epithelial cells, cigarette smoke can induce ER stress (48). Cigarette smoke, therefore, might inhibit differentiation of preadipocytes via induction of ER stress. To examine this possibility, we first investigated whether cigarette smoke causes ER stress in 3T3-L1 preadipocytes. Expressions of GRP78 and CHOP are well-known endogenous markers for ER stress. Northern blot analysis revealed that treatment with CSE induced expression of GRP78 and CHOP within 3 h and peaked at 6 h (Fig. 5*A*), indicating that cigarette smoke triggered ER stress in 3T3-L1 preadipocytes. The induction of ER stress by cigarette smoke was further confirmed by the ES-TRAP assay (21). This assay is based on the fact that activity of the reporter enzyme ES-TRAP is markedly reduced in response to ER stress (21). 3T3-L1 cells were transiently transfected with an *ES-TRAP* gene under the control of a constitutively active viral promoter and treated with CSE for 3–24 h. The culture media and cells were subjected to chemiluminescent assay and formazan assay, respectively. Activity of ES-TRAP was normalized by the number of viable cells estimated by formazan assay. As shown in Fig. 5*B*, ES-TRAP activity was significantly inhibited by CSE at 9 and 24 h, confirming induction of ER stress by CSE.

To examine whether the induction of ER stress is causative of blockade of adipocyte differentiation by CSE, we tested

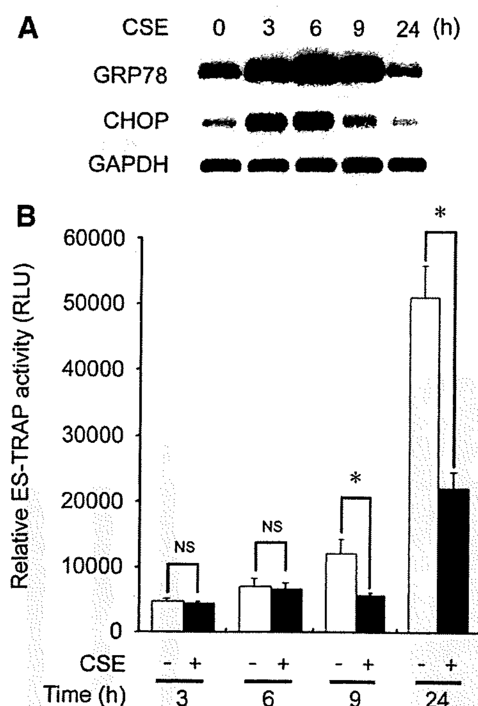

Fig. 5. Induction of endoplasmic reticulum (ER) stress by CSE. *A*: 3T3-L1 preadipocytes were exposed to 4% CSE for indicated time periods, and expression of 78-kDa glucose-regulated protein (GRP78) and C/EBP-homologous protein (CHOP) was examined by Northern blot analysis. *B*: 3T3-L1 preadipocytes transiently transfected with ER stress-responsive alkaline phosphatase (ES-TRAP) were treated with (+) or without (−) CSE for indicated time periods, and cells and culture media were subjected to formazan assay and chemiluminescent assay to evaluate ES-TRAP activity. The values of ES-TRAP activity were normalized by the number of viable cells estimated by formazan assay. Assays were performed in quadruplicate, and data are presented as means ± SE. *Statistically significant differences ($P < 0.05$).

effects of other ER stress inducers including thapsigargin (inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase), A23187 (calcium ionophore), and tunicamycin (inhibitor of protein glycosylation). Treatment of 3T3-L1 preadipocytes with these agents substantially induced ER stress evidenced by expression of GRP78 and CHOP (Fig. 6*A*). The induction of ER stress by tunicamycin was slower than that by thapsigargin and A23187, possibly because accumulation of hypoglycosylated proteins in the ER takes time, or different concentrations were used for individual agents. Using these agents, effects of ER stress on the differentiation of preadipocytes were investigated. 3T3-L1 cells were treated with IDI together with thapsigargin or A23187 for 24 h, or the cells were pretreated with tunicamycin for 6 h and treated with IDI together with tunicamycin for an additional 24 h. The cells were further cultured for an additional 5 days. Microscopic analysis revealed that all ER stress inducers blocked differentiation of 3T3-L1 cells (Fig. 6*B*, *left*). Consistent with this result, accumulation of lipids induced by IDI was also abrogated by any of the ER stress inducers (Fig. 6*B*, *right*). Furthermore, Northern blot analysis revealed that induction of adipocyte markers by IDI was suppressed by either thapsigargin, A23187, or tunicamycin and that suppression of the preadipocyte marker by IDI was reversed by the ER stress inducers (Fig. 6*C*). These results suggested that ER stress interferes with adipocyte differentiation and that CSE blocks adipogenesis, at least in part, via induction of ER stress.

To examine a possibility that activation of AhR is an event downstream or upstream of ER stress, 3T3-L1 preadipocytes were treated with AhR agonists for 6 or 24 h, and expression of ER stress markers was examined by Northern blot analysis. As shown in Fig. 7*A*, all of TCDD, B[*a*]P, and 3MC substantially induced expression of CYP1B1, the marker of AhR activation. However, induction of GRP78 and CHOP was not observed in the cells treated with these AhR agonists. Only B[*a*]P exclusively induced modest elevation of CHOP mRNA at 24 h, but it was not associated with induction of GRP78. We also examined an effect of ER stress on the activation of AhR. 3T3-L1 cells were treated with inducers of ER stress including thapsigargin, A23187, and tunicamycin for 9 or 24 h and subjected to analysis. The result showed that all ER stress inducers upregulated GRP78 and CHOP, whereas induction of CYP1B1 was not observed (Fig. 7*B*). These results suggested that activation of the AhR pathway and induction of ER stress by CSE are independent mechanisms underlying the anti-adipogenic effect of cigarette smoke.

DISCUSSION

Several previous reports suggested that cigarette smoking attenuates body weight gain. Appetite loss by smoking might explain this phenomenon, but direct effects of smoke on adipogenesis have not been reported. In the present investigation, we examined regulatory effects of cigarette smoke on adipogenesis. We found that cigarette smoke has the potential to inhibit differentiation of preadipocytes into adipocytes. Subsequent experiments revealed that cigarette smoke induces activation of AhR and ER stress, both of which play crucial roles in the suppression of adipogenesis by cigarette smoke. Interestingly, AhR agonists did not cause ER stress, and ER stress inducers did not activate AhR. These results disclosed

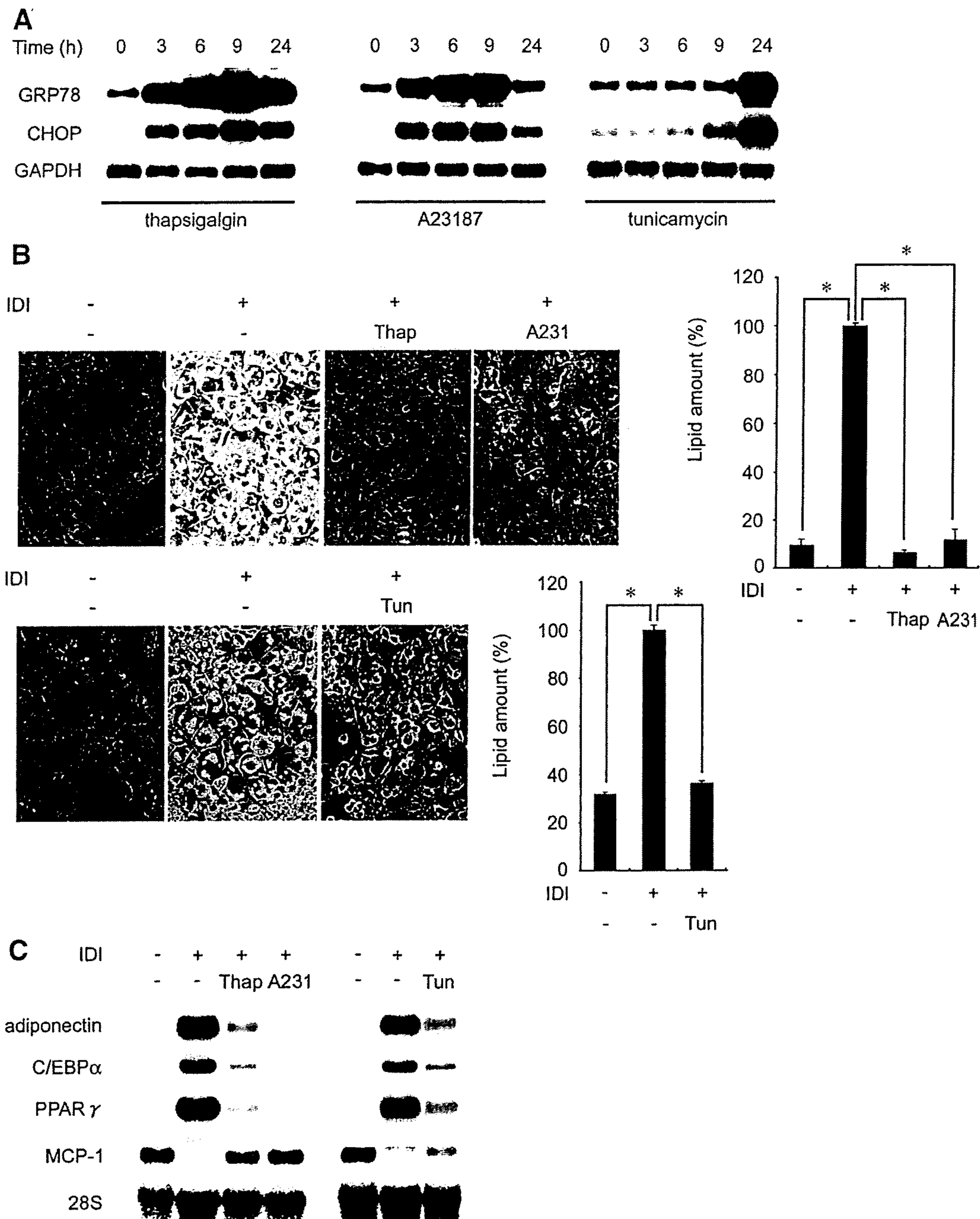

Fig. 6. Blockade of adipocyte differentiation by ER stress. *A*: induction of GRP78 and CHOP by ER stress inducers. 3T3-L1 preadipocytes were treated with thapsigargin (Thap; 20 nM), A23187 (1 μM), or tunicamycin (Tun; 300 ng/ml) for indicated time periods and subjected to Northern blot analysis. *B* and *C*: inhibition of adipocyte differentiation by ER stress inducers. 3T3-L1 preadipocytes were treated with IDI together with Thap or A23187 (A231) for 24 h, or the cells were pretreated with Tun for 6 h and treated with IDI together with Tun for an additional 24 h. After 5 days, the cells were subjected to phase-contrast microscopy (*B*, *left*), quantitative analysis of lipid content (*B*, *right*), and Northern blot analysis (*C*). *B*: assays were performed in quadruplicate, and data are presented as means ± SE. *Statistically significant differences ($P < 0.05$).

that cigarette smoke blocks adipocyte differentiation via dual, independent mechanisms, i.e., through activation of the AhR pathway and induction of the UPR.

Previous reports showed that dioxin inhibited adipocyte differentiation in several cell types, including 3T3-L1 cells, C3H10T1/2 cells, and primary mouse embryonic fibroblasts (MEFs) (3, 8, 40). However, molecular mechanisms underlying the negative regulation of adipogenesis by dioxin have not been fully elucidated. Shimba et al. (46) suggested possible involvement of ERK in the suppression of adipogenesis via AhR, because *1*) 3T3-L1 cells overexpressing AhR exhibited higher ERK activity and 2) treatment with ERK inhibitors

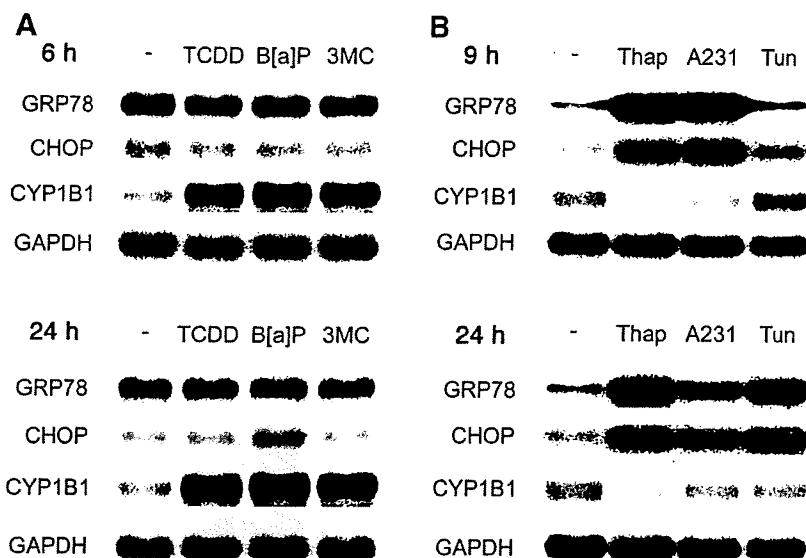

Fig. 7. Lack of linkage between activation of AhR and induction of ER stress triggered by cigarette smoke. 3T3-L1 cells were treated with agonists of AhR (TCDD, B[*a*]P, or 3MC; *A*) or inducers of ER stress (Thap, A23187, or Tun; *B*) for 6–24 h and subjected to Northern blot analysis of GRP78, CHOP, and CYP1B1.

abrogated the anti-adipogenic effect of AhR, possibly through inhibition of PPAR-γ, the crucial factor required for adipogenesis (17). Hanlon et al. (19) also demonstrated that low levels of ERK activation cooperated with AhR-induced factor(s) to generate a suppressor that prevents transcription of PPAR-γ. The similar mechanisms might be involved in the suppression of adipogenesis by cigarette smoke. However, in our experimental setting, we found that activation of ERK was observed in CSE-exposed 3T3-L1 cells, whereas selective inhibition of ERK by PD98059 did not reverse the anti-adipogenic effect of CSE (our unpublished data), excluding this possibility.

Currently, active entities responsible for the activation of AhR by cigarette smoke in preadipocytes have not been fully determined. Previous assessment using gas chromatography-mass spectrometric analysis showed that the levels of dioxins in cigarette smoke were very low. For example, Aoyama et al. (4) evaluated the amount of dioxins (PCDDs, PCDFs, and Co-PCBs) in mainstream and side stream smoke. They reported that the total amount of dioxins in cigarette smoke was ranging from 0.4 to 2.4 pg TEQ/cigarette. On the other hand, several reports showed that various polycyclic aromatic hydrocarbons, another major group of AhR agonists, were contained in cigarette smoke (15, 32) and may contribute to intense activation of AhR (24). These data indicate a possibility that main AhR activators in cigarette smoke are not dioxins but an array of polycyclic aromatic hydrocarbons.

We demonstrated in this study that cigarette smoke caused ER stress in preadipocytes. Currently, it is undetermined what kind of substances in cigarette smoke trigger ER stress. Cigarette smoke contains thousands of chemicals including nicotine, reactive oxygen/nitrogen species, and heavy metals such as cadmium (38). Crowley-Weber et al. (14) previously reported that nicotine had the weak potential to induce activation of the GRP78 and CHOP promoters in human hepatoma cells. Recently, we reported that various heavy metals, especially cadmium, had the potential to induce ER stress in vitro and in vivo (51). We also demonstrated that cadmium induced ER stress via generation of reactive oxygens, especially superoxide anion (52). Similarly, cigarette smoke induced ER stress in pulmonary epithelial cells, which was abrogated by the treatment with antioxidants (48). These findings indicate that nicotine, heavy metals, and reactive oxygen species may be responsible for the induction of ER stress in preadipocytes by cigarette smoke.

In the present report, we showed that ER stress inhibited differentiation of preadipocytes into adipocytes. Several underlying mechanisms may be postulated. First, in eukaryotic cells, secretory proteins enter the subcellular pathway through the ER where immature proteins are folded into native conformation and undergo a multitude of posttranslational modification. Several autocrine factors, including adiponectin, fibroblast growth factor, and midkine, have been identified as adipogenic proteins in preadipocytes (10, 18, 37). ER stress may affect secretion of these autocrine factors and thereby interfere with adipocyte differentiation. Second, a previous report showed that CHOP suppressed adipocyte differentiation by inhibiting function of C/EBP. That is, CHOP had an ability to dimerize with C/EBP, and CHOP-C/EBP heterodimers could not bind to the classical C/EBP binding site (5). As demonstrated in this report, CHOP was markedly induced by ER stress in preadipocytes. Induction of CHOP by cigarette smoke may be involved in the blockade of adipogenesis. Third, ER stress is known to induce activation of the apoptosis signal-regulating kinase-1 (ASK1)-JNK pathway (29). A previous report showed that overexpression of ASK1 suppressed conversion of some tumor cells to adipocytes (13). Another report also indicated that PPAR-γ had a domain phosphorylated by JNK and that phosphorylation of this domain attenuated the transacting potential of PPAR-γ (1). Activation of the ASK1-JNK pathway may mediate the anti-adipogenic effect of ER stress. However, our preliminary results showed *1*) phosphorylation of JNK was not induced by CSE and *2*) selective inhibition of JNK by SP600125 did not reverse the anti-adipogenic effect of CSE (our unpublished data), indicating lack of involvement of JNK in the anti-adipogenic effect of CSE. In the present investigation, we used IDI for the induction of adipogenesis. Previous reports evidenced that dysfunction of insulin receptor substrate-1 (IRS-1) suppressed adipocyte differentiation (43) and

that ER stress interfered with insulin signaling via inhibition of IRS-1 (36). As the fourth possibility, interference with the insulin signaling by ER stress may explain the anti-adipogenic effect of cigarette smoke.

The adipose tissue produces an array of adipocytokines essential for the maintenance of normal tissue structure and function. For example, lipoatrophy characterized by a paucity of the adipose tissue causes depletion of adipocytokines and consequent insulin resistance, diabetes, and cardiovascular diseases (17). From this point of view, suppression of appropriate adipogenesis by cigarette smoke may represent an additional mechanism whereby smoking can be injurious to human health.

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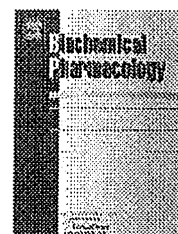

Review

AhR protein trafficking and function in the skin

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ABSTRACT

Because aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor, its nuclear translocation in response to ligands may be directly linked to transcriptional activation of target genes. We have investigated the biological significance of AhR from the perspective of its subcellular localization and revealed that AhR possesses a functional nuclear localization signal (NLS) as well as a nuclear export signal (NES) which controls the distribution of AhR between the cytoplasm and nucleus. The intracellular localization of AhR is regulated by phosphorylation of amino acid residues in the vicinity of the NLS and NES. In cell culture systems, cell density affects not only its intracellular distribution of AhR, but also its transactivation activity of the target genes such as transcriptional repressor Slug, which is important for the induction of epithelial–mesenchymal transitions. These effects of AhR observed in cultured cells are proposed to be reflected on the *in vivo* response such as morphogenesis and tumor formation.

This review summarizes recent work on the control mechanism of AhR localization and progress in understanding the physiological role of AhR in the skin. We propose that AhR is involved in normal skin formation during fetal development as well as in pathological states such as epidermal wound healing and skin carcinogenesis.

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1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor with basic-helix-loop-helix (bHLH)/PER-ARNT-SIM homology region (PAS) family and is constitutively expressed in various mammalian tissues including lung, liver, thymus, and kidney [1]. AhR is involved in skin carcinogenesis by benzo[a]pyrene [2], teratogenesis in cleft palate [3], and hepatotoxicity [4]. Moreover, recent studies suggest that AhR plays a role in physiological function including immunity [5,6] and reproduction [7,8].

When environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholanthrene bind to AhR, the ligand-activated AhR translocates to the nucleus where it binds to its heterodimerization partner AhR nuclear translocator (ARNT) [9]. The heterodimer AhR/ARNT binds to xenobiotic responsive elements, which are enhancer DNA elements located in the 5′-flanking region of the target genes [10]. While AhR activation by exogenous ligands is well investigated, very little is known about the physiological activation of AhR. Many studies using suspension cultures of various cell lines exist to show that AhR-mediated gene expression can be activated in the absence of exogenous ligands [11–13]. When adherent cells are suspended, intracellular signaling may be triggered by the loss of cell–cell contact or cell adhesion. This activation mechanism provides a model to investigate how AhR is activated under the normal physiological conditions.

Exposure to polycyclic aromatic hydrocarbons or topical application of these chemicals elicits inflammatory skin disease [14] as well as tumor formation [15]. This observation suggests that skin may provide clues to elucidate the biological function of AhR. The skin is a dynamic, regenerating organ. When skin is injured, various types of cells including leukocytes, fibroblasts and keratinocytes engage in tissue remodeling [16]. We attempted to study the role of AhR in the wound healing process. In benzo[a]pyrene skin carcinogenesis, it has been reported that AhR−/− mice do not develop tumors [2]. Since stem cells are considered to be the targets for carcinogens, it is very interesting to consider how AhR functions in stem cells. We are also going to discuss the possible roles of AhR on skin carcinogenesis.

2. AhR is a nucleo-cytoplasmic shuttling protein

AhR is a ligand-activated transcription factor and regulates biological responses to a variety of environmental contaminants. When exogenous ligands such as TCDD, benzo[a]pyrene, and 3-methylcholanthrene bind to AhR in cytoplasm, AhR translocates from the cytosol to the nucleus. It is important to investigate the localization of a transcription factor since change in its location is considered to impact gene regulation. Nuclear localization of a lot of nuclear proteins is determined by the nuclear localization signal (NLS) which is used for transport of these proteins to the nucleus through the nuclear pore complex [17,18]. This signal consists of a few short sequences of positively charged amino acid residues, whereas the nuclear export signal (NES) is a short leucine-rich sequence. We identified both the NLS 13–39 amino acid residues and NES 55–75 amino acid residues in the N-terminal region of AhR [19]. AhR shuttles between the cytoplasm and nucleus using these short peptide signals [20,21]. Subcellular localization of the shuttling protein is determined by the balance between nuclear import and nuclear export. It is reported that the localization is regulated by phosphorylation and dephosphorylation especially of amino acids close to the NLS or NES [22,23]. We found that the ligand-dependent nuclear import of AhR is inhibited by the substitution of aspartic acid for serine-12 or Ser-36, which mimics the negative charge conferred by phosphorylation [24]. It is likely that nuclear import of AhR is regulated by phosphorylation of NLS.

Distribution of AhR in a cell is controlled by its binding protein (Fig. 1). The unliganded AhR exists in cytosolic component as a complex [25], composed of AhR, a dimer of hsp90, p23, and the immunophilin homolog XAP2 [26,27]. XAP2 overexpression in cells is shown to enhance cytoplasmic AhR levels, suggesting that XAP2 is able to stabilize and enhance cellular levels of AhR [28,29]. Petrulis et al. [30] studied the mechanism of cytoplasmic retention of the AhR in the presence of XAP2. They showed that XAP2 hinders the binding of importin β to the AhR complex and proposed that XAP2 alters the conformation of the NLS. In addition, Berg and Pongratz [31] identified the other mechanism of XAP2-induced cytoplasmic localization of AhR. In particular, they showed that XAP2 anchors unliganded AhR to actin filaments since an actin inhibitor, cytochalasin B, blocked this effect.

Several reports demonstrate that the AhR is rapidly degraded via the proteasome pathway following exposure to ligands [32,33]. Davarinos and Pollenz [34] evaluated the function of the NES in the context of AhR degradation. They showed TCDD-induced degradation of the AhR was completely inhibited in the HepG2 cells pretreated with leptomycin B, an inhibitor of nuclear transport mediated by CRM1 (chromosomal maintenance factor 1) [35]. Furthermore, expressed AhR ΔNES protein was degraded to a lesser extent than wild-type AhR [34]. These data suggest that ligand-dependent degradation of AhR is mediated by nuclear export of AhR.

3. Cell density affects AhR localization and activity

We have shown that AhR is a nuclear-cytoplasmic shuttling protein. How are these transport mechanisms regulated under the physiological conditions ? We investigated stimuli that affect AhR localization using the human keratinocyte cell line HaCaT in the absence of exogenous ligand [36]. Because growth and differentiation of the cultured keratinocytes is regulated in part by cell density [37,38], effects of cell density on AhR localization were examined. When cells were sparsely inoculated, AhR was predominantly localized in the nucleus. When the cells were subconfluent, AhR was distributed equally both in cytoplasm and nucleus. However, when cells were fully confluent, immunostained AhR was localized predominantly in the cytoplasm. It is suggested that nuclear translocation of AhR is negatively regulated by phosphorylation of serine residues located in its NLS [24]. It is possible that

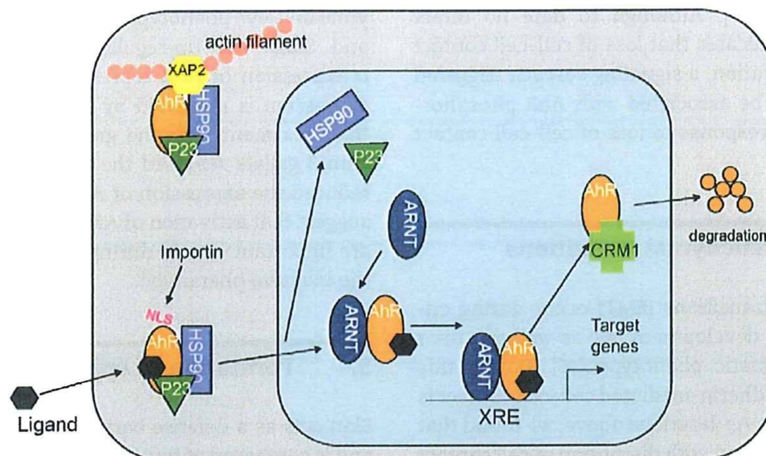

Fig. 1 – Regulation of AhR localization. In cytosol, AhR is complexed with hsp90, p23, and XAP2 (the last of which anchors the ligand-free receptor to the cytoskelton). Ligand binding results in AhR nuclear transport mediated by importins and is followed by dimerization with aryl hydrocarbon nuclear translocator (ARNT). The AhR/ARNT complex binds to xenobiotic responsive element (XRE), and induces transcription of target genes. AhR protein is exported by chromosome region maintenance 1 (CRM1), followed by degradation in cytosol.

these serine residues are phosphorylated to be anchored in cytoplasm under confluent culture. Using immunoblotting analysis, it was also shown that the relative amount of AhR in the nucleus was gradually decreased in proportion to the cell density. These observations led us to examine whether altered intracellular localization of AhR reflects AhR/ARNT-mediated transcription. Reporter analysis using luciferase cDNA connected to the XRE sequence, revealed that AhR activity was also affected by cell density. While the maximal luciferase activity was observed in subconfluent culture, the luciferase activity decreased to the basal level in confluent culture. These observations showed that subcellular localization and transcriptional activity of AhR were regulated by cell density.

Cell density dependent regulation system is reminiscent of contact inhibition. Cell growth is regulated by cell–cell contact in non-transformed cells. When cultured cells are maintained in low density, cells are actively growing. When cells grow to form confluent monolayer, they stop dividing. Critical anti-proliferative signals are mediated by cell–cell contact. It is very interesting to postulate that AhR is involved in regulation of contact inhibition. Recently, Weiss et al. indicated that TCDD treatment in WB-F344 rat liver cells leads to induction of JunD, resulting in up-regulation of cyclinA which triggers a release from contact inhibition via the AhR [39]. While exposure of confluent cells to TCDD-induced further proliferation, subconfluent cells did not respond to TCDD, suggesting that TCDD treatment specifically interferes with the signaling cascade of contact inhibition. It is suggested that this TCDD effect is an AhR-dependent and ARNT-independent reaction since suppression of AhR expression by siRNA abrogates the TCDD effect in sharp contrast with the suppressed expression of ARNT.

Previous reports showed that localization and transcription activity of AhR were altered in several cell lines when cell–cell contact was disrupted [11–13]. Recently, Cho et al. reported that suspension culture of C3H10T1/2 fibroblasts in methylcellulose-containing semisolid media resulted in activation of AhR-mediated transcription [40]. The AhR antagonist α-naphthoflavone blocked ligand-stimulated AhR activity, but did not affect the suspension-induced activation of AhR-mediated transcription, implying that the mechanism of the latter is different from that of former. They found that the activation of AhR by ligands can be clearly distinguished from the activation of AhR by the loss of cellular contact. We have shown that AhR is activated in the cells at the wounded edge in *in vitro* wound healing analysis using green fluorescent protein as a reporter of transcriptional activation by AhR/ARNT complex [36], suggesting that the loss of cell–cell contact leads to AhR activation. Owens et al. indicated the importance of Src family kinases in the disruption of cadherin-dependent cell–cell contact [41]. Src kinase is known to be associated with AhR complex [42]. Ligand binding to the AhR causes Src kinase to dissociate from the AhR complex and translocate from cytoplasm to the membrane [43], thereby increasing its own kinase activity which may be required for promoting destabilization of cell–cell contact. They showed that inhibition of the catalytic activity of the Src kinase stabilizes cadherin-dependent cell contacts, suggesting that Src kinase activity is required to disassemble cell–cell contacts. On the other hand, the Src kinase activity stimulates the epidermal growth factor receptor (EGFR) [44] which is known to play an important role in activation of MAPK pathways and other key signal transduction cascades. Activation of MAPK pathways promotes downstream signaling such as ERK and p38. Our results suggested that loss of cell–cell contact generates signals that increase the phosphorylation level of AhR (i.e., phosphorylation of Ser-68 which is located in the NES), thereby causing AhR to accumulate in the nucleus owing to inhibition of the

export activity. It is likely that activated p38 MAPK is involved in this phosphorylation [36]. Although to date no direct experimental evidence indicates that loss of cell–cell contact is the signal for AhR activation, a signaling cascade triggered by Src kinase appears to be associated with AhR phosphorylation and activation in response to loss of cell–cell contact (Fig. 2).

4. Epithelial–mesenchymal transitions

Epithelial–mesenchymal transitions (EMT) occur during critical phases of embryonic development [45] as well as tumor progression to the metastatic phenotype [46]. During this process, disruption of E-cadherin-mediated cell–cell contact is considered to be a key step. As described above, we found that the AhR activation is associated with disruption of cell contact in keratinocyte. This observation prompted us to examine whether AhR is involved in the regulation of EMT. In many types of cancer, the loss of E-cadherin expression is due to transcriptional repression [47,48]. Transcription factors including a family of zinc finger proteins of the Slug/Snail family are implicated in such repression [49–51]. We have shown that AhR participates directly as a transcription factor in the induction of Slug expression in the context of loss of cell–cell contact, which, in turn, regulates EMT [52]. The induced Slug was associated with reduced level of the epithelial marker, cytokeratin 18 and with increased level of the mesenchymal marker, fibronectin. Belguise et al. [53] investigated the control of EMT in breast cancer. Ectopic coexpression of CK2 and NFκB c-Rel in untransformed mammary epithelial cells was sufficient to induce a mesenchymal invasive phenotype, in association with induction of AhR and Slug. The up-regulation of Slug was abrogated by coexpression of AhR repressor (AhRR), indicating that Slug expression is regulated by AhR. Furthermore, they showed that treatment with the green tea polyphenol epigallocatechin-3 gallate reversed the malignant phenotype as well as reduced the expression of AhR and Slug. Thus, these results suggest that activation of AhR signaling and induction of Slug are important events during the process of progression into the invasive phenotype.

5. Formation of epidermal tissue

Skin acts as a defense barrier against environmental stimuli and is composed of two layers, the dermis and epidermis. The epidermis is divided into several layers and extends from the basement membrane to the outer surface. The basal layer contains the basal keratinocytes where mitosis occurs. As the keratinocytes mature, they form the spinous layer, granular layer, and stratum corneum which has the barrier function (Fig. 3A). Keratinocytes in the spinous and the granular layer produce differentiation-specific proteins including filaggrin and loricrin which are cross-linked by transglutaminase into cornified envelopes [54].

Fernandez-Salguero et al. [55] investigated skin lesions in AhR-null mice and found an association of structurally abnormal hair fibers, rupture of hair follicles and mixed inflammatory cells infiltrate that progressed to acute ulcers. It has been reported that TCDD affects differentiation of

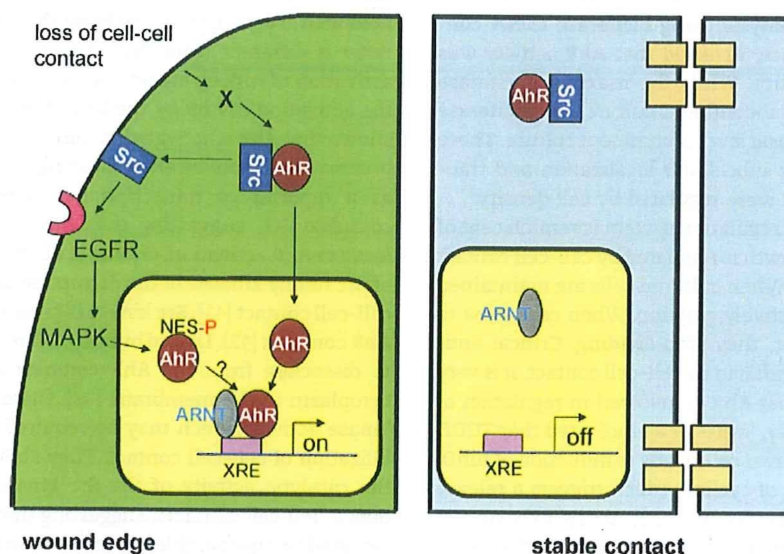

Fig. 2 – A model for AhR localization controlled by cell–cell contact in the *in vitro* wound healing assay. Stable contact (right) mediated by adhesion molecules such as E-cadherin is mechanically disrupted to form wound edge (left). The unknown signal (X) triggered by the loss of cell–cell contact activates AhR, resulting in dissociation of Src from the AhR complex and translocation from the cytosol to the membrane, where its kinase activity required for disruption of cell–cell contact is activated. In addition, Src kinase activity may act as a trigger for the signals such as epidermal growth factor receptor (EGFR)-dependent pathway that induces key signal transduction cascades including MAPK. It is likely that activated p38 MAPK is involved in the phosphorylation of the nuclear export signal (NES) of AhR, which in turn inhibits nuclear export resulting in nuclear accumulation. XRE-mediated transcription is up-regulated by AhR/ARNT complex.

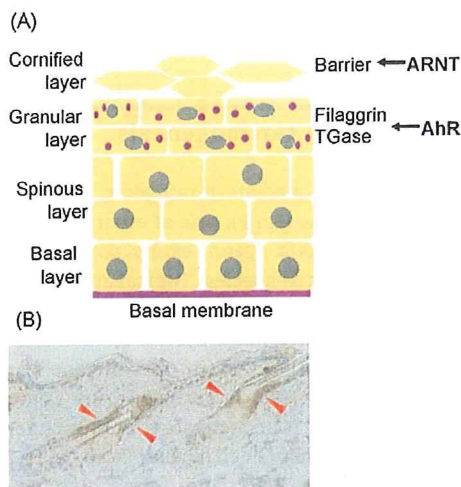

Fig. 3 – (A) Structure of the epidermis, and the possible role of AhR and ARNT. AhR stimulates the expression of filaggrin and transglutaminase 1 (TGase) in granular cells. Barrier formation requires expression of ARNT. (B) Immunohistochemistry for AhR localization on the back skin from 3-wk-old WT C57Bl mice. After excision, the tissue was embedded in O.C.T. compound (Miles, Elkhart, IN) and immediately frozen using liquid carbon dioxide. Frozen tissue was sectioned at 8 μm intervals, and the sections were fixed by 4% formaldehyde before overnight incubation with anti-AhR antibody (BIOMOL, Plymouth Meeting, PA) at 4 °C. Arrowheads indicate the site of immunoreactivity including upper part of hair follicle.

keratinocytes. Loetscher et al. [56] examined the effects of TCDD on developing skin of the C57Bl/6J mouse strain. Examination of mouse fetal skin at embryonic day (E) 16 revealed that expression of filaggrin is accelerated in individuals exposed *in utero* to TCDD at E13. They reported that the two putative XREs are present upstream of the transcription start site of human profilaggrin gene. In addition, Du et al. [57] studied the effect of TCDD on differentiation program of human epidermal keratinocytes and showed the induction of transglutaminase 1 at the mRNA and protein levels. This was further confirmed by the increasing transglutaminase 1-mediated cross-linking activity *in situ*. Since there is no XRE motif in the human transglutaminase 1 promoter region, it is unclear how TCDD regulates transglutaminase 1 expression. These investigations suggest that AhR has roles in the modulation of differentiation of keratinocytes.

The function of the skin barrier is partly dependent on terminal differentiation of keratinocytes. The cells differentiate as they move to the skin surface. The stratum corneum is composed of not only insoluble protein such as involucrine and filaggrin but also lipid complex containing cholesterol, ceramides, and fatty acids. Takagi et al. [58] and Geng et al. [59] reported defects in the barrier function in ARNT-deficient mice. They showed that defects in lipid metabolism resulted in failure of the epidermal barrier function. ARNT-disrupted newborn mice died neonatally of severe dehydration caused by water loss. We are interested to investigate whether AhR is involved in these processes as a heterodimer-partner of ARNT although AhR-null mice have not yet been reported to show such a severe skin phenotype.

To elucidate the AhR function in skin, we examined AhR localization in murine skin by immunohistochemistry. One of the regions in which AhR was detected is the upper part of the hair follicle including the infundibulum (Fig. 3B). Exposure of mammals to TCDD produces an array of pathological manifestations including teratogenesis [3], hepatotoxicity [4], and dermatopathology [60]. Chlorance, a hyperkeratotic skin disorder, is a specific type of acne-like skin disease affecting the hair follicle and inter follicular epidermis and has been used as a hallmark of TCDD exposure in humans. Histopathological analysis of the skin with chloracne has revealed acne-like appearance with hyperkeratosis. In severe cases of acne, the rupture of the infundibulum is associated with inflammation. It is reported that transgenic mice expressing a constitutively active form of AhR in keratinocytes develop severe skin lesions accompanied by inflammation resembling typical atopic dermatitis with increased expression of inflammation-related genes (such as the genes for interleukins and chemokines) [61]. When infundibula maintained in culture were stimulated with interleukin-1α, hyperkeratosis that was similar to that seen in acne was observed [62]. Taken together, these results prompted us to consider that in response to TCDD exposure, AhR expressed in infundibula is aberrantly activated to induce hyperplasia and inflammation, resulting in chloracne.

6. AhR functions in skin wound healing

Skin wound healing is a dynamic three-phase process: inflammation, tissue formation, and tissue remodeling [16]. Diverse cell types including leukocytes, keratinocytes and dermal fibroblasts participate in each phase. For example, in the early phase of healing, inflammatory leukocytes are recruited to the wounded site. Neutrophils cleanse the wound area of foreign particles, and macrophages release cytokines and growth factors. Keratinocytes migrate and proliferate to cover the wound area, and the dermal fibroblasts synthesize extracellular matrix for tissue remodeling. It is reported that AhR is expressed in these cells [40,63–65]. Here, we investigated the role of AhR in skin wound healing using either wild-type mice, mice heterozygously or homozygously deficient for the *AhR* gene.

Our methods of full thickness dorsal skin wounding damaged both the epidermis and the underlying dermis. All mice were 8–10 wk old, anesthetized, and received a single 5-mm-diameter excisional wound on the shaved mid-dorsal skin. Mice were kept separately in cages to prevent fighting. Wound closure was determined and expressed as a percentage of the total surface of the wound (Fig. 4). The wounds closed almost completely at day 12 irrespective of AhR genotype. However, in the early phase of healing, wound area decreased faster in the AhR−/− mice than in the wild-type mice and AhR± mice. The time needed for 50% closure in wild-type, AhR± and AhR−/− mice was 73.7 ± 3.0 h ($n = 50$), 59.0 ± 3.3 h ($n = 32$) and 47.6 ± 3.3 h ($n = 39$), respectively. The difference in time needed for 50% closure between AhR−/−

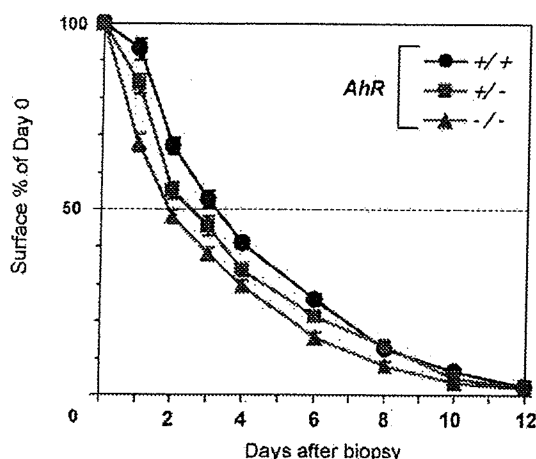

Fig. 4 – Surface areas of the wounds. The back skin of each mouse (8–10 wk) was shaved 1 wk before wounding. A full thickness of mid-dorsal wound (5 mm in diameter) was created and the wound areas were quantified on the indicated days until complete healing, using NIH image software. The surfaces are plotted as percentage of the surface of the wound at day 0 (Mean ± SE). The surface areas of the wounds at day 0 were not significantly different among genotypes.

and wild-type mice was significant (P = 1.97E−08). On the other hand, we did not find any differences in repair processes between wild-type and AhRR−/− mice. These data suggest that inactivation of AhR accelerates wound closure during the early phase, which corresponds to the inflammatory phase, of wound healing. These observations are reminiscent of those observed for peroxisome proliferator-activated receptor (PPAR)-mutant mice. Michalik et al. [66] found that wound healing in PPARα−/− mice was delayed during the first 4 days after injury, suggesting the involvement of inflammation. They further assessed inflammatory infiltration by counting neutrophils and monocytes/macrophages present in the wound bed. Recruitment of neutrophils and monocytes was impaired in the PPARα−/− mice at day 1. AhR expression has been detected in monocytes and macrophages [63,64] and might affect the function of these cells. Tauchi et al. [61] reported that transgenic mice expressing the constitutive active form of AhR in keratinocytes develop severe skin lesions accompanied by inflammation. It is likely that the skin of AhR−/− mice has a reduced inflammatory phenotype. Aschcroft et al. [67] investigated the role of Smad3, a mediator of TGF-β signaling, in skin wound healing. They reported that mice lacking Smad3 show accelerated wound healing accompanied by reduced inflammatory response (i.e., reduced local infiltration of monocytes) leading to reduced level of TGF-β in the wound site. One possible explanation for the increased rate of re-epithelialization in mice lacking Smad3 is the increase in keratinocyte proliferation due to abrogation of the growth inhibitory effect of TGF-β. In our experiments, we attribute this phenomenon (i.e., faster decrease in wound area in AhR-deficient mice in the early phase of wound healing) to reduced inflammation. In wild-type mice, AhR may play a supportive role in the inflammatory response.

7. AhR in skin carcinogenesis

AhR is considered to mediate teratogenic and carcinogenic effects. Benzo[a]pyrene (B[a]P), one of environmental chemicals binding to AhR as a ligand, exerts potent carcinogenic activity in several animal species. Topical application of B[a]P produces skin tumors. It has been revealed that the ultimate metabolite of B[a]P (i.e., benzo[a]pyrene-7,8-diol-9,10-epoxide [BPDE], which is synthesized in the metabolic pathway involving cytochrome P450 isoforms) forms DNA adducts and acts as a mutagen. To investigate the contribution of AhR to carcinogenesis, Shimizu et al. [2] examined the response of AhR-deficient mice to B[a]P and found that no tumors appeared in the AhR-deficient mice. They provided direct evidence that AhR is required for skin tumor induction by benzo[a]pyrene.

An important problem in skin cancer research is the identification of the target cells for chemical carcinogenesis. Evidence is accumulating that a subpopulation known as stem cells are the targets of carcinogenesis [68–70]. A number of investigations revealed that initiated cells persist in the epidermis essentially for the life of the animal, suggesting that the initiated cells are not simply proliferating cells but also stem cells. Morris et al. [15] examined the origin of skin tumors. They completely removed the interfollicular epidermis of carcinogen-initiated mice using an abrasion technique although the hair follicles remained undisturbed. The interfollicular epidermis after abrasion regenerated from cells in the hair follicles. Subsequently, tumor promotion was progressing. Although mice with abraded skin developed papillomas and carcinomas, the number of papillomas was half that of mice with unabraded skin. These results suggest that target of tumor initiation is the cells in hair follicles and, to a lesser degree, in the interfollicular epidermis.

One of the cell surface markers of hair follicle stem cells is CD34. Trempus et al. [68] examined whether CD34 participates in two-stage skin carcinogenesis in CD34 knockout (KO) mice since hair follicle stem cells are thought to be a major target of carcinogens. CD34KO mice failed to develop papillomas, suggesting the requirement of stem cells for skin tumor development. Hair follicle stem cells may be a target for carcinogens. If so, stem cell may express AhR to induce P450 isoforms that metabolically activate carcinogens. It has been shown that hematopoietic stem cells, which express functional AhR, have been shown to be a target of polycyclic aromatic hydrocarbons [71]. Treatment of these cells with B[a]P resulted in impairment of cell expansion and inhibition of cell differentiation into various cell lineages including erythrocyte, granulocyte, macrophage, and megakaryocyte. These toxic effects are related to P450-dependent B[a]P metabolite formation. These results suggest that AhR is functional in the system in stem cells.

Thus, it is implied that stem cells are critical targets of carcinogen metabolites produced by P450 isoforms. These metabolites may be involved in the initiation of the stem cell in

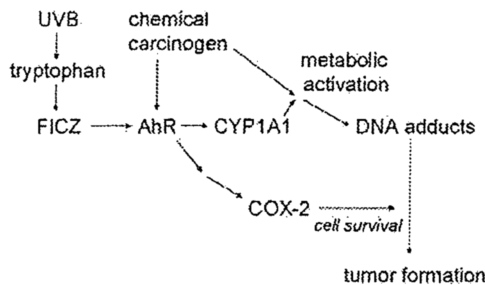

Fig. 5 – Roles of AhR in skin carcinogenesis. CYP1A1 is induced by chemical carcinogens as well as photoproducts of tryptophan [72,73] such as 6-formylindolo[3,2-b]carbazole (FICZ) generated intracellularly by UVB exposure. DNA adducts are formed by reaction with metabolically activated carcinogens. FICZ also induces COX-2 expression through EGFR activation. The COX-2 pathways promote cell survival, resulting in the tumor formation. AhR directly induces CYP1A1 which stimulates generation of initiated cells, and indirectly induces expression of COX-2 leading to tumor promotion.

skin carcinogenesis resulting in the proliferation of the initiated stem cells.

Another risk for skin carcinogenesis is ultraviolet radiation. Photoproducts of tryptophan are known to have high affinity for AhR and are postulated as endogenous ligands [72,73]. The UVB portion (280–315 nm) of the spectrum is a principal risk factor for skin cancer. Fritsche et al. [44] found intracellular formation of the AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ) after UVB irradiation of murine skin and human keratinocyte cell line. AhR activation induces CYP1A1 gene expression and EGFR internalization and subsequent induction of cyclooxygenase-2 (COX-2) gene expression. COX-1 and COX-2 catalyze the first reaction in the conversion of arachidonic acid to prostaglandins. Prostaglandin E_2 is the major product found in UV-exposed skin. In most tissues, COX-1 is constitutively expressed, whereas COX-2 is highly inducible by a variety of inflammatory and tumor-promoting stimuli [74] and is constitutively up-regulated in skin carcinomas [75]. To understand the contribution of COX-1 and COX-2 to UV-carcinogenesis, Fischer et al. [76] performed UV-induced-carcinogenesis experiments using wild-type mice and mice heterogeneously for the COX-1 or the COX-2 gene. While the tumor generations of COX-1± mice and COX-1+/+ mice were essentially similar, the tumor multiplicity in COX-2± mice was reduced to 50–65% and the tumor size was markedly decreased compared with that of wild-type mice. Studies have revealed that UV-induced COX-2 expression contributes to the acquisition of resistance to epidermal apoptosis [77].

In conclusion, UVB exposure induces CYP1A1 and COX-2 in keratinocytes. In this intracellular environment, active carcinogens produced by CYP1A1 may form DNA adducts resulting in generation of initiated cells. Furthermore, as a result of acquiring resistance to apoptosis by induction of COX-2, the initiated cells may be able to clonally expand into detectable skin tumors (Fig. 5).

8. Perspective

Current investigations have revealed that AhR is an important regulator in various tissues even in the absence of exogenous ligands. While identification of endogenous ligands of AhR is one of the most intriguing goals of future study, uncovering the signaling pathway leading to AhR activation in the context of cell–cell contact is also needed. Investigating target genes of AhR/ARNT is also important for elucidation of AhR function. In wound healing, we are interested in the genes that act downstream of AhR in the signaling pathway and are involved in inflammatory agent production such as the release of cytokines. In skin carcinogenesis by benzo[a]pyrene, AhR is believed to be necessary for the induction of CYP1A1 which yields active carcinogens. However, the experimental data indicating that AhR-deficient mice produce no tumors do not exclude the possibility that AhR is involved in cancer progression. It would also be interesting to determine whether induction of epithelial–mesenchymal transitions mediated by AhR is functional in cancer.

Acknowledgements

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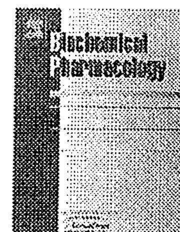

Review

AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions

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ABSTRACT

The arylhydrocarbon receptor (AhR) mediates the adverse effects of dioxins, including modulation of sex steroid hormone signaling. The role of AhR as a transcription factor is well described. AhR regulates the expression of target genes such as CYP1A1; however, the mechanisms of AhR function through other target-selective systems remain elusive. Accumulating evidence suggests that AhR modulates the functions of other transcription factors. The ligand-activated AhR directly associates with estrogen or androgen receptors (ERα or AR) and modulates their function both positively and negatively. This may, in part explain the sex steroid hormone-related adverse effects of dioxins. AhR has recently been shown to promote the proteolysis of ERα/AR through assembling a ubiquitin ligase complex, $CUL4B^{AhR}$. In the $CUL4B^{AhR}$ complex, AhR acts as a substrate-recognition subunit to recruit ERα/AR. This action defines a novel role for AhR as a ligand-dependent E3 ubiquitin ligase. We propose that target-specific regulation of protein destruction, as well as gene expression, is modulated by environmental toxins through the E3 ubiquitin ligase activity of AhR.

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Abbreviations: AhR, arylhydrocarbon receptor; ERα, estrogen receptor; AR, androgen receptor; XRE, xenobiotic-responsive element; ERE, estrogen-responsive element; bHLH/PAS, basic helix-loop-helix/Per–Arnt–Sim; AF-1, autonomous activation function; E_2, 17β-estradiol; 3MC, 3-methylcholanthrene; βNF, β-naphthoflavone; CRL, cullin–RING ubiquitin ligase; SCF, Skp1–CUL1–F-box; CUL4B, cullin 4B; DDB1, damaged-DNA-binding protein 1.

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1. Introduction

Dioxin-type environmental contaminants, such as tetrachloro-dibenzo-p-dioxin (TCDD), exert toxic effects [1]. Some of these toxicities are estrogen- and androgen-related actions [2–7]. The arylhydrocarbon receptor (AhR) is a ligand-dependent transcription factor belonging to the basic helix-loop-helix/Per–Arnt–Sim (bHLH/PAS) family. AhR possesses a variety of biological and toxicological functions [8–11] (Figs. 1 and 2). AhR mediates the toxicological effects of dioxins. In addition, AhR plays a physiological role in various tissues such as the reproductive and immune systems. The transcriptional activity of AhR is regulated by direct binding of its ligands [12,13] (Figs. 1 and 2A). The unliganded AhR is sequestered in the cytosol by interacting with the Hsp90/XAP2 (also called as ARA9 or AIP) chaperon complex [8–11]. Ligand binding to the PAS-B region of AhR is thought to induce conformational changes and subsequent translocation of the AhR complex to the nucleus [8–10]. AhR then dimerizes with the AhR nuclear translocator (Arnt) in the nucleus after dissociating from the chaperon complex, recognizes the xenobiotic-responsive element (XRE), and recruits co-activators such as the histone acetyltransferase p300/CBP, chromatin remodeling factor Brg1, and the mediator (DRIP/TRAP) complex to activate transcription [8–10] (Fig. 1). The AhR/Arnt heterodimer induces the expression of target genes, such as CYP1A1, CYP1A2, and glutathione-S-transferase [1].

The actions of the direct target genes of AhR alone do not fully explain its toxicological and physiological effects. Accumulating evidence suggests that the AhR exhibits its regulatory functions by modulating the function of other transcription factors [2,11], including estrogen receptor ($ER\alpha$ and $ER\beta$) [14–19] and androgen receptor (AR) [18,19] (Fig. 1). These cross-talk pathways are important mediators of the functions of endogenous and exogenous AhR ligands. The liganded AhR recently has been shown to promote the ubiquitination and proteasomal degradation of ERs and AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [18,19]. Thus, complexes of the AhR with ERs or AR appear to regulate transcription as functional units by multiple mechanisms. In this review, we will summarize a novel role for AhR as a component of an E3 ubiquitin ligase complex, which mediates cross-talk of AhR with sex steroid receptors through promotion of proteolysis.

2. Cross-talk of AhR with ERs or AR

2.1. *Transcriptional regulatory mechanism involving nuclear receptors*

ERs and AR belong to the nuclear receptor superfamily of transcription factors [20–22] (Fig. 2). Nuclear receptors, by acting as ligand-dependent transcription factors serve as

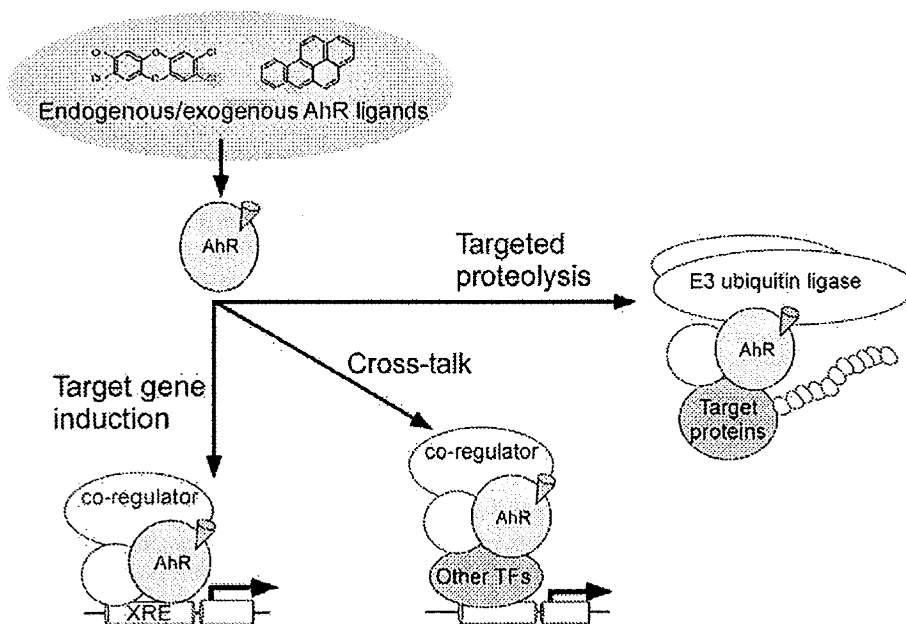

Fig. 1 – Different modes of the AhR signaling pathways. Molecular pathways for AhR-mediated biological actions. AhR may exhibit its biological actions through different modes of pathways as illustrated. Typically, AhR directly binds to its target gene promoters and induces expression of these genes. In addition, cross-talk of AhR with other transcription factors, as well as the function of AhR as an E3 ubiquitin ligase, is considered important for AhR biology. XRE, xenobiotic-response element; TF, transcription factor.

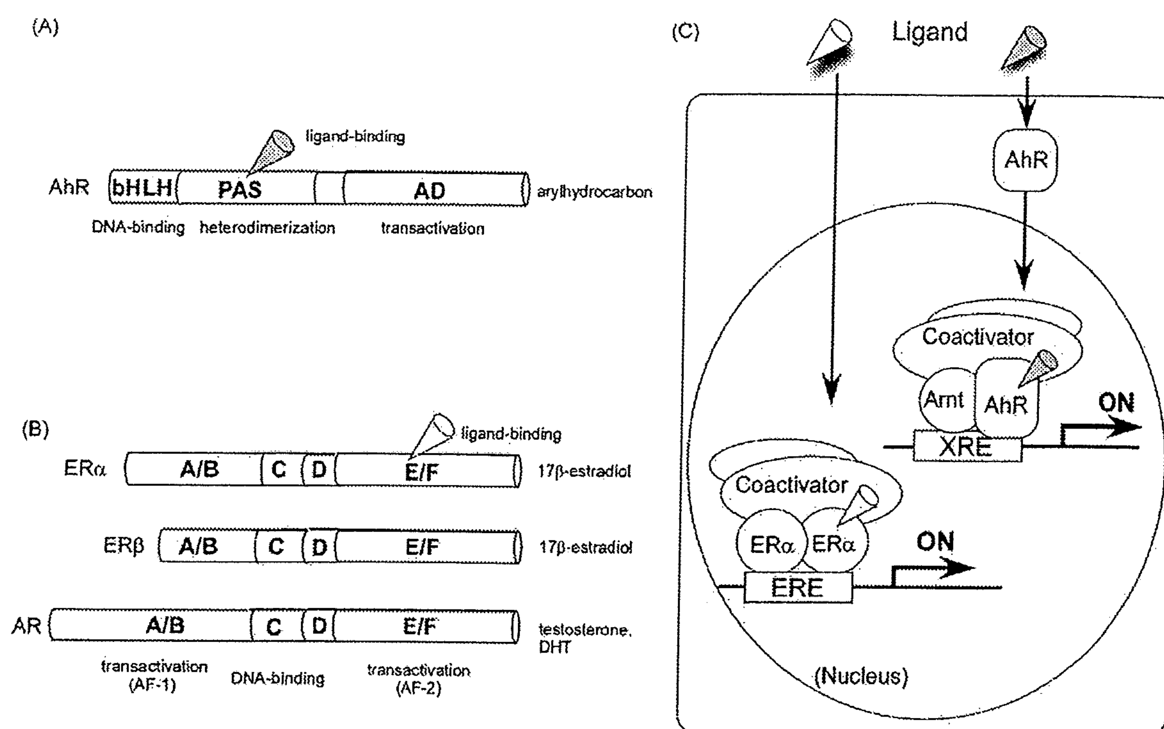

Fig. 2 – Structure and molecular mechanism of AhR and nuclear receptors. A and B domain structures of AhR (A) and nuclear receptors (B). Domain architectures and cognate ligands for these receptors are illustrated. bHLH, basic helix-loop-helix; PAS, Per–Arnt–Sim domain; AD, activation domain; AF, activation function; DHT, dihydrotestosterone. (C) Mechanisms of gene regulation mediated by AhR and nuclear receptors. ERE, estrogen-response element.

sensors for low molecular weight, fat-soluble ligands such as steroids/thyroid hormones, and vitamins A and D [20,21]. Members of the nuclear receptor gene superfamily share a common domain structure with distinct functional domains, designated A–E [21] (Fig. 2B). The ligand-binding domain is located in the C-terminal E domain. The most conserved C domain, located in the middle of the receptor, serves as the zinc finger-type DNA-binding domain. This domain specifically recognizes its cognate response elements in the target gene promoters. The N-terminal A/B domain and the C-terminal E domain are required for ligand-induced nuclear receptor transactivation functions. The autonomous activation function (AF-1) in the A/B domain is constitutively active but is presumably masked in the absence of ligand. The autonomous activation function (AF-2) in the ligand-binding E domain is, in contrast, dependent on ligand binding through the ligand-dependent conformational change of helix 12 and subsequent formation of a hydrophobic surface for the interaction with co-regulators [20] (Fig. 2).

Ligand-bound nuclear receptors recruit a number of transcriptional co-regulators and co-regulator complexes to the target gene promoters to mediate ligand-dependent transcriptional control [21,22] (Fig. 2). These complexes can be classified into three categories according to their functions. The first class of co-regulator complexes modifies histone tails covalently [23]. The amino-terminal tails of histones are subjected to various covalent modifications such as acetylation, methylation, phosphorylation, and ubiquitination by specific histone-modifying enzymes. These post-translational histone modifications are thought to serve as a 'histone code' that fine-tunes the transcriptional state through chromatin structure rearrangement [23]. The second class of complex mediates ATP-dependent dynamic remodeling of chromatin structure [22]. Chromatin remodeling complexes use ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner. These chromatin remodeling complexes support the accessibility of co-regulator complexes and transcription factors to specific promoter regions. The last co-regulator complex class, the mediator complex, directly regulates transcriptional control by physically interacting with general transcription factors and RNA polymerase II. Recent evidence suggests that numerous co-regulators and nuclear receptors are recruited onto the promoters in an ordered manner, associating and dissociating transiently [24,25]. Nuclear receptors, as well as other transcription factors, serve as specific adaptors that connect co-regulator complexes and specific promoter regions.

The ligand-dependent nuclear receptor function is also regulated by other classes of signal transduction pathways. Such cross-talk pathways include at least two mechanisms: functional modulation through post-translational modification, and the association with other classes of transcription factors. MAPK, activated by EGF, phosphorylates ERα at serine 118 [26]. This in turn potentiates the ligand-dependent transactivation function of ERα [26] as well as its rapid turn-over. Phosphorylation-mediated functional modulation has been reported for a number of nuclear receptors to date.

Complex formation-based cross-talk mechanisms are also seen in several nuclear receptors including the glucocorticoid receptor (GR) [27]. GR ligands have an anti-inflammatory action, which is mediated through ligand-dependent repression of AP-1 activity through direct association. More recently, the exchange of different classes of co-regulator complexes has been reported to underlie the signal cross-talk pathway. Ligand-activated PPARγ typically assembles co-activator complexes on its cognate promoters. In the repression of NF-κB activity, PPARγ forms a complex with NF-κB, and this complex stably associates with an NCoR co-repressor complex by inhibiting the degradation of NcoR [28]. A current view of signal cross-talk at the transcription levels is that signal/ligand-dependent transcription factors associate with each other to assemble diverse types of co-regulator complexes. These exchange dynamically and regulate transcription in a manner specific for each cross-talk pathway [22].

2.2. *Molecular mechanisms of cross-talk of AhR with estrogen or androgen receptors*

Signal cross-talk pathways are important mediators of the functions of AhR ligands in various tissues. Dioxin-type environmental contaminants exert both estrogen- and androgen-related effects [1–3,5–7,29–32] (Fig. 3). Dioxins have well-described anti-estrogenic effects, such as the inhibition of estrogen-induced uterine enlargement, MCF-7 cell growth, and target gene induction [3,7]. However, there is also evidence to the contrary as dioxins have also been shown to have estrogenic effects including the stimulation of uterine enlargement [29], induction of estrogen-responsive genes such as *VEGF*, *c-fos*, and *TERT*, and a similar pattern to estrogen of transcriptional regulation in a genome-wide study [6]. In addition, AhR-deficient mice exhibit impaired ovarian follicle maturation [33]. Using AhR-deficient cells, the importance of AhR in the proliferation of mammary cells has been confirmed [34]. These findings suggest that AhR, activated by its endogenous ligand, may modulate the estrogen signaling pathway. Similarly, dioxins exert both androgenic and anti-androgenic effects on prostate development in an age-specific manner [5]. As is true for other cross-talk pathways [22], the AhR appears to modulate estrogen/androgen signaling both positively and negatively depending on cellular context.

The molecular mechanisms of AhR modulation of ERα have been extensively studied, and both direct and indirect regulatory mechanisms have been proposed. First, TCDD/AhR either increases or decreases estrogen levels through an indirect mechanism [2,35]. TCDD promotes the clearance of estrogen, thereby repressing ER transcriptional activity [35]. AhR-deficient mice have decreased estrogen production due to impaired induction of aromatase (CYP19) gene expression [33]. Another indirect mechanism involves competitive DNA binding of AhR and ER on the responsive promoters [2]. AhR and ER, each bound to its own target promoter recruits transcriptional co-regulators

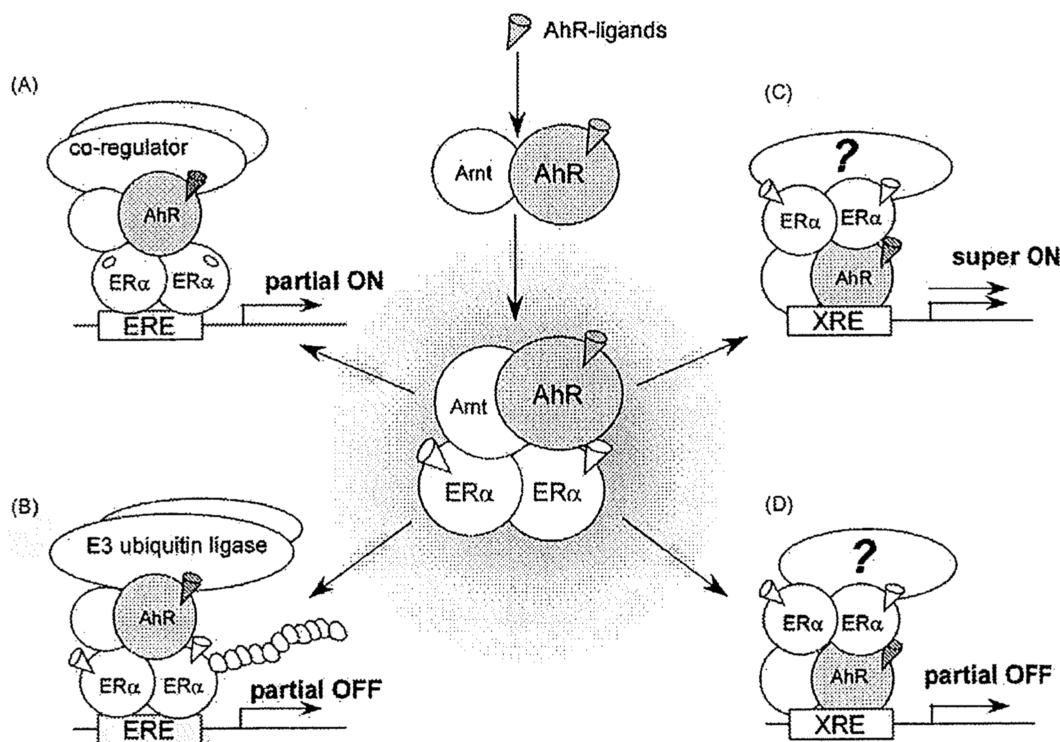

Fig. 3 – Cross-talk of AhR with ERα through direct association. Ligand-bound AhR directly associates with estrogen or androgen receptors (ERα, ERβ, or AR) in the nucleus. This association leads to different types of cross-talk between AhR and ERs/AR, as illustrated (see text for details). (A) Ligand-bound AhR associates with unliganded ERs upon ERE and recruits transcriptional co-activators. (B) Ligand-bound AhR forms E3 ubiquitin ligase complex and recognizes ERs for proteolysis. (C) Ligand-bound ERα associates with AhR and activates transcription through XRE. (D) Association of ERα with AhR results in repression through XRE.

in a competitive manner. This mechanism may be limited to specific genes and conditions since not all of the estrogen-responsive promoters contain XRE.

More recently, direct association of AhR with ERs has been independently reported. Ligand-activated AhR/Arnt associates with ERα and ERβ through the N-terminal A/B region within ERs [14–18] (Fig. 3). By means of this association, the liganded AhR potentiates the transactivation function of 17β-estradiol (E_2)-unbound ERα (Fig. 3A), while it represses E_2-bound ERα-mediated transcription upon the estrogen-responsive element (ERE) [14] (Fig. 3B). The interaction of AhR/ER is induced by different AhR ligands, such as TCDD, 3-methylcholanthrene (3MC), and β-naphthoflavone (βNF). The activation of AhR is thought to be sufficient for the interaction with ERα, as a constitutively active form of AhR [12] modulates ERα function in the absence of AhR ligand [19]. These results suggest that the cross-talk of AhR with ER is initiated primarily through stimulation of AhR. Supporting this, ERα is predominantly located in the nucleus, whereas AhR translocates to the nucleus upon ligand stimulation. The association of AhR/ERα has been shown by several independent approaches, including *in vitro* [36], *in vivo*, and biochemical methods [18]. Moreover, AhR/ERα cross-talk in the transcriptional regulation of ERα-responsive genes is abolished in AhR-deficient mice [10,33], confirming the specificity of the molecular pathway *in vivo* [14]. Reciprocally, E_2-bound ERα associates with XRE-bound AhR to either potentiate [15] (Fig. 3C) or repress [16] (Fig. 3D) AhR-mediated transcription. Considered together, the AhR/ERα complex may be able to bind to either XRE or ERE through the attachment functions of AhR or ERα, respectively. Alternatively, different complex subtypes that contain AhR/ERα may control promoter selectivity (Fig. 3). Reflecting this functional cross-talk, Arnt also acts as a co-regulator for both ERα and ERβ [37].

The proposed mechanism of AhR/ER association is a reasonable explanation for dioxin/estrogen cross-talk. First, this mechanism explains the functional AhR/ER cross-talk irrespective of differences in target gene promoters. Second, ligand-dependent AhR/ER association may result in a rapid cellular response to dioxins in terms of ER activity. The responses of ER transcriptional activity to AhR ligands are observed within a few hours in cultured cells as well as in mice, which supports the existence of direct cross-talk mechanisms. Third, variations in the AhR/ER containing co-regulator complexes may result in the complex, bi-phasic consequences of AhR/ER cross-talk. Given that complexes containing different classes of transcription factors can recruit co-regulator complexes distinct from their cognate associating complexes [22], it is possible that the AhR/ER complex, acting as a functional unit, may recruit different types of complexes depending on the cellular context. A current area of interest is the identification of the molecular determinants by which the activity of the AhR/ER complex is controlled.

3. Ubiquitin ligase activity of AhR

3.1. *The ubiquitin–proteasome system*

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels in response to various cellular contexts in metazoans (Fig. 4A). Whereas the transcriptional regulatory system is targeted by environmental fat-soluble ligands, the involvement of the ubiquitin–proteasome system in the adverse effects of these environmental toxins remains largely unknown. The target selectivity of these systems depends on the recognition of specific DNA elements by sequence-specific transcription factors [20–22] and recognition of degradation substrates by E3 ubiquitin ligases [38–41] (Fig. 4B). These transcription factors and E3 ubiquitin ligases primarily serve as specific adapters to subsequently recruit enzymes such as transcriptional co-

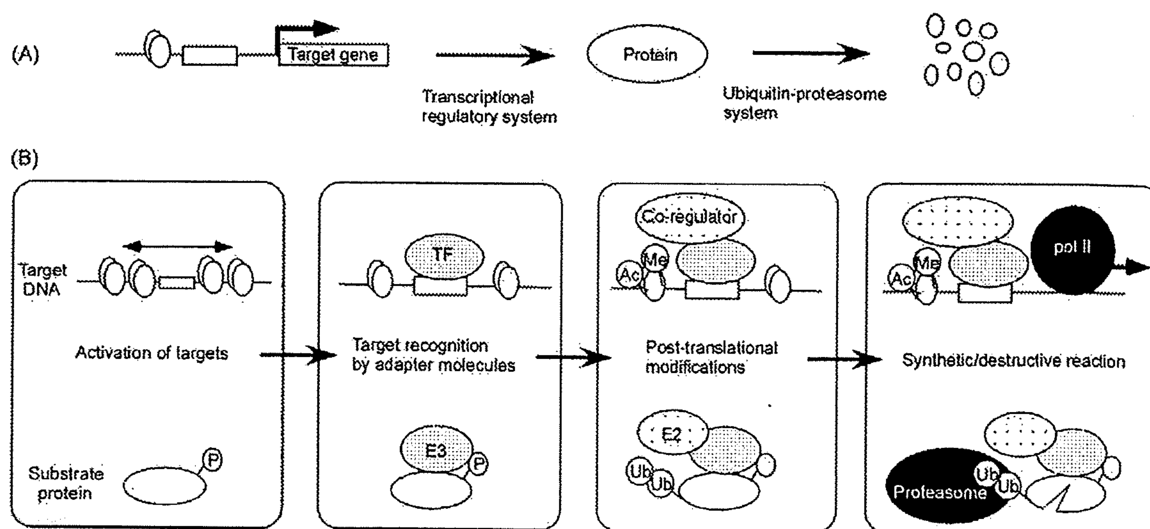

Fig. 4 – The ubiquitin–proteasome system. (A) The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. (B) The transcription factors and E3 ubiquitin ligases primarily serve as target-specifying adapters in these systems. Ub, ubiquitin; P, phosphorylated serine/threonine; Ac, acetylated lysine; Me, methylated lysine; Pol-II, RNA polymerase II.

regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. Considering the functional analogy of E3 ubiquitin ligase and transcription factors, it is possible that E3 ubiquitin ligase also serves as a target of environmental toxins.

The ubiquitin–proteasome system, which regulates cellular protein degradation, plays a pivotal role in cellular homeostasis [38–41]. Ubiquitin is a 76 amino acid polypeptide that is highly conserved among eukaryotes. Ubiquitin is covalently attached to lysine (Lys) residues of substrate proteins. Ubiquitination of proteins is catalyzed by sequential reactions involving ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). Ubiquitin is conjugated either as one molecule (mono-ubiquitination) or as a tandem polymer (poly-ubiquitination). Poly-ubiquitination can occur at any of seven lysine residues in the ubiquitin molecule. The Lys48-linked poly-ubiquitin chain is then recognized by the 26S proteasome for subsequent proteolysis (Fig. 4B).

Among E1, E2, and E3 enzymes, the E3 ubiquitin ligases are most diverse and therefore possess substrate specificity. E3 acts as a bridge between E2 and the substrate, maintaining the appropriate distance. E2 then conjugates ubiquitin to the substrate [38–41]. Of the RING-type E3s, the largest class is comprised of the cullin–RING ubiquitin ligases (CRLs) [40–44]. CRLs are multisubunit complexes that include a cullin (CUL1, 2, 3, 4A, 4B, or 5) subunit, a RING finger protein Rbx1/Roc1 or Rbx2/Roc2, and a substrate-recognition subunit. Cullin serves as a scaffold protein, binding to the substrate-recognition subunit or adapter protein at its N-terminus while binding to Rbx1 at its C-terminus [41]. Rbx1 binds to E2 enzymes through RING finger to support efficient conjugation of ubiquitin to the substrates. Their diverse substrate-recognition subunits enable CRLs to target numerous substrates. The best characterized CRLs are the SCF (Skp1–CUL1–F-box) complexes. In SCF complexes, F-box proteins function as a substrate-recognition subunit by binding to Skp1, which is bound to the N-terminal region of CUL1. F-box proteins and other types of substrate-recognition subunits serve as adapters for target-specific substrates. Therefore, any protein binding to E3 core components can potentially act in a manner similar to substrate-recognition subunits. More interestingly, F-box proteins and other types of substrate-recognition subunits are rapidly degraded through an auto-catalytic mechanism once they are integrated into the CRL core complexes [42]. In this way, CRLs can efficiently ubiquitinate different substrates by associating with different substrate-recognition subunits. This raises the possibility that F-box and F-box 'equivalent' proteins act either as substrates or as adapter components, as in the case of DDB2 in the CUL4-based CRL complex [45–50].

3.2. *AhR is an E3 ubiquitin ligase*

As discussed above, dioxins, through activating the AhR, have well-described effects on the transcriptional regulatory system. TCDD is also reported to decrease the uterine ERα protein level in the rat [51], suggesting that AhR may also be involved in the control of protein stability. Somewhat unexpectedly, our own study has shown that in a ChIP analysis, the ligand-bound AhR does not block co-activator recruitment of liganded ERα. In addition, repression of ERα transcriptional activity by AhR is not observed when ERα is over-expressed in transient reporter assays (Ohtake et al., unpublished data). These observations imply that the ligand-activated AhR has an additional molecular role beyond transcriptional regulation, at least in the modulation of sex hormone signaling.

Exploring the functions of AhR in sex hormone signaling, we found that upon activation of AhR by binding of AhR ligands such as 3MC and βNF, as well as by expression of constitutively active AhR, protein levels of endogenous ERα, ERβ, and AR, were drastically decreased without alteration in mRNA levels [19] (Fig. 5). Since ligand-bound AhR and ERα proteins are ubiquitinated for proteasome-mediated degradation [52–57], we tested whether the functional modulation of ERs and AR by activated AhR is related to this degradation system. 3MC-enhanced degradation of sex steroid receptors is attenuated in the presence of a proteasome inhibitor MG132, and 3MC-enhanced poly-ubiquitination of ERα is consistently observed irrespective of E2 binding. MG132 treatment abrogates the transcriptional modulation of liganded sex steroid receptor function by activated AhR. This indicates that the ubiquitin–proteasome system mediates the repressive AhR–ER cross-talk pathway.

These experiments provide evidence that AhR acts as an E3 ubiquitin ligase component. First, FLAG–AhR immunoprecipitated complexes exert a self-ubiquitination activity in an E1/E2 enzyme-dependent manner *in vitro*. Second, 3MC-dependent recognition of ER and AR by AhR [14] appears to induce ubiquitination of ER/AR. Third, degradation of AhR itself is accelerated upon activation of degradation of sex steroid receptors, which is a typical sign of self-ubiquitination of the E3 component [42]. Taken together, these properties of AhR resemble that of classical adapter components of the E3 ubiquitin ligase complex such as F-box proteins in the SCF complex [39,42], DDB2/CSA in the CUL4A complex [45–49], and VHL in the CUL2 complex [58]. Therefore, we reasoned that activated AhR might serve as an E3 ubiquitin ligase component.

Supporting this idea, an AhR associating ubiquitin ligase complex has been biochemically purified [59] from HeLa cells. This complex includes cullin 4B (CUL4B) [39,60], damaged-DNA-binding protein 1 (DDB1) [61,62], and Rbx1 [39] together with subunits of the 19S regulatory particle (19S RP) of 26S proteasome as well as Arnt and transducin-beta-like 3 (TBL3) (Fig. 5). The core complex appears to constitute a CRL-type E3 ligase, and therefore is referred to as $CUL4B^{AhR}$. Although the typical CUL4B-type CRL complex contains substrate-recognition components having a WDXR/DWD motif [45–49], no such component has been identified in this complex. AhR directly interacts with the N-terminal region of CUL4B in GST pull-down assays. Together with the direct interaction of AhR with ER, it appears that AhR may act as a substrate-recognition component in the $CUL4B^{AhR}$ complex. Using an *in vitro* reconstituted ubiquitination assay, the E3 ubiquitin ligase activity of $CUL4B^{AhR}$ for ERα is dependent only on 3MC, and not on E_2. This suggests that $CUL4B^{AhR}$ has the unique property of being able to respond to ligand signals by complex assembly and ubiquitin ligase activity (Fig. 5). The importance of the $CUL4B^{AhR}$ components for the promotion of ERα ubiquitina-