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A Novel Class of Prolyl Hydroxylase Inhibitors Induces Angiogenesis and Exerts Organ Protection Against Ischemia

Masaomi Nangaku, Yuko Izuhara, Shunya Takizawa, Toshiharu Yamashita, Yoshiaki Fujii-Kuriyama, Osamu Ohneda, Masayuki Yamamoto, Charles van Ypersele de Strihou, Noriaki Hirayama and Toshio Miyata

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A Novel Class of Prolyl Hydroxylase Inhibitors Induces Angiogenesis and Exerts Organ Protection Against Ischemia

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Objective—Hypoxia inducible factor (HIF) plays a pivotal role in the adaptation to ischemic conditions. Its activity is modulated by an oxygen-dependent hydroxylation of proline residues by prolyl hydroxylases (PHD).

Methods and Results—We discovered 2 unique compounds (TM6008 and TM6089), which inhibited PHD and stabilized HIF activity in vitro. Our docking simulation studies based on the 3-dimensional structure of human PHD2 disclosed that they preferentially bind to the active site of PHD. Whereas PHD inhibitors previously reported inhibit PHD activity via iron chelation, TM6089 does not share an iron chelating motif and is devoid of iron chelating activity. In vitro Matrigel assays and in vivo sponge assays demonstrated enhancement of angiogenesis by local administration of TM6008 and TM6089. Their oral administration stimulated HIF activity in various organs of transgenic rats expressing a hypoxia-responsive reporter vector. No acute toxicity was observed up to 2 weeks after a single oral dose of 2000 mg/kg for TM6008. Oral administration of TM6008 protected neurons in a model of cerebrovascular disease. The protection was associated with amelioration of apoptosis but independent of enhanced angiogenesis.

Conclusions—The present study uncovered beneficial effects of novel PHD inhibitors preferentially binding to the active site of PHD. (***Arterioscler Thromb Vasc Biol.* 2007;27:2548-2554.**)

Key Words: hypoxia ■ hypoxia inducible factor ■ structure based drug design ■ stroke ■ ischemia

Oxygen supply declines under ischemic conditions in many human vascular diseases including ischemic heart disease, chronic kidney failure, and stroke. The resulting hypoxia causes functional impairment of cells as well as structural tissue damage and triggers a broad spectrum of cellular defenses such as angiogenesis, erythropoiesis, glycolysis, and antioxidative enzymes.

Hypoxia-inducible factor (HIF), a heterodimeric nuclear factor, is a crucial intermediate in these defensive mechanisms.[1–3] Under normoxic conditions, HIF is constitutively transcribed and translated. Its stability is drastically reduced by the oxygen-dependent enzymatic hydroxylation of proline residues by prolyl hydroxylases (PHD).[4–9] Hydroxylated HIF recruits the E3-ubiquitin ligase, von Hippel Lindau protein (pVHL)[10,11] which, in turn, tags HIF with ubiquitin groups and targets it for degradation by the proteasome.[12,13] Under hypoxic conditions, HIF is not hydroxylated but binds to its heterodimeric partner HIF-1β. The resulting protein complex transactivates in the nucleus a host of genes involved in the adaptation to hypoxic stress.[14]

Activation of HIF may prove therapeutic for vascular disorders. Most treatments for ischemic and hypoxic disorders are currently focused on symptomatic relief and correction of etiologic factors. Drugs dissolving thrombi are also used to restore blood flow in the acute phase. As yet no compound enhancing organ resistance to hypoxia is clinically available. HIF activates a "master gene" switch that results in a broad and coordinated downstream reaction, protecting tissues against the consequences of hypoxia. The availability of less cumbersome non-toxic small molecular activators of HIF should prove very useful for therapeutic intervention.[15,16]

To obtain such novel compounds and to understand a molecular mechanism of PHD inhibition, we performed docking simulation based on the 3-dimensional structure of human PHD2. We further documented the in vitro and in vivo effectiveness of the novel PHD inhibitors we identified.

Materials and Methods

Please see the supplemental data section at http://atvb.ahajournals.org for detailed Methods.

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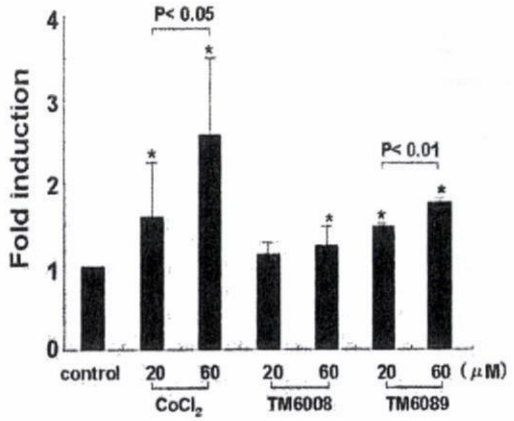

Figure 1. Stimulation of HIF-dependent luciferase reporter gene expression. IRPTC expressing the 7xHRE/Luc plasmid were incubated with the tested compounds. Cobalt chloride was used as a positive control. Results from 3 independent experiments are averaged and shown as fold increase above unstimulated control cells. *$P<0.05$ vs control.

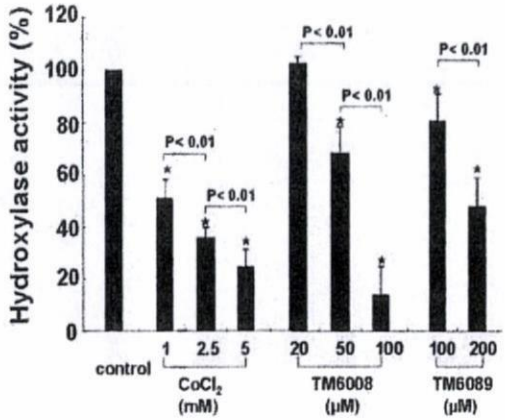

Figure 2. Inhibition of PHD activity. Results from 3 independent experiments are averaged and shown as the percentage of inhibition of ODD-dependent hydroxylase activity. *$P<0.01$ vs control.

Docking Simulations

The X-ray crystal structure of human PHD2 was obtained from the Protein Data Bank[17] (PDB code: 2HBT). Throughout the present study, the software system MOE (Molecular Operating Environment, version 2005.06) and the MMFF94s force field[18] were used. Binding sites were characterized using the alpha site finder function[19] in MOE. The docking of small molecules and the target sites was performed by the program Ph4Dock.[20]

PHD Activity

PHD activity was determined as described by Kaule et al.[21] In brief, mitochondrial fraction of IRPTC homogenates was reacted with the tested compounds and ODD peptide of HIF-1α. ODD-dependent hydroxylase activity was assessed by counting the radioactivity of [1-^{14}C]-succinate converted from [5-^{14}C]-2-OG by PHD.

Transition Metal Chelation

The chelating activity of the tested compounds for transition metal ions was measured by the method of Price et al[22] with some modifications.

Capillary Network Formation

Capillary network formation was examined by Matrigel assays (BD Biosciences) as described previously.[23]

Sponge Assays

Sponge angiogenesis assays were performed as described previously.[24]

Hypoxia-Sensing Transgenic Rat

Stimulation of the HIF-HRE system by systemic administration of TM6008 or TM6089 was evaluated using the hypoxia-sensing transgenic rat strain.[24] Expression of the hypoxia-responsive luciferase gene was estimated by semiquantitative RT-PCR as described previously.[25]

Cerebral Ischemic Injury Model

Transient global ischemia of Mongolian gerbils was achieved by bilateral carotid occlusion.[26] Animals were then randomly divided into 3 experimental groups: Groups 1 (TM6008) and 2 (vehicle) animals underwent transient global ischemia. Group 3 animals were sham-operated and served as controls.

We also measured cortical microperfusion by laser-Doppler flowmetry in gerbil forebrain ischemia treated with TM6008 or vehicle.

Statistics

Differences among groups were assessed by Kruskal-Wallis test or ANOVA. The statistical significance was determined by 2-tailed Mann–Whitney U test or Student t test. Data are expressed as means±SD. Values are considered significant at $P<0.05$.

Results

Identification of Novel HIF-Stimulating Compounds

Thirty-seven compounds that have structural similarities to FG-0041, a previously reported PHD inhibitor supposedly acting through iron chelation,[27] were selected from a chemical database. The chemical structures of these compounds are shown in supplemental Figure I. Their HIF-stimulating activity was tested by an in vitro screening assay which used cells expressing luciferase controlled by hypoxia responsive element (HRE) (supplemental Figure II). Cobalt, a well known chemical mimicker of hypoxia by stabilizing HIF-α subunit,[28] was used as a positive control. Two derivatives, TM6008 and TM6089, exhibited strong HIF-stimulating activities (Figure 1). TM6008 is 6-amino-1, 3-di methyl-5-(2-pyridin-2-yl-quinoline-4-carbonyl)-1H-pyrimidine-2, 4-dione, and TM6089 is 6-amino-1,3-di-methyl-5-[2-(pyridin-2-ylsulfanyl)-acetyl]-1H-pyrimidine-2,4-dione.

PHD Inhibition

The inhibitory effect of our compounds on the oxygen-dependent hydroxylation of HIF-α subunit by PHD was evaluated. All tested compounds inhibited PHD activity in a dose-dependent manner (Figure 2). TM6008 was the most effective, exceeding cobalt chloride.

In Vitro Transition Metal Chelation of PHD Inhibitors

Previously reported PHD inhibitors, such as 3,4-DHB,[29] S956711,[29] and FG-0041,[27] share an iron chelating motif. Although chemical structures of TM6008 and TM6089 differ significantly from previous PHD inhibitors, TM6008 also share this motif. By contrast, TM6089 lacks this motif.

We therefore evaluated their abilities to chelate transition metals in vitro by copper-catalyzed oxidation of ascorbic acid. 3,4-DHB, S965711, and TM6008 chelated transition

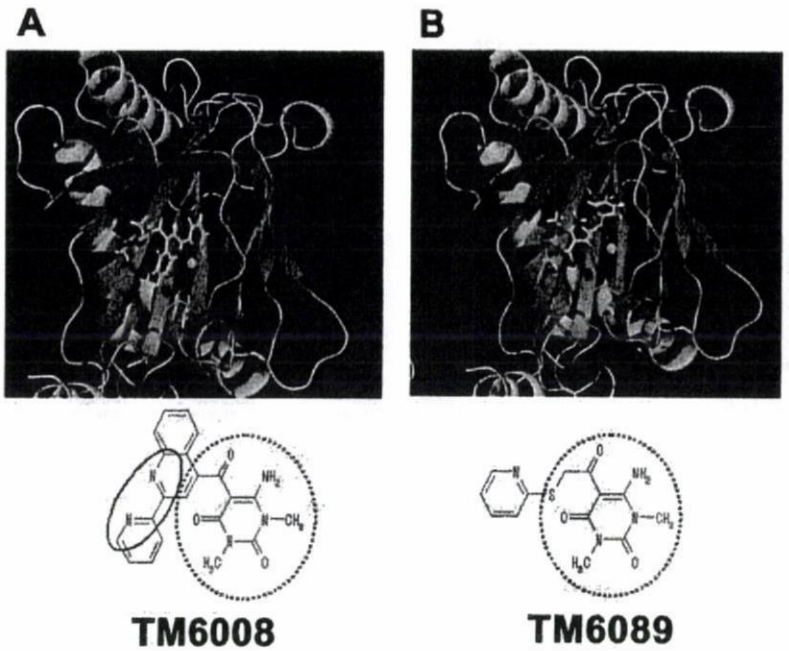

Figure 3. The predicted binding modes of TM6008 (A) and TM6089 (B) in PHD2. TM6008 and TM6089 are drawn by stick models. Sulfur, oxygen, nitrogen, carbon, and hydrogen atoms are shown in orange, red, blue, green, and white, respectively. Fe(II) is shown by an orange sphere. Figures were drawn by the software PyMOL version 0.97 (DeLano Scientific LLC).

metal (copper) and inhibited the autoxidation of ascorbic acid in a dose-dependent manner (IC 50 values were 330, 31.4, and 0.57 μmol/L, respectively). By contrast, TM6089 did not chelate transition metal even at the concentration of 100 μmol/L.

Binding Mode to Human PHD

PHD produces trans-4-hydroxyproline from 2-OG and L-proline (Pro) in the presence of Fe(II). The crystal structure of the catalytic domain of human PHD2, an important prolyl-4-hydroxylase in the human hypoxia response in normal cells, has been recently reported.[30] Based on the 3-dimensional structure of this PHD, we undertook docking simulations between our 2 PHD inhibitors and human PHD2. The docking modes of TM6008 and TM6089 are shown in Figure 3. TM6008 binds to the active site of PHD2 by chelating 2 nitrogen atoms with the iron atom. By contrast, TM6089 binds to the active site by nonchelating mechanism. The sulfur and 1 carbonyl oxygen atom of TM6089 point to the iron atom. The disposition of these 3 atoms, however, is unfavorable to form coordinate bonds. The binding mode of TM6089 demonstrates that TM6089 is a unique inhibitor without iron chelating affinity.

Toxicity and Pharmacokinetics

TM6008 and TM6089 did not exhibit cytotoxicity at the tested concentrations (up to 100 μmol/L). No acute toxicity was observed in mice up to 2 weeks after a single oral dose of 2000 mg/kg for TM6008, whereas the 50% lethal dose of TM6089 was 500 mg/kg. Pharmacokinetics studies in rats given an oral dose of 50 mg/kg of each compound disclosed plasma Tmax, Cmax, and T1/2 values of 3.5 hour, 0.9 μg/mL and 1.5 hour for TM6008, and 1.0 hour, 0.5 μg/mL, and 0.6 hour for TM6089.

Demonstration of the In Vivo Effectiveness

As VEGF is regulated by the HIF-HRE system, we examined whether TM6008 and TM6089 stimulate angiogenesis.

Firstly, we examined whether local injection of our compounds stimulates angiogenesis in vivo. For this purpose, we introduced small sponges under the skin of mice and measured their hemoglobin contents and vessel numbers after 10 days to estimate the stimulation of the HIF-HRE system. Injection of TM6008 increased angiogenesis as demonstrated by an increase of the hemoglobin content, and by an increased vessel number on immunohistochemical evaluation of the sponges. TM6089 also enhanced angiogenesis in the sponge assays (Figure 4A through 4C).

To investigate whether systemic administration of TM6008 and TM6089 stimulates in vivo the HIF-HRE system in various organs, we used the hypoxia-sensing transgenic rats. In the kidney expression of the reporter gene was not detected under basal conditions (amplification of 40 cycles), but expression of the reporter gene was obviously induced after a single oral dose 100 mg/kg of TM6008 and TM6089 (detected at 31.0±0.85 cycles and 31.0±2.05 cycles, respectively). In the liver, expression of the reporter gene, which was undetectable under basal conditions, was also induced after TM6008 administration (detected at 32.3±0.35 cycles), whereas TM6089 was ineffective. In the heart, the reporter gene was detected under basal conditions and both TM6008 and TM6089 increased its expression (1.37±1.00 and 6.69±5.45 fold increase, respectively). No attempt was made to evaluate the expression of the transgene in the brain because the pharmacokinetics studies showed that neither of the tested compounds crossed the blood-brain barrier.

Next, we evaluated capillary network formation by Matrigel assays. When endothelial cells were seeded onto Matrigel at subconfluent density, they developed tube-like structures at 9 hours. Quantification of capillary network formation by measuring the tube length revealed promotion of capillary network formation by TM6008, confirming the results of the sponge assays (Figure 4D).

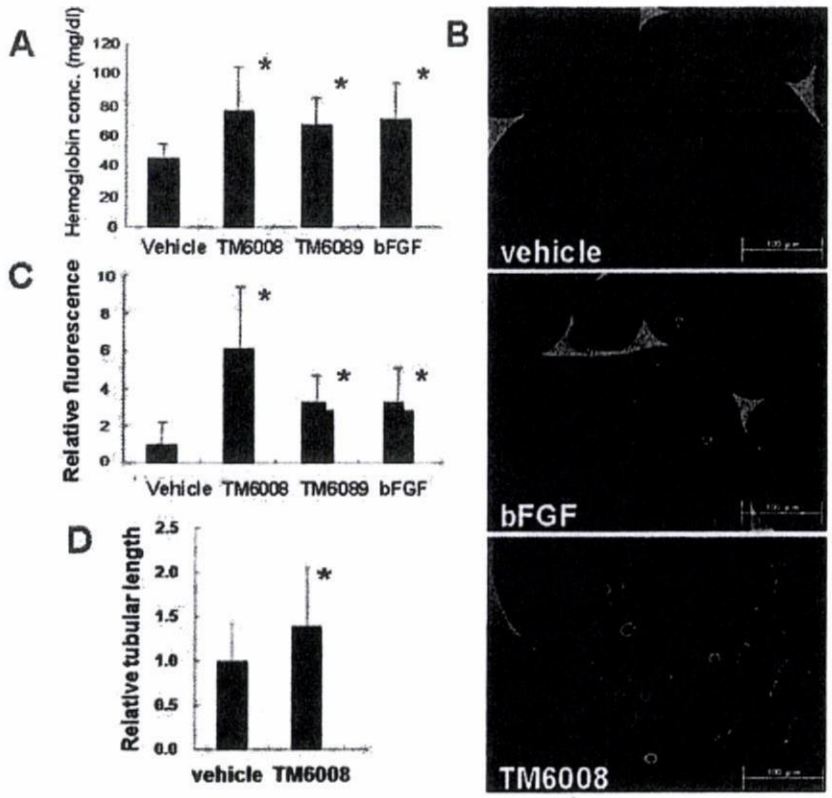

Figure 4. Stimulation of angiogenesis in the mouse sponge model and in the Matrigel assay. To assess the degree of angiogenesis, we measured the hemoglobin content in the sponge (A) and stained for the endothelial cell marker CD31 (B and C). B, Representative immunostaining of CD31 (×200); C, The average number of vessels. D, Capillary network formation on the Matrigel. *$P<0.05$ vs vehicle.

Prevention of Neuronal Cell Death Induced by Hypoxia

PHD inhibitors might protect cells against hypoxic damage. To test this hypothesis we used the delayed neuronal death model in gerbil. Nontoxic TM6008 (100 mg/kg/d) was given orally for 7 days in gerbils after a 5-minute transient global cerebral ischemia. The pathological outcome of neuronal cells was examined after 7 day administration of TM6008 in CA1 hippocampus with light microscopy.

In contrast with nonischemic gerbils (Figure 5A), gerbils subjected to ischemia and given vehicle alone (Figure 5B) exhibited in most pyramidal neurons ischemic cell damage, characterized by shrunken, darkly stained cytoplasm, and pyknotic nuclei with accumulation of glial cells. In the TM6008-treated animals, only a few neurons showed ischemic changes (Figure 5C). The number of viable neurons in the CA1 hippocampus, was higher in the TM6008-treated animals than in the vehicle-treated gerbils (166±73 versus

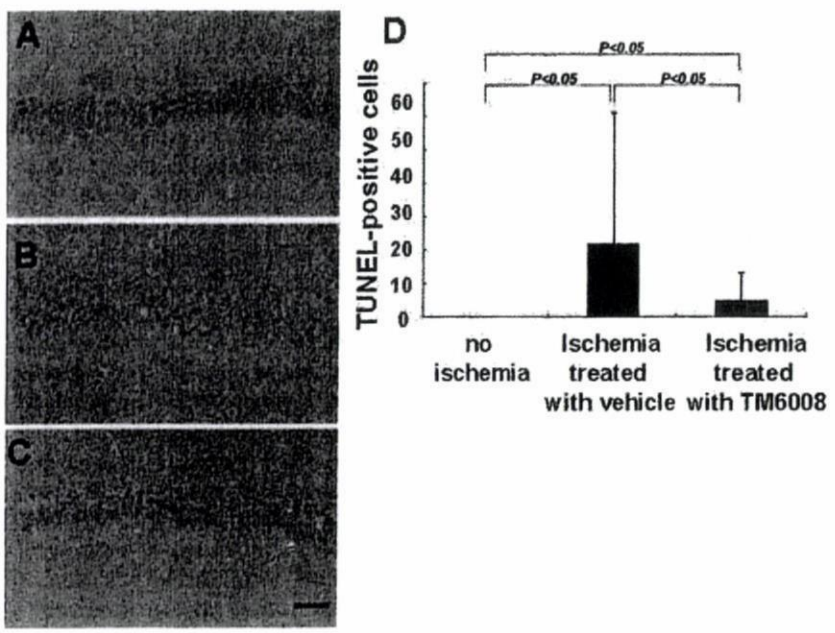

Figure 5. Prevention of hypoxic-induced neuronal cell death. HE staining in CA1 in a nonischemia animal (A), an animal subjected to ischemia and treated with vehicle alone (B), and an animal subjected to ischemia and treated with TM6008 (C). Scale bar=0.1 mm. D, TUNEL-positive cells in CA1.

61±55, $P<0.05$). The number of viable neurons in the CA1 hippocampus of the TM6008-treated animals was not statistically different from that observed in the nonischemia control group (227±50). Further, treatment with TM6008 decreased the number of apoptotic cells (Figure 5D). The final plasma concentration of TM6008 in these experiments was 7.8±2.9 μg/mL. Thus, TM6008 clearly protected against hypoxia-induced apoptotic neuronal death.

Next, we examined whether the protective effect of TM6008 against delayed neuronal death was attributable to enhanced angiogenesis. There was no statistically significant difference of the number of VEGF-positive cells between TM6008- and vehicle-treated groups (10.17±5.02 versus 9.12±1.55, respectively). Further, there was no significant difference of the value of cortical microperfusion at 7 days after occlusion between TM6008- and vehicle-treated groups (25.0±9.3 versus 29.4±8.7, respectively).

To clarify a neuroprotective mechanism of TM6008 in global ischemia models, we immunohistochemically stained with EPO, GLUT-1, and GLUT-3. The number of GLUT-3–positive cells in the CA1 hippocampus was significantly higher in TM6008 treated than in the vehicle-treated gerbils (26.9±7.5 versus 15.3±8.6, $P<0.05$). However, there was no statistically significant difference in EPO or GLUT-1–positive cells in the CA1 hippocampus between TM6008- and vehicle-treated groups (2.9±2.9 versus 3.3±1.7; and 5.5±2.1 versus 6.4±1.8, respectively).

Discussion

We identified novel molecules able to inhibit PHD activity and stabilize HIF. Our docking simulation studies based on the 3 dimensional structure of PHD2 have disclosed the molecular events required to inhibit PHD and therefore stabilize HIF. The target of these PHD inhibitors is the PHD active site.

Most of the PHD inhibitors reported so far, eg, 3,4-DHB, S956711 and FG-0041, are believed to inhibit the enzyme by iron chelating mechanism.[27,29] Iron chelating compounds could have nonspecific binding affinity to the iron containing proteins or iron ions and may not be desirable from the therapeutic point of view because iron is an essential cofactor for a host of important cellular functions, including oxidative phosphorylation and arachidonic acid signaling. To our surprise, the docking simulations demonstrated that TM6089 could preferentially bind to the active site of PHD2 without chelating to the iron atom. Indeed, TM6089 is devoid of iron chelating activity in vitro. Thus, iron chelation is not a necessary intermediate of PHD inhibition. According to our knowledge, TM6089 is the first unique PHD inhibitor which stimulates HIF activity without iron chelation.

The in vivo relevance of our novel PHD inhibitors was first demonstrated by the sponge assay in mice. Previous reports have shown that the hemoglobin contents of the sponge implants and the surrounding granuloma tissue correlated with the degree of angiogenesis.[31] Accordingly, both 3,4-DHB and S956711 were shown to raise the number of vessels in the sponge. In this study, we demonstrated not only an augmented number of vessels by immunohistochemistry but also an increased hemoglobin content in the sponge after local administration of TM6008 and TM6089.

Of great interest, these effects of TM6008 and TM6089 are not restricted locally but extend to several organs. To reach this conclusion, we used a hypoxia-sensing transgenic rat expressing a hypoxia-responsive reporter vector using a HRE of the 5′ VEGF untranslated region.[25] These transgenic rats have the unique asset to allow a sensitive and specific evaluation of HIF stimulation. As a consequence of systemic administration of TM6008 and TM6089 to these rats, expression of the reporter gene was considerably upregulated in the kidney, liver, and heart.

To extend these findings, we used less toxic TM6008 and obtained therapeutically relevant results in studies using gerbils. In gerbils, transient brain ischemia followed by reperfusion results in neuronal death in selectively vulnerable brain regions such as the hippocampal CA1 sector and caudate-putamen. The discovery that, in this model, TM6008 rescued neurons from apoptotic cell death in the CA1 hippocampus is noteworthy. Whereas TM6008 did not cross the blood-brain barrier, TM6008 protected the brain in a model of global cerebral ischemia. This is likely attributable to an increase in permeability of the blood-brain barrier, as previous reports showed that ischemic injury in this model destroys the blood-brain barrier and allows passage of compounds which do not penetrate the barrier under normal conditions.[32]

Mechanisms of neuroprotection by TM6008 can theoretically be multifactorial because HIF regulates a wide range of protective genes such as those involved in erythropoiesis (EPO, transferring, and hepcidin), angiogensis (VEGF), antioxidative stress (HO-1), glycolysis (Glut-1, Glut-3, and aldolase A), and so on. Angiogenic effects of TM6008 shown by the Matrigel assays and sponge assays stimulated us to study whether enhanced angiogenesis played a role in neuronal protection in our model. However, we could not find enhanced angiogenesis in the brain of gerbils treated with TM6008 by counting VEGF-positive vessels or measuring blood flow by laser Doppler flowmetry. Therefore, it is unlikely that the protective effect of TM6008 was related to angiogenesis in the gerbil forebrain ischemia model. This may be explained by different concentrations of TM6008 among the assays. Although we could not measure the local concentrations of TM6008 in the damaged brain, it is likely that the concentration of the gerbil forebrain treated with TM6008 p.o. is lower than those obtained by local administration such as sponge assays and Matrigel assay.

We next focused on effects of TM6008 on neuronal apoptosis. Our terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays demonstrated that TM6008 decreased the number of apoptotic cells in the brain, and other potential neuroprotective mechanisms by TM6008 include antiapoptotic effects mediated by other HIF-regulated genes such as EPO,[33] VEGF,[34] and glucose transporters.[35] EPO is a pleiotropic cytokine[36] and induces neuroprotection via the antiapoptotic signaling cascades like Bcl-X_L through direct binding to the Bcl-X promoter.[37] Antiapoptotic effects of VEGF contribute to reduction of ischemic brain damage in addition to its angiogenic effects.[38]

The glucose transporter GLUT-1 is also positively regulated through HIF-1α, and the microinfusion of virus vectors bearing the GLUT-1 isoform into the brain tissue reduced seizure-induced[39] and ischemic neuronal damage in vivo.[40] However, our immunohistochemical analysis could not demonstrate upregulation of these genes. In contrast, we observed upregulation of Glut-3. Glut-3 is also regulated by HIF,[41] and recent studies suggested a critical role of Glut-3 in protecting against a decline in brain glucose uptake under ischemic conditions.[42]

These results fit with the observations collected during various therapeutic strategies related to HIF target genes. For instance, cobalt chloride has been used as a conventional HIF stabilizer. It is generally believed to replace the iron present in PHD, but recent studies demonstrated that cobalt also depletes intracellular ascorbate,[28] a substrate of PHD. Cobalt is effective in a variety of hypoxia-related disorders including cerebrovascular disease.[23,43,44] In addition to PHD, there are other factors regulating the HIF stability/activity. Factor-inhibiting-HIF (FIH) hydroxylates regulates HIF activation via controlling CBP/p300 recruitment. The phosphoinositide 3-kinase (PI3K)/Akt pathway and the protein kinase C signaling have also been implicated in the regulation of HIF-α. Whether these pathways can be a good target for therapeutic approaches is a future subject to be pursed.

The protective effect of TM6008 against ischemia-induced cerebral lesions suggested its potential usefulness in other ischemic disorders such as cardiac or kidney diseases. It should not be forgotten that HIF stimulation acts as a general switch for several proteins such as VEGF, erythropoietin, etc. Although these proteins are protective under hypoxic conditions, recent demonstration that both erythropoietin and VEGF accelerates diabetic retinopathy independently[45] should call for caution. Their administration during several months warrants long-term experimental studies before concluding to its safety. On the other hand, the short-term use of PHD inhibitors for acute hypoxic damage will probably prove safe.

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Disclosures

None.

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Minireview

Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor

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Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that functions as an intracellular mediator in the xenobiotic signaling pathway. Although a number of studies have examined AhR-mediated *CYP1A1* induction in detail, recent studies of AhR-null mice have revealed that AhR plays important regulatory roles in the normal homeostasis and development of animals. In this short review, we summarize the present state of knowledge about the molecular mechanisms of AhR-mediated CYP1 induction, and we also focus on recent advances in the study of the physiological functions of AhR.

Keywords: Cytochrome P450; CYP1A1; AhR; TCDD; Gene regulation; Nuclear-cytoplasmic transport; Carcinogenesis; Reproduction; Inflammation

Members of the cytochrome P450 (CYP)[1] superfamily of hemoproteins are found across a wide range of species from bacteria to vertebrates [1]. These iron-containing proteins catalyze the monooxygene reaction of various endogenous and exogenous substrates and are classified according to the similarity of their amino-acid sequences. There are 18 CYP mammalian gene families, and four of these families (1, 2, 3, and 4) mainly metabolize foreign compounds including drugs, food additives and environmental pollutants [2]. Members of these CYP families are often specifically induced in response to exposure to foreign chemicals [3].

The molecular mechanisms regulating the induction of CYP proteins have been extensively examined, and a number of different inducers, *cis*-acting DNA elements, *trans*-acting transcription factors, and coactivators have been identified, and these studies have been greatly facilitated by the relatively recent development of novel genetic techniques, such as gene-knockouts, chromatin immunoprecipitation (Chip) and small interfering RNA (siRNA). The induction of CYP1 family member expression is regulated by a heterodimer composed of the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator (Arnt), which contains a characteristic basic-helix–loop–helix (bHLH) and PER-Arnt-SIM (PAS) homology region [4,5]. In contrast, the expression of CYP2, 3, and 4 family members is regulated by the nuclear receptors CAR, PXR (SXR), and PPAR, respectively, all of which possess a nuclear receptor gene family (family NR1) zinc finger motif and form heterodimers with the

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[1] *Abbreviations used:* AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; Chip, chromatin immunoprecipitation; siRNA, small interfering RNA; Arnt, aryl hydrocarbon receptor nuclear translocator; bHLH, basic-helix–loop–helix; PAS, PER-Arnt-SIM; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; NLS, nuclear localization signal; NES, nuclear export signals; RXR, retinoid X-receptor; XRE, xenobiotic responsive element; BTE, basic transcription element; AhRR, AhR repressor; LOH, loss of heterozygosity; PAHs, polycyclic hydrocarbons; CA-AhR, constitutively active form of AhR; SOCS-2, suppressor of cytokine signaling 2.

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retinoid X-receptor (RXR) [3]. In this short review, we summarize our present understanding of the molecular mechanisms controlling the expression of CYP1 family members mediated by AhR with an emphasis on recent studies examining the physiological function(s) of AhR. The mechanisms of NR1-induced expression of CYP2, 3, and 4 family members have been addressed in a number of excellent recent review articles [6–8] , and interested readers should refer to those works.

Molecular mechanisms of CYP1 induction mediated by AhR

Activation of AhR

It is well established that ligation and activation of AhR by endogenous or exogenous compounds such as 3-methylcholanthrene (MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) leads to nuclear transport and transcriptional activation. AhR contains a nuclear localization signal (NLS) in its bHLH region [9], and mice harboring a mutation in the conserved NLS are resistant to TCDD-induced toxicity [10], consistent with results seen in AhR-deficient mice. AhR contains nuclear export signals (NES) in both the bHLH [9] and PAS domains [11] and can shuttle between the cytoplasm and the nucleus. This process is inhibited by LMB, a specific inhibitor of CRM1-dependent nuclear export [12].

In the absence of ligands, AhR is associated with a cytoplasmic protein complex consisting of Hsp90, p23, and ARA9 (XAP2 or AIP). Hsp90 binding is thought to mask the AhR–NLS, and this interaction is essential for the cytoplasmic retention of AhR [13]. Overexpression of ARA9 increases the fraction of AhR found in the cytosol [14], and the LxxLL motif of the NR box in AhR, which was found to mediate protein–protein interactions of transcriptional cofactors with nuclear receptors, is also involved in the cytoplasmic retention of AhR by protein–protein interactions [15]. Additionally, protein kinase C-mediated NLS phosphorylation inhibits the ligand-dependent nuclear import of AhR. Taken together, these findings suggest that the regulation of AhR nuclear import has two distinct components: ligand binding initiates the interaction of the NLS with nuclear import components, and this is negatively regulated by NLS phosphorylation [16] (Fig. 1b).

Several studies have examined the ligand-independent activation of AhR. When several different cell lines, such as Hepa-1 and 10T1/2 fibroblast clonal sublines, are grown in suspension culture, AhR nuclear translocation and the induction of *CYP1A1* or *CYP1B1* mRNA occur in the absence of xenobiotic AhR ligands [17,18]. Additionally, preventing cell–cell interactions of the keratinocyte cell line HaCaT by growing at a low cell density or in Ca^{2+}-deficient S-MEM induces the nuclear accumulation of AhR and promotes the expression of a reporter gene driven by the xenobiotic responsive element (XRE) sequence [19]. In these cells, *Slug*, a member of the snail/slug family of zinc finger transcriptional repressors critical for epithelial–mesenchymal transitions, is transcriptionally activated following AhR nuclear translocation [20]. Furthermore, the second messenger cAMP, an endogenous mediator of hormone and neurotransmitter signaling, is also reported to activate AhR and lead to its nuclear translocation [21]. Finally, omeprazole, a benzimidazole derivative used clinically as an inhibitor of the gastric proton pump, induces CYP1A1 expression in an AhR-dependent manner, but it does not directly bind AhR [22,23]. Ligand-independent AhR activation suggests cross-talk between AhR and other signaling pathways, but further studies are needed to clarify the mechanisms regulating this pathway.

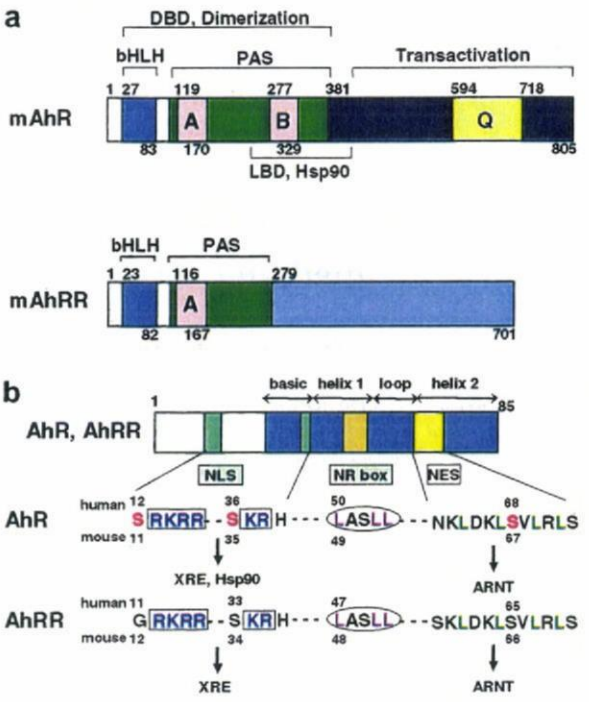

Fig. 1. (a) Schematic representation of functional domain of AhR and AhRR. A, B: weakly homologous repeated regions. Q: glutamine-rich transcription activation region. LBD, Hsp90 ligand binding and Hsp90 interaction domain. DBD DNA binding domain. (b) Schematic representation of the NH_2-terminal functional domains of AhR and AhRR in relation to signals for nuclear import or export. It is noted that a ligand-dependent nuclear import of AhR is inhibited by substitution of aspartic acid for Ser-12 or Ser-36 in human AhR [16], which mimics the negative charge of phosphorylation. On the other hand, a nuclear export of AhR is inhibited by substitution of Ser-68 in human AhR [19].

Transcriptional regulation of CYP1 genes by AhR

The TCDD-induced expression of *CYP1A1* is mediated through the XRE [24]. The core consensus sequence of XRE is 5′-TNGCGTG-3′, and this site is recognized by the AhR/Arnt heterodimer. Approximately 1 kb upstream of the *CYP1A1* gene, a cluster of XREs functions as an enhancer element, and a basic transcription element (BTE), a GC box sequence localized to the proximal

promoter of *CYP1A1*, is also required for the induction of *CYP1A1* [25].

Chromatin remodeling is initiated by liganded AhR/Arnt heterodimer binding to the XREs in the enhancer region, and this leads to increased DNAase sensitivity and the appearance of a DNAase hypersensitive site within 300 bp upstream of the transcription initiation site. BRG1, a component of the SWI/SNF ATP-dependent chromatin-modeling complex, is involved in the TCDD-dependent remodeling of the *CYP1A1* gene [26]. The AhR/Arnt heterodimer transactivates in conjunction with general transcription factors (GTFs) through interactions with coactivator proteins including CBP/p300, SRC-1, NCoA-2 and p/CIP, and the coactivator/corepressor protein RIP140 [27]. In addition, the TRAP/DRIP/ARC/Mediator complex must be recruited to the *CYP1A1* promoter to activate target gene expression in response to xenobiotic stress [28]. However, less is known about the factors regulating the induction of CYP1A2 expression, although the AhR/Arnt heterodimer is clearly required for this to occur. One study suggests that the AhR/Arnt heterodimer may function as a coactivator without directly binding the XRE. Instead, it may interact with other DNA-binding factors of a novel xenobiotic responsive element termed XREII to induce transcription activation [29].

The AhR repressor (AhRR) was identified as a negative regulator of AhR activity. AhRR contains both NLS and NES that are homologous to AhR (Fig. 1b), but AhRR is localized constitutively to the nucleus. Here, AhRR forms a heterodimer with Arnt, but XRE binding by the AhRR/Arnt heterodimer leads to transcriptional repression. Finally, AhRR expression is induced in an AhR-dependent manner, indicating that AhR and AhRR form a regulatory feedback loop [30] (Fig. 2).

Regulation of AhR protein stability

AhR is rapidly degraded both *in vivo* and *in vitro* following ligand binding, and several studies have examined the regulation of AhR degradation. When AhR was fused to the heterologous NLS of nucleoplasmin, it constitutively accumulated in the nucleus and was degraded in a 26S proteasome-dependent manner [31]. Conversely, when nuclear export of AhR was blocked by LMB, AhR accumulated in the nucleus following ligand binding and was not efficiently degraded. In this system, AhR degradation required both an NES and redistribution from the nucleus to the cytoplasm [32]. Although these data are hard to reconcile, a detailed understanding of the factors controlling the degradation of AhR is essential because this is an important component regulating AhR activity. Additionally, it is important to determine whether degradation of AhR is coordinated with cycles of transcriptional activation.

Functional role of AhR in physiology and toxicology

Over the past decade, many studies have examined AhR as a mediator of the adverse cellular response to environmental contaminants, such as TCDD and 3MC. However, the high degree of evolutionary conservation of AhR across a variety of animal species suggests AhR may possess xenobiotic-independent functions [33]. Indeed, a role

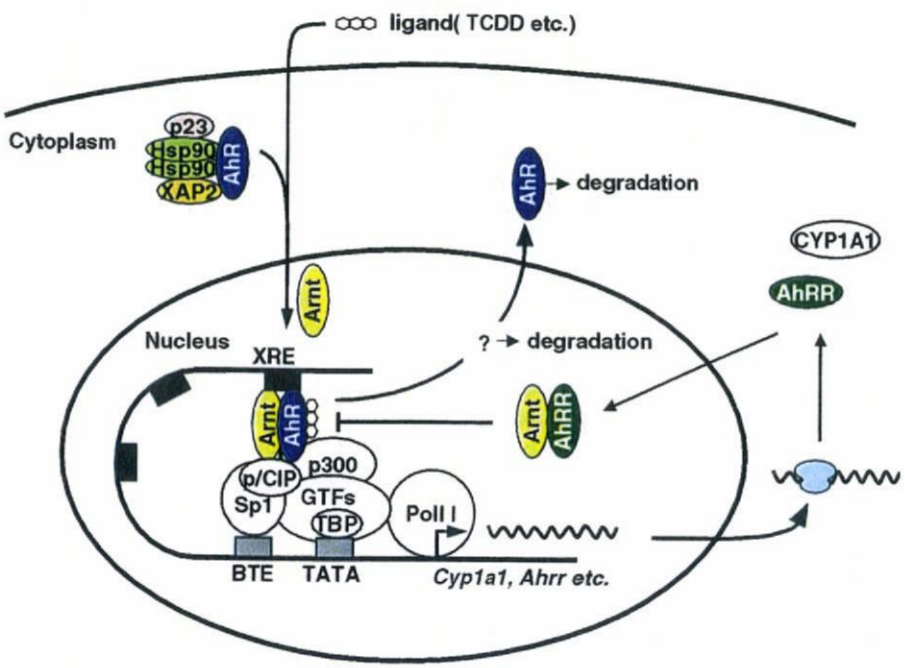

Fig. 2. A model of AhR signaling pathway [5].

for AhR in development was proposed based on the observed expression of AhR and Arnt during mouse embryogenesis [34]. In addition, activation of AhR has been linked to alterations in cell proliferation, apoptosis, adipose differentiation, tumor promotion, and immune and reproductive function. Consistent with these roles, several endogenous compounds, such as bilirubin [35], lipoxin A4 [36] and tryptophan derivatives including FICZ [37], have been isolated as potential natural ligands for AhR. Finally, the generation of AhR-null mice by three independent groups [38–40] has provided strong support for a variety of physiologic roles of AhR, e.g., homeostasis and development.

The role of AhR in carcinogenesis and teratogenesis

A number of papers have examined the role of AhR in regulating the cell cycle and proliferation. However, this remains controversial because studies have reached apparent conflicting conclusions as to whether AhR inhibits or promotes cell cycle progression. One recent report showed that AhR inhibited the growth of epithelial MCF-7 cells, but it promoted the proliferation of HepG2 hepatoma cells. Thus, the precise function of AhR in cell proliferation may differ in a cell type-dependent manner [41]. The constitutive expression of AhR induced tumors in the glandular part of the stomach [42] and increased hepatocarcinogenesis in transgenic B6C3F1 mice following a single injection of *N*-nitrosodiethylamine [43]. Thus, AhR may be oncogenic to varying degrees in different tissues. Consistent with this, AhR-null mice are resistant to benzo[*a*]pyrene-induced tumors [44], directly implicating AhR as a key factor in the development of environmental carcinogenesis. However, the role of AhR in the development of naturally occurring tumors remains largely unknown. In contrast, we recently identified a novel function for AhR as a tumor suppressor in colorectal carcinogenesis (manuscript in preparation).

A role for AhR in renal development has clearly been established. In wild-type mice, exposure to TCDD during development induces hydronephrosis, reduced kidney size, and some developmental renal disorders. In contrast, AhR-deficient mice are completely resistant to these TCDD-induced teratogenic effects [45]. Additionally, in humans with Wilms tumor, a form of renal cancer, there is a relatively high rate of loss of heterozygosity (LOH) at band 7p15-21. A minimal common region of LOH is located between markers *D7S*517 and *D7S*503 [46], and homozygous deletion of this region is frequently found in these tumors [47]. Interestingly, the *AhR* gene maps to this deleted region, suggesting that AhR may be a candidate for a Wilms tumor suppressor gene. Additionally, a recent paper showed that promoter hypermethylation is a novel epigenetic mechanism downregulating AhR activity in hematological malignancies such as ALL, and, in the patients studied, 33% exhibited some degree of AhR promoter hypermethylation [48].

The role of AhR in reproduction and vascular development

The fertility of AhR-null females is reduced, and the phenotype of these mice is similar to that seen with ARKO and ERα/ERβ double knockout mice. The litter size of AhR deficient mice was significantly decreased compared to wild-type mice [49], and this resulted from impaired folliculogenesis and ovulation in AhR deficient females [50]. An *in vitro* reporter gene assay and *in vivo* ChIP assay suggested that AhR synergistically cooperates with the orphan nuclear receptor Ad4BP/SF-1 to activate *CYP19* gene transcription in ovarian granulosa cells. CYP19 is thought to modulate ovarian estradiol concentrations and drive the estrus cycle. Thus, AhR plays a crucial role in female reproduction by regulating the expression of the ovarian P450 aromatase (CYP19), a key enzyme in estrogen synthesis.

Bradfield et al. [51,52] used a Cre-lox system to study AhR signaling in endothelial/hematopoietic cells, and AhR is necessary for the normal developmental closure of the ductus venosus. In mice unable to express AhR in hepatocytes, the patent ductus venosus results in massive portosystemic shunting of blood flow leading to a profound reduction in hepatocyte size. Although these studies clearly identified an important role for AhR in vascular development, the mechanisms of AhR action in this process remain largely unknown.

The role of AhR in inflammation and the immune system

Environmental exposure to polycyclic hydrocarbons (PAHs) may promote inflammatory and/or allergic disorders, and a role for AhR in inflammation has been suggested. Mice specifically expressing the constitutively active form of AhR (CA-AhR) in keratinocytes appeared normal at birth, but they developed severe skin lesions postnatally. These lesions histologically resembled atopic dermatitis, suggesting that the constitutive activation of the AhR signaling pathway is sufficient to trigger inflammatory skin lesions [53]. In contrast, lipoxins are eicosanoids with potent anti-inflammatory effects in many inflammatory diseases. Lipoxin A4 is a natural ligand for AhR, and it controls the migration of dendritic cells and production of interleukin-12 *in vivo*. Lipoxin A4 activates AhR and increases the expression of suppressor of cytokine signaling 2 (SOCS-2) [54]. Thus, the overwhelming activation of AhR may lead to dysregulated inflammation, but, under normal circumstances, AhR may play an anti-inflammatory role. Further studies are needed to clarify the molecular role of AhR in modulating inflammation.

Thymocyte development and T cell-dependent immune reactions are exquisitely sensitive to AhR-dependent TCDD toxicity. To better understand the role of AhR in T cell development and homeostasis, mice were generated transgenically expressing CA-AhR in T cells under the control of the CD2 promoter. AhR activation in T-lineage cells alone directly induced the thymocyte changes, and the

normal increase in splenocyte number following immunization did not occur in these mice. However, the number of resting splenocytes was not affected, suggesting that AhR functions in the growth of activated and proliferating T cells [55].

Using the B6-into-B6D2F1 model of acute graft-vs.-host disease, Kerkvliet et al. [56,57] showed that AhR activation in donor T cells generates a subpopulation of $CD4^+$ $CD25^+$hi T regulatory cells. They suggested that TCDD-mediated AhR activation preferentially activated these regulatory T cells which subsequently dampened the post-immunization T cell proliferation.

M50367, an orally active anti-allergy agent, is a ligand for AhR, and M50367 activation of AhR signaling skews the Th1/Th2 balance toward Th1 dominance, resulting in immunological responses with anti-allergic effects. This is completely abolished in AhR-null mice. Additionally, forced expression of a constitutively active form of AhR suppresses naïve Th cell differentiation into Th2 cells, demonstrating that AhR functions as a modulator of the *in vivo* Th1/Th2 balance through activation of AhR in naïve Th cells [58].

Taken together, these results suggest that AhR is intimately involved in a number of different aspects of immunological responses. The molecular mechanisms controlling AhR function in the immune system will be interesting to determine in future studies.

Conclusion

CYP1A1 is strongly induced by exogenous ligands such as TCDD, and a number of studies have examined the transcription factors and chromatin remodeling factors responsible for CYP1A1 induction. The genetic regulation of CYP1A1 expression is a good model system for the examination of the temporal and spatial recruitment of various transcription factors, nucleosomal remodeling factors and their interactions. Identifying the physiological functions of AhR, and clarifying the mechanisms responsible for its activation in both normal physiology and in response to xenobiotics will provide great insight into a variety of diverse cellular processes. Additionally, modulation of AhR signaling may be a good candidate for the development of therapies targeting endocrine or environmental diseases.

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LETTERS

Dioxin receptor is a ligand-dependent E3 ubiquitin ligase

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Fat-soluble ligands, including sex steroid hormones and environmental toxins, activate ligand-dependent DNA-sequence-specific transcriptional factors that transduce signals through target-gene-selective transcriptional regulation[1]. However, the mechanisms of cellular perception of fat-soluble ligand signals through other target-selective systems remain unclear. The ubiquitin–proteasome system regulates selective protein degradation, in which the E3 ubiquitin ligases determine target specificity[2–4]. Here we characterize a fat-soluble ligand-dependent ubiquitin ligase complex in human cell lines, in which dioxin receptor (AhR)[5–9] is integrated as a component of a novel cullin 4B ubiquitin ligase complex, $CUL4B^{AhR}$. Complex assembly and ubiquitin ligase activity of $CUL4B^{AhR}$ *in vitro* and *in vivo* are dependent on the AhR ligand. In the $CUL4B^{AhR}$ complex, ligand-activated AhR acts as a substrate-specific adaptor component that targets sex steroid receptors for degradation. Thus, our findings uncover a function for AhR as an atypical component of the ubiquitin ligase complex and demonstrate a non-genomic signalling pathway in which fat-soluble ligands regulate target-protein-selective degradation through a ubiquitin ligase complex.

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. This target selectivity depends on the recognition of specific DNA elements by sequence-specific transcription factors[1] and the recognition of degradation substrates by E3 ubiquitin ligases[2–4]. These transcription factors and ligases serve primarily as specific adaptors that subsequently recruit transcriptional co-regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. The selective biological effects of fat-soluble ligands have been reported to be mediated by two classes of sequence-specific transcription factors, nuclear receptors[1] and arylhydrocarbon receptor (AhR) belonging to the basic helix–loop–helix (bHLH)/Per-Arnt-Sim (PAS) family[5–9].

AhR ligands modulate oestrogen and sex hormone, signalling both positively and negatively[8,10–13]. Functional impairments of male and female reproductive organs in AhR-deficient mice indicate the possible importance of AhR in sex hormone signalling[10,14]. Different AhR agonists[9], including 3-methylcholanthrene (3MC) and 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD), modulate oestrogen-dependent oestrogen receptor (ER)-α transactivation through the association of activated AhR/Arnt with ER-α[15]. Similarly, the transcriptional activity of nuclear androgen receptor (AR) was modulated by association with activated AhR (Supplementary Fig. S2a). However, ligand-bound AhR did not block oestrogen-induced co-activator recruitment on the oestrogen-responsive promoter (Supplementary Fig. S2b). This implies another mode of function for ligand-activated AhR beyond transcriptional regulation.

On activation of AhR by 3MC, we observed that protein levels of endogenous ER-α (in mammary tumour MCF-7 cells), ER-β (in ovarian tumour KGN cells) and AR (in prostate cancer LNCaP cells) were drastically decreased (Fig. 1a–c, and Supplementary Fig. 3a) without a change in messenger RNA levels (data not shown), irrespective of the presence of their cognate hormones. Other AhR agonists[9] (namely β-naphthoflavone (β-NF), environmental toxins such as TCDD and benzo[a]pyrene, and the endogenous metabolite indirubin) were similarly effective in protein degradation for ER-α (Fig. 1b) and ER-β/AR (data not shown), in agreement with a previous report on downregulated levels of uterine ER-α protein in rats treated with TCDD[16]. An AhR partial agonist/antagonist α-naphthoflavone (α-NF) was unable to accelerate the degradation of either AhR or ER-α (Fig. 1b, and Supplementary Fig. S3b).

AhR ligand-induced degradation (Fig. 1a–c) and functional repression (Supplementary Fig. S2c, d) of sex steroid receptors were abrogated in the presence of a proteasome inhibitor MG132. Consistently, poly-ubiquitination of ER-α was promoted by the activated AhR regardless of the presence of oestrogen (Fig. 1d, and Supplementary Fig. S3c). Pulse-chase kinetic analysis indicated that 3MC-induced degradation of ER-α was coupled to that of AhR[8,17,18] (Supplementary Fig. S3d). Moreover, the self-ubiquitination activity of the ligand-bound AhR immunocomplex was detected in an E1/E2-dependent manner (Supplementary Fig. S3e). Together with 3MC-dependent recognition of sex steroid receptors by AhR[8,12,13,15], these properties of AhR resemble those of classical adaptor components of the E3 ubiquitin ligase complexes, such as F-box proteins[3] or von Hippel–Lindau protein[19]. We therefore reasoned that activated AhR might act as an E3 ubiquitin ligase complex component.

To address this idea, AhR-containing complexes were purified from HeLa cells expressing Flag–AhR treated with 3MC or α-NF[15,20]. AhR formed large complexes in the presence of 3MC (Supplementary Fig. S4a–c). Further purification revealed five major 3MC-dependent complexes containing AhR (Fig. 1e). Complexes A and C contained well-known co-activators TRAP220/DRIP205/Med220 and p300 (ref. 1) (Supplementary Fig. S4d, e). Endogenous ER-α was detected in complexes B and C; however, ubiquitinated components were seen only in complex B (Fig. 1f, g).

Complex B was composed of the ubiquitin ligase core components cullin 4B (CUL4B)[3,21,22], damaged-DNA-binding protein 1 (DDB1)[23–27] and Rbx1 (Roc1)[3], together with subunits of the proteasomal 19S regulatory particle (19S RP), Arnt and transducin-β-like 3 (TBL3) (Fig. 1h). These components eluted with AhR in the presence of 3MC but not in the presence of α-NF (Fig. 1i, and Supplementary Fig. S4f). Neither CUL4A nor known substrate-specific adaptor components of CUL4A, such as DDB2, CSA and DET1[23,24], were present

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in the AhR–CUL4B complex. As the cullin amino terminus binds adaptor components and the carboxy terminus interacts with an E2 enzyme-binding subunit Rbx1 (ref. 3), we performed tandem purification of the AhR–CUL4B complex with glutathione *S*-transferase (GST)-tagged CUL4B-N (N-terminal domain of CUL4B) and Flag–AhR. This led to the identification of a core complex consisting of five components: DDB1, AhR, Arnt, TBL3 and CUL4B (Fig. 1j). Together with Rbx1, this complex is denoted by $CUL4B^{AhR}$.

Immunoprecipitation of AhR together with endogenous CUL4B from MCF-7 and LNCaP cells was observed only in the presence of 3MC (Fig. 2a, b). Consistently, ligand-dependent co-localization of AhR with CUL4 was seen in MCF-7 cells (Fig. 2c). Whereas CUL4B seemed to act as a scaffold mediating DDB1–TBL3 and AhR–DDB1 interactions in $CUL4B^{AhR}$ (Fig. 2d, lane 4), ligand-activated AhR induced the assembly of complex components (Fig. 2d, lanes 1–3). DDB1 did not bridge CUL4B association with TBL3 or AhR, apparently because of the absence of the signature WDXR/DWD box[22,25–27] of either TBL3 or AhR, which is essential for DDB1 binding (Fig. 2d, lane 5, and Supplementary Fig. S5a). Consistently, specific and 3MC-dependent interaction of the conserved C-terminal acidic domain of AhR with the N-terminal region of CUL4B, but not with DDB1, was observed in a GST pulldown assay (Supplementary Figs S5b and S6). Because a constitutively active AhR mutant (AhRΔPASB)[9] interacted with CUL4B in the absence of ligand (Supplementary Fig. S5b), ligand-dependent structural alteration presumably induces AhR–CUL4B interaction. An AhR mutant lacking the CUL4B-binding

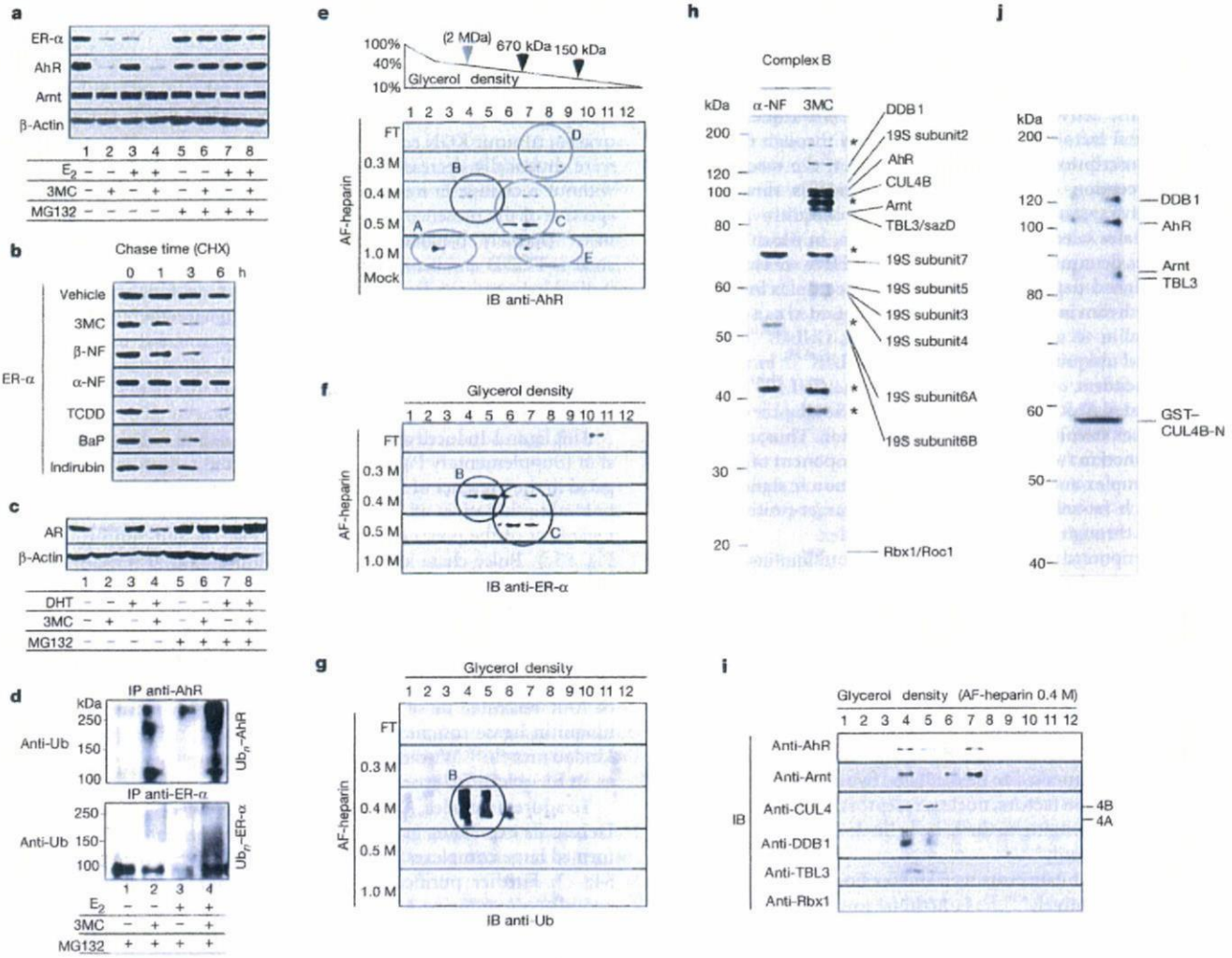

Figure 1 | Activated AhR acts as an E3 ubiquitin ligase. a–c, AhR-ligand-induced proteasomal degradation of ER-α (**a**, **b**) and AR (**c**). MCF-7 cells (**a**, **b**) and LNCaP cells (**c**) were incubated as indicated with E_2 (10 nM), DHT (10 nM) and/or 3MC (1 μM), β-NF (1 μM), benzo[a]pyrene (BaP; 100 nM), TCDD (10 nM), indirubin (10 nM) and α-NF (1 μM) in the presence or absence of MG132 (10 μM) and cycloheximide (CHX; 5 μM) for 3 h (**a**, **c**) or the indicated durations (**b**). Cell lysates were subjected to western blotting with specified antibodies. **d**, AhR-ligand-induced ubiquitination of ER-α. MCF-7 cells were incubated with the indicated ligands for 6 h. Western blots were subjected to dark exposure to detect poly-ubiquitinated forms of the receptors. IP, immunoprecipitation; Ub, ubiquitin. **e**, **f**, Biochemical separation and identification of AhR-associated complexes. Flag–AhR-associated proteins in the presence of 3MC or α-NF from HeLa cells stably expressing Flag–AhR were first fractionated by glycerol-density-gradient centrifugation (top, fractions 1–12), and then separated by Toyopearl AF-heparin column chromatography with the indicated KCl concentrations (FT, 1.0 M KCl). Samples from the 3MC-treated cells were resolved into five distinct complexes. IB, immunoblotting. **g**, Components of an AhR-associated complex are highly ubiquitinated. Western blots with anti-ubiquitin antibody. **h**, Identification of AhR-associated CUL4B ubiquitin ligase complex components. Components from complex B in **e** (fractions 4 and 5 from the glycerol-density-gradient centrifugation, eluted from an AF-heparin column at 0.4 M KCl) were resolved by SDS–PAGE, silver-stained and identified by matrix-assisted laser desorption ionization–time-of-flight MS analysis. **i**, Co-elution of the complex B components as a large complex. **j**, Association of activated AhR with the CUL4B complex. The $CUL4B^{AhR}$ complex from Flag–AhR-expressing HeLa cells treated with 3MC was affinity purified with GST-tagged N-terminal domain of CUL4B followed by anti-Flag antibody column fractionation.

acidic domain (AhRΔacid; Supplementary Fig. S6a) was indeed unable to promote ER-α ubiquitination *in vivo*, although the mutant retained 3MC-dependent transactivation function (Supplementary Fig. S5c). This indicates that the ubiquitin ligase function of AhR is independent of its transactivation function.

With two separately prepared components of recombinant AhR and CUL4B/DDB1/Rbx1 purified from *Spodoptera frugiperda* (Sf9) cells (Supplementary Fig. S7a), complex assembly *in vitro* was also dependent on 3MC (Fig. 2e). Furthermore, by *in vitro* ubiquitination assay (Supplementary Fig. S7b), the E3 ubiquitin ligase activity of $CUL4B^{AhR}$ for ER-α was dependent on 3MC but not on 17β-oestradiol (E_2) (Fig. 2f). These data indicate that both the complex assembly and the ubiquitin ligase activity of $CUL4B^{AhR}$ may be dependent on AhR agonists.

We then examined whether the recognition of sex steroid receptors for 3MC-dependent ubiquitination is indeed mediated by AhR. Co-immunoprecipitation analyses indicated that ligand-activated AhR was required for the recruitment of ER-α (Fig. 2a, d) or AR (Fig. 2b, and data not shown) to $CUL4B^{AhR}$. TBL3 and DDB1 did not seem essential for ER-α recruitment but stabilized the association of ER-α with $CUL4B^{AhR}$ (Fig. 2d). Moreover, knockdown of $CUL4B^{AhR}$ components (Supplementary Fig. S8) impaired the 3MC-induced ubiquitination and degradation of ER-α (Fig. 3a–d, and Supplementary Fig. S9a, b) and AR (Fig. 3e, Supplementary Fig. S9c and data not shown), and abolished the AhR-ligand-induced repression of ER-α transactivation (Supplementary Fig. S10a). Recognition of ER-α by activated AhR was retained, but ubiquitination of AhR-bound ER-α was abrogated, by knockdown of the other $CUL4B^{AhR}$ components (Fig. 3d). An ER-α ΔA/B mutant[15] that lacks interaction with AhR, and an ER-α K7R mutant in which seven lysine residues had been replaced with arginine (Supplementary Fig. S6b), were resistant to AhR-dependent ubiquitination and transrepression (Fig. 3f, and Supplementary Fig. S10b). Taken together, these data suggest that ligand-activated AhR functions as a substrate-specific adaptor component of $CUL4B^{AhR}$. AhR is therefore a unique and atypical substrate-specific component of a cullin-based E3 complex, because AhR bears no known interaction motif with cullin complexes yet associates directly with CUL4B. Ubiquitination of ER-α-associated AhR was similarly abolished by the knockdown, and the overall ubiquitination and degradation of AhR[8,17,18] were partly affected (Supplementary Fig. S11a, b). This implies the existence of $CUL4B^{AhR}$-dependent (self-ubiquitination[3]) and $CUL4B^{AhR}$-independent pathways for AhR degradation.

Human ER-α (hER-α) degradation is reportedly accelerated by the binding of E_2 (ref. 1) or the phosphorylation of Ser 118 (ref. 28), whereas a partial antagonist, tamoxifen, has been shown to stabilize ER-α[1]. Nevertheless, 3MC-activated AhR efficiently induced the ubiquitination and subsequent degradation of tamoxifen-bound ER-α and ER-α-S118A mutant (Fig. 3f). Reciprocally, AhR was dispensable for E_2-dependent ER-α degradation (Supplementary Fig. S11c). These results indicate that the $CUL4B^{AhR}$ system may act independently of innate protein degradation system(s) for ER-α. XAP2/ARA9/AIP[7,8,17], a chaperone that modulates the stability of unliganded AhR, seemed unlikely to mediate the accelerated degradation of ER-α by activated AhR (Supplementary Fig. S11d).

Last, we addressed the physiological significance of $CUL4B^{AhR}$ for sex hormone signalling in intact animals. Injection with either 3MC (Fig. 4a) or β-NF (Fig. 4c) did not affect the expression of ER-α or AR mRNA (data not shown) but caused a decrease in protein levels of uterine ER-α in ovariectomized female wild-type mice and of prostate AR in castrated male wild-type mice (Fig. 4b) regardless of their treatment with cognate sex hormones. However, AhR deficiency ($AhR^{-/-}$ mice)[9,14] abolished such effects of AhR ligands but did not affect the modulation of stability of sex steroid receptors by their respective hormones (Fig. 4a, b). As a result of reduced sex steroid receptor levels after pretreatment with 3MC, E_2-dependent induction of *c-fos* in the uterus[15] and dihydrotestosterone (DHT)-dependent induction of *Probasin* in the prostate[10] were severely impaired (Fig. 4a, b). Cellular proliferation and gene induction in response to sex hormones in primary cultured epithelial cells from normal mouse uterus and prostate were consistently suppressed by 3MC (Supplementary Fig. S12a, b) and β-NF (Supplementary Fig. S12c), but no effect was detected in $AhR^{-/-}$ cells (Supplementary Fig. S12a, b). The significance of $CUL4B^{AhR}$ complex components in the AhR-mediated suppression of sex hormone effects

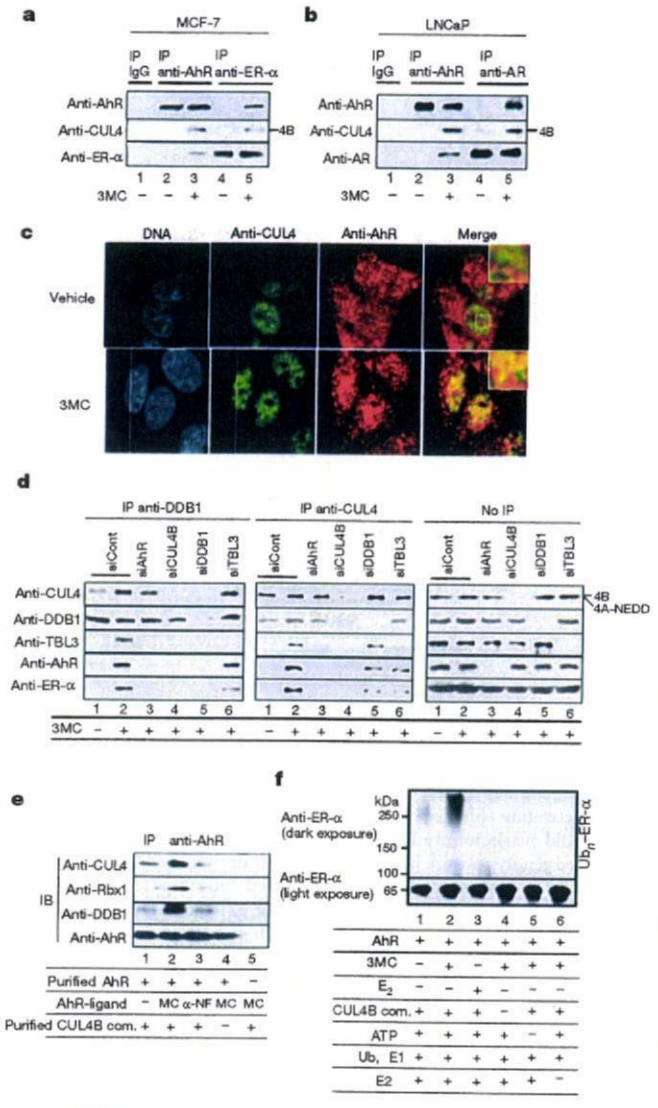

Figure 2 | AhR ligand-dependent assembly and ubiquitin ligase activity of $CUL4B^{AhR}$. **a**, **b**, 3MC-dependent association of endogenous CUL4B and AhR with ER-α and AR. Co-immunoprecipitation analyses from MCF-7 (**a**) and LNCaP (**b**) cells incubated with ligand and MG132 for 2 h. IP, immunoprecipitation. **c**, 3MC-dependent co-localization of AhR with CUL4. MCF-7 cells incubated with 3MC and MG132 for 2 h were immunostained with the indicated antibodies. **d**, Formation of the $CUL4B^{AhR}$ complex. MCF-7 cells were transfected with specified short interfering RNAs (siRNAs) for 48 h, treated with 3MC and MG132 for 2 h, and immunoprecipitated with the indicated antibodies. **e**, Assembly of the CUL4B complex components with AhR is dependent on 3MC *in vitro*. Immunoprecipitation with anti-AhR antibodies of the indicated recombinant CUL4B complex components (CUL4B com.) was observed only in the presence of 3MC. IB, immunoblotting. **f**, $CUL4B^{AhR}$ ubiquitinates ER-α *in vitro*. ER-α protein was incubated with and without recombinant $CUL4B^{AhR}$ E3 complex components, ubiquitin (Ub), ATP, E1 and E2 enzymes as indicated, then subjected to western blotting.

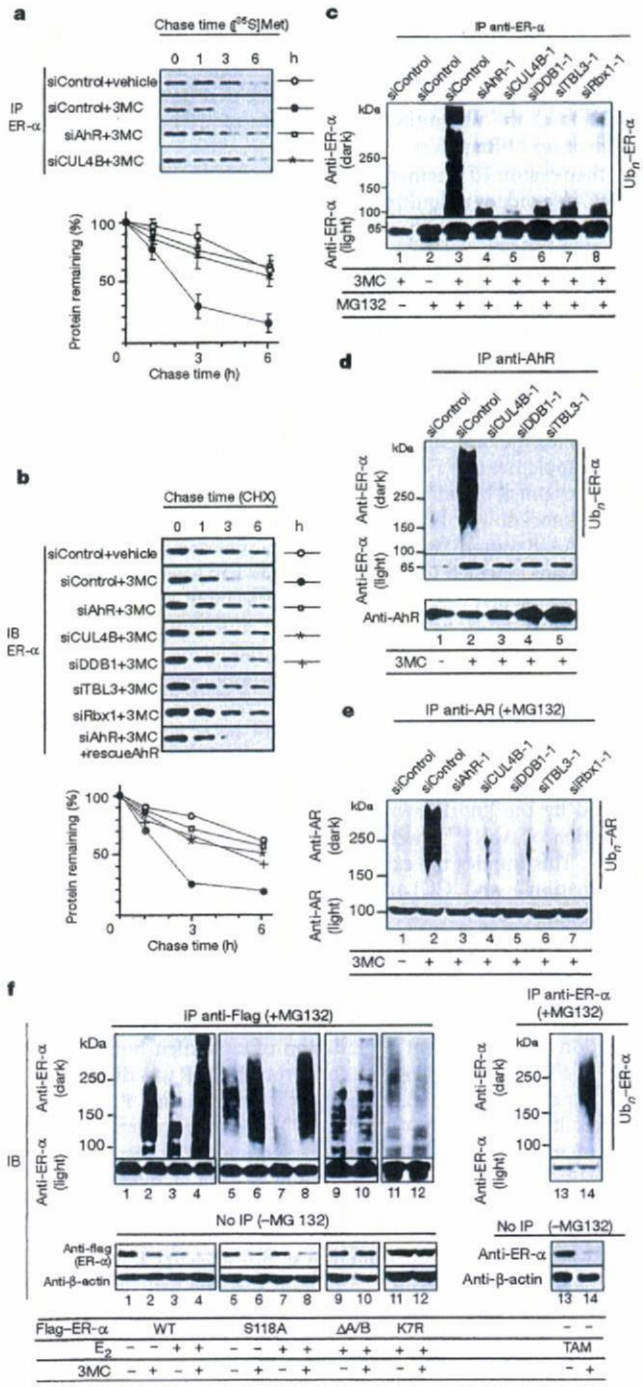

Figure 3 | Activated AhR is a substrate-specific adaptor component of the $CUL4B^{AhR}$ complex. **a–c**, Components of $CUL4B^{AhR}$ are required for 3MC-dependent ubiquitination and degradation of ER-α. MCF-7 cells were transfected with indicated siRNAs for 48 h, then used in pulse-chase analysis as in Supplementary Fig. S3d (**a**), in cycloheximide (CHX) chasing (**b**) and in the *in vivo* ubiquitination assay with ligand incubation for 6 h (**c**). All values are shown as means ± s.d. ($n = 3$) (**a**) or as means ($n = 3$) (**b**). The knockdown efficiency in the same lysates was confirmed in Supplementary Fig. S9a. IB, immunoblotting; IP, immunoprecipitation. **d**, AhR is the substrate-specific adaptor in the targeting of ER-α by $CUL4B^{AhR}$. MCF-7 cells transfected with the indicated siRNAs were lysed in TNE buffer and immunoprecipitated with anti-AhR antibody in the presence of MG132. Ubiquitination of the ER-α co-immunoprecipitated with AhR was detected by western blotting. **e**, LNCaP cells were subjected to the same analysis as in **a–c**. **f**, AhR-ligand-induced ER-α ubiquitination requires intact lysine

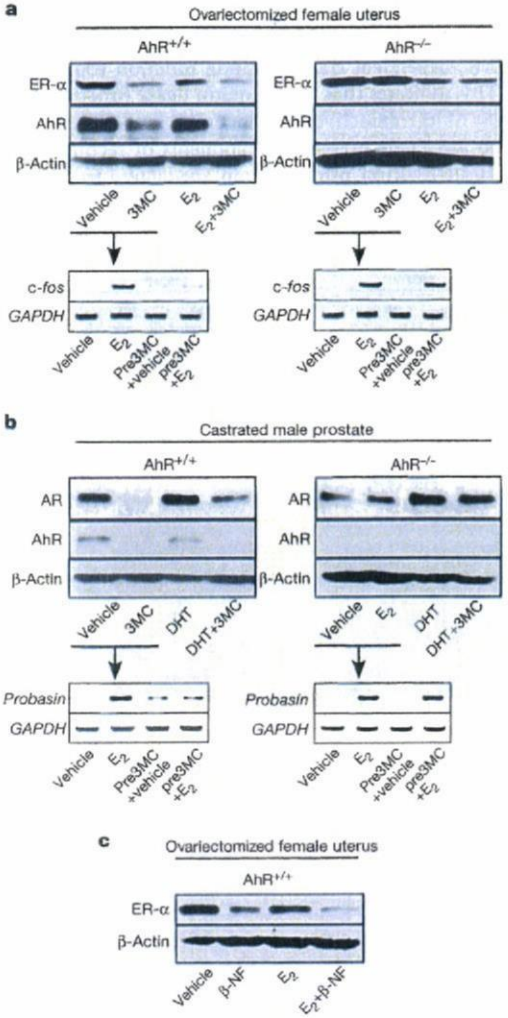

Figure 4 | Ligand-dependent ubiquitin ligase function of AhR *in vivo*. **a**, **b**, AhR activation enhances the degradation of ER-α and AR *in vivo*. Top: nine-week-old ovariectomized female mice (**a**) or castrated male mice (**b**) of the indicated genotypes were injected with vehicle or indicated ligands. After 4 h, uterus (**a**) or ventral prostate (**b**) was isolated and subjected to western blotting. Bottom: mice pretreated with vehicle or 3MC for 8 h were injected with either vehicle or E_2 (**a**), or DHT (**b**). After 4 h, the uterus or prostate was isolated for reverse transcriptase PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **c**, Other AhR agonists produce a similar effect on oestrogen signalling to that of 3MC.

(Supplementary Fig. S12a, b) and the promotion of ER-α degradation in uterine cells (Supplementary Fig. S12d) was verified by knockdown of the components.

Here we have shown that a known sequence-specific transcription factor AhR acts as a ligand-dependent CUL4B-based E3 ubiquitin ligase for selectively targeting sex steroid receptors to bring about accelerated protein degradation. The transcription and ubiquitination functions of AhR seem to be responsible for a distinct set of biological events caused by endogenous and exogenous AhR ligands. In ubiquitin ligase complexes, substrate recognition by known

residues and is independent of oestrogen binding or S118 phosphorylation of hER-α. Intact MCF-7 cells (right) or cells transfected with Flag–hER-α, AhR and their derivatives (left) were treated with the indicated ligands in the presence (top) or absence (bottom) of MG132 for 6 h, then subjected to western blotting. TAM, tamoxifen; WT, wild type.

substrate-specific components is generally evoked by substrate modifications[2–4]. However, the recognition and subsequent ubiquitination of sex steroid receptors by AhR requires dioxin-type compounds as ligands but does not require the phosphorylation or ligand binding of sex steroid receptors. We have therefore shown that fat-soluble ligands directly control the function of a ubiquitin ligase complex for targeted protein destruction in animals (see Supplementary Fig. S1). In plants, auxin was recently found to control protein destruction through the auxin receptor SCF^{TIR1} (refs 29, 30). However, whereas SCF^{TIR1} is regulated by ligand-dependent substrate recognition by TIR1, $CUL4B^{AhR}$ is primarily regulated by the assembly of a ligand-dependent complex as well as substrate recognition. Considered together, ubiquitin-ligase-based perception mechanisms of fat-soluble ligands may be diverse in different species. It is possible that other nuclear receptors and binding proteins for fat-soluble ligands also serve as key components of ubiquitin ligases to mediate a non-genomic pathway of fat-soluble ligands to regulate target-protein-selective destruction.

METHODS

More detailed descriptions of all materials and methods are supplied in the Supplementary Information.

Biochemical purification and separation of AhR-associated complexes. The nuclear extracts preparation, anti-Flag affinity purification and mass spectrometry were performed as described previously[15,20]. For purification of the core $CUL4B^{AhR}$ complex, the nuclear extracts were first bound to the GST–CUL4B-N (amino acid residues 1–318) columns before being loaded on anti-Flag columns[20].

***In vitro* ubiquitination assay.** The *in vitro* ubiquitination assay was performed as described previously[23]. Purified Flag–AhR (0.2 μg) was incubated either with 3MC (10 μM) or vehicle (dimethylsulphoxide) for 30 min at 25 °C, then mixed with Flag–CUL4B/DDB1/Rbx1 complex (0.2 μg), and after further incubation for 30 min at 25 °C the substrate, ER-α (Calbiochem), was added.

Plasmids, antibodies, immunoprecipitation, *in vivo* ubiquitination, pulse-chasing, ligand responses in mice, and RNA-mediated interference experiments. Detailed methods used in this study can be found in the Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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