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Review

Ah receptor and NF-κB interplay on the stage of epigenome

Yanan Tian *

Interdisciplinary Graduate Program of Toxicology, Department of Veterinary Physiology and Pharmacology, Mail Stop 4466, Texas A&M University, College Station, TX 77843-4466, United States

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family. Its ligands include many natural and synthetic compounds, some of which, such as polyhalogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons, are important environmental contaminants. NF-κB is a pleiotropic factor that regulates many physiological and pathophysiological processes including the immune and inflammatory responses. In the past decade, accumulating evidence suggests close interactions between AhR and NF-κB pathways, and these interactions are potentially important mechanisms for many pathological processes such as the chemical-induced immune dysfunctions, carcinogenesis and alteration of xenobiotic metabolism and disposition. AhR–NF-κB interaction has become a mechanistic linchpin linking certain pathological responses induced by environmental insults. Furthermore, the AhR–NF-κB interaction provides basis for therapeutic applications of certain AhR ligands to treat human diseases. The effects of AhR–NF-κB on the epigenome are an important area that is not well understood. In this review, I highlight current research regarding the AhR–NF-κB(RelA) interactions with emphasis on the epigenetic impacts of these interactions on chromatin modifications and transcription elongation control.

Contents

* Tel.: +1 979 458 3599; fax: +1 979 862 4929.
E-mail address: ytian@cvm.tamu.edu.

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1. Introduction

Gene and environment interactions are vital forces that constantly shape the living organisms, direct their evolutionary paths, and ultimately determine their evolutionary fates. Evolutionary pressure necessitates the development and preservation of defense mechanisms to ward off insults from xenobiotics and pathogenic microbes. In dealing with harmful xenobiotics and endobiotics, mammals have evolved a defensive network governed by the xenobiotic receptors/xeno-senors, including aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR) and constitutive androstane receptor (CAR). When encountering infectious agents, organisms have enlisted the innate and adaptive immune defensive systems, in which the nuclear factor kappa B (NF-κB) plays an essential regulatory role. This Darwinian evolution-inspired view of the roles of AhR and NF-κB places AhR, NF-κB and their interactions in the context of gene–environment interaction. However, the AhR and NF-κB are now known to play important roles in diverse physiological/pathophysiological processes beyond meeting the challenges of toxicant and microbial insults (as shown in various topics discussed in this special issue). Within individual organism, a delicate balance exists between AhR and NF-κB regulated pathways and this balance may be adversely affected by environmental and genetic factors leading to diseases. On the other hand both AhR and NF-κB pathways can be modulated therapeutically and AhR–NF-κB interactions are being explored for therapeutic potentials in treating human diseases [1–3].

The evolutionarily conserved AhR and NF-κB are inducible transcription factors, each governing the expression of distinct sets of genes that are important for normal physiology as well as pathophysiological responses. The Ah receptor plays a pivotal role in mediating detoxification of xenobiotics as well as toxic responses induced by dioxin (or 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) and related compounds [4–6]. NF-κB is a key transcription factor in regulating the immune system and inflammatory responses, combating infections, and in regulating the response to cellular stresses such as hypoxia and oxidative stress (reviewed in Refs. [7–9]). In 1999, we reported that AhR and NF-κB physically interact and functionally modulate each other's activities [10]. An overarching hypothesis driving our research on AhR–NF-κB interactions is that immune/inflammatory pathways and detoxification pathways are interconnected, evolutionarily conserved biological and chemical defenses. These pathway "cross-talks" are important mechanisms for pathological processes such as the chemical-induced immune dysfunctions, carcinogenesis and alteration of xenobiotic metabolism and disposition. Many other laboratories have also investigated the interactions between AhR-regulated pathways and NF-κB-regulated pathways with characterization of the interactions in different tissues/cell types and different components of the NF-κB-regulated signaling network including RelA [10], RelB [11], c-Rel [12] and p50 [13].

Completion of the human genome project ushers biomedical research into a new era. Investigators are now seeking a comprehensive view of the epigenetic changes that determine how genetic information is stored, retrieved and made manifest across tissue/cell types, various developmental stages and disease states. The term epigenetics here refers to heritable changes in gene expression caused by environmental factors, not by changes in the underlying DNA sequence and epigenome refers to the total epigenetic state of a cell. We have just begun to understand the "nuts and bolts" of the epigenetic machinery regarding AhR-regulated gene expression. There is no doubt this is a critical area of research for understanding the mechanisms of TCDD-induced toxicity. In this review, my discussion will be focused on AhR-regulated histone modification and transcription elongation. Other aspects of epigenetic regulations of gene expression including non-protein-coding RNAs and DNA methylation are not discussed in this review.

1.1. *AhR and AhR-regulated gene expression*

The AhR was defined biochemically in the 1970s [14] and the gene was first cloned from mouse liver in the early 1990s [15,16]. Analysis of the AhR gene revealed that it consists of domains shared by Drosophila transcription factors Per, Sim and AhR nuclear translocator (ARNT) [5,17]. This group of transcription factors, now known as the PAS proteins, represents a large family of transcription factors that play important roles in regulation of various physiological functions including circadian rhythnm, responses to low oxygen tension (hypoxia), and detoxification of xenobiotics [17]. The AhR is the only member of the family that is known to be activated by xenobiotics, including polyhalogenated aromatic hydrocarbons (e.g. TCDD and polychlorinated biphenyls or PCBs) and polycyclic aromatic hydrocarbons (PAHs). Importantly, many natural compounds, synthetic drugs and endogenous metabolites are ligands for the AhR, which have therapeutic potentials for use in treating diseases through pathway "cross-talk" [18].

Unliganded AhR is normally located in the cytoplasm in association with heat shock protein 90 [19,20]. Upon ligand binding, the AhR translocates into the nucleus and forms a heterodimer with ARNT and binds to the xenobiotic response element (XRE) which consists of conserved core sequences 5′-(A/T)NGCGTG-3′ [21,22]. However, recent studies indicated that AhR nuclear translocation can be induced by cAMP [23]. Thus, endogenous signals as well as AhR ligands can both cause AhR nuclear translocation which is a critical step leading to the activation of AhR-regulated gene expressions.

The XRE sequences are found in the regulatory regions of genes involved in the metabolism of xenobiotics, including *cyp1a1*, *1a2*, *1b1*, glutathione-S-transferase Ya and NAD(P)H-quinone oxidoreductase. Analysis of the regulation of these genes, especially *cyp1a1*, has provided a basis for understanding the mode of action of TCDD and related compounds [24,25]. Moreover, AhR knockout studies showed that this gene is involved in a wide range of physiological functions in addition to induction of detoxification genes [4].

1.2. *NF-κB classic and alternative pathways*

The core components of NF-κB pathway consist of membrane receptors, the receptor-associated adaptors, IκB kinases (IKKs), inhibitory kappa Bs (IκBs) and NF-κB dimers (such as p65/p50), which bind to DNA sequences (κB sites) to regulate gene expression [7].

In mammals there are five related NF-κB proteins which form homo/heterodimers with each other: RelA (also known as p65), RelB, c-Rel, p50 and p52. The NF-κB dimeric complexes regulate gene expression through binding to a variety of related target DNA sequences known as the κB sites found in the regulatory regions of targets genes. RelA, RelB and c-Rel contains transcriptional activation domains and therefore are transcriptionally active. The p50 and p52 proteins form a transcriptionally active heterodimer with RelA, RelB or c-Rel. However, p50 and p52 themselves lack a transcriptional activation domain and p50/50 and p52/52 homodimers are transcripionally inactive, and may, in fact, inhibit transcriptional activity of active NF-κB. The p65/p50 heterodimer was the first NF-κB dimer identified as a nuclear transcription factor that binds to enhancers controlling the gene expression of immunoglobulin (Ig) κ light chains in B cells [26]. Therefore, the term NF-κB usually refers to this classic p65/p50 heterodimeric transcriptional complex, which is fundamentally important for innate immunity and inflammatory responses. RelB/p52 dimer has recently been shown to regulate ''the alterative pathway'' which is required for the generation of secondary lymphoid organs, and for B-cell maturation and survival [27,28].

The transcriptional activity and cellular compartmentalization of NF-κB proteins are primarily regulated through interaction with inhibitory proteins referred to as the IκBs. Inactive NF-κB is localized in the cytoplasm in association with the IκBs. The mammalian IκB family consists of IκBα, IκBβ, IκBγ, IκBε, Bcl-3 as well as the precursors of NF-κB1 (p105) and NF-κB2 (p100). IκB proteins possess ankyrin repeats which mediate binding to the Rel homology domain, masking the nuclear translocation/DNA binding sequences. Under physiological conditions, IκB proteins associate with Rel proteins and with IκB kinases subunit, forming a high molecular weight, multi-protein complex. NF-κB activation by a variety of cytokines, growth factors, immune activation stimuli, or cellular stresses results in the phosphorylation of IκBs by IκB kinases. The best studied system is phosphorylation of IκBα by IKKβ at serines 32 and 36 of the IκBα protein and this results in rapid degradation by the ubiquitin-proteasome system, leading to nuclear translocation of the RelA p65/p50 dimer, thus activating gene expression. This pathway of NF-κB activation is known as the canonical NF-κB pathway. In the non-canonical (or alternative) pathway, cytoplasmic RelB is in association with p100 (an IκB protein) and stimulus-activated IKKα phosphorylates p100 which is then cleaved to give rise to p52. The RelB/p52 dimer then translocates into the nucleus to function as the DNA binding transcription factor. The classic RelA/p50 and alternative RelB/p52 NF-κB proteins regulate overlapping and distinct sets of genes [28].

1.3. *Significance of AhR and NF-κB interactions*

Investigation of interactions between AhR and various components of NF-κB pathways are important because it reveals novel mechanisms for the physiological and pathophysiological processes that may be co-regulated by the two pathways. For example, NF-κB–AhR interaction is an important mechanism for suppression of AhR activity by proinflammatory agents through the action of NF-κB both as a sensor for various inflammatory and stress stimuli and as an effector for repressing transcription of important xenobiotic metabolizing enzymes (reviewed in [29–31]).

Since NF-κB was found to modulate AhR signaling, it has become apparent that manipulation of NF-κB activity by immune stimuli may modulate TCDD-induced toxicity. For example, it has been shown that through induced-inflammatory responses by inoculation of mice with the tuberculosis vaccine BCG, which activates NF-κB as a key step in its immune stimulatory action [32,33], the animals were protected against TCDD-induced cleft palate [34,35]. This would be a logical outcome following our reasoning i.e. NF-κB activation suppresses AhR-mediated pathways, thereby reducing the teratogenic effects of TCDD. Moreover, following the same reasoning, simultaneous suppression of NF-κB (e.g. by potent immune suppressor hydrocortisone) and activation of AhR by TCDD would result in synergistic induction of cleft palate, and synergism of this birth defect was observed in mice co-treated with TCDD and hydrocortisone [36,37]. These studies suggest that AhR and NF-κB interact and function to check and balance each other and disruption of this balance may lead to diseases. For example, a recent study by Thatcher et al. [38] showed that AhR-null mice develop heightened inflammatory responses to cigarette smoke and endotoxin associated with rapid loss of the NF-κB RelB suggesting an important role of AhR–NF-κB interactions for homeostasis of lung function. Interactions between AhR and RelB are discussed in detail in this special issue by other contributors.

Genotoxicity induced by polycyclic aromatic hydrocarbons (PAHs), such as BaP, is mediated by the AhR; however, recent evidence indicates that AhR-null mice and cyp1 null mice exhibit extensive PAH-induced DNA damage, particularly in the hematopoietic system, suggesting roles of AhR and AhR-regulated genes in protecting mammals from xenobiotics-induced mutagenic damages [39,40]. Based on these observations and accumulating evidence indicating that NF-κB and NF-κB-mediated inflammatory activities are involved in carcinogenesis [41], it is possible that NF-κB-mediated inflammatory responses play a role in carcinogenesis through suppressing AhR-regulated detoxification. In addition, since the AhR plays an important role in cell proliferation and cell cycle control [42–45], its AhR–NF-κB interactions will necessarily impact on cell proliferation and may be involved in tumor

promotion. However, hitherto most studies have focused on the oncogenic roles of various components of NF-κB signaling pathways. Here, I hypothesize that the roles of NF-κB activation in carcinogenesis and tumor promotion need to be investigated in the context of the ''two stage carcinogenesis'' model where AhR–NF-κB interactions may play a role in both carcinogenic initiation and tumor promotion.

2. AhR–NF-κB interplays in inflammation and detoxification pathways

NF-κB is a pleiotropic transcription factor that participates in many physiological responses that are affected by TCDD and related compounds, suggesting cross-talk between these two pathways. We and others have analyzed the interactions between the critical components of AhR–NF-κB signaling pathways. Using co-immunoprecipation experiments, we demonstrated that AhR associated with the RelA(p65) subunit of NF-κB. There are reciprocal inhibitory effects between these two pathways. Therefore, it appears that the classic (canonical) NF-κB pathway interacts with AhR through direct association of p65 and Ah receptor (ARNT is not directly associated with p65) [10]. The effects of TCDD on immune/inflammatory pathways have been reported earlier. Olnes et al. [46,47] showed that TCDD treatment of rat thymocytes suppressed prostaglandin G/H synthase (PGHS), which is regulated by NF-κB [48] and a transient induction (2 h) of NF-κB/DNA binding activity was detected by electrophoretic mobility shift assay (EMSA) [46]. Sulentic et al. reported TCDD suppresses IgM expression [49] which is potentially regulated by NF-κB–AhR interactions in the enhancer region of the IgM gene [12]. Recent studies by Hollingshead et al. [50] showed that in MCF-7 cells IL-1β and PMA treatments suppress the TCDD-induced *cyp1a1* gene expression. However, TCCD synergized IL-1β-induced IL-6 gene expression and AhR and RelA are both required for the synergism [50]. In an earlier study Kim et al. [51] observed that AhR and NF-κB are both highly elevated and constitutively active in breast cancer cells. Furthermore, these investigators demonstrated physical and functional association between RelA and AhR, resulting in the activation of *c-myc* gene expression in breast cancer cells. These investigators proposed a mechanism in that AhR in cooperation with NF-κB activates *c-myc* and resulting in increased proliferation and tumorigenesis of mammary cells [51]. There are differences in the published literature regarding the consequences of AhR–NF-κB interactions and subsequent downstream effects. For example, AhR activation in some cases resulted in activation of NF-κB, while others reported suppression of NF-κB activity. These differences may be due to cell type/tissue context [52] and differences in RelA and RelB in regulating the canonical or non-canonical NF-κB pathways [11,53]. In addition, the different types of AhR ligands used in the studies may also cause different responses. The prototypic AhR agonist is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which is not metabolized and TCDD exerts its action at very low concentration typically in the nano- or picomolar range, and its activity is strictly AhR-dependent. Whereas, other AhR agonists, such as benzo-[a]-pyrene (BaP), are metabolized (in some cases made more toxic) and also directly act on NF-κB and other signaling pathways in some cases activating and other cases suppressing NF-κB. These ligand differences may account for the observed differences in responses. For example, the AhR regulates the generation of Treg and TH17 cells in mice. AhR activation by TCDD induces Treg cells that suppress experimental autoimmune encephalomyelitis (EAE) by a TGF-β1-dependent mechanism, whereas AhR activation by 6-formylindolo[3,2-b]carbazole (FICZ) interferes with Treg cell differentiation, boosted TH17 cell differentiation and worsened EAE [54]. FICZ is metabolized by AhR-regulated enzymes [55]. NF-κB has been found to play an important role in EAE [56]. The effects of FICZ or its metabolites on NF-κB pathway however, have not yet been investigated.

3. AhR–NF-κB interactions impact on epigenome through histone modifications

3.1. *Chromatin sets the stage for eukaryotic transcriptional regulation*

Completion of sequencing of human genome has provided foundation to begin to understand the organization of human genome in terms of primary DNA sequences. How these genetic alphabets are read and genetic blueprint is implemented rely on the epigenetic mechanism which we are only beginning to understand. In the eukaryotic nucleus, the DNA template is packaged into a nucleoprotein complex known as chromatin, which consists of a roughly 2:1 mass ratio of protein to DNA. The major proteins that associate with chromatin are the histone core octamer consisting of H2A, H2B, H3, H4 and the linker histone H1. The fundamental repeating unit of chromatin is the nucleosome, which consists of 147 bp of DNA wrapped around an octamer of histone molecules. The core histones are predominantly globular except for their N-terminal ''tails'', which are unstructured. The primary structure of the chromatin template has a ''bead-on-a-string'' appearance (Fig. 1). A striking feature of the histone N-terminal tails is the large number of amino acid residues that are potentially subjected to various kinds of covalent modifications. It has been proposed that higher-order chromatin is established by the covalent modifications of the histone tails, such as trimethylation of H3K9, and subsequent formation of chromosomal subdomains by non-histone modifying factors, such as heterochromatin proteins (HP), which further compact chromatin into higher chromosomal structures. It remains unclear how nucleosomal arrays containing linker histone H1 further twist and fold the chromatin fiber into a progressively more compacted filament leading to defined higher order chromosomal structures.

Fig. 1 – ''Beads-on-a-string'' model of nucleosome.

DNA templates that are packed into nucleosomal structures are inaccessible for transcription factors and therefore, transcriptionally ''silenced''. The nucleosome is a structural, and more importantly, a functional unit of chromatin with N-terminal domains of histones protruding outside the nuclear core complex for modifications. Histone modifications, notably, acetylation change histone-DNA association by neutralizing the charges of histone tails and facilitate ''unwrapping'' of the nucleosomal unit. Transcriptional co-activators, such as p300/CBP, p300/CBP associated factor (PCAF) and steroid receptor coactivators (SRCs), harbor histone acetyltransferase (HAT) activities and often collectively contribute to histone acetylation. However, their recruitment to genes is a dynamic and orderly process. Whereas acetylation and phosphorylation appear as transient modifications, histone methylations, notably, methylation of lysine 9 of histone H3 (H3K9), may be a relatively stable modification that may be suited to the longer-term maintenance of epigenetic chromatin state [57].

The epigenetic marking system based on histone modifications potentially increases regulatory repertoires through controlling access to the genetic information stored in the linear arrangements of the DNA sequences. More importantly, epigenetics, imposed at the level of DNA-packaging proteins (histones) is a critical feature of a genome-wide mechanism of information storage and retrieval. Some investigators have proposed that the combinatorial modifications of histones give rise to a ''histone code'' which greatly extends the genetic information stored in the linear arrangements on the DNA sequences [58]. Investigation of these epigenetic modifications is rapidly becoming an important area of research in understanding the nature of gene regulation (reviewed in [57–60]).

4. Regulation of AhR transcriptional activity through chromatin modifications

4.1. *AhR-regulated transcription initiation*

Acetylation and deacetylation are the opposing forces in shaping the chromatin into accessible and inaccessible structures for transcription. To analyze the effects of AhR–NF-κB interactions on histone acetylation, in earlier studies, we performed chromatin immunoprecipitation (ChIP) assays and EMSA to dissect the steps of AhR binding to XREs and subsequent histone modification (acetylation) [61,62]; our results suggest that ligand-induced activation of *cyp1a1* expression is a series of interconnected, yet distinct events. The trans-activator (AhR) binding to the enhancer sequences is separable from the subsequent assembly of the preinitiation complex, which includes the recruitment of coactivators bearing HAT activities, chromatin remodeling complexes and transcription mediator complexes [63–65] as well as transcription elongation factor [62]. Interestingly, TCDD-induced binding of AhR to the consensus XRE sequences is not affected by TNF-α which is a classic NF-κB inducer as judged by either EMSA or ChIP assays. NF-κB activation by TNF-α however, strongly inhibits histone acetylation without affecting AhR binding to the XRE sequences in either EMSA or ChIP assays [61]. These observations indicate that binding by an activator to an enhancer sequence is necessary but not sufficient for transcriptional activation and furthermore, binding to the DNA sequences and recruitment of coregulators are distinct steps subject to regulations. This model heightens the awareness of the roles of chromatin modifications in *cyp1a1* regulation, pointing out the potential pitfall of using EMSA as the sole gauge for transcriptional activation.

4.2. *Suppression of AhR-regulated cyp1a1 gene expression by NF-κB*

Suppression of heptic cytochrome P450 following activation of immune responses by infectious or inflammatory stimuli has been known for more than 30 years [30]. The interaction between inflammatory cytokines and P450 transcriptional regulation is important for the potential drug–drug interaction and drug toxicity [30,66]. Inflammatory stimuli such as TNF-α, IL-1β and lipopolysaccharide (LPS) cause suppression of cytochrome P450 including P450 1A1 and 1A2 gene expressions. In addition, H_2O_2 treatment [67], and hypoxic conditions also suppress *cyp1a1* expression [68]. Although physiological responses induced by these stimuli may vary, one common characteristic of these stimuli is their induction of NF-κB. Based on the above observations we hypothesized and demonstrated that NF-κB plays an important role in suppressing AhR-regulated *cyp1a1* transcription. Using a super repressor IκBα in which serine 32 and 36 were mutated to alanine and is resistant to degradation and constitutively active as the NF-κB suppressor, we demonstrated that the suppressive effects of TNF-α and LPS on the *cyp1a1* expression are due, in part to direct NF-κB action [61]. Recent studies by Hollingshead et al. [50] also demonstrated specific NF-κB involvement in the IL-1β, and PMA imposed suppression of *cyp1a1* expression by small interfering RNA (siRNA) knockdown of the NF-κB RelA. Taken together, these studies [10, 50, 61] demonstrated that NF-κB (p65/p50) plays a pivotal role in mediating *cyp1a1* suppression by proinflammatory cytokines.

NF-κB can be activated by a large number of stimuli. Transcriptional activation by NF-κB is usually through its binding to the κB-enhancer sequences, and this binding can be analyzed by EMSA. However, in general, activator/enhancer binding in and of itself does not always result in transcriptional activation, as demonstrated by the suppressive effects of NF-κB on AhR-regulated *cyp1a1* promoter activity. Inhibition of transcriptional activity was seen without effects on the AhR binding to the XRE sequences [61].

The transcriptional activity of the AhR and NF-κB are modulated by the transcriptional co-regulators (coactivators and corepressors). Steroid receptor coactivator-1 (SRC-1), p300/CBP, receptor-interacting protein 140 (RIP140) [69] and GRIP1-associated coactivator 63 (GAC63)[70] are known transcriptional co-activators for the AhR. p300/CBP and the SRC family of coregulators are also required for NF-κB pathway transactivation. It is conceivable that there may be competition for coactivator binding, so that when one pathway is activated, the other will be repressed through competition for the co-regulator availability, i.e. ''squelching'' mechanism [71-73]. However, co-regulators such as p300/CBP are utilized by numerous transcription factors regulating a variety of pathways, the mechanistic detail is lacking for the apposing factors to directly compete for the commonly shared critical

co-regulators to achieve specific mutual repression between pathways.

4.3. *Interplays of histone acetylation and methylation controlled by AhR–NF-κB interactions*

The AhR/ARNT interact with histone modification cofactors such as p300/CBP and the protein arginine methyltransferases (PRMTs) such as PRMT1 and PRMT4(CARM1) (Fig. 2 and our unpublished results) and PRMT1, PRMT2 and PRMT4 were shown to be nuclear receptor coactivators [74–77]. These enzymes regulate gene expression through methylating histone and non-histone proteins, and the methylation marks are important for nuclear/steroid receptors-mediated transcriptional activity.

PRMT1 methylates arginine 3 of histone H4 (H4R3) and is a major methyltransferase in mammalian cells. Increasing evidence indicates that PRMT1 plays vital roles in physiological and pathophysiological processes including development, nuclear receptor regulated gene expressions and oncogenesis [77–80]. Interestingly, recent evidence suggests that histone modifications by PRMT1 set the stage for subsequent histone modifications [81] and there is an intricate interplay between PRMT1 and other histone modifications. For example, arginine methylation (H4R3) by PRMT1 facilitates H4 acetylation but H4 acetylation inhibits methylation of H4R3 [82]. These observations suggest a unidirectional, sequential process of histone modifications and to complete the transcription, methylated H4R3 has to be demethylated, followed by acetylation and then deacetylation by regulatory complexes containing histone deacetylases (HDACs) for histone to be methylated at H4R3 to begin the transcription cycle again (see Fig. 3 for illustration). What could be the advantage to have such an elaborate interplay between methylation and acetylation? One possibility is that it is a feedback mechanism where gene activation begins with PRMT1-mediated methylation and followed by acetylation, which in turn tunes down the activation by reducing (inhibition) H4R3 methylation.

To investigate the roles of histone acetylation and arginine methylation (PRMT1) in AhR-regulation gene expression, we performed ChIP assay to analyze the effects of the AhR agonist TCDD and effects of NF-κB activation by TNF-α on these histone modifications. Our studies revealed an interesting interplay between marks of histone H4 acetylation and arginine methylation at H4R3 in the binary ''SGRGK'' modification cassette (Figs. 2 and 3). The concept of the binary modification cassette is described in ref [83]. Our ChIP assay for the histone acetylation and methylation at ''SGRGK'' modification cassette showed that the AhR agonist TCDD treatment caused dramatic acetylation of H4K5 with reduction of H4R3 methylation marks, while TNF-α treatment caused the opposite. In a time course study, TCDD treatment induced a time-dependent increase of H4 acetylation with a concomitant decrease of H4R3 methylation (Fig. 2B). In transient transfection assays, expression of PRMT1 cDNA transfection caused significantly enhanced AhR-regulated luciferase reporter gene expression indicating PRMT1 is a co-activator for AhR (data not shown). Based on these results and the existing literature [81–83] we reason that the seemingly diametrically opposing

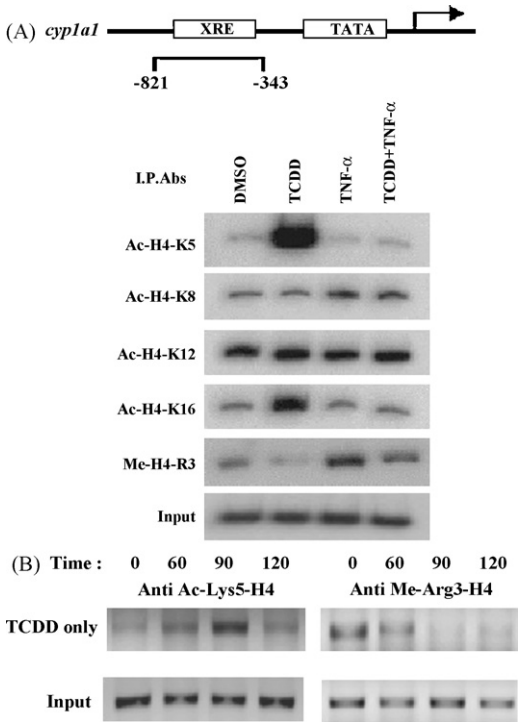

Fig. 2 – Histone modification at *cyp1a1* regulatory regions in response to AhR and NF-κB activation in Hepa1c1c7 cells. (A) Cells were dosed with TCDD (10 nM) only, TNF-α (5 ng/ml) only or both together for 2 h. The cells were then harvested for analysis by ChIP assay using indicated antibodies. B. Time course studies for AhR-activation associated acetylation of H4K5 and dimethylation of H4R3. Cells were treated with TCDD (10 nM) for the indicated durations. Acetylation and methylation of histone H4 were assayed by ChIP analysis with indicated antibodies.

interaction of AhR and TNF-α-induced H4 acetylation and H4R3 methylation determined by ChIP (Fig. 2) is a snapshot of the dynamic interplay between the positive and negative regulations by AhR and NF-κB interactions (Fig. 3). Based on positive and negative interaction between AhR and NF-κB in *cyp1a1* regulation, I propose a model of a ''transcription cycle'' marked by interplay between histone acetylation and arginine methylation which is described as follows: methylation of the H4R3 initiates the ground state for transcriptional activation. H4R3 methylation is also required for subsequent histone acetylation including H4K5 by coregulator complexes containing HAT activity, such as p300/CBP. The methylation mark is then removed to leave acetylation as the sole modification. This represents the ''active state'' and the gene transcription is permitted at the highest rate. The acetylation of histone H4 also serves as a negative feedback mechanism through inhibition of H4R3 methylation to prevent excessive activation. ''Turning off'' the transcription begins with removal of the acetylation mark from lysine 5 and possibly other histion lysines by HDACs containing corepressor complexes such as SMRT and NcoR. Histone H4 is then reset to the ''ready state'' with remethylation of the H4R3 by PRMT1. Based on this

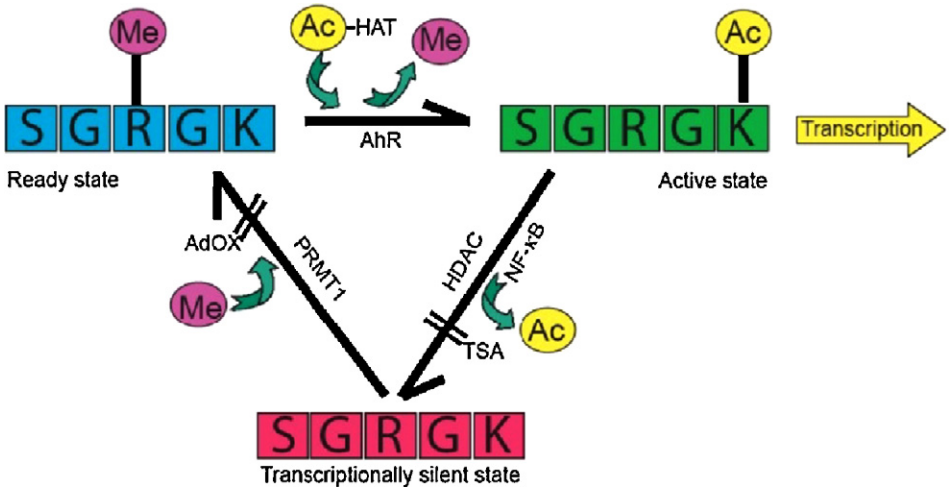

Fig. 3 – Schematic illustration of the epigenetic control and its impacts on *cyp1a1* transcriptional activity through histone modifications. Methylation on the H4R3 initiates the ground state for transcriptional activation. H4R3 methylation is required for subsequent acetylation of H4K5 by coregulator complexes containing HAT activity, such as p300/CBP. The metylation mark is then removed to leave acetylation as the sole modifications. This represents the "active state" and the transcription of the gene is permitted at highest rate. "Turning off" the transcription begins with the removal of the acetylation marks including lysine 5 by histone deacetylases containing corepressor complexes such as SMRT and NcoR. H4 is then reset to the ready state with remethylation of the H4R3 by PRMT1.

model, the "transcriptional cycle" proceeds in a unidirectional manner, since while methylation facilitates the acetylation, acetylation subsequently inhibits the H4R3 methylation [82]. This model accommodates the existence of the arginine demethylation processes (enzymes), which have been demonstrated. This model is also consistent with the observation that HDAC inhibitors such as TSA alter the epigenome in a way that sensitize most genes for activation. The model further predicts that breaking the transcription cycle by inhibition of demethylation should suppress gene expression.

5. AhR–NF-κB interaction converges on the *cyp1a1* transcriptional elongation

5.1. *Transcription elongation control by phosphorylation of RNA Pol II C-terminal domain (CTD)*

Research on transcriptional regulation in the past has been mainly focused on formation of the preinitiation complex at the proximal promoter region. It is now recognized that transcription elongation by RNA Pol II is a stage which consists of discrete biochemical steps that are potential targets for regulation [84]. After assembly of the preinitiation complex for RNA synthesis at the promoter, transcription elongation by RNA Pol II is subjected to negative regulation and Pol II CTD is hypophosphorylated, which only generates short RNA transcripts ("abortive transcription"), a phenomenon called "proximal promoter pausing". This phenomenon can be clearly determined on the AhR-regulated *cyp1a1* promoter [62]. Two factors holding back processive elongation have been identified: DSIF (DRB sensitivity-inducing factor) [85,86] and NELF (negative elongation factor (NELF), which cooperates with DSIF to repress Pol II transcription elongation. DRB (5,6-dichloro-1-D-ribofuranosylbenzimidazole) is a classic inhibitor of transcription elongation by RNA polymerase II. The repression imposed by DSIF and NELF on Pol II elongation is reversed by the positive transcription elongation factor b (P-TEFb)-dependent phosphorylation of the pol II C-terminal domain. Therefore, P-TEFb plays an important role in facilitating Pol II to transcribe DNA template in a processive transcription elongation (reviewed in Ref. [84]). Without P-TEFb, only short (20–50 bp) transcripts will be produced and RNA Pol II is said to engage in an "abortive transcription". P-TEFb is a cyclin dependent kinase complex that contains a "T type" of cyclin and a cyclin-dependent kinase 9 (CDK9). A typical P-TEFb complex contains two subunits, the cyclin T1 and CDK9. The CDK9 phosphorylates the C-terminal domain (CTD) of the large subunit of RNA Pol II [87] as well as NELF and DSIF [88].

Proximal promoter pausing is a distinct step of transcriptional elongation, which has been observed in both highly transcribed genes as well as genes of low transcription level. It may serve as a regulatory point to coordinate transcriptional elongation with pre-mRNA processing such as capping [88]. At the pausing point the RNA Pol II CTD undergoes step-wise phosphorylation by P-TEFb which facilitate the RNA Pol II to transcribe beyond the proximal promoter region (promoter escape).

RNA Pol II CTD has a unique feature in that it has multiple repeats of "YSPTSPS" amino acid sequence motif. In mammalian cells, this motif is repeated 52 times and in yeast cells the same motif is repeated about 25 times. The serine residues in the motif can be phosphorylated by the CDK9 subunit of P-TEFb which targets RNA Pol II CTD. Therefore, a hyperphosphorylated Pol II CTD (IIO form) is indicative that RNA Pol II is engaged in productive transcription elongation. A hypophosphorylated CTD (IIA form) indicates that RNA is not

committed to productive transcription elongation, even though the RNA Pol II may have engaged (loaded) on the promoter. The mechanism for elongation control by CTD phosphorylation is thought to be in part due to the changes of charges. RNA Pol II CTD in the preinitiation complex is hypophosphorylated, making contact with position 16 downstream from transcription start site [89]. Phosphorylation of the CTD disrupts the CTD–DNA interactions by introducing negative charges to the CTD which are repelled by the phosphate groups on the DNA helix. Thus, phosphorylation of the CTD during initiation can destabilize extensive downstream contact between the polymerase and DNA template.

5.2. *AhR regulates cyp1a1 transcriptional elongation*

In 1992, Morgan and Whitlock [90] reported that TCDD induced changes in the nucleosomal positions in both the promoter and transcribed regions of mouse *cyp1a1* gene with an interesting difference: the nucleosomal changes in the transcribed regions were sensitive to inhibition by actinomycin D, while the TCDD-induced nucleosomal changes in the promoter were insensitive to the same treatments. Actinomycin D binds to DNA and blocks the movement of RNA PII, thus inhibiting transcription. These results suggest that within a single *cyp1a1* gene, transcription is regulated by two interconnected but distinct mechanisms involving controls of initiation and elongation. In the last two decades, the transcriptional processes leading to initiation have been investigated extensively, but little is known about the process of transcription elongation of *cyp1a1*. In an earlier study, we reported that transcriptional elongation of *cyp1a1* is a highly regulated step with the AhR directly interacting with the pivotal transcriptional elongation factor P-TEFb and interestingly, TNF-α treatment inhibits CDK9 kinase activity suggesting a negative regulation of the transcriptional elongation of *cyp1a1* by NF-κB [62]. Using the ChIP assay, we found that TCDD treatment of Hepa1c1c7 cells induced binding of the AhR complex to the regulatory region of *cyp1a1* followed by promoter occupancy by RNA PII and recruitment of P-TEFb to the promoter region. The association of P-TEFb with the promoter was correlated with strong phosphorylation of serine 2 and serine 5 of the RNA PII CTD (see Fig. 4 for working model). Interestingly, TNF-α cotreatment with TCDD caused strong inhibition of serine 2 phosphorylation of RNA PII CTD without affecting the phosphorylation of serine 5 which is a substrate of CDK7 in the TFIIH complex.

These above results suggest that in addition to the transcription initiation, *cyp1a1* is regulated at phase of transcription elongation by both positive and negative signals such as TCDD and TNF-α, respectively. Accumulating evidence indicates that a negative signal-induced pause of RNA Pol II at the proximal promoter region may result in the transcription machinery poised for even higher magnitude of activation (potentiation) [91]. This phenomenon is somewhat reminiscent of the AhR–NF-κB interaction on the methylation and acetylation of histones where negative signal TNF-α treatment causes the deacetylation of histone H4 allowing methylation of H4R3 which in turn facilitates another round of transcription.

Taken together, investigation of AhR–NF-κB cross-talk at the epigenetic level allows us to gain mechanistic insights of

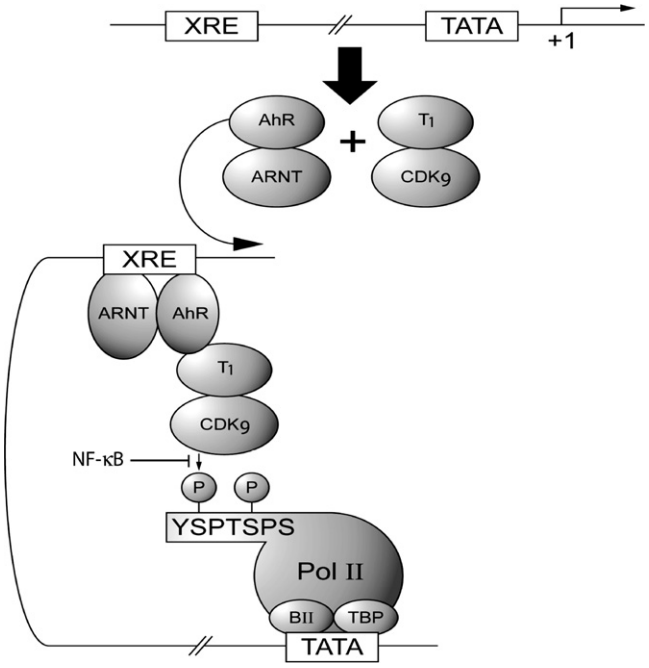

Fig. 4 – A working model for AhR-regulated transcription elongation. Ligand-activated AhR binds to XRE sequences, which leads to the assembly of the preinitiation complex at the *cyp1a1* promoter. Through its ability to associate with cyclin T1, the XRE-bound AhR also brings the P-TEFb complex to the vicinity of the *cyp1a1* promoter. The CDK9 subunit of P-TEFb phosphorylates the CTD of RNA PII resulting in processive elongation. NF-κB activation inhibits serine 2 phosphorylation.

how mammalian homeostasis is achieved by the balancing act of different signal transduction pathways. Because of the ubiquitous presence of the AhR and NF-κB in different tissue/cell types, and their pleiotropic functions in controlling various physiological responses, their physical and functional interactions create a critical link for the mechanistic understanding of various toxic responses induced by TCDD. It has been postulated a quarter century ago that alterations of gene expression play a key role in various toxic responses caused by TCDD [92]. Now in the genomic era, we are in position to investigate the molecular details of AhR–NF-κB interactions on the epigenome and through these efforts we will not only learn about how these toxic compounds cause harm, but also delineate pathways in which AhR–NF-κB interaction can be utilized to produce benefits for human health.

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