THE EFFECTS OF DIOXIN ON THE EPIDERMAL BARRIER

Thomas R. Sutter, Sridevi Bodreddigari, Christina Campion, Ryan S. Wible, Carrie Hayes Sutter
University of Memphis, Biological Sciences, W. Harry Feinstone Center for Genomic Research

Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the toxic effects, including carcinogenicity and teratogenicity, of a large class of environmental pollutants known as 'dioxinlike' compounds, named after the most potent congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin). Studies in *Ahr* null allele mice show that the AHR is necessary for most of the toxic effects of TCDD. However, the mechanism(s) of toxicity downstream of AHR activation remain(s) elusive. As dioxin toxicity does not require concordant exposure to xenobiotics, and dioxin is not metabolized to a reactive intermediate, additional physiologic processes must be disrupted by exposure to TCDD. In humans exposed to dioxin, the most often observed and best studied toxic response is chloracne. Chloracne is manifested in the skin as hyperkeratinization of the interfollicular squamous epithelium, hyperproliferation and hyperkeratinization of cells of the hair follicle, as well as a metaplastic response of the ductular sebaceous glands. How AHR-mediated changes in gene expression lead to toxicity is not well understood for any organ system including skin [1, 2].

Recently, dioxin via the AHR, was shown to enhance human keratinocyte differentiation and to elevate the expression of three genes involved in cornification, a late part of the differentiation process [3]. Formation of the outermost layer of the skin, the stratum corneum, is essential to epidermal barrier function and provides protection against the outside environment and loss of water from the body. Of importance, epidermal growth factor (EGF) receptor (EGFR) signaling was shown to block both TCDD-AHR-mediated gene expression and cell differentiation. These results may in part explain how dioxin affects epidermal homeostasis and identify one mechanism by which EGFR signaling can repress AHR-mediated transcription in keratinocytes. Here we report on studies to test the hypothesis that in cultures of normal human epidermal keratinocytes (NHEKs), TCDD-activated AHR disrupts the transcription of filaggrin (FLG), a gene involved in the formation and function of the stratum corneum, and that EGFR signaling acts to repress this effect.

As FLG is part of the human epidermal differentiation complex (EDC) found on chromosome 1, we measured mRNAs from an additional 18 genes located in the EDC for their regulation by TCDD. Of these genes, 14 were increased by TCDD. Immunoblot assays demonstrated that the proteins of FLG, as well as that of another prodifferentiation gene, small proline rich protein 2 (SPRR2), were increased by TCDD. *In utero* exposure to TCDD accelerated the formation of the epidermal barrier in the developing mouse fetus by approximately one day. These results indicate that the epidermal permeability barrier is a functional target of the TCDD-activated AHR.

Materials and methods

Keratinocyte Cell Culture. Neonatal foreskin NHEKs, purchased from Lonza (Walkersville, MD), were grown in keratinocyte serum-free media [(KSFM) Invitrogen, Carlsbad, CA]. Confluent fifth passage NHEKs were pretreated in basal medium with or without EGF for 24 h. Treatments (with or without EGF and with or without TCDD) were carried out for 24 h unless otherwise indicated.

Reverse Transcriptase (RT) real-time PCR. Total RNA was isolated using RNA Stat-60 (Tel-Test, Friendswood, TX). RT real-time PCR was carried out in an iCycler (Bio-Rad) with M-MLV RT (Invitrogen), and iQ SYBR Green Supermix (Bio-Rad). Cyclophilin (PPIA) was used as the reference for sample normalization. Levels of mRNA (n=3 +/- SD) were plotted relative to DMSO control sample, given a value of one.

Western blots. Protein was isolated by boiling cells in lysis buffer [2% SDS, 1% beta-mercaptoethanol, Tris-HCl (pH 6.8)] for 10 min. Samples were separated by SDS-PAGE, transferred to PVDF membrane and incubated with FLG (Leica, Buffalo Grove, IL) or SPRR2 (Enzo, Plymouth Meeting, PA) antibodies and species appropriate HRP-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Signals
Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were performed as in Beischlag et al [4] except that DNA was purified using Qiaquick DNA purification kit (Qiagen). The AHR antibody and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primers amplified the sequence from -1565 to -1368, relative to the FLG transcriptional start site at +1.
Luciferase Reporter Constructs. To generate pGL4.23-1A1 (-815/-1216), a 402 bp region of genomic DNA containing 2 minimal and 3 canonical XREs was amplified using the following primer sequences: 5'-TATGGTACCGCAGAAGCCACACGCAGACC-3', containing a Kpn I site, and 5'-TATGAGCTCGGGGGCCACGGAAAGACTC-3', containing a Sac I site. For the creation of pGL4.23-FLG (-1346/-2058), a 712 bp region containing 2 canonical XREs was amplified. PCR amplified fragments were digested with Kpn I and Sac I and ligated into Kpn I- and Sac I-digested pGL4.23 (Luc2/minP) vector (Promega, Madison, WI). Mutagenesis of the two XRE regions of FLG was conducted using the Quick Change II Site Directed Mutagenesis Kit (Agilent Stratagene, Santa Clara, CA).
Luciferase activity. NHEKs were cotransfected with 0.1 μg of *Renilla* luciferase DNA pGL4.74 (Promega), and 0.9 μg of the following *Firefly* luciferase plasmids: pGL4.23 (Promega), pGL4.23-1A1, pGL4.23-FLG, pGL4.23-FLG M1, pGL4.23-FLG M2, or pGL4.23-FLG M1 & M2. Transient transfections of NHEKs were performed using Lipofectamine™ 2000 (Invitrogen). Dual luciferase assays (Renilla and Firefly) were performed according to the manufacturer's instructions (Promega, Madison, WI).
XRE sequence analysis. Gene sequences, 5 kb upstream to 2 kb downstream of the transcriptional start sites (TSS), were obtained from NCBI Human Genome Build 37.2 and analyzed for the presence of XRE core consensus sequence, GCGTG, using Lasergene (DNASTAR, Madison, WI).
Skin permeability assay. Time-mated, presumed pregnant C57BL/J6 mice (Jackson Laboratories) were fed Harlan Teklad No 216C Cerified Global 16% Protein Rodent Diet (Harlan Teklad, Madison, WI) *ad libitum*. Mice were treated with TCDD (10 μg/kg) or vehicle control in corn oil on gestational day (GD)12 or GD13 by gavage and sacrificed on GD15 and GD16, respectively (n=3/group). The skin permeability assay was carried out according to published methods [5]. Fetuses were immersed in X-gal for 18 h before photographs were taken.
Statistical analyses. Statistical analyses were performed using ANOVA followed by pairwise comparison by the Sidak-Bonferroni's multiple comparison test ($p<0.05$).

Results and discussion

Exposure of confluent NHEKs to TCDD (10 nM, 24 h) caused a significant increase in the FLG mRNA. TCDD-dependent FLG RNA increases were repressed by activation of the EGFR. Immunoblots demonstrated that the TCDD-mediated increase in FLG RNA resulted in an increase in FLG protein. Statistically significant increases were detected after 48 and 72 h of TCDD treatment (10 nM). Both the control levels of FLG protein as well as the TCDD-mediated increased levels were decreased by the presence of EGF, correlating with the mRNA.

Analysis of the promoter region (-1 to -5000 kb) of the human FLG gene revealed two XRE consensus sites (5'-CACGCNA-3'), one at -2011 to -2005 and the other at -1402 to -1396, relative to the transcription start site at +1. ChIP assays demonstrated that after treatment with TCDD there was an increase in the amount of AHR protein bound to the XRE consensus site found at -1402 to -1396. The additional XRE consensus site, located at -2011 to -2005, was not analyzed by ChIP. To determine whether FLG was transcriptionally regulated by the AHR, luciferase reporter assays were carried out in NHEKs treated with TCDD with or without EGF. The wild type FLG reporter construct contains the FLG promoter with both of the XRE consensus sites. The TCDD-mediated increase in relative

transcriptional activity of the wild type FLG promoter (3.2-fold) was similar to that of CYP1A1 (4.6-fold), a gene well-characterized for its regulation by the AHR.

As TCDD is known to accelerate keratinocyte differentiation and FLG is part of the EDC located on chromosome one, 18 other genes in this complex were examined for their regulation by TCDD. TCDD exposure significantly increased mRNA expression of 14 genes besides FLG ($p< 0.05$). These included repetin (RPTN) (2.1-fold), hornerin (HRNR) (3.6-fold), FLG (1.5-fold), FLG2 (1.9-fold), late cornified envelope (LCE)3E (7.3-fold), LCE3A (36.6-fold), LCE2B (3.0-fold), LCE2A (2.2-fold), LCE1C (2.0-fold), small proline rich (SPRR)1A (2.0-fold), SPRR2A (11.0-fold), SPRR2B (9.2-fold), S100A9 (3.8-fold), S100A12 (4.6-fold), and S100A7 (8.5-fold). Compared to TCDD alone, TCDD plus EGF decreased the following RNA levels, RPTN (4.3-fold), HRNR (16.1-fold), FLG (10.7-fold), FLG2 (20.0-fold), LCE3E (4.3-fold), LCE3A (1.6-fold), SPRR1A (1.9-fold), SPRR2A (2.9-fold), SPRR2B (1.6-fold), S100A9 (3.4-fold), S100A12 (6.4-fold), and S100A7 (3.2-fold).

Immunoblot assays using a SPRR2 antibody which detects both SPRR2A and SPRR2B proteins demonstrated that the SPRR2 proteins levels corresponded to the mRNA levels, as SPRR2 protein level was increased (2-fold) by TCDD alone and this increase was repressed by EGF (3-fold).

To investigate the effect of TCDD-mediated activation of the AHR on skin development *in utero*, pregnant mice were treated with TCDD (10 μg/kg) on GD12 or GD13 by gavage and sacrificed on GD15 and GD16, respectively. Fetuses were subjected to an epidermal permeability assay. After submerging fetuses in X-gal, the substrate for endogenous β-galactosidase, the color of the fetus is assessed. TCDD exposure caused a significant acceleration in epidermal barrier formation in fetuses from mice treated on either GD12 or GD13 and sacrificed on GD15 and GD16, respectively. TCDD accelerated the barrier formation by one day, as the TCDD-treated GD15 mice have a similar barrier to the control GD16 fetuses.

The importance of FLG to proper keratinocyte differentiation is demonstrated by human genetic studies that have linked loss-of-function mutations with causation of the disease ichthyosis vulgaris. In addition, these FLG mutations have been associated with atopic dermatitis [6, 7]. Besides FLG, numerous genes in the EDC are implicated in human skin diseases. Psoriatic samples have increases in S100A2, S100A7, S100A8, S100A9, S100A15, SPRR2A, and SPRR1B, and these genes are candidate susceptibility genes [8]. S100A7 is elevated in samples of atopic dermatitis skin [9]. The SPRR and S100 proteins are important components of the cornified envelope. We studied the expression of three of the genes associated with psoriasis, S100A7, S100A9, and SPRR2A, and each was increased by TCDD. Taken together, a number of genes that TCDD increased are associated with diseases such as psoriasis and atopic dermatitis, which are characterized by abnormal skin phenotypes, increased inflammation, and a compromised epidermal barrier. Increases in these genes of the EDC by TCDD may contribute to hyperkeratosis, a thickening of the cornified and granular epidermal layers, observed in the interfollicular skin of humans exposed to TCDD, as well as to the atopic dermatitis and inflammation observed in the skin of mice expressing constitutively active AHR [10].

The regulation of epidermal barrier formation *in utero* is complex. In mice, a barrier initiates dorsally at GD16.5, spreads ventrally, and is completed by GD18 [5]. Several transcription factors are essential to establishing a prenatal epidermal barrier in mice including distal-less homeobox 3 (DLX3) [11], Kruppel-like factor 4 (KLF4) [12], GATA binding protein 3 (GATA3) [13] and ARNT [14, 15]. Although we show here that activation of the AHR *in utero* is able to accelerate epidermal barrier formation, we know that the AHR is not essential to epidermal barrier formation as AHR knock out mice survive, albeit with dermatological lesions characterized by epidermal hyperplasia, hyperkeratosis, fibrosis and anagenic hair follicles [16]. With respect to barrier formation, the AHR is not an essential dimerization partner of ARNT, yet activation of the AHR modulates the expression of a number of genes in the EDC, which likely contribute to toxicity by TCDD or other AHR ligands.

Acknowlegments
This research was funded by NIEHS grant ES017014 and the University of Memphis W. Harry Feinstone Center for Genomic Research. We thank Edward R. Frizell of Huntingdon Life Sciences (Somerset, NJ) for aiding us in animal

studies and the Molecular Resource Core for sequence analysis (University of Tennessee, Memphis, TN), as well as Gaylene Stevens, Quynh T. Tran, Shirlean Goodwin, and Sandra Leon Carrion for helpful discussions.

References

1. Swanson, H.I. and C.A. Bradfield, The AH-receptor: genetics, structure and function. Pharmacogenetics, 1993. 3(5): p. 213-30.
2. Suskind, R.R., Chloracne, "the hallmark of dioxin intoxication". Scand J Work Environ Health, 1985. 11(3 Spec No): p. 165-71.
3. Sutter, C.H., et al., EGF receptor signaling blocks aryl hydrocarbon receptor-mediated transcription and cell differentiation in human epidermal keratinocytes. Proc Natl Acad Sci U S A, 2009. 106(11): p. 4266-71.
4. Beischlag, T.V., et al., Recruitment of thyroid hormone receptor/retinoblastoma-interacting protein 230 by the aryl hydrocarbon receptor nuclear translocator is required for the transcriptional response to both dioxin and hypoxia. J Biol Chem, 2004. 279(52): p. 54620-8.
5. Hardman, M.J., et al., Patterned acquisition of skin barrier function during development. Development, 1998. 125(8): p. 1541-52.
6. Smith, F.J., et al., Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. Nat Genet, 2006. 38(3): p. 337-42.
7. Palmer, C.N., et al., Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet, 2006. 38(4): p. 441-6.
8. Bowcock, A.M., et al., Insights into psoriasis and other inflammatory diseases from large-scale gene expression studies. Hum Mol Genet, 2001. 10(17): p. 1793-805.
9. Glaser, R., et al., The antimicrobial protein psoriasin (S100A7) is upregulated in atopic dermatitis and after experimental skin barrier disruption. J Invest Dermatol, 2009. 129(3): p. 641-9.
10. Tauchi, M., et al., Constitutive expression of aryl hydrocarbon receptor in keratinocytes causes inflammatory skin lesions. Mol Cell Biol, 2005. 25(21): p. 9360-8.
11. Morasso, M.I., N.G. Markova, and T.D. Sargent, Regulation of epidermal differentiation by a Distal-less homeodomain gene. J Cell Biol, 1996. 135(6 Pt 2): p. 1879-87.
12. Segre, J.A., C. Bauer, and E. Fuchs, Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet, 1999. 22(4): p. 356-60.
13. de Guzman Strong, C., et al., Lipid defect underlies selective skin barrier impairment of an epidermal-specific deletion of Gata-3. J Cell Biol, 2006. 175(4): p. 661-70.
14. Takagi, S., et al., Alteration of the 4-sphingenine scaffolds of ceramides in keratinocyte-specific Arnt-deficient mice affects skin barrier function. J Clin Invest, 2003. 112(9): p. 1372-82.
15. Geng, S., et al., Targeted ablation of Arnt in mouse epidermis results in profound defects in desquamation and epidermal barrier function. J Cell Sci, 2006. 119(Pt 23): p. 4901-12.
16. Fernandez-Salguero, P.M., et al., Lesions of aryl-hydrocarbon receptor-deficient mice. Vet Pathol, 1997. 34(6): p. 605-14.