HHS Public Access

Author manuscript

Lab Invest. Author manuscript; available in PMC 2009 December 01.

Published in final edited form as:

Lab Invest. 2009 June ; 89(6): 695–707. doi:10.1038/labinvest.2009.24.

Ah receptor represses acute phase response gene expression without binding to its cognate response element

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Abstract

Repression of the NF-κB pathway has been extensively researched due to its pivotal role in inflammation. We investigated the potential of the aryl hydrocarbon receptor (AHR) to suppress NF-κB regulated gene expression, especially acute phase genes, such as serum amyloid A (*Saa*). Using AHR mutants, it was determined that nuclear translocation and heterodimerization with ARNT are essential, but DNA-binding is not involved in AHR-mediated *Saa* repression. A number of AHR ligands were capable of repressing *saa3* expression. AHR activation leads to a decrease in RELA and C/EBP/β recruitment to and histone acetylation at *Saa3* gene promoter. A battery of acute-phase genes (e.g. C-reactive protein and haptoglobin) induced by cytokine exposure was repressed by AHR activation in mouse hepatocytes. Dietary exposure to an AHR ligand represses cytokine induced acute-phase response in liver. Use of a human liver-derived cell line revealed similar repression of *Saa* mRNA levels and secreted protein. Repression of AHR expression also enhanced *Saa* induction in response to cytokines, suggesting that AHR is capable of constitutively repressing *Saa* gene expression. These results establish a role for AHR in inflammatory signaling within the liver, presenting a new therapeutic opportunity, and signify AHR's ability to function in a DNA-independent manner.

Keywords

Ah receptor; Serum amyloid; TCDD; inflammation; acute phase response; liver

The acute phase response (APR) represents a major adaptive physiological first-line reaction to potentially deleterious environmental stresses including infection, inflammation, chemical stress and neoplastic growth. Homeostatic disruption by such factors initiates the APR, primarily within the liver, resulting in a complex but highly coordinated change in the pattern of hepatic gene expression. The stimulation and repression of a subset of

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predominantly secreted hepatic factors known as the positive and negative acute phase proteins (APP) respectively, signals to the body the need to respond to a perceived stress. Numerous proteins have been classified as belonging to the APP, including; plasminogen, fibrinogen, C-reactive protein (CRP), α1-antitrypsin, α2-macroglobulin, and serum amyloid A (SAA)1. Many APP are pleiotropic in nature and generally serve to modulate the immune system and metabolic processes to counteract a perceived stress. However, persistent pathophysiological conditions such as cancer or autoimmune diseases (e.g. rheumatoid arthritis) can lead to chronic stress and sustained APR induction with subsequent deleterious effects on immune signaling, catabolism, cachexia and amyloidosis. Consequently, clarification of the transcriptional regulation of specific acute phase proteins and the potential to modulate their expression has obvious clinical benefits.

Induction of the APR is principally driven through cytokine signaling and activation of transcription factors such as, nuclear factor-κB (NF-κB); signal transducer and activator of transcription-3 (STAT3) and CCAAT-enhancer binding protein β (C/EBPβ or NF-IL6). The transcriptional activity of NF-κB can be influenced by numerous factors, both such as post-translational modification of NF-κB subunits, or through cross talk with other transcription factors (e.g. nuclear receptors)2. Recently, evidence has been provided implicating the aryl hydrocarbon receptor (AHR) or dioxin receptor as a modulator of NF-κB activity3. AHR, a ligand-activated transcription factor belonging to the basic helix-loop-helix PAS protein family has an established role in xenobiotic metabolism, driving the expression of detoxification enzymes, CYP1A1 being a prime example. AHR adheres to the paradigm of a ligand-activated transcription factor; ligand binding promotes the dissociation of AHR from a cytoplasmic chaperone complex thus facilitating nuclear translocation of AHR4. Nuclear AHR readily forms a heterodimer with AHR-nuclear translocator (ARNT) thus forming a competent transcription factor capable of binding cognate DNA dioxin response elements (DRE) and stimulating the expression of AHR target genes5. Recent evidence suggests that AHR activity is not restricted solely to xenobiotic metabolism but may also exert modulatory effects upon diverse cellular processes through the phenomenon of receptor cross-talk6.

Here, evidence is presented highlighting the potential of AHR to negatively regulate the transcriptional activity of factors that regulate the inflammation response with particular emphasis upon suppression of acute phase protein expression in the liver. Utilizing a murine in vivo model in conjunction with mouse and human cell culture systems we demonstrate, the capacity of ligand activated AHR to directly attenuate cytokine-mediated induction of the APR component SAA mRNA and protein, as well as other APR genes. Furthermore, we demonstrate that this attenuation occurs in the absence of direct interaction of AHR and its cognate response element. This represents the first report documenting the ability of AHR to repress inflammatory signaling in a non-traditional fashion and highlights the potential of AHR as a target for the therapeutic management of inflammatory disease.

MATERIALS AND METHODS

Reagents and Mice

Anti-RELA antibody (sc-372) and anti-C/EBPβ (sc-150) were purchased from Santa Cruz Biotechnology Inc. and anti-acetyl-histone H4 (Lys5) antibody was purchased from Upstate. Affinity-purified anti-AHR rabbit polyclonal antibody was obtained from BioMol. Recombinant interleukins were purchased from PeproTech Inc. Hepa1c1c7 and Huh7 cells were obtained from American Type Culture Collection and Curt Omiecinski (Penn State University), respectively. C57BL6/J were obtained from Jackson Laboratory, while $Ahr^{fx/fx}Cre^{Alb}$ and $Ahr^{-/-}$ mice were gifts from Chris Bradfield (University of Wisconsin, Madison).

AHR Ligand Dietary Exposure

Female C57BL6/J and $Ahr^{-/-}$ mice, 10–12 weeks old mice with mean weights 19.8 ± 0.7 g and 16.9 ± 1.4 g respectively, were used in this study. Mice were maintained on a standard 12 h light/dark cycle with *ad libitum* access to standard chow and water. Mice were given *ad libitum* access to purified AIN93M (Dyets, Inc.) or purified diet supplemented with 0.4 g/kg β-NF for 18 h (overnight). The next day mice were given intra-peritoneal injections of vehicle (phosphate-buffered saline) or 10 μg/kg murine IL1B/IL6 as indicated. 4 h post- *i.p.* injection mice were sacrificed by asphyxiation with CO_2 and hepatic tissue harvested.

Cell Culture

Hepa1c1c7 and Huh7 established cell-lines were cultured in α-minimum essential medium with 8% fetal bovine serum (FBS). SV-40 virus immortalized $mAHR^{-/-}$ hepatocytes were maintained in αMEM, 10 nM dexamethasone and 4% FBS at 34°C. The cells were grown in the absence of dexamethasone during experiments. Primary bone marrow (BM) cells were isolated from lower limb bones of 8–12 week old C57BL/6J mice, BM cells were cultured overnight in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 8% FBS and penicillin/streptomycin. Non-adherent cells were centrifuged and plated in DMEM supplemented with 10 ng/ml granulocyte-monocyte colony stimulating factor and 2 mM glutamine. Half the volume of medium was replaced every day for 4 days prior to treatment.

Primary Hepatocyte Isolation

Primary murine hepatocytes were isolated by the *in situ* two-step perfusion method from mice7. Hepatocytes were maintained in culture media (Hepatozyme-SFM (Invitrogen)/2.5% DMSO/10 nM dexamethasone/100 IU/ml penicillin and 100 μg/ml streptomycin). Cells were cultivated for 5 d before treatment.

Expression Constructs

Plasmid constructs pcDNA3-mAhR, pcDNA3-ARNT-HA, pEYFPmAhR, pCI-XAP2, pGudLuc 6.1 were generated previously. The mAhR mutant constructs, pcDNA3-mAhR ΔH1 (Δ43–51) and pEYFP-mAhR ΔH1 were generated using loop-out mutagenesis with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Transient Transfections and Luciferase Assays

SV-40 virus immortalized mAHR$^{-/-}$ hepatocytes8 were transfected using Lipofectamine 2000 according to the manufacturer's protocol.

Antibodies and Protein Blot Analysis

AHR was detected using mouse monoclonal antibody RPT1 (Affinity Bioreagents). Primary antibodies were detected with a biotinylated rabbit anti-mouse antibodies (Jackson Immunoresearch). Biotinylated secondary antibodies were detected using either ^{125}I-streptavidin (Amersham Biosciences) or ECL.

RNA Isolation and Real-Time PCR

RNA was isolated from cells with TRI® reagent (Sigma) and reverse transcribed with a High Capacity cDNA Archive kit (Applied Biosystems). Quantitative real-time PCR was performed on iQ systems (BioRad) using iQ SYBR Green master mix (BioRad), according to the manufacturer's protocol. Expression values of genes of interest were normalized to that of ribosomal protein L13a (RpL13a) or β-actin. The sequence of the primers used in real-time PCR are listed in Table S1.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays (ChIP) were performed as described previously9. Briefly, cells cultured in 150 mm culture dishes were crosslinked with 1% formaldehyde for 8 min at 37°C and sonicated using a Branson Sonifier 450 to generate 500 – 700 bp fragments. The sonicated lysate was diluted to two A260 units and 1 ml of this lysate was subjected to immunoprecipitation with the appropriate antibodies given above and protein A sepharose. The level of enrichment of promoter fragments was determined by PCR or real-time PCR. The *Saa3* primers used were 5′-GCGCAATCTGGGGAAAGAAGATGT and 5′-TGAGTGGCTTCTGTCCTTTGCTGA (forward and reverse, respectively); for Saa2, 5′-TACTACACCCCAGAAGATTGCCAC and 5′-AGGTGAGAGGAGGCAGGCATTTAT.

siRNA Transfections

Repression of AHR expression was performed with siRNA oligos purchased from Dharmacon RNAi Technologies. Approximately 60% confluent Huh7 cells were transfected with 120 nM scrambled or anti-*AHR* oligos using Dharmafect-1 transfection reagent following manufacturer's protocol. Culture medium was changed after 24 h and the cells were allowed to recover for an additional 12 h before treatment.

ELISA

Huh7 cells were treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and interleukins for 10 h or 24 h under serum-free conditions. Culture media was analyzed for SAA protein levels using a human SAA ELISA kit purchased from Anogen (Yes Biotech Laboratories Ltd.) according to the manufacturer's protocol. The level of SAA in mouse serum was determined using a mouse SAA ELISA kit (Immunology Consultants Lab, Inc. Newberg, OR). Statistical comparison of treatments was performed using the Student's *t*-test (α=0.05).

DNA Microarray Analysis

RNA was isolated from 10^6 sorted cells using TRI® reagent and further purified with RNeasy® columns. RNA integrity was confirmed by Bioanalyzer (Applied Biosystems). Samples were then hybridized to Affy mouse 2.0A genome chips. Labeling, hybridization and washing were performed at the microarray core facility, the Pennsylvania State University. Data was processed and significantly altered genes were identified using GeneChip Operating Software (GCOS). Genes were declared as increased or decreased in AHR and DNA-binding mutant AHR (A78D-AHR) transfected cells, as compared to control transfections.

RESULTS

Identification of DNA-binding Independent Effects Mediated by the AHR

The established mechanism of AHR function involves binding of the AHR-ARNT heterodimer to DNA bearing the DRE sequence. In order to investigate the possibility of a DNA-binding independent manner of AHR function, simian virus 40 (SV40) immortalized AHR null mouse hepatocytes were transfected with either the AHR or DNA-binding defective mutant (A78D) AHR expressing plasmid or control vector. The A78D-AHR mutant has previously been shown to bind ligand, translocate to the nucleus and heterodimerize with ARNT, yet it fails to bind DNA at its response element10. Green fluorescent protein (GFP) expressing plasmid was cotransfected along with AHR plasmids in a ratio of 1:3 and a high level of transfection efficiency was obtained (Fig. S1a). GFP expressing transfected cells were subjected to FACS and RNA and protein were isolated. AHR expression was confirmed by Western blot (Fig. 1a) and the RNA was used for microarray experiments. Single samples were analyzed on individual DNA microarrays. Data analysis was targeted to identify the subset of genes altered in WT-AHR as well as A78D-AHR transfected cells but not in the control. Multiple APR genes were identified to be repressed by the wild-type and DNA-binding defective mutant AHR (Table 1). Maximum repression was observed for *Saa3* mRNA, which was selected for further analysis.

Generation and Characterization of an AHR Heterodimerization Mutant

It is important to examine whether heterodimerization of AHR and ARNT is essential for the DNA-independent effects of AHR. An AHR-heterodimerization mutant (ΔH1-AHR) was constructed by deleting the DNA sequence that encodes for amino acids 43 – 51, which encompass helix-1 of the helix-loop-helix domain (Fig. 1b). Immunoprecipitation of HA-tagged ARNT failed to co-precipitate the ΔH1-AHR, demonstrating the inability of this mutant to heterodimerize with ARNT in contrast with WT-AHR (Fig. S2a). Consequently, ΔH1-AHR was unable to drive the expression of DRE-driven luciferase in Cos-1 cells (Fig. S2b) as well as that of endogenous *Cyp1a1* mRNA in BP-8 cells, a rat hepatoma-derived cell-line deficient in AHR (Fig. S2c). However, a photoaffinity ligand binding assay demonstrated that ΔH1-AHR was still capable of binding ligand as efficiently as WT-AHR (Fig. S2d). TCDD treatment of yellow fluorescent protein (YFP)-tagged ΔH1-AHR and WT-AHR transfected Cos-1 cells demonstrated that ΔH1-AHR is capable of translocating into the nucleus upon ligand activation (Fig. S2e). Thus, the ΔH1-AHR is selectively

deficient in its ability to heterodimerize with ARNT and drive classical DRE-dependent gene expression.

Saa3 Repression Requires AHR Heterodimerization and Nuclear Translocation

The conventional AHR pathway requires nuclear translocation and heterodimerization with ARNT. A previously described K14A-AHR mutant incapable of translocating to the nucleus11 and the ΔH1-AHR mutant (Fig. 1b), were expressed in SV40-immortalized AHR null cells along with GFP-plasmid. *Saa3* expression was repressed by the WT-AHR and A78D-AHR, but not by K14A-AHR and ΔH1-AHR (Fig. 1c). Thus, DNA-binding independent gene regulation by AHR appears to require nuclear translocation and heterodimerization. As expected, *Cyp1a1* – the prototypic AHR target gene, was induced in the cells transfected with WT-AHR, but not with A78D-AHR or ΔH1-AHR (Fig 1d). K14A-AHR minimally induced *Cyp1a1*; however, this may be due to overexpression. It should be noted that activation of AHR by TCDD did not alter the extent of *Saa3* repression in SV40-immortalized AHR null hepatocytes (data not shown).

Saa3 Repression Under Different Experimental Conditions

The AHR-mediated *Saa3* repression observed in immortalized AHR null cells was confirmed in other model systems. *Saa3* transcription was induced in Hepa1c1c7 cells, a mouse hepatoma derived cell-line, by treatment with different pro-inflammatory cytokines such as interleukin-1β (IL1B), interleukin-6 (IL6), tumor necrosis factor-α (TNFA) or a combination of IL1B and IL6 (Fig. 2a and 2b). Activated AHR repressed the induction of *Saa3* by ~50% for each cytokine treatment. AHR ligand dependent repression of *Saa3* induction by AHR may provide an interesting therapeutic approach for chronic inflammatory diseases. However, it is essential to confirm that *Saa3* repression is not limited to only high doses of an AHR ligand. To this end, repression of cytokine-induced *Saa3* was studied with decreasing doses of TCDD in Hepa1c1c7 cells. TCDD was able to effectively repress *Saa3* even at the lowest dose tested (200 pM) (Fig. 2e). Also, Hepa1c1c7 cells were treated with different AHR ligands to determine if *Saa3* repression was a TCDD-specific effect. The established AHR ligands, benzo[a]pyrene (B[a]P), β-naphthoflavone (β-NF), α-naphthoflavone (α-NF) and M50354 were all able to repress *Saa3* induction (Fig. 2c). M50354 is a recently described AHR agonist compound capable of attenuating atopic allergic responses12. Interestingly, all ligands tested were effective at repressing *Saa3*, while TCDD was far more effective at inducing CYP1A1 (Fig. 3d). These results would support the concept that an AHR ligand may be found that is highly selective in eliciting a gene repression response.

AHR Mediated Saa3 Repression is a Direct Transcriptional Effect

In order to ascertain that AHR directly effects the transcription of *Saa3*, Hepa1c1c7 cells were pretreated with cycloheximide, a translation inhibitor. Though cycloheximide treatment elevated the constitutive level of Saa3 expression, it did not alter its repression by AHR activation (Fig. 2g). This indicates that AHR-mediated *Saa3* repression is a direct effect and not secondary to changes in the expression of another protein. Gene repression can be mediated by a decrease in transcription rate or by alteration of mRNA stability. After

challenging with TCDD and ILB/IL6, Hepa1c1c7 cells were treated with Actinomycin D, a transcription inhibitor, and the level of *Saa3* mRNA was assessed over 4 h. The decay rate of *Saa3* mRNA was not significantly altered by AHR activation over the time period examined (data not shown).

AHR Activation Represses Other Saa Family Member Genes

All members of the SAA family are upregulated simultaneously in an acute phase response. Hence, we examined the effect of AHR activation on the expression of *Saa1* and *Saa2* in Hepa1c1c7 cells (Fig. 3a and 3b). Cytokine-mediated induction of both *Saa1* and *Saa2* was repressed by AHR activation by 75 and 85 percent, respectively. This is significant as *Saa1* and *Saa2* are the major hepatic serum amyloid isoforms. Interestingly, *Saa1* and *Saa2* did not appear to be repressed in the previous microarray results from WT-AHR or A78D-AHR transfected SV40-immortalized mouse hepatocytes, the reason for which is not clear.

Saa Repression is AHR Dependent

AHR-deficient or AHR-expressing primary hepatocytes were isolated from $Ahr^{fx/fx}Cre^{Alb}$ (hepatocyte-specific conditional AHR null)13 or C57BL6/J mice respectively. *Saa* transcription was highly induced in these cells by IL1B/IL6 treatment. TCDD was able to restrict the induction of *Saa1* and *Saa2* in AHR-expressing (Fig. 3c and 3d), but not in AHR-deficient, hepatocytes (Fig. 3e and 3f). This, along with the observation that AHR transfection in AHR null cells is required for suppressing *Saa3* (Fig. 1c), clearly establishes that AHR is essential for *Saa* repression by TCDD.

AHR Can Repress Saa Induction Mediated by Complex Inflammatory Medium

Different cytokines can have counter-regulatory effects on various aspects of an inflammatory response. Thus, it is possible that IL1B and IL6 mediated *Saa* induction might not simulate the exact response obtained with a combination of cytokines, as expected in an inflammatory response in vivo. To confirm the ability of AHR to repress *Saa* induction under such circumstances, primary bone-marrow cells were isolated from C57BL6J mice and were cultured to promote differentiation into macrophages. Following a three-day LPS challenge, the conditioned culture medium was collected from the macrophages and used to treat Hepa1c1c7 cells. To differentiate the effect of secreted cytokines from those of LPS, LPS-containing culture medium was incubated in the absence of macrophages and used as a control. AHR-activation was able to repress the induction of *Saa1* in response to the macrophage-conditioned-media or LPS alone (Fig. 3g). Similar results were obtained examining *Saa2* repression by TCDD (data not shown). This demonstrates AHR's ability to repress *Saa* induction under a physiologically attainable concentration/combination of cytokines.

Mechanistic Insights into AHR-mediated Saa Repression

The fact that AHR directly represses *Saa3* and that the K14A-AHR (nuclear localization) mutant failed to repress *Saa3* induction, suggests that AHR likely affects the formation of a transcription complex within the nucleus and perhaps at the *Saa* promoters. Chromatin immunoprecipitation (ChIP) assays in Hepa1c1c7 cells demonstrate that activated AHR

appears to reduce the presence of the RELA (p65) subunit of NF-κB at the *Saa3* and *Saa2* promoters in response to interleukin treatment (Fig. 4a, 4b). In addition, C/EBPβ presence on the SAA3 promoter after cytokine treatment was greatly inhibited after TCDD co-treatment (Fig. 4a). AHR has previously been shown to physically interact with RELA14, which might then contribute to preventing RELA recruitment to *Saa* promoters in response to IL1B/IL6 treatment. Chromatin immunoprecipitation with an acetylated-histone 4 antibody demonstrated that AHR activation also reduced histone acetylation at *Saa2* and *Saa3* promoters (Fig. 4a, 4b).

AHR Mediated Suppression Extends to Other APR Genes

After confirming the repression of *Saa1* and *Saa2* in primary mouse hepatocytes, expression of other acute phase response genes was also examined by real-time PCR (Fig. 5a–f). AHR activation was able to repress induction of a number of acute phase genes; including, C-reactive protein (CRP), LPS-binding protein (LBP), haptoglobin, alpha-2-macroglobulin, and alpha-1-acid glycoprotein-1. This suggests that AHR represses the acute-phase response through a common transcriptional regulatory mechanism.

AHR Activation In Vivo Leads to Attenuation of Cytokine Mediated Acute Phase Response

In order to test whether activation of the AHR leads to repression of cytokine-mediated acute-phase response, we reasoned that continual exposure to an AHR ligand would be more effective, thus a dietary route of exposure was chosen. After a preliminary dose-response experiment with dietary β-NF indicated that 0.4 g of β-NF/kg yielded about 10% of the *Cyp1a1* inducibility observed at higher doses of β-NF in mice, BNF concentration was chosen for subsequent experiments (data not shown). C57BL6/J mice were fed a semi-purified control diet or a diet containing 0.4 g of β-NF/kg overnight. Mice were then *i.p.* injected with IL1B and IL-6, and after 5 h liver RNA was isolated. The level of *Cyp1a2* mRNA induction was measured in C57BL6/J and *Ahr*-null mouse liver in the presence and absence of IL1B/IL-6 as a measure of AHR activation (Fig. S4). These results revealed that β-NF induced *Cyp1A2* mRNA in C57BL6/J mice indicating that dietary β-NF effectively induced AHR activity. Real-time PCR analysis revealed that *Saa1*, *Saa2* and *Crp* mRNA induction after cytokine treatment were all significantly reduced by the presence of an AHR ligand (Fig. 6). In contrast, dietary β-NF had no effect on cytokine-mediated acute-phase gene expression in *Ahr*-null mice, indicating that the observed repression in C57BL6/J mice is AHR-mediated (Fig 3S). Interestingly, β-NF treatment of *Ahr*-null mice leads to an increase in cytokine-mediated induction of certain acute-phase response genes (e.g. *Lpb*). As expected, IL1B/IL6 exposure in mice leads to the induction of *Il-8* and *Nfkbia* mRNA in liver, two genes regulated by inflammatory signaling (Fig. 6e–f). In addition, β-NF failed to significantly influence the level of induction of *Il-8* and *Nfkbia* (commonly known as IκBα) mRNA, suggesting that the AHR's ability to alter inflammatory gene regulation is gene context specific. However, *Il8* mRNA levels did show a non-statistically significant repressed response to β-NF exposure.

AHR Mediated Repression of SAA is Observed in Human Huh7 Cells

Another important question to address is whether human cells would elicit a similar response to AHR activation in the presence of cytokines. *SAA3* is not expressed in human liver15, while *SAA1* and *SAA2* have a very high sequence similarity, thus making it difficult to design unique primer-set for detecting *SAA2*. Hence, *SAA1* mRNA levels were monitored to assess the effect of AHR activation on the acute phase response. Huh7 cells, a human hepatocarcinoma derived cell line, were treated with vehicle or TCDD to activate the AHR, followed by treatment with human IL1B and IL6. Under these conditions, activation of AHR repressed *SAA1* mRNA induction by 75% (Fig. 7a). Changes in the level of secreted SAA protein were determined by ELISA and were found to mimic changes in mRNA (Fig. 7b). Since it is not possible to differentiate between different SAA family members by ELISA, this repression of SAA reflects the changes in the levels of all secreted SAA family members.

Although TCDD exerts its effects almost exclusively through the AHR, we wished to confirm that the observed TCDD-mediated *Saa1* repression in human cells is indeed AHR dependent. Repression of AHR expression in Huh7 cells was accomplished using *AHR* siRNA oligonucleotides. As expected, diminished AHR expression resulted in a loss of *SAA1* repression with TCDD treatment (Fig.7c and 7e). AHR repression was verified by the loss of its ability to induce a transcriptional target gene, *CYP1A1* (Fig. 7d). Interestingly, the loss of AHR resulted in an enhanced induction of *SAA1* with IL1B/IL6 treatment (Fig. 7c). In order to confirm that this was not an off-target effect of the *AHR* siRNA oligo sequence, a second anti-*AHR* siRNA oligo was transfected into Huh7 cells by electroporation. Loss of AHR expression blocked TCDD-mediated repression of cytokine induced SAA1 expression (Fig. 7e). Repression of AHR expression by this second *AHR* siRNA also resulted in enhanced *SAA1* induction by IL1B/IL6 treatment (Fig. 7f). This suggests that AHR may function to constitutively suppress the level of *SAA1* transcription.

Whether AHR-mediated repression of inflammatory genes is a universal phenomenon or a promoter specific effect was examined. Two known NF-κB regulated genes, *Il-8* and *Nfkbia* were induced by IL1B/IL6 and both remained unaffected upon co-treatment with TCDD (data not shown). Thus, AHR-mediated repression of cytokine-mediated induction of acute phase gene expression is context specific and does not appear to occur at every target gene regulated by NFκB.

DISCUSSION

A dysregulated inflammatory/immune response appears to be the underlying cause of many diseases, such as multiple sclerosis, asthma, lupus and rheumatoid arthritis. An acute phase response dominates the initial reaction to perceived insults and commences a series of biochemical and neuroendocrine changes that facilitate mounting an inflammatory/immune response. Acute phase proteins largely expressed by the liver serve various tasks in this process. However, persistent activation of the APR has its own perils. Elevated CRP is associated with increased cardiovascular risk and has been proposed to be a better clinical marker of atherosclerosis and related events than lipid levels16. SAA is an apolipoprotein for high-density lipoproteins (HDL) and influences cholesterol metabolism leading to

enhanced inflammation. Conversely, constant elevation of SAA, and even alpha-2-macroglobulin, leads to extracellular amyloid plaques that interfere with organ function and underlie the pathology of diseases such as Alzheimer's17. In about 5% of rheumatoid arthritis patients AA amyloid deposition occurs, which can then lead to renal dysfunction and other adverse complications18. Therapies that can selectively reduce circulating SAA levels should be useful in managing amyloidosis in these patients.

Recent studies have demonstrated that SAA can contribute to systemic inflammation19. For example, SAA has been shown to serve as a ligand that activates TLR2, RAGE and FPRL1 receptors leading to an enhanced inflammatory response20–22. Collectively, signaling through these receptors can lead to activation of MAPKs, p42/44, JNK, and p38 kinases, as well as the transcription factors AP-1 and NFκB. The ability of SAA to activate FPRL1 receptor in synoviocytes can result in synovial hyperplasia as well as endothelial angiogenesis, two common phenotypes observed in rheumatoid arthritis22. Interestingly, elevated levels of the AHR have been observed in synovial tissue of rheumatoid arthritis patients, suggesting another possible target tissue for modulating AHR activity23.

Transcription of SAA and most other APPs, are primarily regulated by NF-κB, NF-IL6 (C/EBP-β) and STAT324. The inhibitory effects of the glucocorticoid receptor (GR), and estrogen receptor (ER) on NF-κB induced gene transcription has received wide attention25. NF-κB signaling allows multiple levels of regulation, which have been utilized by NRs to interact with this pathway. Cytokines engage distinct receptors on the cell surface, leading to recruitment and activation of a family of adaptor proteins through various post-translational modifications. Eventually, signaling from different cytokine receptors converges on phosphorylation-dependent activation of the IKK complex (IκB-kinase complex), which in turn releases NF-κB to translocate into the nucleus. Activated AHR may inhibit any of these cell-surface receptors or the immediate downstream cytoplasmic signaling to repress NF-κB activity. However, in the context of APR gene regulation, AHR effectively repressed *Saa3* mRNA when induced separately by IL1B, IL6 and TNFA. Also, K14A-AHR the nuclear localization mutant, was unable to repress *Saa3* mRNA induction. This demonstrates that AHR-mediated NF-κB suppression is not due to an effect on upstream cytokine signaling, but is primarily a nuclear phenomenon.

In ChIP assay, activation of AHR diminished cytokine-induced association of the RELA subunit of NF-κB with its response elements in *Saa2* and *Saa3* promoters. Other groups have previously demonstrated the ability of AHR to physically interact with RELA14. While a direct physical interaction between the two proteins might certainly explain the reduction in RELA recruitment to *Saa* promoters, it cannot be the sole mechanism for AHR-NF-κB cross-talk, because AHR activation is unable to universally repress NF-κB driven gene expression (Fig. 7G and 7H). Also, we did not observe a significant reduction in RELA or p50 protein levels upon AHR activation (data not shown). Yet another possible mechanism could be the ability of the AHR to interact with RelB and bind to RelB-like response elements, as has been observed in U937 macrophages26. However, we have been unable to detect the AHR at the *SAA3* promoter by ChIP analysis.

The degree of APR repression observed in mice in this study is not as marked as that observed in cell culture experiments. There may be many reasons for this observation, although we believe that treatment of mice with an AHR agonist will have a dual effect on inflammatory signaling. There are two basic mechanisms that can modulate inflammatory signaling that probably are cell-type specific; the first is the ability of the AHR/ARNT heterodimer to repress gene transcription, which is the subject of this report. The second is the ability of various AHR ligands to enhance inflammatory signaling, apparently through a DRE-mediated mechanism. For example, IL1B and TCDD co-treatment of MCF-7 cells leads to a syngeristic induction of *Il6* expression27. The use of an AHR ligand that can effectively dissociate the repressive effects from the DRE-driven transactivation effects should tilt the overall AHR-mediated responses towards anti-inflammatory effects. Exposure of Hepa 1 cells to the partial agonist α-naphthoflavone reveals that this compound is effective at repressing cytokine-mediated SAA3 induction yet is a relatively poor inducer of DRE-driven transcriptional activity (Fig. 3C). This result would support the concept that a highly selective AHR ligand can be identified that would yield anti-inflammatory activity.

Ligand-activated receptors have multiple domains that impart different functionalities, such as DNA-binding, ligand-binding, dimerization and co-regulator recruitment. However, depending on the manner of activation and the physiological context, the functionalities of soluble receptors can be dissociated from their biological roles, as in the case of GR28 and ER29. Here, we demonstrate for the first time that DNA-binding is not essential for AHR-mediated repressive effects on NF-κB transactivation, while heterodimerization with ARNT and nuclear translocation are required. Aside from xenobiotic metabolizing enzyme induction, this is also the first report identifying a functional molecular role for AHR in a biological process, and not just regulation of individual genes. The data presented in this report demonstrate the functional interplay between AHR and inflammatory signaling pathways to regulate the expression of multiple APR genes, an important aspect of the hepatic inflammatory response. This report identifies a novel physiological function performed by the AHR in murine as well as human systems. AHR-mediated transcriptional repression is not conducted in the classical DRE-dependent fashion, but most likely involves multiple protein-protein interaction mechanisms. The fact that dietary exposure to the AHR ligand β-NF effectively represses cytokine-mediated APR in liver, as seen in figure 6 underscores the possibility of utilizing the AHR as a therapeutic target for treatment of inflammatory/autoimmune disease. However, in order to therapeutically utilize the ability of AHR to function as a repressor of APR, and possibly other inflammatory phenomena, it is necessary to identify selective ligands that would not also induce xenobiotic metabolism.

Supplementary Material

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ACKNOWLEDGEMENTS

We thank Dr. Chris Bradfield for the $Ahr^{fx/fx}Cre^{Alb}$ and $Ahr^{-/-}$ mice. This work was supported by Public Health Service grant ES04869 from the National Institute of Environmental Health Sciences.

ABBREVIATIONS

APR	acute phase response
AHR	aryl hydrocarbon receptor
ARNT	AHR-nuclear translocator
β-NF	β-naphthoflavone
IL1B	interleukin 1β
SAA	serum amyloid A
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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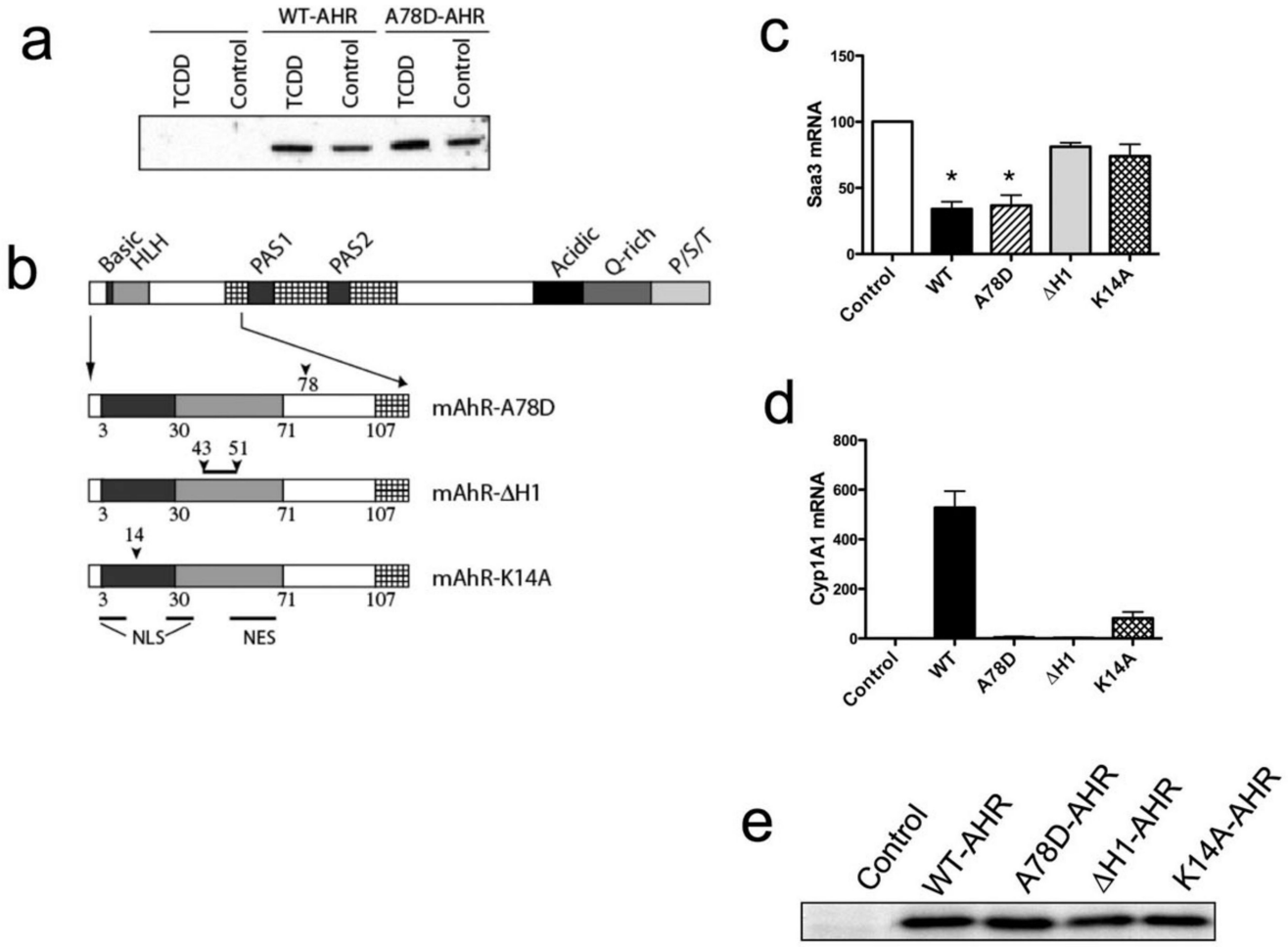

Figure 1.

Functional dissociation of the AHR function involved in Saa3 repression. (**a**) Western blot analysis of WT-AHR and A78D-AHR protein expression in SV40 immortalized AHR-null mouse hepatocytes transfected with a combination of GFP and WT-AHR/A78D-AHR/ control vector in a ratio of 1:3, using Lipofectamine2000 transfection reagent. Cells were sorted for GFP expression using FACS analysis. (**b**) Schematic representation of murine WT-AHR domains and the deletion/mutation (arrowheads) for each AHR mutant. (**c**) and (**d**) Real-time PCR on RNA isolated from SV40 immortalized AHR-null hepatocytes transfected with WT-AHR or various AHR mutants for 24 h. (**e**) Western blot analysis of the WT-AHR and AHR mutants expressed in experiment depicted in panels c and d. WT, wild-type; A78D, DNA-binding mutant; ΔH1, heterodimerization mutant; K14A, nuclear localization mutant. Data represent mean induction ± SEM (n = 3/treatment group) and were analyzed by one-way ANOVA to determine significance (*$P < 0.05$).

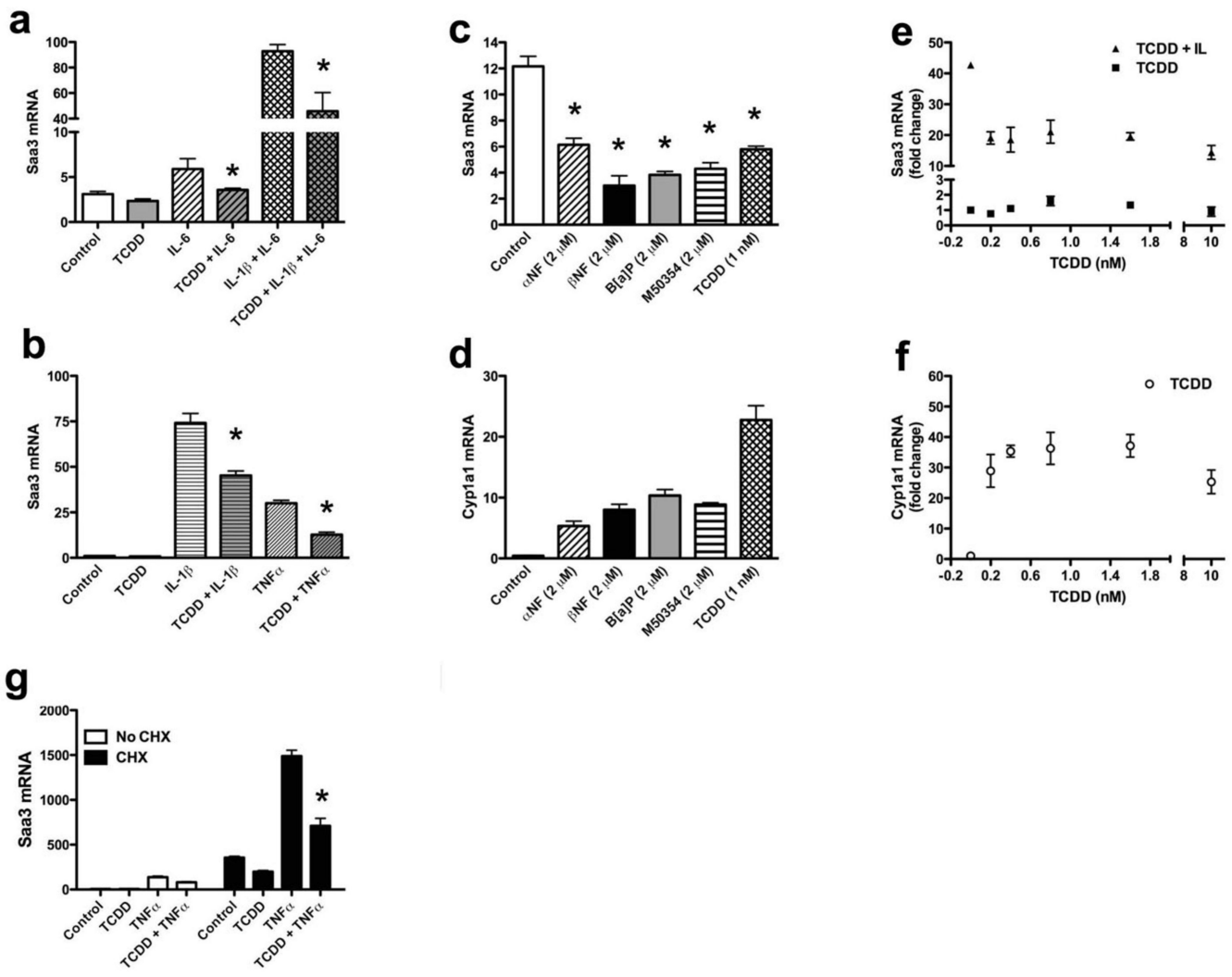

Figure 2.

AHR directly represses *Saa3* transcription in a dose-responsive and ligand-dependent manner. (**a** and **b**) AHR represses *Saa3* induction by various cytokines. Hepa1c1c7 cells were treated with 10 nM TCDD or vehicle control. After 30 min, cells were treated with 2 ng/ml IL1B, IL6, TNFA or a combination of IL1B and IL6 for an additional 6 h. (**c** and **d**) Various classes of AHR ligands can suppress *Saa3*. Hep1c1c7 cells were treated with different AHR ligands at the described doses for 30 min prior to interleukin (IL1B + IL6, 2 ng/ml each) treatment. *Saa3* mRNA (**c**) and *Cyp1a1* mRNA (**d**) were measured by real-time PCR. (**e** and **f**) Analysis of TCDD dose-response of AHR-mediated *Saa3* repression. Hepa1c1c7 cells treated with increasing doses of TCDD (0.2 nM to 10 nM) for 30 min prior to interleukin (IL1B + IL6, 2ng/ml each) treatment. (**e**) Closed triangles represent repression of IL-induced *Saa3* mRNA by various doses of TCDD, as determined by real-time PCR. Closed squares represent uninduced *Saa3* mRNA levels, as a control. (**f**) TCDD-driven *Cyp1a1* mRNA induction, as measured by real-time PCR. (**g**) AHR-mediated *Saa3* repression is due to direct transcriptional inhibition. Real-time PCR on RNA from Hepa1c1c7 cells treated first with (black bars), or without (open bars), the translational

inhibitor – cycloheximide (10 μg/ml) for 30 min, then with TCDD (10 nM) for 30 min and finally with TNFA for 6 h. Data represent mean induction ± SEM (n = 3/treatment group) and were analyzed to determine significance (*P < 0.05).

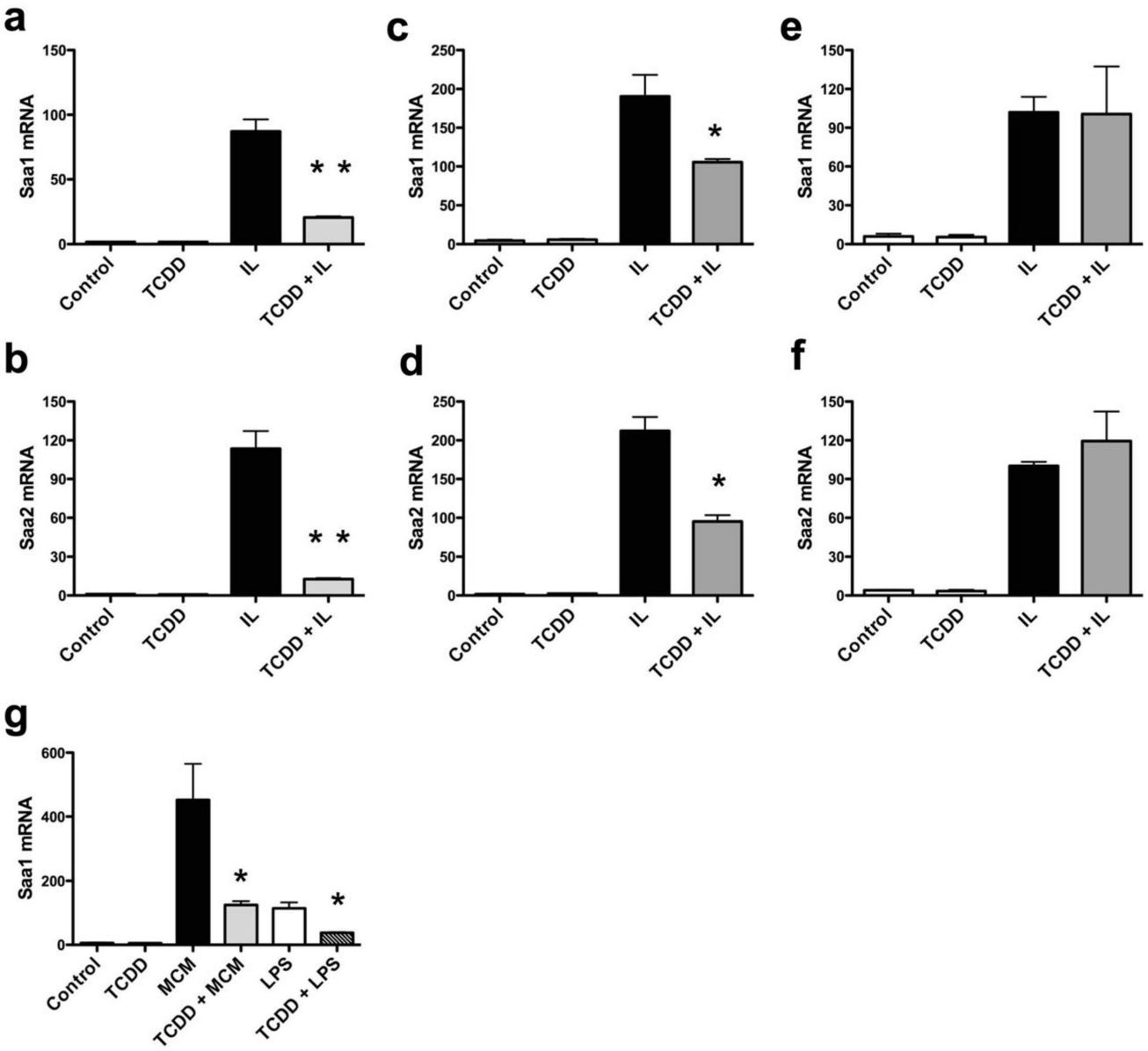

Figure 3.

AHR activation represses gene expression of several Saa-family members. (**a** and **b**) Real-time PCR on RNA from TCDD (10 nM, 30 min) followed by interleukin (2 ng/ ml each of IL1B and IL6) treated Hepa1c1c7 cells. Effect of AHR activation on induction of other SAA family members, *Saa1* (**a**) and *Saa2* (**b**), was determined. Data represents the mean +/− S.D. of triplicate measurements. Experiment was repeated thrice with similar results. (**c** and **d**) Real-time PCR on RNA from primary mouse hepatocytes treated with TCDD (10 nM for 30 min) followed by interleukin (2 ng/ ml each of IL1B and IL6) for 24 h. Prior to treatment, cells were transferred to α-MEM with 1 mg/ml bovine serum albumin for 24 h. Repression of *Saa1* (**c**) and *Saa2* (**d**) mRNA was measured. (**e** and **f**) Real-time PCR measurement of *Saa1* (**e**) and *Saa2* (**f**) mRNA, as described above, in AHR-deficient primary mouse hepatocytes isolated from $Ahr^{fx/fx}Cre^{Alb}$ mice. (**g**) Primary murine bone marrow cells were

cultured to promote differentiation into macrophages, as outlined in the text. After a 3 d LPS challenge, the conditioned media was collected from macrophage containing plates (MCM–macrophage conditioned media) and used to treat Hepa1c1c7 cells for 6 h following TCDD (10 nM, 30 min) pre-treatment. 'LPS' refers to LPS-spiked media that was maintained under similar culture conditions in the absence of any cells, and thus was devoid of any cytokines secreted by macrophages. *Saa1* mRNA levels were determined by real-time PCR. Data represent mean induction ± SEM (n = 3/treatment group) and were analyzed to determine significance (*$P < 0.05$; **$P < 0.01$).

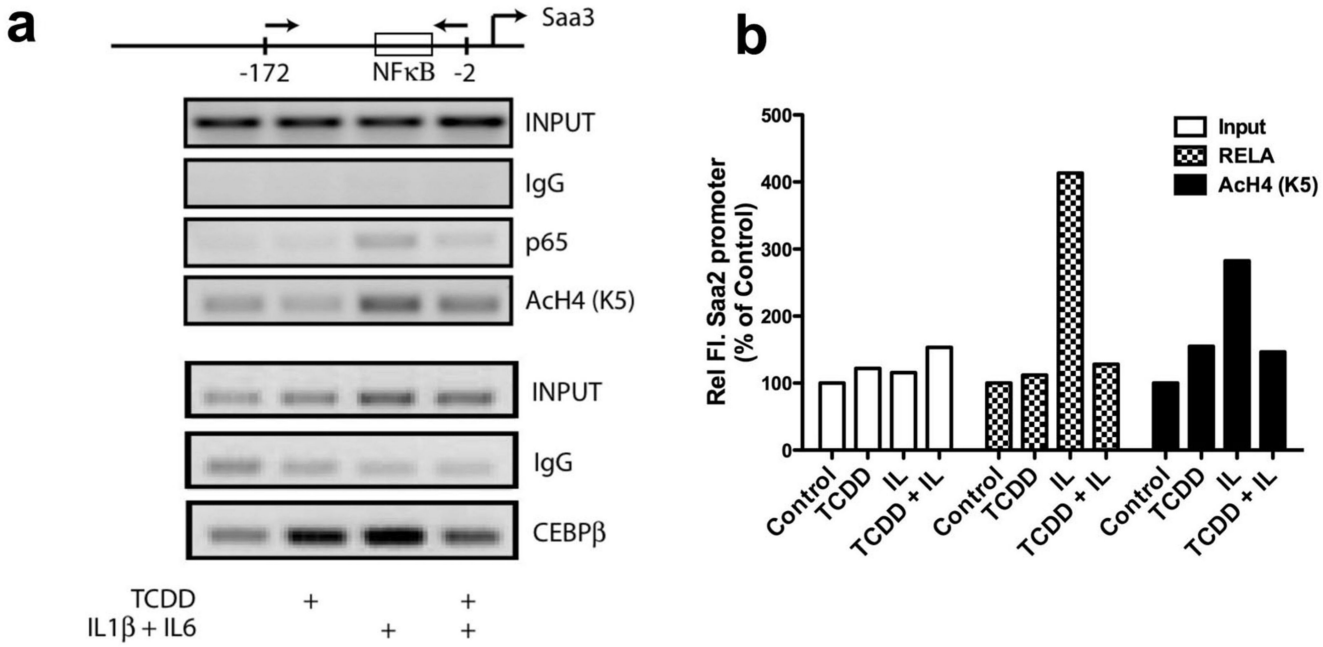

Figure 4.

AHR directly represses *Saa* transcription. (**a** and **b**) ChIP assay to determine the effect of AHR activation on *Saa2* and *Saa3* promoters. Hepa1c1c7 cells were treated with TCDD (10 nM for 30 min) prior to interleukin treatment (2 ng/ml each of IL1B and IL6 for 20 min). Immunoprecipitation was performed with antibodies for RELA and acetylated histones (K5). Changes at the *Saa3* promoter were assessed by PCR (**a**), while changes at *Saa2* promoter was analyzed by real-time PCR (**b**). Data represents one of three independent experiments.

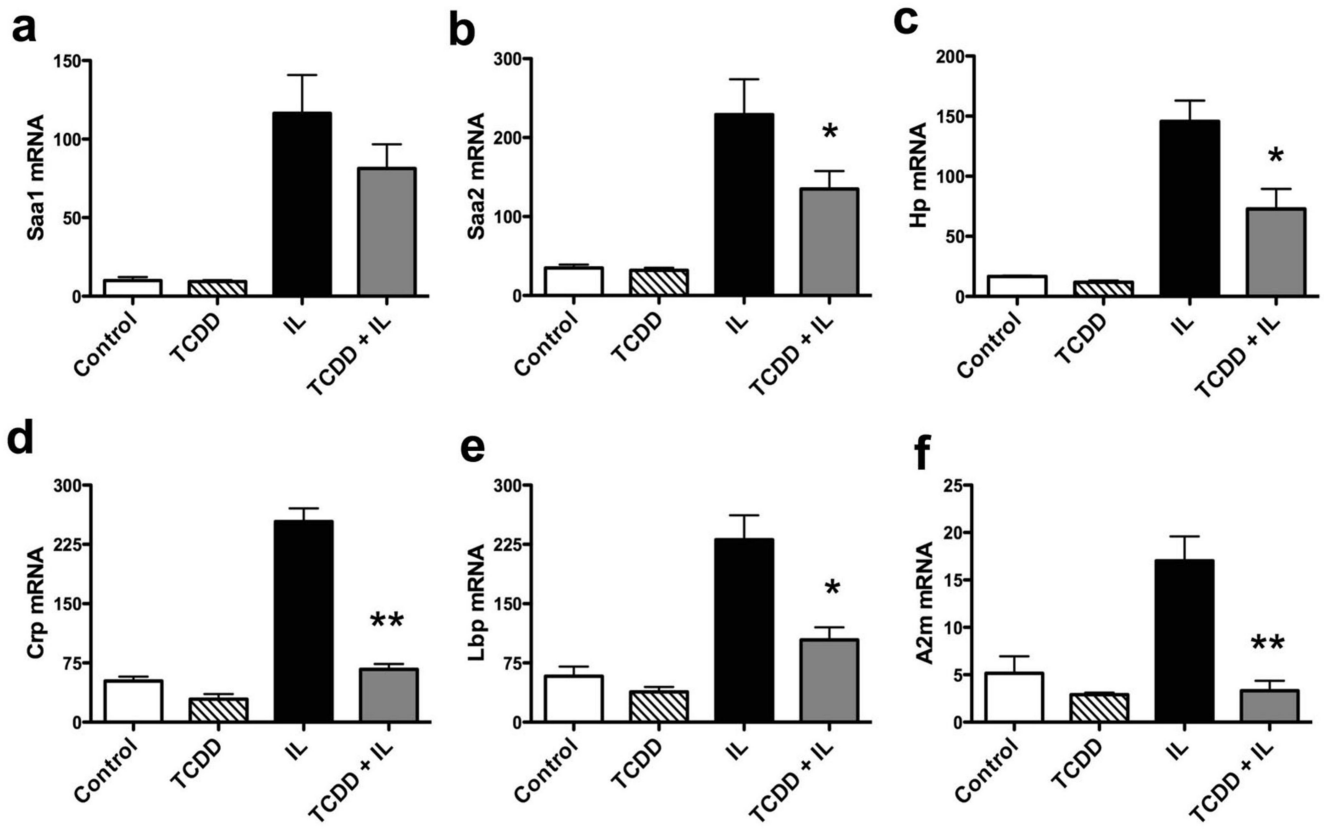

Figure 5.

AHR activation represses a battery of APR genes. Real-time PCR on RNA from primary mouse hepatocytes treated with TCDD (10 nM, 30 min) followed by interleukin (2 ng/ml each of IL1B and IL6) for 24 h. Repression of various APR genes was assayed. CRP, C-reactive protein; LBP, LPS-binding protein; A2m, alpha-2-macroglobulin; Hp, haptoglobin. Data represent mean induction ± SEM (n = 3/treatment group) and were analyzed to determine significance (*P < 0.05; **P < 0.01).

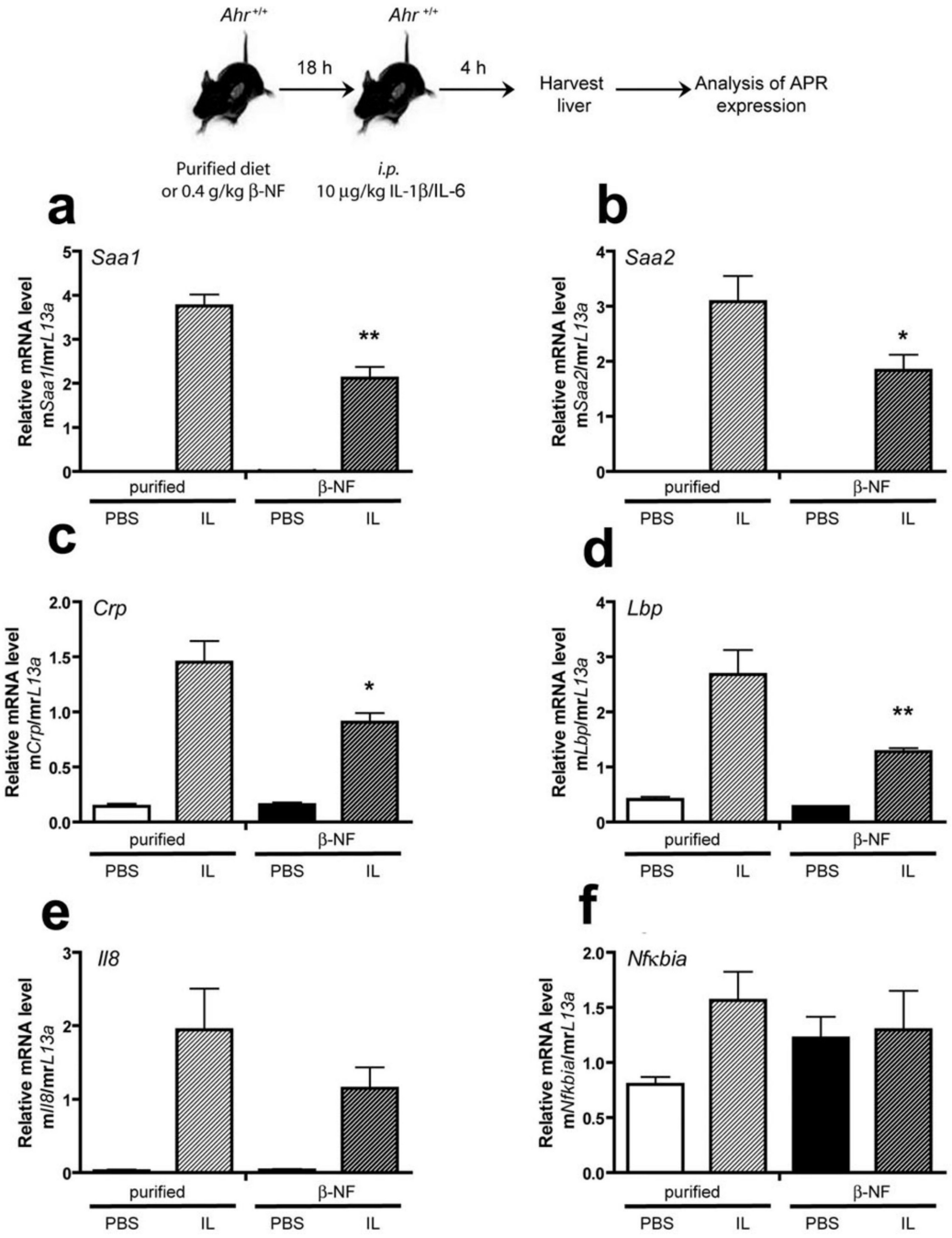

Figure 6.

β-NF attenuates cytokine-mediated APR expression *in vivo*. Female C57B6/J mice were given *ad libitum* access to purified chow ± 0.4 g/kg β-NF for 18 h. After 18 h mice were given *i.p.* injection of vehicle (PBS) or 10 mg/kg murine IL1B/IL6 as indicated. 4 h post-*i.p.* injection mice were sacrificed and hepatic tissue harvested for RNA isolation and subsequent quantitative PCR analysis of APR and pro-inflammatory genes *Saa1*, *Saa2*, *Crp*, *Lbp*, *Il-8* and *Ikb* (*Nfkbia*) (**a–f**). Data represent mean induction ± SEM (*n* = 3/treatment

group) and were analyzed by one-way ANOVA to determine significance (*$P < 0.05$; **$P < 0.01$).

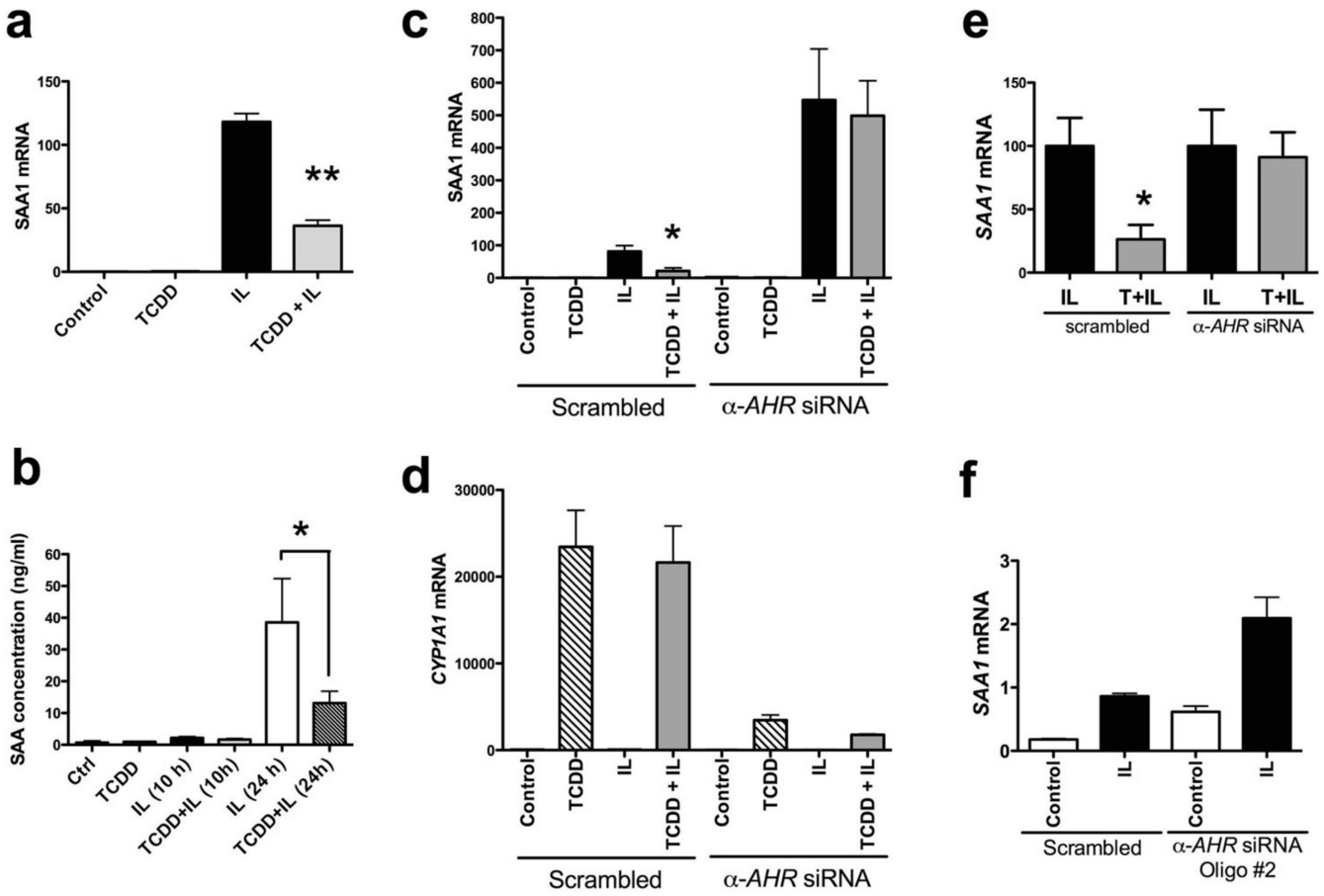

Figure 7.

AHR-mediated repression of SAA in human cells. (**a**) Real-time on RNA from Huh7 cells treated with TCDD (10 nM, 30 min) followed by human interleukins (2 ng/ml each of IL1B and IL6) for 6 h. Human *SAA1* mRNA abundance was assayed. (**b**) ELISA to quantify SAA protein secreted by Huh7 cells, treated for 10 h or 24 h with TCDD and interleukins. Just prior to treatment, cells were transferred to serum-free medium. (**c** and **d**) siRNA-driven AHR knock-down in Huh7 cells. 36 h after siRNA transfection, cells were treated with TCDD and interleukins, as in (A). *SAA1* mRNA (**c**) and *CYP1A1* (**d**) mRNA levels were determined by real-time PCR. (**e**) An alternate representation of data from (**c**). *SAA1* induction, upon interleukin exposure of scrambled and anti-AHR siRNA transfected cells, was scaled to 100 units. (**f**) Real-time PCR to assess *SAA1* mRNA induction in Huh7 cells electroporated with a different anti-AHR siRNA oligo (Dharmafect). Data represent mean induction ± SEM (n = 3/treatment group) and were analyzed to determine significance (*P < 0.05; **P < 0.01).

Table 1

Genes regulated by transiently expressed A78D-AHR or WT-AHR in immortalized AHR-null murine hepatocytes compared to control transfected cells.

Gene Title	Gene Symbol	Ratio A78D vs Control	Ratio WT vs Control
aryl-hydrocarbon receptor	Ahr	2.6	2.6
trimethyllysine hydroxylase, epsilon	Tmlhe	2.1	2.7
LIM domain containing preferred translocation partner in lipoma	Lpp	1.7	2.2
acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	Anp32a	1.7	1.8
denticleless homolog (Drosophila)	Dtl	1.6	1.6
DNA segment, Chr 9, ERATO Doi 306, expressed	D9Ertd306e	1.5	1.6
PREDICTED: LIM domain only 7 [Mus musculus], mRNA sequence	Lmo7	1.5	1.7
germ cell-less homolog (Drosophila)	Gcl	1.4	1.6
zinc finger protein 207	Zfp207	1.4	1.6
WD repeat domain 26	Wdr26	1.4	1.6
protein tyrosine phosphatase, receptor type, J	Ptprj	1.4	1.7
RIKEN cDNA 3300001H21 gene	3300001H21R ik	0.7	0.6
zinc finger protein 179	Zfp179	0.7	0.6
Ceruloplasmin	***Cp***	***0.7***	***0.5***
procollagen, type VI, alpha 1	Col6a1	0.7	0.6
matrilin 2	Matn2	0.7	0.6
interferon, alpha-inducible protein 27	Ifi27	0.7	0.5
vanin 3	Vnn3	0.7	0.6
Transferring	***Trf***	***0.7***	***0.6***
procollagen, type VI, alpha 2	Col6a2	0.7	0.6
procollagen, type VI, alpha 2	Col6a2	0.7	0.6
myxovirus (influenza virus) resistance 1	Mx1	0.7	0.6
complement component factor h	***Cfh***	***0.7***	***0.6***
complement component 1, s subcomponent	***C1s***	***0.7***	***0.5***
slit homolog 3 (Drosophila)	Slit3	0.7	0.6
Kruppel-like factor 10	Klf10	0.7	0.6
lipocalin 2	Lcn2	0.6	0.6
lipopolysaccharide binding protein	***Lbp***	***0.6***	***0.5***
serine (or cysteine) peptidase inhibitor, clade A, member 3M	Serpina3m	0.6	0.6
proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	Prg4	0.6	0.5
complement component 3	***C3***	***0.6***	***0.5***
FBJ osteosarcoma oncogene	Fos	0.6	0.4
serine (or cysteine) peptidase inhibitor, clade A, member 3N	Serpina3n	0.5	0.5

Gene Title	Gene Symbol	Ratio A78D vs Control	Ratio WT vs Control
STEAP family member 4	Steap4	0.5	0.4
serine (or cysteine) peptidase inhibitor, clade A, member 3G	Serpina3g	0.5	0.6
serum amyloid A 3	***Saa3***	***0.4***	***0.4***