The Tryptophan-Derived Endogenous Aryl Hydrocarbon Receptor Ligand 6-Formylindolo[3,2-b]Carbazole Is a Nanomolar UVA Photosensitizer in Epidermal Keratinocytes

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Endogenous UVA chromophores may act as sensitizers of oxidative stress underlying cutaneous photoaging and photocarcinogenesis, but the molecular identity of non-DNA key chromophores displaying UVA-driven photodynamic activity in human skin remains largely undefined. Here we report that 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan photoproduct and endogenous high-affinity aryl hydrocarbon receptor (AhR) agonist, acts as a nanomolar photosensitizer potentiating UVA-induced oxidative stress irrespective of AhR ligand activity. In human HaCaT and primary epidermal keratinocytes, photodynamic induction of apoptosis was elicited by the combined action of solar-simulated UVA and FICZ, whereas exposure to the isolated action of UVA or FICZ did not impair viability. In a human epidermal tissue reconstruct, FICZ/UVA cotreatment caused pronounced phototoxicity inducing keratinocyte cell death, and FICZ photodynamic activity was also substantiated in a murine skin exposure model. Array analysis revealed pronounced potentiation of cellular heat shock, endoplasmic reticulum stress, and oxidative stress response gene expression observed only upon FICZ/UVA cotreatment. FICZ photosensitization caused intracellular oxidative stress, and comet analysis revealed introduction of formamidopyrimidine-DNA glycosylase (Fpg)-sensitive oxidative DNA lesions suppressible by antioxidant cotreatment. Taken together, our data demonstrate that the endogenous AhR ligand FICZ displays nanomolar photodynamic activity representing a molecular mechanism of UVA-induced photooxidative stress potentially operative in human skin.

INTRODUCTION
The causative role of UV photons in skin photoaging and photocarcinogenesis has been firmly established. UVA (320–400 nm) radiation results in little photoexcitation of DNA directly, and cutaneous generation of reactive oxygen species (ROS) and organic free radicals is now a widely accepted mechanism of UVA phototoxicity (Agar et al., 2004; Wondrak et al., 2006; Cadet et al., 2009; Zastrow et al., 2009; Marionnet et al., 2010). In addition to various molecular sources including nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase and mitochondrial electron leakage that may contribute to cutaneous ROS formation in response to UV exposure, skin chromophores may act as endogenous photosensitizers causing either direct substrate oxidation (type I) or activation of molecular oxygen (type II) upstream of cellular oxidative stress (Scharffetter-Kochanek et al., 1997; Baier et al., 2006; Wondrak et al., 2006). In human skin, various chromophores have been proposed as endogenous UV sensitizers, including protoporphyrin IX (Kennedy and Pottier, 1992), urocanic acid (Menon and Morrison, 2002), riboflavin (Sato et al., 1995), B6 vitamers (Wondrak et al., 2004), melanin precursors (Kipp and Young, 1999), collagen crosslinks (Wondrak et al., 2003), and advanced glycation and lipid peroxidation end products (Lamore et al., 2010a). However, molecular identity and causative involvement of relevant endogenous skin photosensitizers remain poorly understood (Wondrak et al., 2006).

In addition to a role in the generation of ROS through excitation of endogenous preexisting photosensitizers, we have recently demonstrated that acute exposure to solar UV can also cause the formation of potent photosensitizers in...
human skin (Williams et al., 2014). Recently, the UVB-driven formation of the 1-tryptophan-derived photoprodut 6-formylindolo[3,2-b]carbazole (FICZ) in human HaCaT keratinocytes has been demonstrated (Fritsche et al., 2007). Importantly, FICZ displays activity as a high-affinity aryl hydrocarbon receptor (AhR) agonist more potent than 2,3,7,8 tetrachlorodibenzo-p-dioxin (Wincent et al., 2009), and the significance of FICZ-dependent AhR signaling in immune modulation, circadian rhythm, xenobiotic metabolism, as well as skin barrier function and photocarcinogenesis has been explored (Fritsche et al., 2006; Wincent et al., 2009, 2012). Importantly, in addition to UVB-induced formation from tryptophan, recent research has demonstrated that FICZ is a common metabolite of Malassezia yeasts detectable in skin scale extracts from patients with Malassezia-associated diseases including seborrhic dermatitis (Magiatis et al., 2013). In contrast to metabolically inert synthetic AhR ligands such as 2,3,7,8 tetrachlorodibenzo-p-dioxin that cause sustained AhR signaling, FICZ undergoes rapid metabolic inactivation by CYP1A1, and it has been suggested that light-driven formation of this endogenous AhR ligand followed by transient AhR signaling and rapid enzymatic turnover represents the components of a photobiological signaling cascade operative in human skin (Rannug and Fritsche, 2006; Wincent et al., 2012).

In contrast to prior investigations that have examined AhR-directed activities of FICZ detected in keratinocytes and human skin (Fritsche et al., 2007; Magiatis et al., 2013), no research has explored the possibility that photoexcitation of this indolo[3,2-b]carbazole chromophore may be associated with photobiologically relevant effects that occur independently of AhR-related mechanisms. Here we present experimental evidence suggesting that FICZ displays nonmolar photodynamic activity as a photosensitizer targeting human epidermal keratinocytes (HEKs) and reconstructed human epidermis with pronounced potentiation of UVA-induced oxidative, proteotoxic, and genotoxic stress responses irrespective of AhR ligand activity.

**RESULTS**

Array analysis identifies FICZ as a sensitizer of UVA-induced stress response gene expression in human keratinocytes

First, mass spectrometric, UV-visible, and fluorescence spectroscopic characterization of the commercial FICZ preparation used in our experiments was performed, indicating that FICZ displays pronounced absorptivity and fluorescence throughout the UVA-1 and blue visible portions of the solar spectrum (Figure 1a).

Next, it was demonstrated that FICZ treatment causes rapid and transient nuclear translocation of AhR in human HaCaT keratinocytes observable within 15 minutes of continuous exposure (Figure 1b), together with pronounced upregulation of protein levels of CYP1A1, an established major AhR target gene (Figure 1c).

Using the Human Oxidative Stress RT² Profiler PCR Expression Array technology we then explored the possibility that FICZ treatment may modulate UVA-induced stress response gene expression (Figure 1d and Supplementary Figure S1a online). Array analysis compared transcriptional profiles elicited in human HaCaT keratinocytes in response to the combined or isolated exposure to FICZ (100 nM) and solar-simulated UVA (6.6 J cm⁻²). We observed that only in response to exposure to the combined action of FICZ and UVA was pronounced stress response gene expression induced. Genes upregulated in response to combined FICZ/UVA treatment were indicative of induction of oxidative (e.g., HMOX1, AKR1C2, SQSTM1, TXNRD1, GPX3) and proteotoxic stress responses (e.g., HSPA6, HSPA1A, DDIT3). In contrast to stress response gene expression that was only responsive to the combined action of FICZ and UVA, pronounced upregulation of the AhR target gene CYP1A1 was observed in all groups exposed to FICZ (FICZ only and FICZ/UVA) irrespective of UVA exposure.

**FICZ causes UVA-induced oxidative stress and impairment of genomic integrity**

Next, we observed the rapid induction of stress response signaling elicited in HaCaT keratinocytes by FICZ/UVA cotreatment within 1 hour, including dual activation of phosphorylation of p38 (Thr180/Tyr182) mitogen-activated protein kinase and inhibitory phosphorylation of eIF2α (eukaryotic translation initiation factor), established hallmarks of cell stress signaling in skin cells greatly potentiated by FICZ (Figure 2a). Likewise, upregulation of cellular NAD(P)H-quinone oxidoreductase and heme oxygenase 1 protein levels was also observed in response to FICZ/UVA cotreatment (Figure 2b and Supplementary Figure S1b online), but protein levels of thioredoxin reductase 1, upregulated at the mRNA level (Figure 1d), remained unchanged. We also observed that FICZ/UVA cotreatment impaired mitochondrial transmembrane potential (ΔΨm), observable by JC-1 flow cytometry within 1 hour (Figure 2c).

The nature of FICZ/UVA-induced cytotoxicity was further explored by assessing photodynamic induction of oxidative stress (Figure 2d–l). Flow cytometric analysis of FICZ/UVA-treated cells (Figure 2d) indicated the generation of reactive species of sufficient longevity, such as protein peroxides (Wright et al., 2003), capable of oxidizing the indicator dye 2′,7′-dichlorodihydrofluorescein during cell loading after irradiation, an effect suppressed if photodynamic treatment occurred in the presence of the singlet oxygen quencher NaN₃. Moreover, direct formation of superoxide anions by UVA/FICZ photosensitization was demonstrated using the
FICZ Is a Nanomolar UVA Photosensitizer

SL Park et al.

**Figure 1.** The aryl hydrocarbon receptor (AhR) agonist 6-formylindolo[3,2-b]carbazole (FICZ) is a sensitizer of UVA-induced stress response gene expression in HaCaT keratinocytes. (a) Left: Electrospray MS-MS (tandem mass spectroscopy; positive ion mode); molecular ion (m/z = 284); ∆m 28 u: loss of carbonyl; middle: UV-visible spectrum; right: fluorescence spectra (excitation spectrum (λex = 530 nm); emission spectrum (λem = 390 nm)). (b) AhR nuclear translocation (FICZ: 100 nM; ≥ 240 minutes; bar = 10 μ). (c) CYP1A1 western blot analysis (16 hours). (d) Oxidative Stress RT2 Profiler PCR Expression Array analysis. After irradiation (UVA: 6.6 J cm⁻²), cells were rinsed and then cultured in medium (6 hours), followed by analysis; left: FICZ/UVA-induced gene expression (versus untreated); cutoff lines: 3-fold up- or downregulation; right: numerical expression changes (UVA only; FICZ only; FICZ/UVA versus untreated (n = 3, mean ± SD; P < 0.05)).

chemical nitro blue tetrazolium (NBT) reduction assay performed in the absence or presence of superoxide dismutase (Figure 2e). UVA-driven formation of the NBT reduction product NBF occurred as a function of FICZ concentration and was suppressed when UVA exposure occurred in the presence of superoxide dismutase, consistent with NBT photoreduction downstream of FICZ-sensitized formation of superoxide anions (Wondrak et al., 2002).

We also observed that FICZ is a sensitizer of UVA-induced phototoxic damage targeting isolated macromolecules (peptides and plasmid DNA) as well as genomic DNA in HaCaT keratinocytes (Figure 2f–l). Sensitization of macromolecular damage by FICZ was studied examining photooxidation of melittin (C₁₃₁H₂₂₉N₃₉O₃₁, Mw = 2,845.80, monoisotopic peak), previously used as a model target in studies on peptide oxidation and radiation damage (Figure 2f; Lamore et al., 2010a). The peptide melittin remained either untreated or underwent UVA irradiation (3.3 J cm⁻²) in the presence or absence of FICZ (100 nM). Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry revealed that only UVA exposure in the presence of FICZ induced the formation of a reaction product displaying a mass increase of 32 u (2,877.74–2,845.76 u, monoisotopic peaks), suggesting the FICZ-sensitized introduction of molecular oxygen into the target, a mass increase completely suppressed if exposure occurred in the presence of the singlet oxygen quencher NaN₃ (Lamore et al., 2010a).

Next, photodynamic introduction of DNA damage was examined using a chemical plasmid cleavage assay enhanced by formamidopyrimidine (Fpg)-endonuclease digestion visualizing sites of DNA base oxidation including 8-oxodG through enzymatic generation of strand breaks (Figure 2g–i) (Wondrak et al., 2002; Lamore et al., 2010b). Indeed, only the combined action of UVA and FICZ followed by Fpg digestion was sufficient to cause the formation of nicked target DNA. Dose–response analysis revealed that low nanomolar concentrations of FICZ are sufficient to induce the UVA-driven formation of Fpg-sensitive sites, and photodynamic cleavage of plasmid DNA was completely suppressed in the presence of antioxidants known to act as excited state quenchers and singlet oxygen antagonists (NaN₃, 1,4-diazabicyclo[2.2.2]octane, l-histidine).

Genotoxic consequences of FICZ/UVA exposure were also observed in HaCaT keratinocytes employing the comet assay enhanced by Fpg digestion performed at various timepoints after treatment (0–6 hours; Figure 2j–l). Consistent with
displayed a NaN₃-suppressible increase in comet tail moment. HaCaT keratinocytes exposed to the combined (but not the UVA (6.6 J cm⁻²); NaN₃ (10 mM); 1 hour). (d) Induction of mitochondrial transmembrane potential (Δψm; 10 nM FICZ; UVA (6.6 J cm⁻²); 1 hour); numbers: percentage of cells inside the circle with intact Δψm (mean ± SD, n = 3). (e) Intracellular oxidative stress by flow cytometry of 2',7'-dichlorofluorescein (DCF) fluorescence (UVA (3.3 J cm⁻²); FICZ (20 nM); NaN₃ (10 mM); 1 hour). (f) MS analysis of peptide photooxidation. Monoisotopic mass peaks are indicated. (g-i) FICZ/UVA-induced PHX-174-plasmid cleavage enhanced by formamidopyrimidine (Fpg) digestion (OC: open circular; CC: closed circular; FICZ: 10 nM; UVA: 6.6 J cm⁻²). (h) With inclusion of chemical antioxidants (10 mM each); (i) dose–response (UVA: 6.6 J cm⁻²). (j-l) Fpg-enhanced Comet assay (FICZ: 100 nM; UVA: 6.6 J cm⁻²; 20 mM NaN₃ (analysis post treatment: j: 1 hour; k-l: 0–6 hours). (l) Tail moment analysis (n ≥ 100 per group; mean ± SEM).

Photodynamic introduction of oxidized DNA base lesions, HaCaT keratinocytes exposed to the combined (but not the isolated) action of FICZ and UVA followed by Fpg digestion displayed a NaN₃ suppressible increase in comet tail moment. A statistically significant increase in comet tail moment was already detectable when analysis was performed immediately after exposure (“0 hours”) and increased further over an extended incubation period (“6 hours”), an observation consistent with the known amplification of photooxidative damage by subsequent free radical chain reactions downstream of the initial oxidative insult.

**FICZ is a nanomolar photosensitizer of UVA-induced keratinocyte cell death**

Photodynamic induction of skin cell death by FICZ/UVA cotreatment was examined by morphological analysis.
FICZ is a nanomolar UVA photosensitizer

6-Formylindolo[3,2-b]carbazole (FICZ) is a nanomolar sensitizer of UVA- and blue light–induced apoptosis. (a) HaCaT keratinocytes were exposed to the isolated or combined action of FICZ (100 nM) and UVA (6.6 J cm$^{-2}$) or remained untreated (control). Transmission electron (upper panels; 2,650-fold magnification; bar = 2 μm) or light microscopy (bar = 10 μm) after 6 hours incubation in medium. (b) Flow cytometric analysis of AnnexinV-FITC/propidium iodide (PI)-stained cells (24 hours after treatment as in a). Numbers indicate viable cells (AV–/PI–) as a percentage of total gated cells (mean ± SD, n = 3). (c) Flow cytometric detection of cleaved caspase-3. (d) Left: cytoprotection against FICZ-induced cell death by zVADfmk (40 μM; 1 h pretreatment) or NaN$_3$ (10 mM; coinubation during UVA; FICZ: 20 nM; UVA: 1 J cm$^{-2}$); right: bar graph summarizing viability as assessed by flow cytometry (mean ± SD, n = 3). (e) Glutathione depletion–enhanced apoptosis (buthionine sulfoximine (BSO): 1 mM, 24 hours pretreatment; conditions as in d). (f) FICZ-induced apoptosis assessed as in b. (g) FICZ dose–response relationship (UVA: 6.6 J cm$^{-2}$). Photodynamic induction of cell death (UVA: 6.6 J cm$^{-2}$) using riboflavin (B$_2$) and protoporphyrin IX (PPIX) is also depicted. (g) UVA dose–response relationship (FICZ: 100 nM). (h) Blue light (LED 460 nm; 2.5 J cm$^{-2}$)–induced apoptosis (FICZ: 100 nM; zVADfmk/NaN$_3$ as above; mean ± SD, n ≥ 3).

Next, the dose–response relationship underlying FICZ sensitization of UVA-induced cell death was explored, indicating that FICZ concentrations as low as 5 nM and small doses of UVA (≥ 0.33 J cm$^{-2}$) were sufficient to cause pronounced photodynamic effects (Figure 3f-g). In contrast, UVA-preirradiation of FICZ followed by incubation of unirradiated cells using preirradiated FICZ did not mimic the effects of FICZ/UVA coexposure, suggesting that photodynamic induction of HaCaT cell death depends on the formation of a short-lived cytotoxic factor (such as singlet oxygen or other ROS) that is absent from the preirradiated FICZ preparation (Figure 3f; 'pre'). Remarkably, UVA-driven photodynamic activity of FICZ surpassed that of established endogenous photosensitizers including protoporphyrin IX and riboflavin (vitamin B$_2$) that were active only in the upper nano- (100 nM) or lower micromolar (10 μM) range, respectively (Figure 3f).
Next, on the basis of absorbance in the blue light region we employed a monochromatic visible photoexcitation source (460 nm, LED) in order to assess the feasibility of achieving visible light-driven FICZ-dependent photodynamic effects (Figure 3h). Indeed, FICZ displayed pronounced blue-light-driven photodynamic activity that was antagonized by either NaN₃ cotreatment or preincubation using zVADfmk, observations matching the blockade of FICZ/UVA-induced cell death as detected above (Figure 3h).

The deformylated FICZ derivative indolo[3,2-b]carbazole (ICZ) does not display photodynamic activity
In order to explore the structure activity relationship of FICZ-induced photodynamic effects, we also examined the FICZ derivative ICZ, a close structural analog and potent AhR agonist devoid of the carbaldelyde function contained in FICZ (Supplementary Figure S2a online; Wincent et al., 2009). Consistent with the established AhR ligand activity of ICZ, we observed ICZ-induced upregulation of CYP1A1 expression at the protein and mRNA level irrespective of UVA exposure (Supplementary Figure S2c and g online). In contrast, ICZ was devoid of any UVA-driven photodynamic activity as indicated by morphological inspection (transmission electron and light microscopy; Supplementary Figure S2b online), flow cytometric analysis of viability (Supplementary Figure S2d online), and oxidative stress induction (Supplementary Figure S2e online). Likewise, in response to exposure to the combined action of ICZ and UVA, stress response gene expression remained unchanged at the mRNA and protein levels (NAD(P)H-quinone oxidoreductase and heme oxygenase 1; Supplementary Figure S2f and g online).

Photodynamic activity of FICZ can be observed in dermal fibroblasts, primary HEKs, and an organotypic epidermal skin reconstruct
UVA-induced photodynamic activity of low nanomolar concentrations of FICZ was also observed when human dermal fibroblasts were exposed to the combined action of FICZ and UVA (6.6J cm⁻²; Figure 4a), accompanied by changes at the mRNA level indicative of stress response gene expression (HMOX1, DDIT3, and HSPA6; Figure 4b). In accordance with earlier reports that document constitutive attenuation of AhR signaling in dermal fibroblasts, we observed that FICZ exposure (FICZ only or FICZ/UVA combination) failed to upregulate CYP1A1 mRNA levels, indicating again that FICZ photodynamic effects occur in the absence of AhR-mediated signaling. Next, photodynamic effects of FICZ were confirmed in primary HEKs (Figure 4c and d) and reconstructed human epidermis undergoing short-term culture in growth medium supplemented with FICZ (Figure 4e). One day after UVA exposure, only the tissue reconstructs that had received FICZ/UVA combination treatment displayed pronounced phototoxicity as evident from detection of pycnotic/eosinophilic features and caspase-3 positivity, changes consistent with photodynamic induction of cell death that affected 100% of keratinocytes situated in the basal layer of the epidermis. Photodynamic effects of FICZ were also observed in an acute exposure model in murine skin (Figure 4f). When SKH-1 mice received FICZ topical treatment followed by UVA exposure, photodynamic induction of pronounced epidermal necrosis, together with Hsp70 upregulation, was observed, molecular changes that were not detected in skin exposed to the isolated action of either topical FICZ or UVA. Moreover, as early as 48 hours after FICZ-UVA photodynamic treatment, signs of regenerative re-epithelialization and tissue remodeling originating from the hair follicles were observable.

DISCUSSION
UVA sensitization by specific cutaneous chromophores is an important mechanism of skin cell photooxidative stress that contributes to photoaging and carcinogenesis (Wondrak et al., 2006). Here we demonstrate that the l-tryptophan-derived photopродuct and AhR ligand FICZ display nanomolar UVA-driven photodynamic activity as substantiated by (i) photooxidation of isolated macromolecules (Figure 2f-i), (ii) photooxidative stress and cytotoxicity in cultured human skin cells (Figures 2a–d, j–l, 3, and 4a–d), and (iii) tissue damage observable in human reconstructed epidermis (Figure 4e) as well as murine skin (Figure 4f).

Remarkably, FICZ photosensitization is observable in the low nanomolar concentration range, suggesting that FICZ exhibits extraordinary photodynamic potency (Figure 3f). Comparative sensitization experiments demonstrated that the UVA-induced photodynamic potency of FICZ surpasses that of established endogenous photosensitizers including riboflavin and protoporphyrin IX, an observation that to the best of our knowledge supports the conclusion that FICZ is the most potent endogenous UVA photosensitizer identified as of today. We also observed that FICZ photodynamic activity could be elicited by either UVA or blue light photoexcitation, a finding consistent with its UV-visible absorption characteristics (Figure 1a).

Prior investigations have demonstrated the UV-driven photochemical transformation of l-tryptophan leading to the formation of specific photoproducts, some of which display activity as endogenous photosensitizers, such as N-formylkynurenine known to be a micromolar sensitizer of photoxidative stress during aging of the human lens (Andley and Clark, 1989). However, in spite of an extensive body of evidence that explores the emerging role of the tryptophan photoproduct FICZ in AhR-dependent modulation of skin barrier function and photocarcinogenesis, no research has focused on the direct photochemical and photobiological reactivities of this UVA-visible chromophore. FICZ photodynamic potency surpasses that of other tryptophan-derived micromolar sensitizers including N-formylkynurenine and xanthurenic acid by more than 1,000-fold, but the specific mechanistic involvement of FICZ versus other tryptophan-derived metabolites and photoproducts in mediating skin photooxidative stress remains to be elucidated. In this context, it should be emphasized that independent of its solar UV-driven origin FICZ has now been identified as a common metabolite of commensal Malassezia yeasts involved in the causation of cutaneous inflammatory pathologies such as seborrheic dermatitis, and detailed liquid chromatography–mass spectrometry analysis has demonstrated unambiguously that a microbiome-derived pool of FICZ (and other tryptophan...
metabolites with unexplored phototoxicity) exists constitutively in human skin (Magiatis et al., 2013).

Importantly, photodynamic activity of FICZ observed by us in skin cells and tissue does not depend on AhR signaling, as supported by the fact that (i) FICZ photoreactivity associated with ROS formation and substrate oxidation can be observed in cell-free chemical systems, (ii) FICZ-induced stress response signaling, introduction of photooxidative DNA damage, and loss of mitochondrial transmembrane potential can be observed within minutes of FICZ/UVA exposure, and (iii) FICZ-sensitized UVA photodamage occurs in fibroblasts displaying strongly attenuated or absent AhR signaling. Finally,
(iv) the deformylated FICZ derivative ICZ, an established AhR agonist of comparable potency, was completely devoid of UVA-driven photodynamic activity (Supplementary Figure S2 online). The differential photodynamic activity of FICZ and ICZ suggests that it is the excited state chemistry associated with the carbaldehyde moiety (attached to the common indolocarbazole UVA chromophore) that allows efficient intersystem crossing and triplet state formation underlying phototoxicity, a reactivity documented with numerous carbonyl group–containing UVA photosensitizers such as benzophenone-related chromophores. However, the complex UVA-driven photochemistry of FICZ that may involve the combined mode of type I and type II photosensitization awaits further photochemical investigations.

In summary, our experimental data suggest that two molecular pathways may operate simultaneously mediating FICZ-dependent effects in human skin downstream of its formation as a cutaneous microbial metabolite or solar UVB-induced tryptophan photooxidation product (Supplementary Figure S3 online). First, as demonstrated earlier, FICZ may modulate skin cell function through an AhR-dependent pathway (Fritsche et al., 2007; Magiatis et al., 2013), a mechanism that may contribute to inflammatory dysregulation and carcinogenesis as suggested before (Agostinis et al., 2007; Gaitanis et al., 2012; Esser et al., 2013; Tigges et al., 2014). In addition, as demonstrated in this prototype study, photoexcitation of FICZ by solar UVA (and potentially visible) photons causes photodynamic effects leading to the induction of cutaneous oxidative stress that may potentially exacerbate AhR-dependent cutaneous pathologies. However, it remains to be shown that in human skin, exposed to full spectrum solar UV, initial UVB-driven formation of the AhR agonist FICZ from tryptophan is followed by UVA-dependent photosensitization reactions, an emerging mechanism of skin phototoxicity that integrates photosensitizer generation driven by one spectral fraction of solar UV (UVB) with photosensitizer activation driven by another fraction (UVA). Future experimentation will aim at examining the occurrence of UV-driven versus microbial FICZ formation and its mechanism in AhR-dependent and -independent human skin photobiology.

**MATERIALS AND METHODS**

**Chemicals**

FICZ (CAS#:172922-91-7) was purchased from Enzo (Plymouth Meeting, PA); ICZ (CAS#: 6336-32-9) was purchased from Tractus (Perinneville, NJ). All other chemicals were from Sigma (St Louis, MO).

**Spectroscopy and mass spectrometry**

Mass spectrometric analysis and UV-visible/fluorescence spectroscopy were performed as described in Supplementary Materials and Methods online (Lamore et al., 2010a).

**Irradiation with solar-simulated UVA and blue light**

Irradiation with solar-simulated UVA and blue light occurred as described in Supplementary Materials and Methods online (Wondrak et al., 2003; Lamore et al., 2010a).

**Cell culture**

Dermal neonatal foreskin Hs27 fibroblasts from ATCC and human immortalized HaCaT keratinocytes were cultured in DMEM containing 10% fetal bovine serum (Wondrak et al., 2008). Primary HEKs (adult HEKa (C-005-5C)) were cultured on collagen matrix protein-coated dishes using Epilife medium (EDCS growth supplement; Life Technologies, Carlsbad, CA).

**Human epidermal skin reconstructs**

EpiDerm tissues (EPI-200, 9 mm diameter, six-well format; MatTek, Ashland, MA) were treated with FICZ (100 nm final concentration in 0.9 ml EPA-200-ASY media per well), followed by culture at 37°C for 6 hours. Before irradiation, inserts were rinsed with PBS and then UVA-exposed. After irradiation, tissue inserts were cultured for another 24 hours in media. Tissue was then processed for paraffin embedding followed by hematoxylin and eosin staining and immunohistochemical analysis.

**Immunocytochemical detection of AhR nuclear translocation**

For immunocytochemistry, cells were pelleted by centrifugation and processed for paraffin embedding. For AhR detection (sc-5579, Santa Cruz Biotechnology, Santa Cruz, CA), staining was performed using a streptavidin biotin peroxidase system with a phosphatase substrate and a hematoxylin counter stain.

**Human Oxidative Stress RT2 Profiler PCR Expression Array analysis**

Preparation of total cellular RNA, reverse transcription, and Human Oxidative Stress RT2 Profiler PCR Expression Array (Super-Array, SABiosciences, Frederick, MD) profiling were performed as described in Supplementary Materials and Methods online (Lamore et al., 2010b; Qiao et al., 2013).

**Detection of intracellular oxidative stress**

Induction of intracellular oxidative stress by photosensitization was analyzed by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate as a non-fluorescent precursor dye following a published standard procedure (Wondrak et al., 2004; Lamore et al., 2010c).

**Mitochondrial transmembrane potential**

Mitochondrial transmembrane potential (ΔΨm) was assessed using the potentiometric dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyanobenzimidazolyl-carbocyanine iodide (JC-1; Sigma, T4069) following our published procedure (Qiao et al., 2013).

**Immunoblot analysis**

Immunoblot analysis was performed following our published standard procedures. The following primary antibodies were used: CYP1A1 (sc-20772, Santa Cruz Biotechnology), total p38 (#9212, Cell Signaling, Danvers, MA), CYP1C1 (sc-377222, Santa Cruz Biotechnology), heme oxygenase 1 (#5833, Cell Signaling). Use of horseradish peroxidase-conjugated goat anti-rabbit (111-035-144) or goat anti-mouse secondary antibody (115-035-146, Jackson Immunological Research, West Grove, PA) was followed by visualization using enhanced chemiluminescence detection reagents.
Plasmid cleavage assay
DNA strand breakage was measured by the conversion of supercoiled 4X-174 RFI double-stranded DNA (New England Biolabs, Ipswich, MA) to open circular form as described elsewhere (Wondrak et al., 2002).

Comet assay (alkaline single-cell gel electrophoresis)
The alkaline Comet assay was performed according to the manufacturer's instructions (Trevigen, Gaithersburg, MD) as described in Supplementary Materials and Methods online (Wondrak et al., 2003; Lamore et al., 2010b).

Transmission electron microscopy
Specimens were examined using a CM12 transmission electron microscope (FEI, Hillsboro, OR) operated at 80 kV with digital image collection as described recently (Qiao et al., 2013).

Flow cytometric analysis of cell viability
Induction of cell death was confirmed by AnnexinV-FITC/propidium iodide dual staining of cells using an apoptosis detection kit according to the manufacturer's specifications (APO-AF, Sigma).

Caspase-3 activation assay
Treatment-induced caspase-3 activation was examined using a cleaved/activated caspase-3 (asp 175) antibody (Alexa Fluor 488 conjugate, Cell Signaling) followed by flow cytometric analysis as published recently (Lamore et al., 2010a).

Superoxide assay
Chemical formation of superoxide radical anions during FICZ photodynamic treatment was determined using the photometric NBT reduction assay, confirmed by scavenging of superoxide using superoxide dismutase as detailed in Supplementary Materials and Methods online (Wondrak et al., 2002).

Mouse skin photodynamic treatment using FICZ
SKH-1 hairless female mice (Charles River Laboratories, Wilmington, MA) were maintained under 12-hours light/dark cycles receiving water and food ad libitum. At the beginning of the experiment, 8-week-old mice (n = 12) were divided into four groups (n = 3): (i) control (DMSO only), (ii) UVA + DMSO, (iii) FICZ in DMSO, (iv) UVA + FICZ in DMSO. Solar-simulated UVA dose was 6.6J/cm². ‘FICZ’ (in DMSO; 1 mM final concentration) or ‘DMSO only’ were applied topically to dorsal skin areas (20μl total volume). After 10 minutes, UVA or mock irradiation was performed. Mice were maintained for another 48 hours and then euthanized. Dorsal skin was harvested and processed for (histo)-pathological examination and further immunohistochemistry analysis (Hsp70). Animal experimental procedures and protocol have been reviewed and approved by the University of Arizona Institutional Animal Care and Use Committee (PHS Assurance No. A-3248-01; #11-316).

Hematoxylin and eosin staining and immunohistochemistry
Reconstructed skin and mouse skin specimens were stained using hematoxylin and eosin according to standard procedures. Immunohistochemistry analysis was performed as previously described (cleaved caspase-3 (Asp 175; 9664, Cell Signaling); Hsp70 (C92F3A-2, Enzo); Lamore and Wondrak, 2011). Detection of primary antibody was performed on a Discovery XT Automated Immunostainer (Ventana Medical Systems, Tucson, AZ) using a biotinylated-streptavidin-horseradish peroxidase and 3′,3′-diaminobenzidine system. Hematoxylin counterstaining was also performed online. Images were captured using an Olympus BX50 camera (Olympus, Center Valley, PA).

Statistical analysis
The results are presented as means (± SD) of at least three independent experiments. Selected data sets were analyzed employing one-way analysis of variance with Tukey’s post hoc test using the Prism 4.0 software (Prism Software Corp., Irvine, CA). In all bar graphs, means without a common letter differ (P<0.05).

CONFLICT OF INTEREST
The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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