinity yet reported and is also an ideal substrate for the CYP1 enzymes, providing a potential mechanism for control of its own steady-state level under physiological conditions. This suggests two possible mechanisms underlying the ability of many exogenous and endogenous substances to activate the AhR: either being structurally similar to this highly planar and lipophilic molecule or by increasing its presence through formation in situ or attenuating its metabolic clearance.

These considerations provide an explanation for the early observations by Daniel Nebert and co-workers of AhR activation even in the absence of exogenous activators, leading those investigators to suggest the existence of a natural ligand.[16,17] Moreover, we propose that in most cases the activation of AhR in the absence of added exogenous ligands, e.g., following addition of fresh Trp-containing medium to cell cultures,[18] during

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oxidative stress in rats,[19] or in cells exposed to the stress of hydrodynamic shear, involves FICZ.[20,21] Furthermore, FICZ may also play a direct or indirect role in recently reported AhR activation by chemical CYP1 inhibitors or complex environmental samples.[15,22,23]

A large number of experiments have demonstrated the involvement of FICZ in many basic biological processes. These include adaptive responses to ultraviolet light;[24−26] alterations in circadian rhythms;[27] enhanced genomic instability;[28] the differentiation and growth of T cells, innate lymphoid cells, and hematopoietic stem cells;[29−35] protection against immune-related diseases;[36−39] and NK cell-mediated control of tumor growth.[40]

We and other investigators have demonstrated earlier that FICZ is formed from Trp upon exposure of aqueous solutions, including cell culture media, to ultraviolet and visible light,[8,9,14,41,42] as well as upon exposure of cultured human skin cells loaded with high levels of Trp to ultraviolet B light.[25] The presence of FICZ *in vivo* has been confirmed by the detection of sulfate-conjugated metabolites of this compound in human urine,[14] as well as of FICZ itself in extracts of skin cells from patients with vitiligo[43] or skin disorders caused by pathogenic species of *Malassezia* yeast.[44] However, the potential of biological processes other than excitation of Trp with light to form FICZ and the rates of such formation remain largely unknown.

In our original study on the UV-dependent formation of FICZ from Trp, we proposed that indole-3-acetaldehyde (I3A) was a precursor.[9] Here, we set out to examine this proposal in detail and to identify potential alternative biological pathways for the formation of FICZ via I3A. In the latter connection, an apparent possibility is oxidative deamination of tryptamine (TRA) by the mitochondrial enzyme monoamine oxidase (MAO), two isoforms of which, MAO-A and B, have been detected in humans and shown to produce I3A.[45] Similarly, decarboxylation of indole-3-pyruvate (I3P) could generate FICZ. TRA and I3P were used as starting materials in the present study. Both compounds are tryptophan metabolites formed by decarboxylation and oxidative deamination, respectively. Since H_2O_2 is formed both during irradiation of Trp and in the MAO-catalyzed reactions of TRA, we also studied whether H_2O_2 alone could generate FICZ from Trp. Here, we report for the first time light-independent formation of FICZ and present the most likely chemical mechanisms involved in these reactions producing FICZ via I3A.

EXPERIMENTAL PROCEDURES

Materials. The following compounds and enzymes were obtained from the sources indicated in parentheses: FICZ (Syntastic AB); indolo[3,2-*b*]carbazole-6-carboxylic acid (CICZ) (synthesized by Wahlström et al.[46]); glutathione (GSH), Trp, TRA, riboflavin, I3A, indole-3-acetic acid (I3AA), I3P, H_2O_2, bovine catalase, resorufin, 7-ethoxyresorufin (EOR), fluorescamine, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), and 2-phenylethylhydrazine (phenelzine) (Sigma-Aldrich); and human recombinant monoamine oxidase (MAO) A and B obtained from a Baculovirus expression system (supersomes) (BD Bioscience). The bicistronic plasmid encoding human NADPH-cytochrome P450 reductase together with CYP 1A1 was a generous gift from Dr. Fred Guengerich (Vanderbilt University School of Medicine, Nashville).

Formation of FICZ via Deamination of TRA. Reaction mixtures (1 mL) containing 100 μM TRA in 100 mM sodium phosphate buffer (pH 7.4), 50 μg of MAO-A or 125 μg of MAO-B per mL, with or without the addition of 1.0 μM GSH, were incubated at 37 °C for 30 min (MAO-A) or 120 min (MAO-B). In control experiments, phenelzine, an irreversible inhibitor of MAO, was also added. The incubation mixtures were divided into 120 μL aliquots, and the reaction was terminated by addition of 45 μL of 2 N NaOH and 15 μL of 75% formic acid or by freezing. Subsequently, the aliquots were incubated at different temperatures (37, 55, 60, 65, 75, or 85 °C) or at two different pH values. After different time points, the samples were centrifuged (10 000*g*) for 3 min. The supernatant was transferred to a new tube, and the pellet was washed with 30 μL of acetonitrile and centrifuged again in the same manner. The combined supernatants were immediately subjected to analysis by HPLC-FL and MS (QToF). In addition, the HPLC fraction containing I3A was stored for 5 days at 37 °C in the dark and thereafter analyzed for FICZ by LC-MS-QqQ.

Formation of FICZ by Irradiation of Trp. Solutions (30 mL) of 0.16 mM of Trp were irradiated for different lengths of time, either with visible light (a standard 100 W bulb (1.4 J/cm^2)) at a distance of 18 cm or by ultraviolet light from a high-pressure mercury lamp (Black-Ray long-wave UV lamp model B-100A) at a distance of 15 cm. Prior to this irradiation, differing amounts of H_2O_2 and/or catalase (2 μg/mL) were added to some of the samples. Following irradiation, the solutions (500 μL) were immediately injected onto the HPLC column and analyzed for the presence of FICZ and CICZ.

Formation of FICZ upon Exposure of Trp to H_2O_2. We incubated 0.16 mM Trp with different concentrations of H_2O_2 in the dark at 37 °C for 14 days (to ensure that the concentrations of FICZ were high enough), after which samples were taken for analyses of FICZ by HPLC-FL and LC-MS-QqQ (MRM).

Synthesis of FICZ and CICZ by *Malassezia furfur*. The lipophilic *Malassezia furfur* strain CBS 1878 was grown on agar medium,[47] with Trp as the single nitrogen source, for 7 days at 32 °C, following which the agar from each plate was cut into smaller pieces and extracted with acetonitrile. This crude extract was fractionated by HPLC-FL and analyzed for FICZ and CICZ by LC-MS/MS.

Formation of FICZ from I3P. Solutions of 1.50 mM I3P (50 mL), prepared as described by Chowdhury et al.,[48] were incubated for 5 days at 37 °C in amber glass vials in the dark, concentrated by solid-phase extraction (SPE, SepPak eluted with 3 mL of MeOH), and analyzed immediately thereafter for FICZ by HPLC-FL. The HPLC fraction containing FICZ was subjected to LC-MS analysis for confirmation.

Dehydrogenation of I3P with DDQ. I3P (404 mg, 2 mmol) was dissolved in dioxane (20 mL), and a solution of DDQ (454 mg, 2 mmol) in dioxane (20 mL) was added dropwise at 65−70 °C to the stirred solution, which resulted in quick formation of DDQ-2H as a precipitate. After 1 h, the reaction mixture was allowed to cool, and the precipitate of DDQ-2H was filtered off. The mother liquor was evaporated and treated with Na_2CO_3 (aq, 2%). The brownish solid was washed with water, dried, and then analyzed with HPLC-FL and MS/MS.

Inhibition of CYP1A1 Activity. Human CYP1A1 and NADPH cytochrome P450 reductase were expressed in *Escherichia coli* DH5α, and bacterial membranes containing these recombinant enzymes were prepared according to Guengerich.[49] The level of CYP protein was calculated on the basis of the carbon monoxide (CO) difference spectrum.[50] The ethoxyresorufin deethylase (EROD) activity of the recombinant CYP1A1 (10 nM) was assayed after preincubation with the samples of interest for 10 min followed by addition of EOR (1 μM) and NADPH (0.5 mM).[15] The ability of 10 fractions from the I3P incubations (at three to four concentrations) to inhibit the CYP1A1 activity was determined. One such fraction contained CICZ, and in this case the inhibitory effect of pure CICZ was tested instead.

Chemical Analyses. Most of the HPLC analyses were performed with a Merck Hitatchi LaCrom instrument equipped with an L-7100 pump, an L-7455 diode array detector, and a Shimadzu RF-535 fluorescence HPLC monitor. The excitation/emission wavelengths 240/320, 390/525, and 390/455 nm were used for I3A, FICZ, and CICZ, respectively. Fractions from the I3P incubations to be tested for inhibition were obtained utilizing an YL9100 HPLC equipped with an Agilent 1200 fluorescence detector and a Foxy R1 fraction collector. All HPLC-FL separations were achieved on a 250 mm long, 5 μm particle size reverse-phase C18 column using a 40 min linear mobile phase gradient from 5% B to 100% B (A, 1.5 mM formic acid in H_2O; B, 1.5 mM formic acid in acetonitrile) at a flow rate of 1.0 mL/min. Both CICZ

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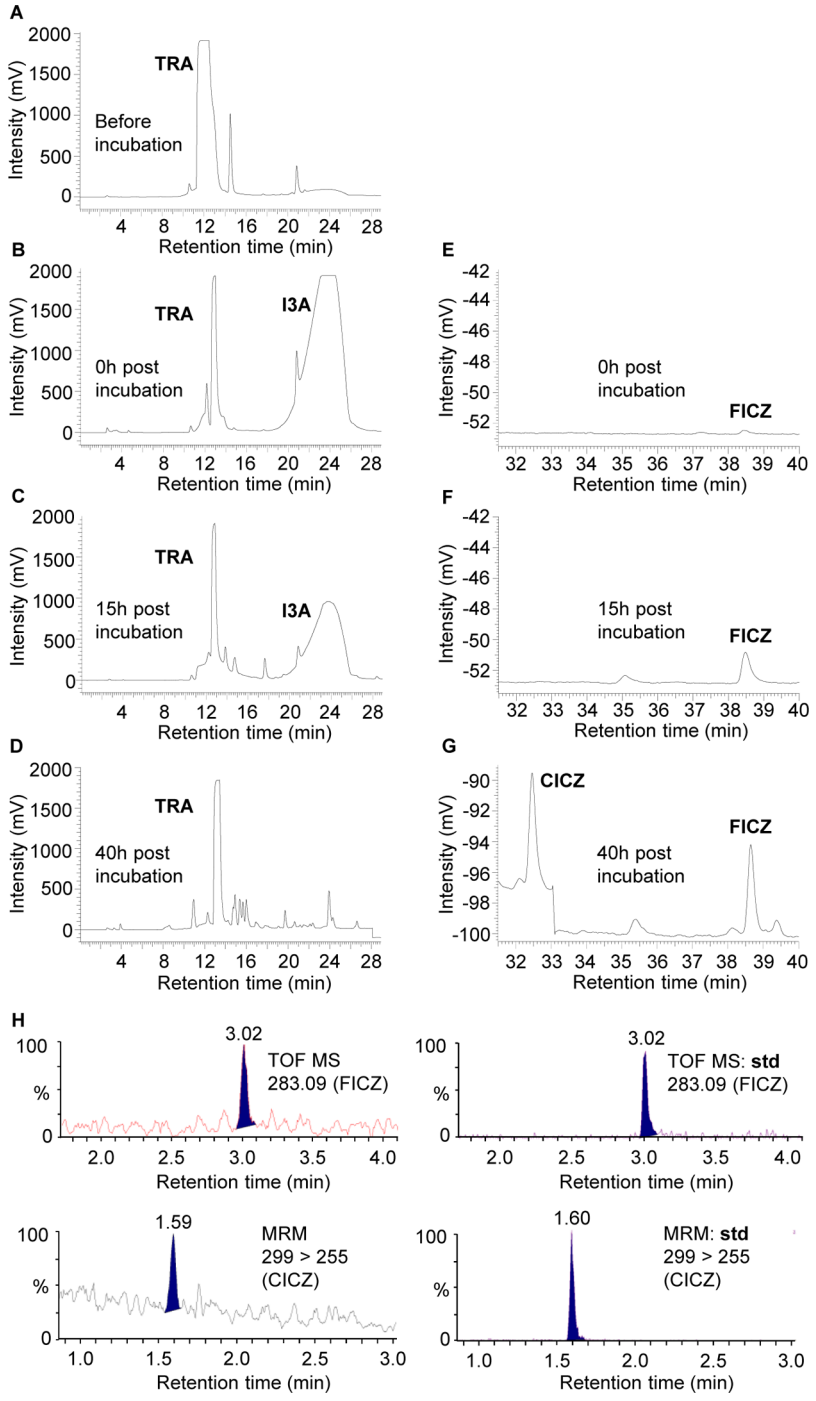

Figure 1. HPLC and MS analyses of the time-dependent formation of FICZ and CICZ from I3A produced by MAO-A-catalyzed deamination of TRA (for 30 min at 37 °C). (A–D) HPLC-FL analyses of the time-dependent reduction in the level of TRA and concomitant increase in and subsequent disappearance of I3A. (A) Incubation mixture directly after addition of TRA and MAO-A. (B–D) Time-dependent increase and decrease of the polar precursor I3A at 0, 15, and 40 h postincubation at 37 °C. (E–G) Time-dependent formation of FICZ and CICZ at 0, 15, and 40 h postincubation at 37 °C. (H) LC-MS-QToF confirmation of the identity of FICZ (TOF upper panel) and LC-MS-QqQ confirmed the identity of CICZ (MRM lower panel) following 40 h of incubation, alongside the corresponding chromatograms for authentic standards (std).

and FICZ were detected on the basis of their fluorescence and retention times.

Two different LC-MS systems equipped with essentially identical UHPLC (ultra high-performance liquid chromatography) pumps (Acquity UPLC, Waters, Milford, MA) were used. In both cases, the separation column was an Acquity HSS T3 C18 (2.1 × 100 mm, dp 1.8 μm) with a VanGuard precolumn (Waters, Wexford, Ireland). For detection, the first system employed a triple-quadrupol (QqQ) mass spectrometer (Xevo TQ-S, Waters, Manchester, UK) operated in the electrospray ionization negative-ion mode (ESI−), with multiple reaction monitoring (MRM); detailed settings are given in Supporting Information Table S1. The other system involved a quadrupole time-of-flight (QToF) high-resolution mass spectrometer (QToF Premier, Waters, Manchester, UK) in the full-scan ESI mode.

For elution in the first system, 50% A (5% acetonitrile, 10 mM acetic acid in water) was replaced linearly during a period of 2 min by 100% B (5% water, 10 mM acetic acid in acetonitrile), followed 0.5 min later by a reversed gradient back to 50% A during a period of 0.5 min, with a final

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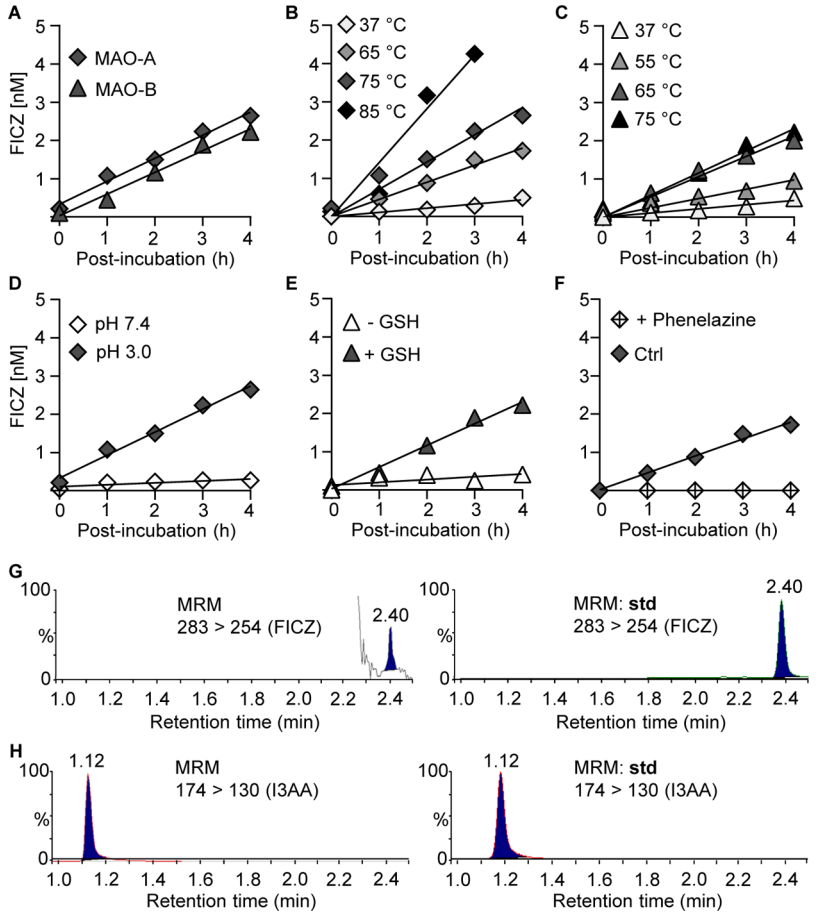

Figure 2. Influence of the isoform of MAO, time, temperature, pH, GSH, and phenelzine on formation of FICZ post incubation following deamination of TRA by MAO-A or -B. (A) Formation at 75 °C. (B, C) Formation at different temperatures after MAO-A and -B catalysis, respectively. (D) pH-dependent formation. (E, F) Formation following initial MAO-B-catalyzed deamination of TRA in the presence or absence of GSH or the inhibitor phenelzine, respectively. Confirmation by LC-MS-QqQ (MRM) of the identity of (G) FICZ and (H) I3AA formed in an I3A fraction stored in the dark for 5 days at 37 °C, alongside the corresponding chromatograms for authentic standards (std).

2.5 min of re-equilibration. The flow rate was 0.4 mL/min, and the column temperature was 40 °C. The MS settings employed are documented in Supporting Information Table S1. The details regarding the other system are provided in ref 15 (although the in-line sample enrichment described there was not used here).

RESULTS

Formation of FICZ via Deamination of TRA. To confirm that I3A is an essential precursor of FICZ, a series of experiments was performed. First, the enzymatic generation of I3A by MAO-A and -B was studied. In Figure 1, the reactions with MAO-A can be followed over time. At each time point, the polar part of the chromatograms shows the fate of the precursors, whereas the formation of the products, FICZ and CICZ, are shown in the nonpolar part of the same chromatograms. Immediately after mixing TRA with MAO-A, only TRA could be detected by HPLC with fluorescence detection (HPLC-FL) (Figure 1A), whereas after 30 min of incubation at 37 °C, I3A was the dominant peak, and the peak decreased thereafter with time (Figure 1B–D). Moreover, after the 30 min incubation with MAO-A, a small amount of FICZ was detected (Figures 1E and 2A), whereas incubation with MAO-B for 120 min resulted in no detectable levels (cf. Figure 2A). Post incubation at 37 °C, the amount of FICZ increased (Figure 1F,G), and, in addition, an HPLC peak demonstrating the fluorescent properties and retention time of CICZ, the oxidation product of FICZ, appeared at the later time points (Figure 1G). LC-MS (QToF) analyses confirmed the presence of both FICZ and CICZ (Figure 1H).

The influence of various conditions during the postincubation period for the formation of FICZ was investigated in greater detail (Figure 2). The rate of FICZ formation at 4 h postincubation at 75 °C was the same irrespective of whether MAO-A or -B was the initial catalyst (Figure 2A). This rate increased with temperature, being approximately 0.5, 2, and 5 pmol/mL after 4 h at 37, 75, and 85 °C, respectively (Figure 2B,C). More FICZ was formed at pH 3.0 than 7.4 after this incubation (the latter being the same pH as that used for the enzymatic reactions) (Figure 2D). An addition of GSH during the MAO-catalyzed formation of I3A also favored the production of FICZ (Figure 2E). The requirement for enzymatic formation of I3A from TRA for FICZ formation was further confirmed by the finding that inhibition of MAO activity with phenelzine at a concentration of 5 μM prevented this formation completely (Figure 2F). Moreover, after storage of the HPLC fraction containing I3A for 5 days at 37 °C, MRM (LC-MS-QqQ) analysis revealed formation of both FICZ (Figure 2G) and I3AA, the oxidized form of I3A (Figure 2H). It can be concluded, therefore, that the MAO-catalyzed reactions produce the necessary precursor I3A, which then generates FICZ in several nonenzymatic steps favored by low pH or increased temperature.

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Formation of FICZ from Trp. Light-dependent formation of FICZ from Trp was also time-dependent (Figure 3A–C) and required H_2O_2, since catalase prevented the formation of FICZ evoked by both visible and UV light (Figure 3B,C). Adding H_2O_2 (0.5 to 2.5 mM) enhanced the yield of FICZ maximally at 1 mM (data not shown). These findings led us to incubate Trp with different concentrations of H_2O_2 in the dark, and, indeed, formation of FICZ was detected by both HPLC-FL (Figure 3D) and MRM (Figure 3E). This formation was maximal at a H_2O_2/Trp ratio of approximately 10.

Synthesis of FICZ and CICZ by *Malassezia furfur*. When a crude acetonitrile extract from an agar culture of this yeast was fractionated by HPLC-FL, both FICZ and CICZ were detected and their identities were subsequently confirmed by LC-MS-QqQ (Figure 4).

Formation of FICZ from I3P. Since spontaneous decarboxylation of I3P yields I3A, formation of FICZ from I3P was investigated by incubating I3P as described by Chowdhury et al. and analyzed.[48] Both HPLC-FL and LC-MS (QToF) analyses verified the presence of FICZ (Figure 5, panels A and C, respectively).

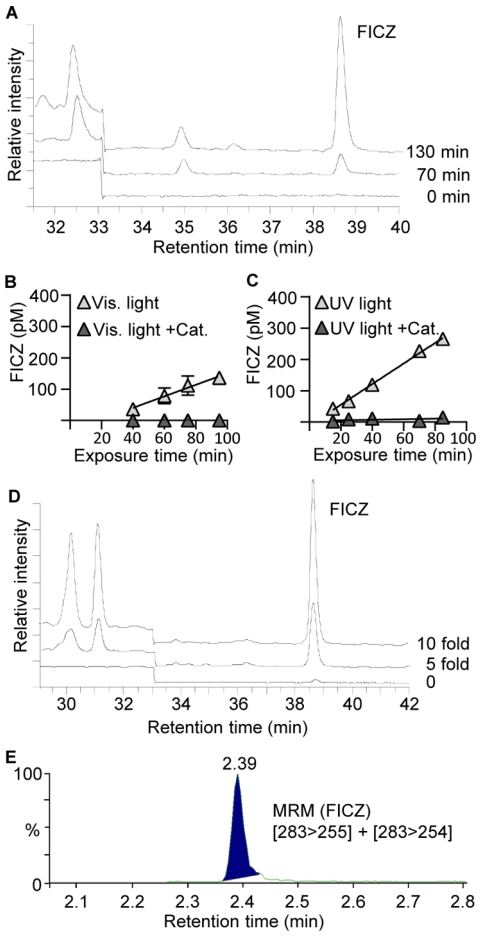

Figure 3. Formation of FICZ from Trp upon irradiation with visible or UV light or in the dark in the presence or absence of H_2O_2. (A) HPLC-FL chromatogram depicting the amount of FICZ formed after exposure of Trp to visible light for different periods of time. (B, C) Effect of catalase on FICZ formation in response to visible light or UV light, respectively. (D) H_2O_2-dependent formation of FICZ from Trp in the absence of light; the ratio of H_2O_2 to Trp was 5:1 or 10:1. (E) LC-MS-QqQ confirmation of the identity of FICZ formed in (D).

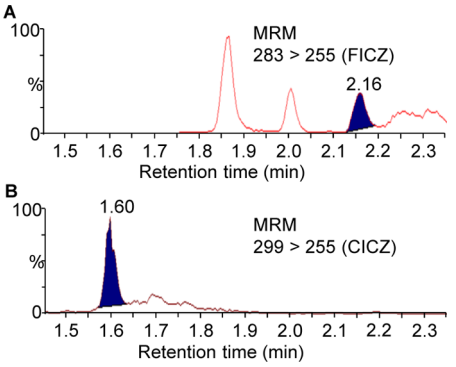

Figure 4. MS confirmation of formation of FICZ and CICZ in *Malazzessia furfur*. A crude acetonitrile extract from an agar culture of *Malassezia furfur* (CBS 1878) grown with tryptophan as the single nitrogen source, for 7 days at 32 °C, was fractionated by HPLC and subjected to MS analyses. (A) LC-MS-QqQ confirmation of the identity of FICZ; (B) LC-MS-QqQ confirmation of the identity of CICZ.

Inhibition of CYP1A1 Activity. I3P incubations resulted in complex chromatograms with several fractions containing indole-related substances. Our previous results showed that CYP1-inhibiting compounds may potentiate the effect of FICZ. We therefore set out to test the CYP1-inhibiting properties of the I3P-derived fractions. Ten such fractions (excluding the FICZ and CICZ fractions) from the I3P incubation inhibited CYP1A1 activity in a dose-dependent manner, albeit with differing potencies (Figure 5A,B). Fractions 1 and 5 were particularly potent, inhibiting this activity by 80% or more at the highest concentration tested (corresponding to 15 μL of the undiluted fraction). It should be noted, however, that each fraction may have contained more than one substance and at different concentrations, making quantitative comparison problematic. Pure CICZ itself also inhibited CYP1A1 activity (IC_{50} 9.8 nM) (not shown).

Proposed Mechanism for FICZ Formation. The results above strongly suggest that I3A is the common denominator in the different reactions leading to FICZ. A multistep synthesis was chosen to obtain the desired products, FICZ and CICZ, and to demonstrate the proposed mechanism. I3P (**1a**) rather than the more unstable I3A (**4**) was used as the starting material. The strong oxidant DDQ was used here as the reagent for dehydration and oxidative couplings and cyclization reactions, which we postulate to be the main reactions in the production of FICZ from I3P. In this context, it should be noted that in the equilibrium between tautomers **1a** and **1b** the enol form (**1a**) predominates and that in earlier studies the presence of the keto tautomer **1b** could not be detected by NMR.[51] It was thought that dehydrogenation of **1a** with DDQ might yield the hypothetic product **2**, which in turn should undergo decarboxylation to the likewise hypothetical aldehyde **3** (Scheme 1).

From a theoretical point of view, I3A (**4**) should similarly be expected to form **3** after dehydrogenation (Scheme 2), which should be expected to accept still intact **4** in a Michael fashion (Scheme 3) to form the still unknown key molecule **5**.

In this context, it is interesting that treatment of 2-methylindole **6** with DDQ yields indolo[3,2-*b*]carbazole (ICZ) **7** (Scheme 4) in a relatively low yield, as previously shown.[52]

9, a molecule of principally similar structure to the dialdehyde **5**, has previously been obtained by oxidative coupling of **8**[53] (Scheme 5).

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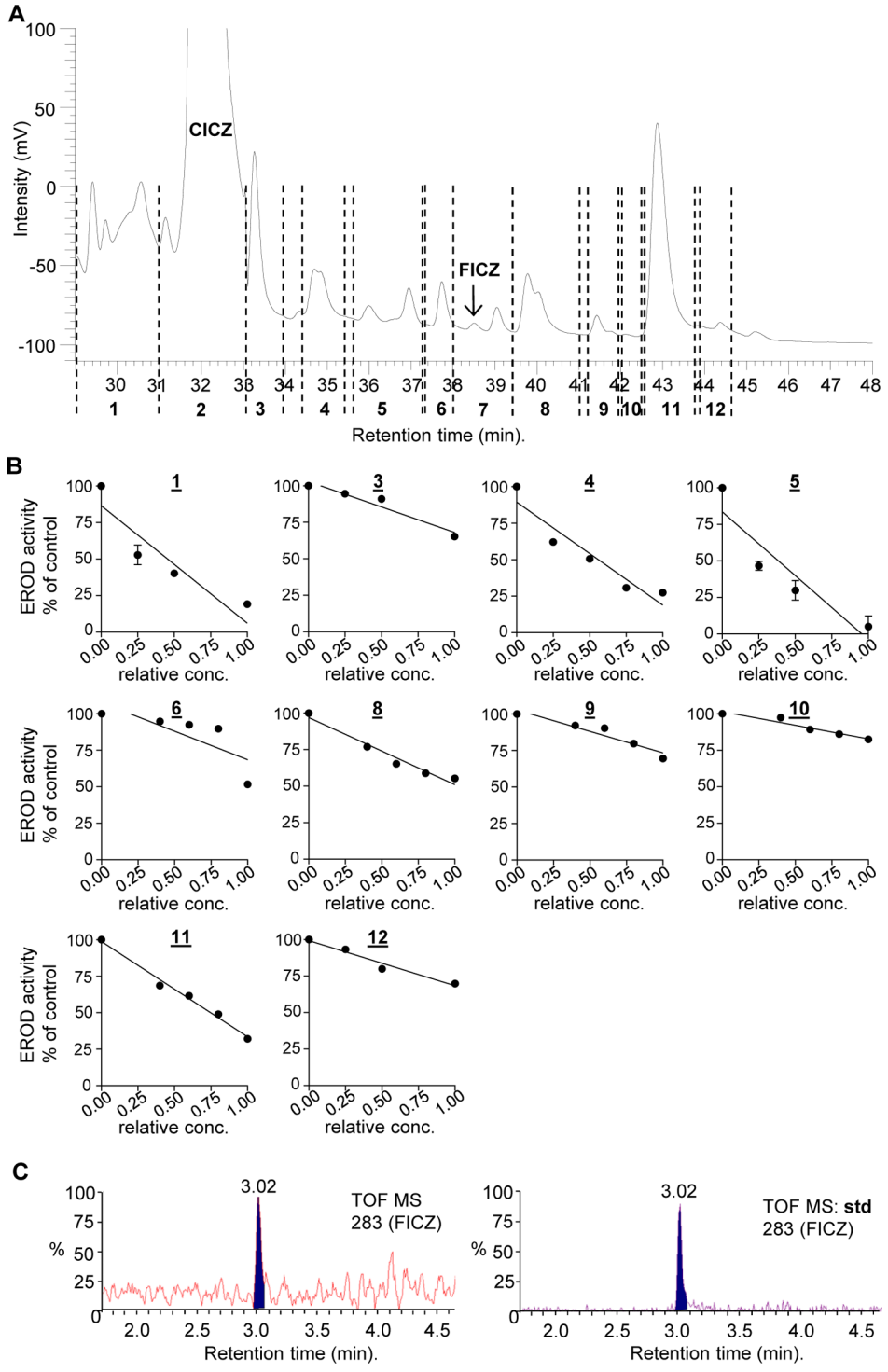

Figure 5. Detection and characterization of I3P-derived products. (A) HPLC-FL chromatogram showing nonpolar fractions generated from I3P. (B) Inhibition of CYP1A1 activity by 10 I3P-derived fractions. Each fraction was tested at three or four concentrations (1.00 = undiluted). The fractions containing CICZ (2) and FICZ (7) were not tested. (C) Formation of FICZ from the HPLC-FL fraction containing I3A derived from incubation of I3P, as confirmed by LC-MS-TOF. An authentic FICZ standard is shown on the right of (C).

Both **5** and **9** should be good substrates for oxidative coupling to, e.g., **10** (Scheme 6).

The initially formed compound **10** should readily undergo further dehydrogenation to the known molecule 6,12-diformylindolo[3,2-*b*]carbazole, dFICZ **12**, but it should also have the capacity to directly yield FICZ (**11**) via elimination of formaldehyde from **10**.[54] The two end products, FICZ and dFICZ (**11** and **12** in Scheme 6), were thus expected to be found in the complex mixture from the synthesis presented in Scheme 1. Indeed, FICZ (**11**) was identified by HPLC-FL and MRM together with larger amounts of its oxidation product CICZ (Figure 6). Compound **12** (dFICZ) was not detected, but its oxidation product, 12-formyl-5,11-dihydroindolo[3,2-*b*]-carbazole-6-carboxylic acid (malasseziazole C), was tentatively identified as a major product of the synthesis (Figure S1). The multistep reactions involved in this mechanism are summarized

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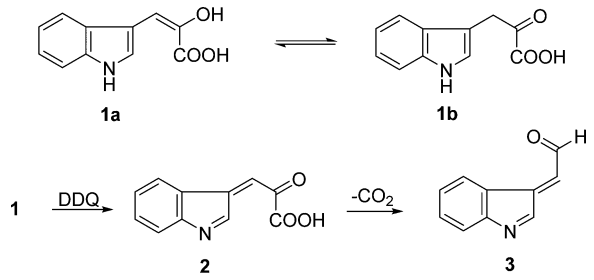
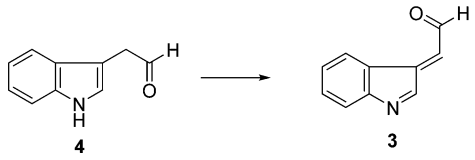
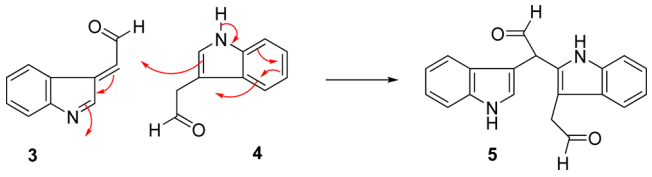
Scheme 1



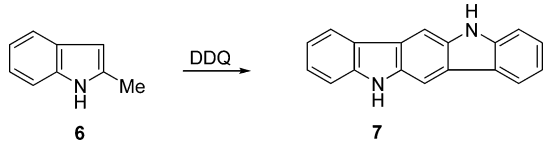
Scheme 2



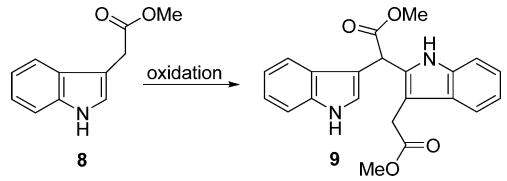
Scheme 3



Scheme 4



Scheme 5



in Schemes 1, 3, and 6 and supported by the analogous reactions presented in Schemes 2, 4, and 5.

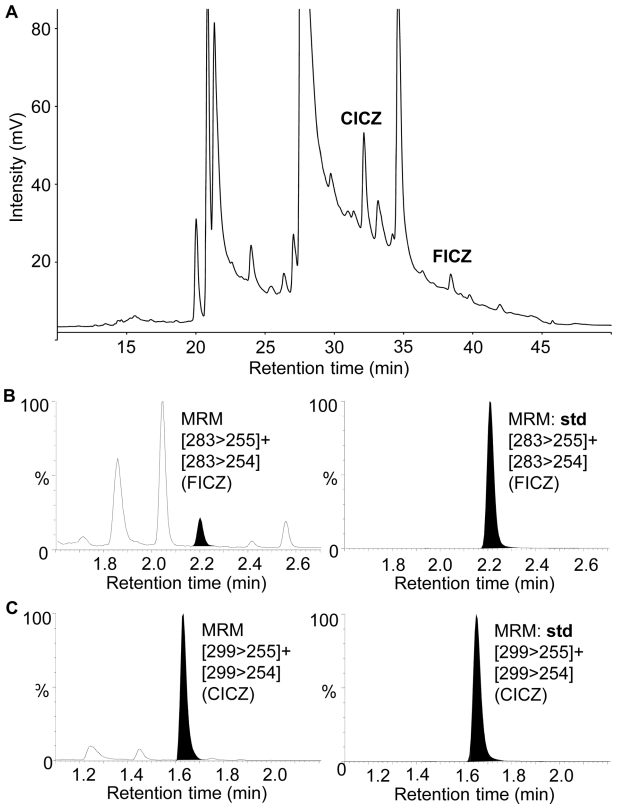
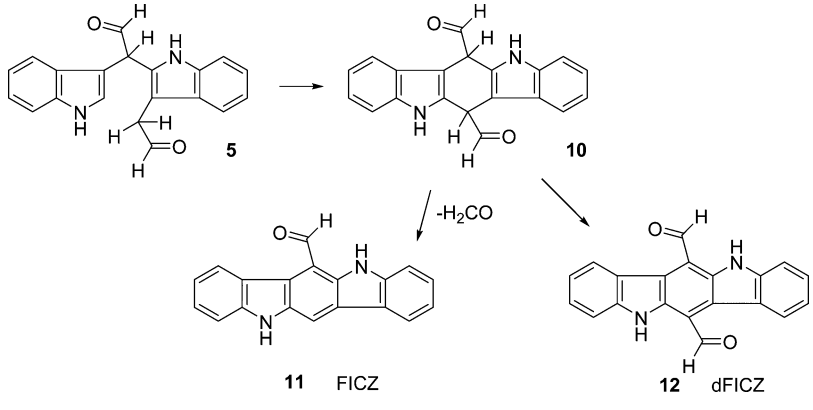


Figure 6. Formation of FICZ and CICZ after dehydrogenation of I3P by DDQ. (A) HPLC-FL chromatogram demonstrating the presence of FICZ and its oxidation product CICZ in the crude extract from the dehydration reaction. MRM confirmation of the identity of (B) FICZ and (C) CICZ together with the corresponding chromatograms for authentic standards (std).

DISCUSSION

Our present findings provide evidence for novel pathways, in addition to the originally described light-dependent pathways, for endogenous formation of FICZ (summarized in Figure 7). These results will have a considerable impact on our conceptualization of the role of FICZ and AhR signaling. The early findings indicated that AhR might act as a sensor of light, as do other members of the PAS family of sensor proteins, but our subsequent experiments now demonstrate that the function(s) of the FICZ−AhR interaction is not limited to or dependent on exposure to light.

Scheme 6



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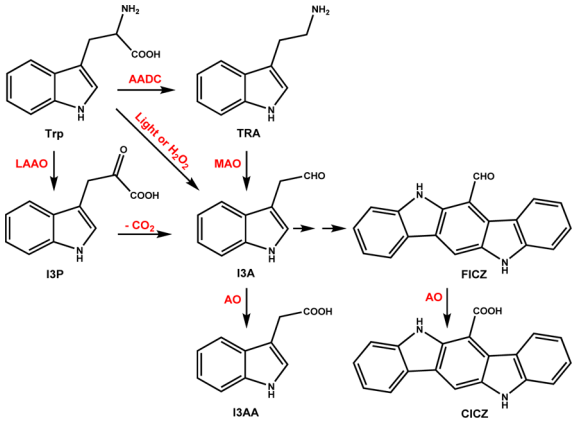

Figure 7. Pathways known to result in formation of FICZ. Tryptophan (Trp) can be converted to indole-3-pyruvate (I3P), tryptamine (TRA), and indole-3-acetaldehyde (I3A), which is the ultimate precursor for FICZ and CICZ. I3A can also be oxidized to indole-3-acetic acid (I3AA). The reactions or enzymes suggested to be involved are shown. AADC, L-amino acid decarboxylase (DOPA decarboxylase); AO, aldehyde oxidase; LAAO, L-amino acid oxidase; $-CO_2$ depicts decarboxylation.

In 1995, I3A was proposed to be the precursor for FICZ,[9] and, indeed, we found here that the MAO-catalyzed oxidative deamination of TRA results in formation of FICZ, which probably explains the reported AhR activation by MAO and TRA.[55,56] Moreover, the I3A produced by this deamination subsequently generated FICZ nonezymatically (Figure 2G). It is interesting to note that the deamination reaction also produces H_2O_2, which is another important player in the generation of FICZ (cf. below). The yield of FICZ was elevated when GSH was present during the enzymatic reaction with MAO, indicating that oxidation of I3A to the corresponding acid I3AA is a competing reaction. A hypothesized molecular mechanism yielding FICZ through dehydrogenations was tested using the more stable compound I3P and was found to generate FICZ and CICZ (Figure 6). We did not detect the end product dFICZ but rather its oxidation product 12-formyl-5,11-dihydroindolo[3,2-*b*]-carbazole-6-carboxylic acid. Earlier, Bradfield and co-workers reported that I3P gives rise to AhR-activating products, and they recently identified two of these, 1,3-di(1*H*-indol-3-yl)propan-2-one (Malassezione) and 1-(1*H*-indol-3-yl)-3-(3*H*-indol-3-ylidene)propan-2-one, as AhR agonists.[48,57] However, no other AhR ligands were mentioned. Here, we add FICZ and CICZ to the list of products. Considered together, these observations highlight the importance of I3A as a precursor of FICZ and possibly of other AhR agonists as well.

Even though FICZ fulfills all of the criteria of an endogenous AhR ligand, some other attractive candidates have been suggested. Several substances that are formed via catalytic breakdown of Trp or via enzymatic degradation of plant glucosinolates have been demonstrated to activate AhR signaling. The AhR activators kynurenine, kynurenic acid, and cinnabarinic acid are degradation products of Trp along the indoleamine-2,3-dioxygnease (IDO)- and tryptophan-2,3-dioxygenase (TDO)-catalyzed kynurenine pathways.[58−61] These molecules exhibit relatively low affinity for binding to the receptor, but activation of the kynurenine pathways is functional for many AhR-modulating immune responses as well as for malignant tumor progression (for references, see ref 2). The plant-derived compound indole-3-carbinol (I3C) is another important natural AhR activator. I3C was described early on as a dietary anticarcinogen that increases the metabolic turnover of known carcinogens through induction of cytochrome 450 enzymes.[62] Recently, I3C has been found to play important roles in immune surveillance in the gut.[63,64] However, I3C binds to the AHR with very low affinity; therefore, it is possible that its effects on gut immunity are explained by the conversion to medium- and high-affinity ligands such as 3,3′-diindolylmethane (DIM) and indolo[3,2-*b*]carbazole (ICZ) in the acidic milieu of the stomach.[65] The compound 2-(1′*H*-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), originally isolated from swine lung, and indigo, which together with indirubin was isolated from human urine and bovine serum, are other indoles exhibiting high affinity for the AhR and that have been presented as possible endogenous AhR ligands.[66,67] In comparison with the above-mentioned substances indolo[3,2-*b*]carabazoles, FICZ in particular still appears to be a key endogenous ligand, especially taking into account its properties as an exceptionally good substrate for the AhR-regulated cytochromes P450 (CYP) 1A1, 1A2, and 1B1.[14]

In this connection, it is remarkable to note that *Malassezia* yeast species, which constitute a part of the normal skin microbiota and which also have been associated with skin diseases in humans, can produce a number of AhR ligands from Trp, with FICZ among them.[44,68,69] Formation of FICZ was confirmed in the present study, and, in addition, we demonstrated the presence of CICZ in the *Malassezia* samples. It is interesting to note that the *Malassezia* metabolite malasseziazole C (12-formyl-5,11-dihydroindolo[3,2-*b*]-carbazole-6-carboxylic acid) identified by Irlinger et al. 2005 is, in fact, a partly oxidized dFICZ molecule (cf. Scheme 6 and Figure S1).[70] It seems reasonable to assume that the presence of FICZ and related substances in the skin is a common phenomenon. However, the concentrations of FICZ may vary with the conditions of the skin, e.g., oxidative status or bioavailability of Trp or other precursors involved in the different pathways leading to FICZ. Furthermore, the high AhR levels in all skin cell types point to some physiologic purpose.[71] Important roles of AhR in immune cells of the barrier organs, such as skin, are discussed in recent reviews.[2,4]

It should be noted that all of the reaction mixtures examined here, whether generated from Trp, TRA, or I3P, were associated with complex and overlapping HPLC chromatograms, indicating that a large portion of the substances present are likely to be identical. In earlier investigations on light-dependent oxidation of Trp by ourselves and others, numerous fractions, several of which activated AhR, were also obtained. Furthermore, Öberg et al. showed that in irradiated cell culture media not only the FICZ-containing fraction but also three other nonpolar fractions induced EROD significantly.[41] Rifkind's group also reported that, in addition to FICZ, sunlight also generates several other Trp photoproducts that can induce EROD both *in vitro* and *in vivo*.[42] Later, one of these was identified as 1-(1*H*-indol-3-yl)-9*H*-pyrido[3,4-*b*]indole, with moderately strong affinity for binding to AhR.[72] In their original study of I3P condensation products, Bradfield and co-workers obtained multiple fractions capable of activating AhR.[57] It is clear that all of these different fractions contain indole derivatives with different states of oxidation and condensation, and an important question now is whether they activate AhR signaling via the same or a different mechanism. In this context, the simplest hypothesis is that all of the fractions contain substantial amounts of a high-affinity AhR ligand, but this seems to be unlikely. Alternatively, fractions may contain several low-affinity ligands that can activate alone or in additive or synergistic fashion, or they may contain molecules that, during

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sample preparation or analysis, are converted to high-affinity ligands, such as FICZ. However, our results indicate that AhR activation by all of these different fractions is indirect (cf. below).

Previously, we reported that a large number of structurally diverse chemicals that activate AhR exhibit little or no affinity for the receptor but are strong inhibitors of CYP1 (reviewed in ref 15). Some of these do not activate AhR in medium free from background levels of FICZ, providing further support for an indirect mechanism.[15,73] Here, we demonstrate, in agreement with the findings by Bradfield and colleagues,[57] that several fractions obtained from the I3P reactions inhibited CYP1A1 activity and might, therefore, activate AhR indirectly by inhibiting metabolic degradation of FICZ. It is intriguing that conditions that favor formation of FICZ produce at the same time a large number of substances that inhibit CYP1A1, thereby reducing the clearance and augmenting the effects of FICZ. The significance of such an indirect mechanism *in vivo* will depend on the level and availability of the endogenous ligand.

The fact that AhR is activated by a multitude of exogenous and endogenous compounds with diverse chemical structures has led Denison's group to formulate the alternative proposal that the binding pocket of this receptor is extremely unspecific and promiscuous (reviewed in ref 74). Recently, Soshilov and Denison employed site-directed mutagenesis of the ligand-binding domain of AhR to obtain one particular mutant, I319K, that was highly specific, being activated only by FICZ in a luciferase reporter system.[75] With this mutant, they tested several AhR ligands; however, none of these ligands had low or no affinity for the receptor. As these substances did not show any activity with the mutant, the authors concluded that indirect activation via FICZ could not be involved. Since the activation of this mutant receptor was tested only at a relatively high concentration (0.1 μM) of FICZ, rather than at the picomolar concentrations present in cell culture media under normal conditions,[15,41] it is not known whether the mutant was more sensitive to FICZ at lower concentrations also. Furthermore, the tested AhR ligands were not shown to be CYP1A1 inhibitors, which is one essential mechanism for indirect activation. Therefore, indirect activation via FICZ cannot be ruled out on the basis of the observations presented for this mutant.

The observation that FICZ formation upon exposure of Trp to light is dependent on H_2O_2 and, more importantly, that H_2O_2 alone can convert Trp to FICZ are of particular interest, since H_2O_2 appears to be formed and present in most or all human cells.[76] There are multiple sources of H_2O_2 in the cell, in most cases as a byproduct of enzymatic activities. One exception is the members of the NADPH oxidase family of NOX enzymes, which produce H_2O_2 in a regulated manner.[77,78] A large number of stimuli can activate NOX activity, and we have recently reported that As^{3+} can induce AhR signaling via H_2O_2 produced by NOX.[79] Moreover, Trp is easily converted to FICZ in cell culture media and other solutions. Several microbiota, both on the human skin and in the gut, can convert Trp to a large number of indoles and indolocarbazoles such as FICZ, CICZ, or ICZ. The processes involve common human enzymes as well as non-enzymatic reactions. Therefore, it is likely that these processes would take place in mammalian and human cells *in vivo*. Together, these data suggest ubiquitous formation/presence of FICZ, albeit at low levels, in most cell types under normal conditions. Furthermore, considering the large number of physical and chemical agents that may inhibit CYP1A1, indirect activation of AhR in humans may be more common than is presently realized. Such activation by raising the cellular level of FICZ may be harmful if it is sustained or functional if it is transient. In addition to the detection of FICZ or its metabolites in humans,[14,43,80] rapidly accumulating observations in animal models or human cells *in vitro* (cf. above) indicating or suggesting physiological role(s) of FICZ make further analyses of the formation, occurrence, and functions of this compound increasingly urgent.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00416.

MS (QqQ) settings, capillary voltage, source temperature, and desolvation temperature; MS/MS spectrum of 12-formyl-5,11-dihydroindolo[3,2-*b*]carbazole-6-carboxylic acid (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

AHR, aryl hydrocarbon receptor; CICZ, indolo[3,2-*b*]carbazole-6-carboxylic acid; CYP1A1, cytochrome P4501A1; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; EROD, ethoxyresorufin-*O*-deethylase; FICZ, 6-formylindolo[3,2-*b*]carbazole; H_2O_2, hydrogen peroxide; I3A, indole-3-acetaldehyde; I3P, indole-3-pyruvate; MAO, monoamine oxidase; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; TRA, tryptamine; Trp, tryptophan

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