Aryl Hydrocarbon Receptor Repressor (AhRR) Function Revisited: Repression of CYP1 Activity in Human Skin Fibroblasts Is Not Related to AhRR Expression

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The skin reacts to environmental noxae by inducing cytochrome P450 (CYP)-catalyzed reactions via activation of the aryl hydrocarbon receptor (AhR). A drawback of this response is the generation of oxidative stress, which is especially dangerous for postreplicative cells such as dermal fibroblasts, in which damage may accumulate over time. Accordingly, in dermal fibroblasts, CYP1 expression is repressed and it has been proposed that this is due to the AhR repressor (AhRR), which is supposedly overexpressed in fibroblasts as compared with other skin cells. Here, we revisited this "AhRR hypothesis", which has been mainly based on ectopic overexpression studies and correlation analyses of high AhRR gene expression with CYP1A1 repression in certain cell types. In primary human skin fibroblasts (NHDFs) of 25 individuals, we found that (i) the AhRR was expressed only at moderate RNA copy numbers and that, against the common view, (ii) in some fibroblast strains, CYP1A1 mRNA expression could be induced by AhR activators. However, even the highest induction did not translate into measurable CYP1 enzyme activity, and neither basal expression nor mRNA inducibility correlated with AhRR expression. In addition, enhancement of CYP1A1 mRNA expression by trichostatin A, which inhibits AhRR-recruited histone deacetylases at the *CYP1A1* promoter, failed to induce measurable CYP1 activity. Finally, *AhRR*-deficient ($^{-/-}$) mouse embryonic fibroblasts were not induced to biologically relevant CYP1 enzyme activity despite impressive mRNA induction. These data clearly indicate that repressed CYP1 activity in NHDFs is not causally related to AhRR expression, which may serve a different, yet unknown, biological function.

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INTRODUCTION

The aryl hydrocarbon receptor (AhR) repressor (AhRR)—like the AhR itself—belongs to the group of basic helix-loop-helix–homologs of Period/ARNT (AhR nuclear translocator)/Single-minded proteins and is an integral part of the AhR signaling machinery (Mimura *et al.*, 1999). The AhR is a ligand-dependent transcription factor, which, in its unligated state, rests as a multiprotein complex in the cytoplasm of most cells of the body, including skin (Bickers *et al.*, 1984; Fujii-Kuriyama *et al.*, 1992). Upon ligand binding, the receptor sheds its cofactors, translocates into the nucleus where it dimerizes with its partner ARNT, binds to xenobiotic response elements in the promoter region of AhR-dependent genes, and initiates transcription (Fujisawa-Sehara *et al.*, 1987; Rowlands and Gustafsson, 1997; Abel and Haarmann-Stemmann, 2010). Besides genes that are involved in xenobiotic metabolism, one gene of the AhR gene battery is the *AhRR* (Mimura *et al.*, 1999). Overexpression studies suggested that the AhRR also forms heterodimers with ARNT binding to xenobiotic response elements and blocking AhR target gene transcription (Mimura *et al.*, 1999; Oshima *et al.*, 2007). This transcriptional hindrance is due to the lack of a transactivation domain in the AhRR protein that is present in the AhR (Sogawa *et al.*, 1995; Mimura *et al.*, 1999). Thereby, the AhRR is thought to form a negative feedback loop on the AhR gene battery, including its own

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Abbreviations: 3-MC, 3-methylcholanthrene; AhR, aryl hydrocarbon receptor; AhRR, AhR repressor; ARNT, AhR nuclear translocator; B(a)P, benzo(a)pyrene; CYP, cytochrome P450; EROD, ethoxyresorufin O-deethylation; HDAC, histone deacetylase; LOQ, limit of quantification; MEF, mouse embryonic fibroblast; NHDF, normal human dermal fibroblast; NHEK, normal human epidermal keratinocyte; RT–PCR, reverse-transcriptase PCR; TSA, trichostatin A

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transcription. Such AhR signaling was found in almost all cells of the body, including skin (Das *et al.*, 1986; Ahmad *et al.*, 1996).

The skin is the largest organ of the human body and represents the body's protective surface as the first and outermost contact site for environmental noxae (Ahmad and Mukhtar, 2004; Swanson, 2004; Merk *et al.*, 2006; Oesch *et al.*, 2007). In this regard, it is important to note that lipophilic chemicals, such as polycyclic aromatic hydrocarbons and polyhalogenated hydrocarbons, or physical stressors, such as UV radiation, may overcome the physical barrier of the skin. Chronic exposure to the classical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, for example, leads to chloracne, a chronic inflammatory disease of the skin, which results in the formation of hyperplasia of some skin epithelial structures, and therefore indicates a physiological relevance of the AhR in pathologies of the skin (Panteleyev and Bickers, 2006). It is therefore not surprising that similar to liver—as the first-pass organ for oral exposure—skin as the first-pass organ for dermal exposure possesses capacities for xenobiotic metabolism (extensively reviewed in Oesch *et al.*, 2007; Gotz *et al.*, 2012a, b). Accordingly, the ability of skin to metabolize polycyclic aromatic hydrocarbons has long been described *in vivo* in animals and humans. Skin cells express the AhR, and AhR activation by polycyclic aromatic hydrocarbons causes induction of cytochrome P450 (CYP)1A1 and 1B1, which hydroxylate such xenobiotics (Levin *et al.*, 1972; Alvares *et al.*, 1973; Bickers *et al.*, 1984). In contrast to inducible CYP enzymes, phase 2 drug metabolism enzymes such as glutathion S-transferase, N-acetyltransferase, or UDP-glucuronosyltransferase are constitutively present in skin and thus guarantee detoxification of hydroxylated metabolites (Oesch *et al.*, 2007; Gotz *et al.*, 2012b). The fact that the same drug metabolism is also essential for skin after UV exposure is a relatively new realization. We and others showed *in vitro* and *in vivo* that upon UV irradiation, natural AhR ligands are formed intracellularly from free tryptophan (Rannug *et al.*, 1987; Wei *et al.*, 1999; Bergander *et al.*, 2004; Fritsche *et al.*, 2007; Wincent *et al.*, 2009). Among those, 6-formylindolo-3,2b-carbazole is the most potent AhR ligand (Rannug *et al.*, 1987, 1995). 6-Formylindolo-3,2b-carbazole is metabolized by CYP enzymes and thus induces their expression (Wei *et al.*, 1998, 2000; Bergander *et al.*, 2003, 2004; Wincent *et al.*, 2009). Hence, AhR signaling is indispensable for skin xenobiotic metabolism of polycyclic aromatic hydrocarbons and UV photoproducts.

The drawback of such CYP monooxygenase–catalyzed metabolic reactions is the generation of oxidative stress (Puntarulo and Cederbaum, 1998; Morel *et al.*, 1999). A tissue with a high cellular turnover rate is, up to a certain limit, sparsely harmed by oxidative stress because structural cellular damages such as mitochondrial DNA mutations or other macromolecular modifications do not accumulate over time. Such a tissue, in which cells constantly proliferate, is the epidermis mainly consisting of keratinocytes. A completely different situation is given in the dermis. Dermal fibroblasts are postreplicative and rest in the dermis for decades, producing extracellular matrix. For them, accumulation of damage is pathogenic and causes tissue degeneration. Therefore, there is a need for fibroblasts to keep their oxidative stress level low. As one source of reactive oxygen species production is xenobiotic metabolism (reviewed in Gonzales, 2005), repression of metabolism is a necessary consequence for the fibroblast, especially with regard to the fact that the epidermis as the outer barrier is metabolically competent (Pendlington *et al.*, 1994; Afaq and Mukhtar, 2001; Swanson, 2004; Du *et al.*, 2006). Consequently, in human skin fibroblasts, CYP1 expression was found to be repressed (Gradin *et al.*, 1993; Haarmann-Stemmann *et al.*, 2007). In addition, it has been proposed that this repression is mediated by the AhRR (Gradin *et al.*, 1993; Mimura *et al.*, 1999; Oshima *et al.*, 2007). This assumption was based on the observation that the AhRR was overexpressed in fibroblasts as compared with other skin cells (Akintobi *et al.*, 2007). In these studies, however, AhRR expression was not analyzed (i) in adult primary human fibroblasts, and (ii) the data were in large part obtained from overexpression experiments and at least in part from mRNA analysis as fold induction rather than actual copy numbers. In the present study, we therefore revisited this ''AhRR hypothesis''.

RESULTS

Expression of AhR signaling components in human skin cells and MEFs

Here we used primary human skin fibroblasts from 25 individual breast reduction donors belonging to five different age groups (Figure 1). Real-time reverse-transcriptase (RT)–PCR analyses revealed for one that mRNA steady-state levels of components of the AhR signaling pathway (AhRR, AhR, and ARNT) were each expressed in similar copy numbers among the 25 individuals. The five different age groups also did not differ markedly from each other. Thereby, the expression levels of AhRR, AhR, and ARNT did not exceed 1, 6.7, and 17.9 copies/10^4 transcripts β-actin, respectively, in those cells (Figure 1a–c). Comparison of these expression patterns with the distribution of AhR signaling components in primary human keratinocytes (normal human epidermal keratinocytes (NHEKs)), as well as the keratinocyte cell line NCTC 2544 and $AhRR^{+/+}$ and $AhRR^{-/-}$ mouse embryonic fibroblasts (MEF), revealed that normal human dermal fibroblasts (NHDFs) express significantly more AhRR than all the other tested cells (Figure 1d; vs. NHEK: $P=0.00000005$; vs. NCTC: $P=000003$; vs. $AhRR^{+/+}$ MEF: $P=0.03$; vs. $AhRR^{-/-}$ MEF: $P=00000003$). A different result was obtained for the expression of AhR (Figure 1e); whereas NHEKs ($P=0.008$) and $AhRR^{-/-}$ MEFs ($P=0.02$) express significantly more AhR transcripts (up to 1.5/10^4 β-actin) compared with NHDFs (6–7 copies/10^4 β-actin), NCTCs express significantly less AhR ($\sim$2.5 copies/10^4 β-actin; $P=0.0004$). ARNT expression on the other hand (Figure 1f) is significantly less in keratinocytes ($\sim$5/10^4 β-actin) compared with NHDFs (vs. NCTCs: $P=0.03$), whereas $AhRR^{-/-}$ and $AhRR^{+/+}$ MEFs express one order of magnitude more ARNT ($\sim$2000 transcripts/10^4 β-actin; $AhRR^{-/-}$ MEF: $P=0.009$; $AhRR^{+/+}$ MEF: $P=0.003$). As shown, as examples for the age groups 20–29 and >60 years, the interindividual differences inside the different age groups

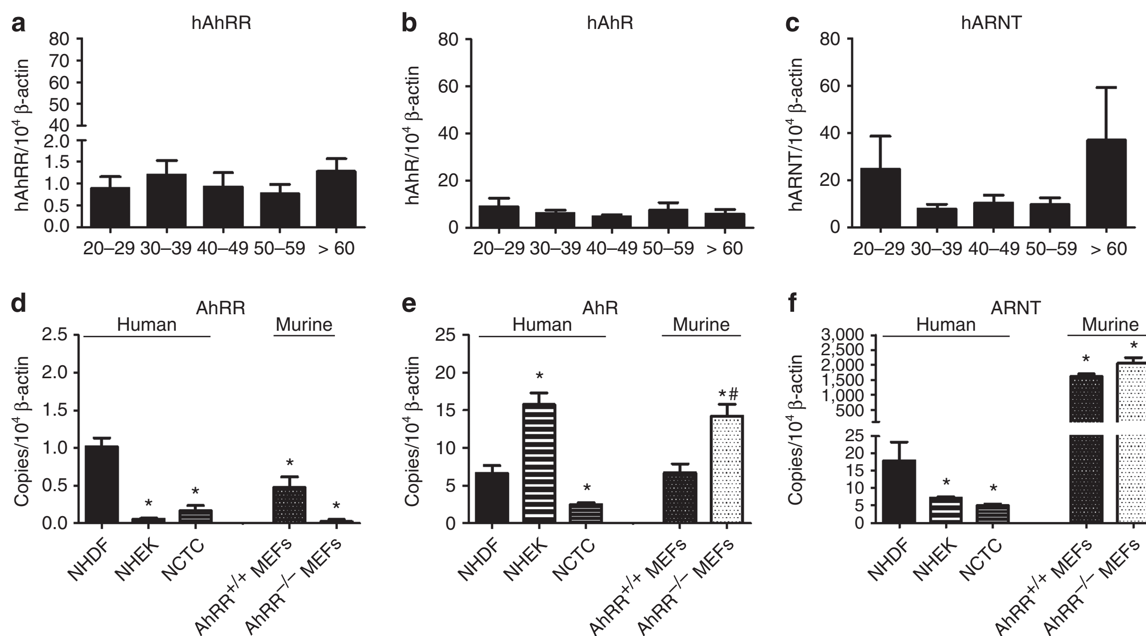


Figure 1. Expression of aryl hydrocarbon receptor (AhR) signaling components in human skin cells and mouse embryonic fibroblasts (MEFs). Real-time reverse-transcriptase PCR (RT–PCR) detection of AhR repressor (AhRR), AhR, and AhR nuclear translocator (ARNT). Copy numbers of AhR signaling components were detected in different cells after 48 hours in culture; the expression of each gene was normalized to 10^4 transcripts β-actin. Mean ± SD is plotted; *$P<0.05$ versus normal human dermal fibroblast (NHDF); $^{\#}P<0.05$ versus *AhRR*$^{+/+}$ MEFs. (**a**–**c**) Expression of AhRR (**a**), AhR (**b**), and ARNT (**c**), respectively, in NHDF cells from individuals of five different age groups, each bar representing five individuals ($n=25$). (**d**–**f**) Comparison of AhRR (**d**), AhR (**e**), and ARNT (**f**) expression in NHDFs ($n=25$), *AhRR*$^{+/+}$ MEFs ($n=3$), *AhRR*$^{-/-}$ MEFs ($n=3$), normal human epidermal keratinocytes (NHEKs; $n=3$), and NCTCs ($n=3$). Expression is normalized to 10^4 copies transcripts β-actin.

were bigger than the differences between the different ages, but did not reach statistical significance (Supplementary Figure S1 online, Figure 1a and b).

Inducibility of AHR signaling by AHR agonists in human skin cells and MEFs

To verify that AhR signaling in fibroblasts is not functional (reviewed in Haarmann-Stemmann and Abel, 2006; Evans *et al.*, 2008), we challenged the 25 different NHDF strains with 250 nM of the AhR agonist benzo(a)pyrene (B(a)P; Figure 2a–f). Indeed, B(a)P did not increase CYP1A1 copy numbers significantly. However, plotting the obtained data as x-fold of solvent control (Supplementary Figure S2 online) disclosed a significant increase in CYP1A1 induction for the age groups 40–49, 50–59, and >60 years. In the age group of 50–59 years, preincubation of cells with the competitive AhR antagonist 3′methoxy-4′-nitroflavone (10 μM) inhibited this CYP1A1 induction significantly. However, Figure 2b–f, which show the detailed analysis of Figure 2a (each graph representing one age group), clearly demonstrates that the inducibility of CYP1A1 after treatment with B(a)P displayed large interindividual differences. We next asked whether these differences in inducibility of CYP1A1 in NHDF cells correlated with the respective AhRR content of the cells. Linear regression analyses revealed a coefficient of determination (r^2) of 0.014 (Figure 2j, basal) and 0.006 (Figure 2k, induced), indicating no correlation between basal or inducible CYP1A1 and AhRR expression in NHDFs.

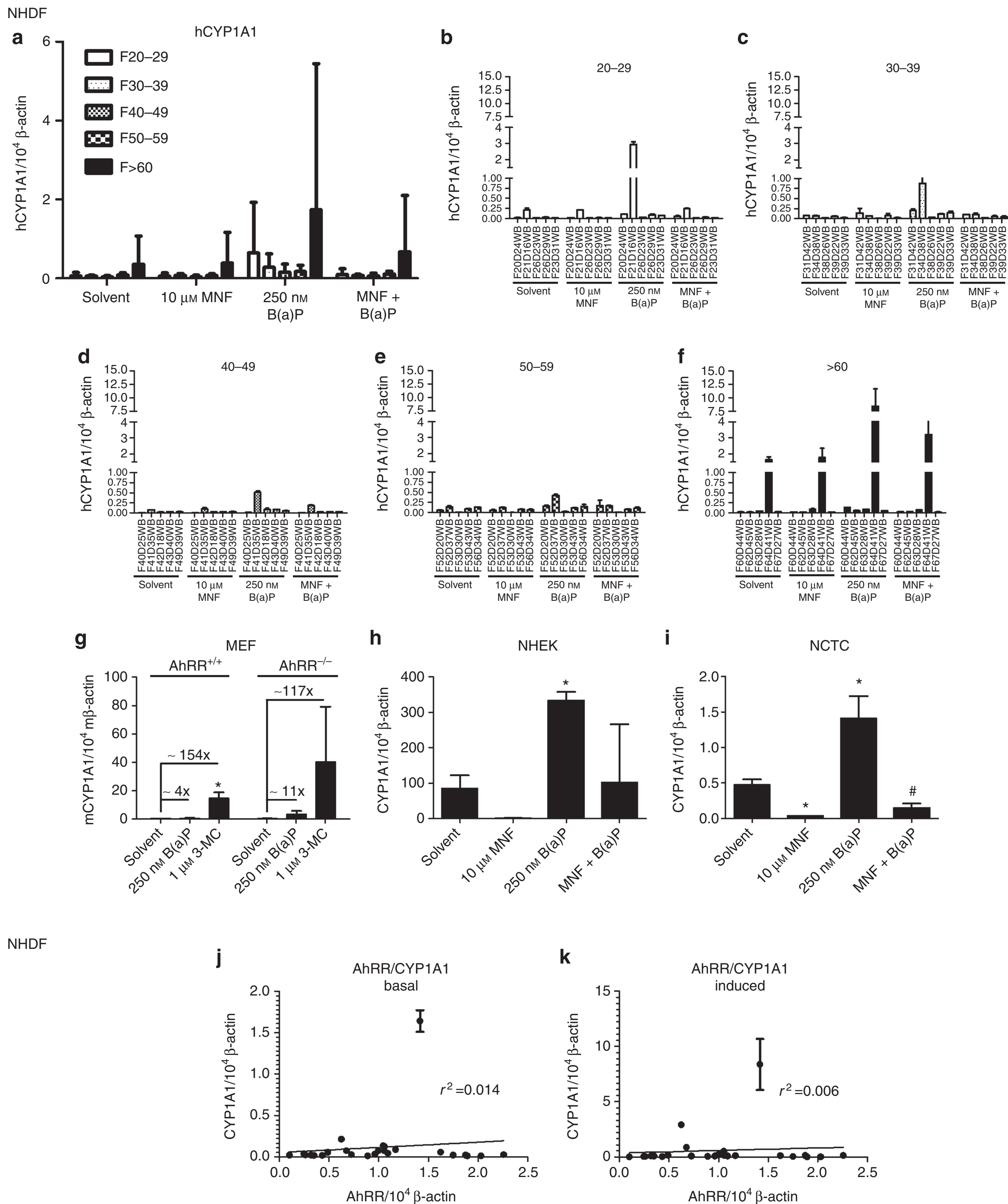
As the AhRR theory has partly been studied in MEFs (Oshima *et al.*, 2007), we next studied CYP1A1 mRNA expression in MEFs obtained from *AhRR*-knockout (*AhRR*$^{-/-}$), as well as *AhRR*-proficient (*AhRR*$^{+/+}$), mice. In *AhRR*$^{-/-}$ MEFs, CYP1A1 mRNA expression was 11-fold induced by 250 nM B(a)P and even 117-fold in the mean by 1 μM of the synthetic AhR-agonist 3-methylcholanthrene (3-MC) after 48 hours (Figure 2g). However, the SD was very high and therefore significance was not reached. In *AhRR*$^{+/+}$ MEFs, CYP1A1 expression was induced 154-fold after treatment with 1 μM 3-MC. In contrast, in NHEKs and NCTCs, CYP1A1 mRNA expression was significantly induced by 250 nM B(a)P ~3-fold (from ~100 to ~300 copies/10^4 β-actin in NHEKs and from 0.5 to 1.5 copies/10^4 β-actin in NCTCs). In case of NCTCs, induction of the CYP1A1 gene expression by B(a)P was significantly inhibited by pre-incubation of the cells with 10 μM of the AhR agonist, 3′-methoxy-4′-nitroflavone.

CYP1A1 enzyme activity in NHDFs compared with *AhRR*$^{+/+}$ and *AhRR*$^{-/-}$ MEFs

Functional relevance of CYP1A1 induction reflects in CYP1 enzyme activity. Therefore, we measured ethoxyresorufin O-deethylation (EROD) activity in NHDFs, *AhRR*$^{+/+}$, and *AhRR*$^{-/-}$ MEFs (Figure 3), which, to the best of our knowledge, has only been done for human skin fibroblasts earlier in one study, wherein Quan *et al.* (1995) observed no measurable CYP1 activity upon B(a)P-trans-7,8-dihydrodiol

treatment, but did not associate this finding with a repressor of AhR signaling at the time.

For NHDFs, three individuals from the lowest (1) and highest (2) age group, respectively, which displayed the largest CYP1A1 mRNA induction upon AhR activation or overall CYP1A1 copy numbers (Figure 2), were chosen for the functional analyses (Figure 3). NCTC 2544 cells, a keratinocyte cell line with known CYP1 induction upon AhR



activation (Gotz *et al.*, 2012a), were used as a positive control. B(a)P and 3-MC (0.01, 0.1, 0.25, 1, and 10 μM) did not induce EROD activity above the limit of quantification (LOQ; indicated by dotted line) in any samples of the NHDFs under any condition tested, whereas NCTC 2544 cells presented a significantly inducible EROD substrate turnover (from basal 1 to induced 100–200 pmol min^{-1} mg^{-1}; Figure 3a–c). EROD activity was also not detectable in *AhRR*$^{+/+}$ MEFs upon any treatment (Figure 3d), and 1 μM B(a)P or 3-MC resulted in a marginal EROD activity at the level of the LOQ at 0.8 pmol min^{-1} mg^{-1} in *AhRR*$^{-/-}$ MEFs (Figure 3e). Compared with keratinocytes, this turnover rate is negligible.

Effect of HDAC inhibition on the expression of CYP1A1 and AhRR mRNA and EROD activity in NHDFs

Previous work from our own laboratory (Haarmann-Stemmann *et al.*, 2007) and that of others (Gradin *et al.*, 1999; Oshima *et al.*, 2007) showed that histone deacetylase (HDAC) inhibition leads to a superinduction of CYP1A1

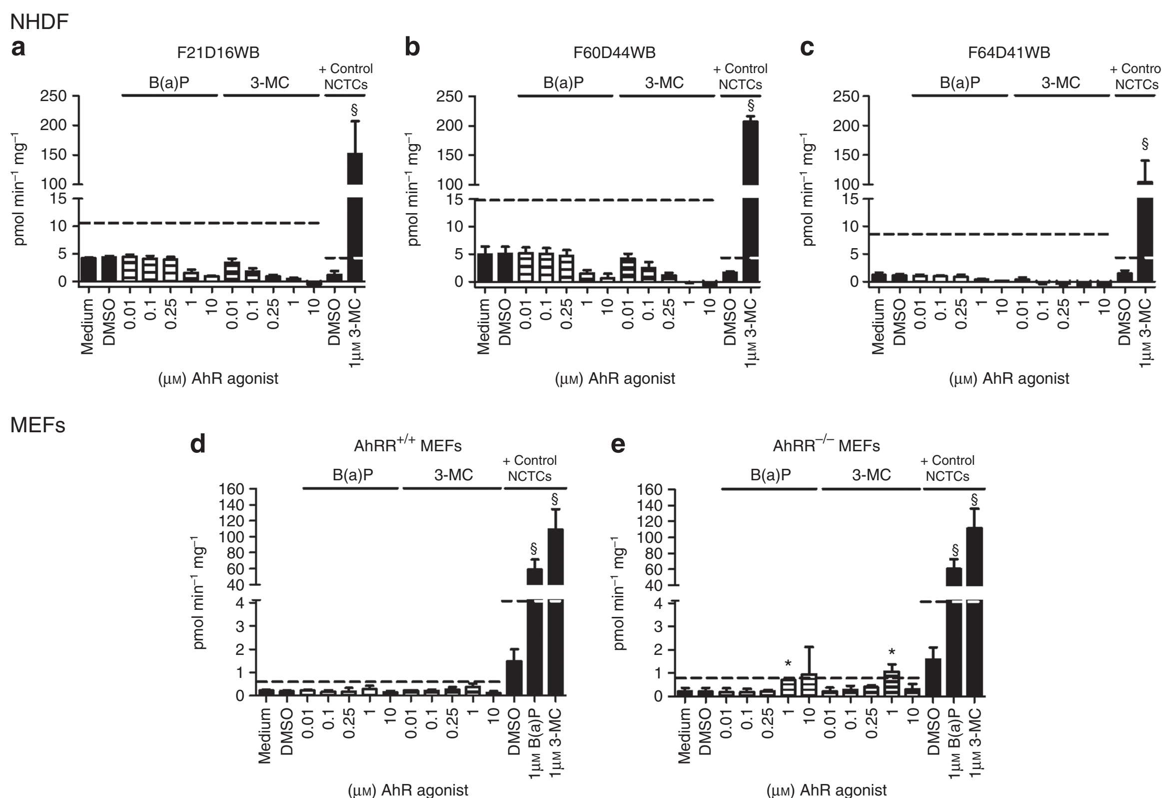


Figure 3. Induction of ethoxyresorufin O-deethylation (EROD) activity in normal human dermal fibroblast (NHDF) cells and mouse embryonic fibroblasts (MEFs) by different concentrations of the aryl hydrocarbon receptor (AhR) agonists 3-methylcholanthrene (3-MC) and benzo(a)pyrene (B(a)P; 0.01–10 μM). The asterisks indicate significant differences ($P<0.05$) to solvent (DMSO) control. NCTCs were used as a positive control; § indicates significant differences ($P<0.05$) to solvent (DMSO) control of NCTCs. The dotted line indicates the limit of quantification (LOQ) for each cell strain. Each graph represents three independent experiments. (**a–c**) Induction of EROD activity in NHDFs in 21- (**a**), 60- (**b**), and 64- (**c**)-year-old female donors induced by different concentrations of 3-MC and B(a)P. Activity is shown in pmol min^{-1} mg^{-1}. (**d, e**) Induction of EROD activity in wild-type MEFs (*AhRR*$^{+/+}$) (**d**) and *AhRR*-deficient MEFs (*AhRR*$^{-/-}$) (**e**) induced by different concentrations of 3-MC and B(a)P. Activity is shown in pmol min^{-1} mg^{-1}.

Figure 2. Inducibility of aryl hydrocarbon receptor (AhR) signaling by AhR agonists in human skin cells and mouse embryonic fibroblasts (MEFs). Real-time reverse-transcriptase PCR (RT–PCR) detection of cytochrome (CYP)1A1 in normal human dermal fibroblasts (NHDFs) of donors of different ages (**a–f**), *AhRR*$^{+/+}$ and *AhRR*$^{-/-}$ MEFs (**g**), as well as normal human epidermal keratinocytes (NHEKs) (**h**) and NCTCs (**i**). Expression of CYP1A1 is normalized to 10^4 transcripts β-actin; *$P<0.05$ versus solvent control (DMSO); $^{\#}P<0.05$ versus 250 nM benzo(a)pyrene (B(a)P). (**a**) Summary of inducibility of CYP1A1 in NHDF cells from individuals of five different age groups (20–29, 30–39, 40–49, 50–59, and >60 years), each bar graph representing five different individuals. Real-time RT–PCR detection of CYP1A1 was performed after pretreatment with 10 μM 3′-methoxy-4′-nitroflavone (MNF) for 1 hour, followed by 48 hours of incubation with B(a)P (250 nM). (**b–f**) Graphs show detailed analysis of **a**, each graph representing one age group (**b**: 20–29 years, **c**: 30–39 years, **d**: 40–49 years, **e**: 50–59 years, and **f**: >60 years). (**g**) Inducibility of CYP1A1 in *AhRR*$^{+/+}$ and *AhRR*$^{-/-}$ MEFs. (**h, i**) Inducibility of CYP1A1 in NHEKs (**h**) and NCTC 2544 (**i**). Real-time RT–PCR analysis of CYP1A1 was performed after pretreatment with 10 μM MNF for 1 hour followed by 48 hours of incubation with B(a)P (250 nM), $n=3$. (**j, k**) Correlation of AhRR and CYP1A1 (**j**: basal, **k**: induced by 250 nM B(a)P) expression in NHDFs. Basal AhRR expression is plotted on the *x* axis, and basal or induced CYP1A1 expression is plotted on the *y* axis. Linear regression line and coefficient of determination (r^2) values are shown, $n=25$. As the experiments were performed in duplicates, the error bars represent the minimum/maximum of each data point.

mRNA expression in fibroblasts. HDACs are recruited by the AhRR and are necessary for its transcription inhibitory activity (Gradin *et al.*, 1999). We therefore next treated the same three NHDF cell strains already used for the induction experiments in Figure 3 with the HDAC inhibitor trichostatin A (TSA; 0.5 μM). None of these treatments decreased cell viability (Figure 4d). As expected, TSA increased CYP1A1 mRNA expression, whereas in the same samples AhRR mRNA expression was diminished (Figure 4a and b). However, EROD enzyme activity analyses in these cells demonstrated that ''superinduction'' of CYP1A1 by HDAC inhibition did not lead to an increase in measurable EROD activity above the LOQ (Figure 4c).

DISCUSSION

For a decade now, it is thought that the AhRR represses AhR-dependent xenobiotic metabolism in HeLa cells or fibroblasts (reviewed in Haarmann-Stemmann and Abel, 2006; Evans *et al.*, 2008). This assumption is founded on overexpression studies, electrophoretic mobility shift assay, and chromatin immunoprecipitation analyses as well as, in part, on mRNA expression analyses as fold of control (Gradin *et al.*, 1993; Mimura *et al.*, 1999; Haarmann-Stemmann *et al.*, 2007) done in fibroblasts and MEF cells (Oshima *et al.*, 2007). Therefore, we revisited this common knowledge ''AhRR hypothesis'' by expanding the database for (i) primary human skin fibroblasts from 25 different donors, (ii) physiological stoichiometry of AhR signaling compounds and involvement of HDACs in these not genetically manipulated cells, and (iii) a functional readout, CYP1 activity. To confirm our data, we also included $AhRR^{+/+}$ and $AhRR^{-/-}$ MEFs, and used epidermal keratinocytes that are known to possess approximately 10% of liver CYP1 activity (Smith and Hotchkiss, 2001) as positive controls. This thorough revisiting of the ''AhRR hypothesis'' revealed that—at least in adult primary human fibroblasts—the AhRR does not control AhR-dependent CYP activity.

Previous studies showed that two cell types, fibroblasts and keratinocytes, within the same organ, i.e., skin, might contain high and low levels of AhRR expression, respectively (Akintobi *et al.*, 2007). In the present study, we analyzed

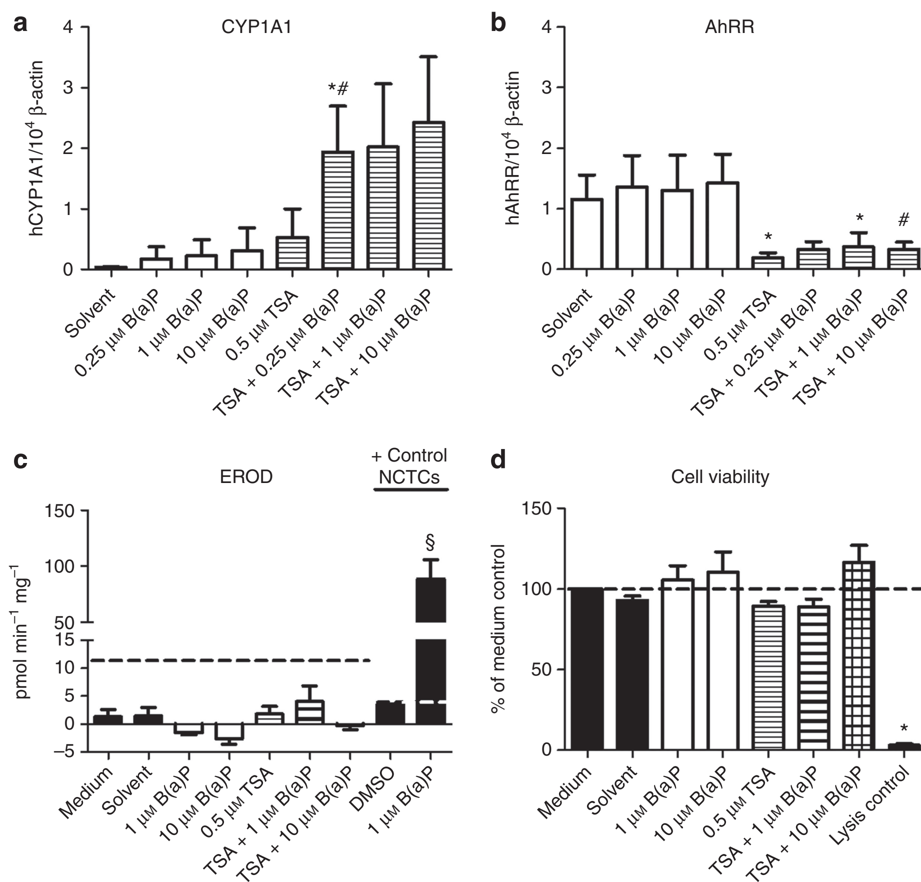


Figure 4. Effect of histone deacetylase (HDAC) inhibition on the expression of cytochrome (CYP)1A1 and aryl hydrocarbon receptor repressor (AhRR) mRNA, as well as ethoxyresorufin O-deethylation (EROD) activity in normal human dermal fibroblasts (NHDFs). Each graph represents three independent experiments performed in three different NHDF strains (F21D16WB, F60D44WB, F64D41WB). (**a**, **b**) CYP1A1 and AhRR transcripts were quantified using real-time reverse-transcriptase PCR (RT–PCR) and normalized to 10^4 transcripts β-actin. Cells were treated for 16 hours with benzo(a)pyrene (B(a)P; 0.25, 1, and 10 μM) or cotreated with trichostatin A (TSA; 0.5 μM), respectively. (**c**) EROD in NHDF cells by B(a)P (1 and 10 μM) or cotreated with TSA (0.5 μM). NCTCs were used as a positive control: $^{\S}P<0.05$ compared with solvent control of NCTCs. The dotted line indicates the limit of quantification (LOQ); activity is shown in pmol min^{-1} mg^{-1}. (**d**) Change in cell viability in NHDF cells by different concentrations of B(a)P (1 and 10 μM) alone or cotreatment with TSA (0.5 μM). As a control lysis of the cells was achieved by adding 9% Triton X (1:50). The dotted line marks the approximately 100% cell viability of untreated (medium) cells.

the AhRR, AhR, and ARNT amount expressed in human fibroblasts and keratinocytes by real-time RT–PCR (Figure 1). In accordance with those previous findings, we identified gene expression levels for AhRR one order of magnitude higher in NHDFs compared with NHEKs, and for AhR, a two-fold higher expression in keratinocytes compared with fibroblasts. Our findings are in contrast to previous work of Akintobi *et al.* (2007) who found a six-fold higher expression of AhRR versus AhR in human fibroblasts than in human keratinocytes. These differences might be due to interindividual or gender variation (Supplementary Figure S1 online).

Gene induction experiments with B(a)P from this study are in agreement with the work from Hosoya *et al.* (2008). Although the CYP1A1 was induced up to 8-fold by B(a)P in NHDFs from this study (Supplementary Figure S2 online), it was 25-fold induced by B(a)P in murine skin fibroblasts. Differences in magnitude of induction are possibly due to different B(a)P concentrations (250 nM vs. 1 μM), incubation times (48 vs. 24 hours), species, or cellular age, as our donors were 20- to 60-year-old females, and the Hosoya study used skin fibroblasts from neonatal mice. In contrast, Gradin *et al.* (1993) did not observe CYP1A1 induction by 2,3,7,8-tetrachlorodibenzofuran in fibroblasts derived from neonatal foreskin of human donors, which may be due to the method of detection, because real-time RT–PCR is more sensitive than northern blot analysis, the age of the donors, or even the skin location where fibroblasts were gained from. Fibroblasts of the same donor, prepared from skin from different parts of the body, display differences in their expression profile (Chang *et al.*, 2002). We also have to point out that CYP1A1 expression in all tested NHDF samples had CT values well behind the last measured CYP1A1 standard (3.75×10^2 copies of CYP1A1 μl^{-1}) and therefore must be considered as close to the limit of detection and handled with care. The role of the AhRR in magnitude of CYP1A1 induction in fibroblasts was tackled by the use of *AhRR*-proficient and -deficient mouse fibroblasts. The study by Hosoya *et al.* (2008), as well as this study (Figure 2g), observed high CYP1A1 induction after treatment with an AhR agonist in $AhRR^{-/-}$ cells (150-fold (Hosoya *et al.* (2008)) and 117-fold (this study), respectively).

To critically challenge the role of the AhRR in control of CYP1A1 expression in fibroblasts, we took a step back from ''fold of gene induction'' to the raw data, i.e., AhRR copy numbers, which were derived from a product-specific copy number standard. These analyses revealed that copy numbers of this gene were low, with 1 and 0.5 copies/10^4 transcripts β-actin in fibroblasts and MEFs, respectively. As the actual gene copy numbers corrected for the housekeeping gene *β-actin* for the AhRR have not been reported previously, we cannot compare our data with any other study. However, within our data, we found that fibroblasts and MEFs expressed the AhRR in the same low order of magnitude (with MEFs ~50% lower than NHDF; Figure 1d). Keratinocytes expressed the AhRR at much lower levels (Figure 1e). Despite this low expression level in fibroblasts, we wanted to know whether there is any functional relevance of the AhRR on AhR signaling. Therefore, we used two strategies: first, we correlated gene copy numbers of basal and induced CYP1A1 expression with AhRR expression, and second, we measured CYP1 enzyme activity with the EROD assay in primary fibroblasts as well as in $AhRR^{+/+}$ and $AhRR^{-/-}$ MEFs. Expression levels of the AhRR did not correlate with neither basal nor induced CYP1A1 gene expression levels (Figure 2j and k). However, CYP1A1 did not correlate with AhR or ARNT expression either (data not shown). AhRR expression and inducibility of CYP1A1 also showed no association in nine different tumor cell lines in an earlier study (Tsuchiya *et al.*, 2003). Therefore, these data suggest that in the physiological stoichiometry of the cell, the AhRR does not (necessarily) determine AhR signaling. The fact that this suggestion is true for adult human fibroblasts is strongly supported by our functional data. Measurements of enzyme activity in human fibroblasts (chosen were the individuals with highest CYP gene expression/induction) and in $AhRR^{+/+}$ and $AhRR^{-/-}$ MEFs clearly showed that in the copy number range of CYP1A1 gene expression (up to 10 copies hCYP1A1/10^4 transcripts β-actin), there was no EROD activity measurable above the LOQ (Figure 3a–c). In the case of NCTCs, even lower CYP1A1 transcript numbers (only up to 1.5/10^4 β-actin; Figure 2i) resulted in an impressive CYP1 induction of up to ~200 pmol min^{-1} mg^{-1} (Figure 3). These data strongly suggest that there is no correlation between CYP1A1 mRNA expression and enzyme activity. In fact, it has been described by Gry *et al.* (2009), who compared RNA and protein profiles of 1,066 gene products in 23 human cell lines, that only one-third of the tested genes show a significant correlation between mRNA and protein expression. This phenomenon has also been described for CYP enzymes earlier (Swanson, 2004; Svensson, 2009). Wild-type MEFs also displayed no EROD activity, whereas $AhRR^{-/-}$ MEFs just reached the LOQ (at 1 pmol min^{-1} mg^{-1} ethoxyresorufin) with inducer concentrations up to 1 μM (Figure 3d and e). As this enzyme activity was very low, especially in comparison with keratinocytes (100–200 pmol min^{-1} mg^{-1} ethoxyresorufin; Figure 3, Gotz *et al.*, 2012a), we doubt the physiological relevance of CYP1A1 activity in fibroblasts.

Previous work identified the association of AhRR function with HDAC activity (Gradin *et al.*, 1999; Haarmann-Stemmann *et al.*, 2007; Oshima *et al.*, 2007). As this was supposed to be the molecular mechanism of CYP1A1 expression control, we treated our three best inducible individuals with the HDAC inhibitor TSA in the presence or absence of B(a)P (Figure 4). Our study reproduced the previous results that CYP1A1 transcripts are strongly induced upon TSA exposure, which in our cells correlated with a downregulation of AhRR expression. However, on measuring EROD activities in those cells, it was found that the substrate turnover never reached the LOQ, clearly showing that CYP1A1 mRNA expression was overall too low to be translated into physiologically relevant CYP1 activity in these cells.

Taken together, by revisiting the ''AhRR hypothesis'' in primary human fibroblasts from 25 human individuals, we found that (i) the AhRR is expressed only at moderate RNA copy number levels and that, against the common view, (ii) in some of the investigated fibroblast strains, CYP1A1 mRNA

expression can be induced by AhR activators. However, even the highest induction did not translate into measurable CYP1 enzyme activity, and neither basal nor induced CYP1A1 mRNA expression correlated with AhRR expression. $AhRR^{-/-}$ MEFs were not induced to biologically relevant CYP1 enzyme activity despite impressive mRNA induction. Finally, inhibition of HDAC activity by TSA failed to induce measurable CYP1 activity. Thus, although we agree that CYP activity in primary human fibroblasts is repressed, our data give strong indications that (i) not the AhRR but a so far unidentified factor mediates the repression of CYP activity in these cells and that (ii) the AhRR may, at least for primary human adult fibroblasts, serve a different, yet unknown, biological function. One possible task of the AhRR in fibroblasts might be the involvement in proliferation required for wound healing. Wounds in $AhR^{-/-}$ animals had elevated numbers of fibroblasts, which secreted higher levels of active transforming growth factor-β that increased keratinocyte migration in culture and led to faster wound healing in the $AhR^{-/-}$ mice (Carvajal-Gonzalez *et al.*, 2009). We observed a different proliferative behavior of $AhRR^{-/-}$ MEFs compared with the wild types *in vitro* (unpublished observations). Whether the AhRR is truly involved in the determination of fibroblast proliferation is further investigated.

Practically, this is of relevance not only for environmentally induced skin diseases but also for therapeutical interventions, as AhR-modifying compounds are used for photoprotection of human skin (property right EP99 12 3929.4 (1999-12-02); Symrise AG (Holzminden, Germany); property right DE59913409.7 1998-12-11) as well as in therapeutics. Coal tar, e.g., which contains a variety of AhR agonists, has been used for psoriasis treatment (Goeckerman, 1931), or the antifungal ketoconazole has recently been identified as an AhR agonist (Tsuji *et al.*, 2012). To understand the effects and side effects of such compounds on different skin cell types on a molecular basis, knowledge of regulation of AhR signaling in either cell type is essential. This work contributes to the comprehension of AhR signaling in primary human fibroblasts.

MATERIALS AND METHODS

Chemicals and materials

All chemicals, if not otherwise specified, were purchased from Sigma-Aldrich (St Louis, MO) and were of the highest purity available. Cell culture media were obtained from PAA (Pasching, Austria) and PromoCell (Heidelberg, Germany). The CBQCA protein quantification kit was purchased from Life Technologies (Paisley, UK). The Cell Titer-Blue cell viability assay kit was purchased from Promega (Madison, WI). Multi-well plates and cell culture devices were obtained from Greiner Bio One (Frichenhausen, Germany) and Carl Roth GmbH (Karlsruhe, Germany).

Preparation of primary fibroblasts

Normal human dermal fibroblasts. Dermal fibroblasts from 25 healthy volunteers were prepared from skin samples from breast reduction surgery obtained from the hospital Kaiserswerther Diakonie in Düsseldorf, Germany. The samples were taken from female patients of five different age groups (20–29, 30–39, 40–49, 50–59, and >60 years; five each) and unknown pharmacological background. Patients were informed beforehand and gave written consent to donate removed tissue for scientific purpose. The preparation of primary human fibroblasts has been fully approved by the Ethics Committee, Heinrich-Heine-University of Düsseldorf (Project-Nr.: TOX_EF_D01/2008). This study was conducted in compliance with the Declaration of Helsinki Principles. Skin samples were collected immediately after surgery, kept cold during the transport (<1 hour), and processed immediately. Briefly, subcutaneous fat was removed before skin samples were cut into $\sim 0.5\,cm^2$ pieces. Skin pieces were washed in 70% ethanol, followed by sterile phosphate-buffered saline. Skin pieces were incubated with dispase ($10\,mg\,ml^{-1}$ in phosphate-buffered saline, sterile filtered) at 37 °C and 5% CO_2 for 2 hours; thereafter, epidermal sheets were removed and dermal pieces were plated on cell culture dishes to dry for 30 minutes, and then the medium was added. Fibroblasts started to migrate out of the dermal pieces after approximately 1–2 weeks. Cells were used for experiments in passages 2–7. Cell labeling: F, fibroblast; number, age; D + number, internal identification number; W, female (German, weiblich); B, breast.

MEFs. Mouse embryonic fibroblasts (MEF) were generated from embryos of wild-type and *AhRR*-deficient mice (HW and IF, unpublished) at embryonic day 14. Cells were maintained in DMEM containing 10% fetal calf serum, 1% glutamine, 0.1% 2-mercaptoethanol, and 1% penicillin streptomycin and were used at passage 3 for all experiments.

Cell culture

NHDFs were cultured in DMEM high glucose with stable glutamine containing 10% (v/v) fetal calf serum and 1% (v/v) antibiotic–antimycotic solution (PAA, Pasching, Austria; catalog number P11-002). MEFs were cultured as mentioned above. NCTC 2544 cells were cultured in minimum essential medium containing 10% (v/v) fetal calf serum and 1% (v/v) antibiotic–antimycotic solution. Primary NHEK-c (PromoCell GmbH, Heidelberg, Germany) from a 29-year-old female donor (breast) were cultured in full KGM2 Media (PromoCell) supplemented with supplement mix and $50\,\mu g\,ml^{-1}$ gentamycin (PAA) and $2.5\,\mu g\,ml^{-1}$ amphotericin (PAA). All cells were maintained under standard conditions at 37 °C and 5% CO_2. Treatment of cells was performed 24 hours after seeding in six-well plates (RNA analysis) or 48-well plates (EROD/Cell Titer-Blue) in the respective media. For subsequent EROD assay, the respective media were used without fetal calf serum.

RNA isolation, RT–PCR, and real-time RT–PCR

Total RNA was isolated from cells using the PeqLab Total RNA Kit (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. RNA concentration was assessed by spectrophotometry at 260 nm. Reverse transcription was performed as follows: 500 ng of total RNA, 1 μg of p(DT)15 primer (Roche, Basel, Switzerland), and 5 mM solutions of each deoxyribonucleotide triphosphate were dissolved in 10 μl of H_2O and heated for 5 minutes at 65 °C. The samples were chilled, and 4 μl of 4 × reverse transcription buffer (250 mM Tris HCl, 375 mM KCl, 15 mM $MgCl_2$) and 200 U of M-MLV reverse transcriptase (Promega, Fitchburg, WI) were added to a final volume of 20 μl. The samples were reverse transcribed at 37 °C for 50 minutes, and the reaction was inactivated at 70 °C

for 15 minutes. Real-time RT–PCR was performed using the Rotor Gene Q device (Qiagen, Hilden, Germany). The PCR mix consisted of 1/10 volume of Quanti Tect SYBR Green FAST PCR Master Mix (Qiagen), 0.5 μM solutions of each primer, and 2.5 μl of complementary DNA (after RT–PCR diluted 1:2.5 with H_2O), in a final volume of 15 μl. The application started with an initial incubation step of 7 minutes at 95 °C to activate the DNA polymerase. The conditions for PCR amplifications were as follows: 47 cycles of 10 seconds at 95 °C for denaturation, and 35 seconds at 60 °C for primer annealing, elongation, and fluorescence detection. PCR-primer sequences for human and murine CYP1A1, AhRR, AhR, ARNT, and β-actin are given in the Supplementary Table S1 online. The quantification of PCR products was estimated from fragment-specific standard curves and was calculated with the Rotor Gene Q 1.7 (Qiagen) software. Standard curves were prepared using 1.5×10^2 to 1.5×10^7 complementary DNA copies per μl and amplified as described above.

EROD activity

For measuring CYP1A1 activities in living monolayer cultures, ethoxyresorufin (dissolved in DMSO) was used according to a protocol described by Rolsted *et al.* (2008). Resorufin as the reaction product in the respective media or assay solution was used to generate standard curves. Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA). Suitability of the EROD assay for both species, human and mouse, was shown by comparing EROD activities of liver microsomes for the two species (Gotz *et al.*, 2012a). Cells were treated with the synthetic AhR agonist 3-MC and the environmentally relevant AhR agonist B(a)P dissolved in DMSO. The standard incubation period for induction was 24 hours, as derived from an EROD time course (Supplementary Figure S3 online), and the final maximum solvent concentration was 0.2% unless otherwise stated. All experiments were carried out three times in triplicate using each three independent cell lots unless otherwise stated. For experiments with HDAC inhibitor, TSA (0.5 μM in EtOH) cells were coincubated with the respective inhibitor and B(a)P or respective solvent for 16 hours.

Cell viability and protein content assessment

Assay kit for measurement of cell viability (Cell Titer Blue, Promega) was applied as described by the manufacturer. Protein in monolayer cell culture was determined using the CBQCA protein quantification kit (Molecular Probes/Invitrogen) using bovine serum albumin as reference protein at excitation and emission wavelength of 465 and 550 nm, respectively, on a Thermo Ascent Fluoroscan plate reader.

Statistics

All experiments were conducted at least three times. Statistical analyses for significance were performed using Student's unpaired *t*-test; $P < 0.05$ was considered significant. Data are presented as means ± SD. LOQ was defined as the mean of blank measurements plus nine times SD of the blank. Correlation analyses were performed by using the GraphPad Prism 5.00 statistical software (GraphPad Software, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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