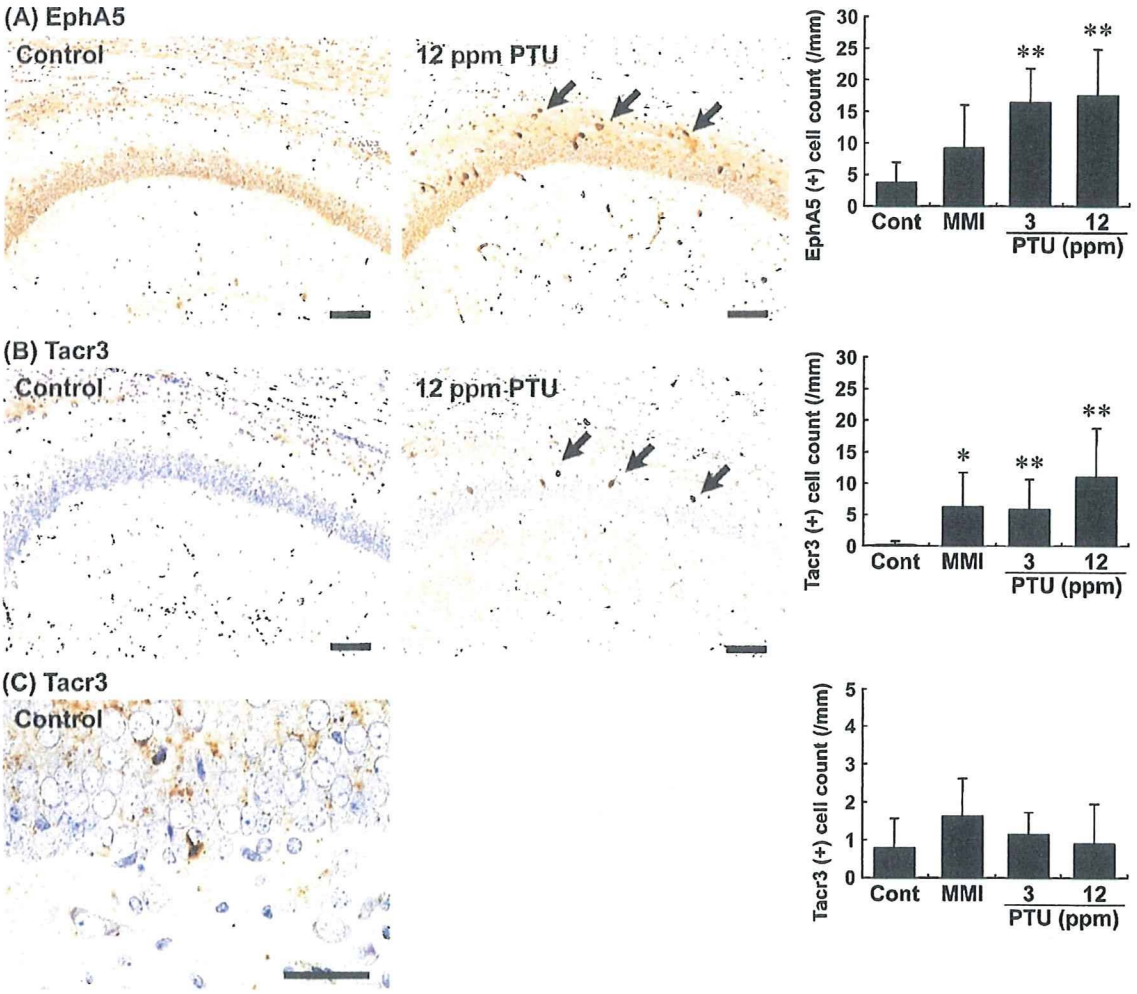

Fig. 3. Distribution of immunoreactive cells for EphA5 and Tacr3 in the hippocampal formation in rats at PND 20 after maternal exposure to anti-thyroid agents. (A) EphA5-immunoreactive cells with strong intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Note the higher number of EphA5-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Bar =100 μm. The graph shows the number of EphA5-positive cells/unit length (mm) of the CA1 region of the bilateral hemispheres. ** $P<0.01$ versus untreated controls (Student's *t*-test). (B) Tacr3-immunoreactive cells with strong intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Note the higher number of Tacr3-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Bar =100 μm. The graph shows the number of Tacr3-positive cells/unit length (mm) of the CA1 region of bilateral hemispheres. * $P<0.05$, ** $P<0.01$ versus untreated controls (Student's *t*-test). (C) Tacr3-immunoreactive cells located in the subgranular zone of the dentate gyrus. Bar =50 μm. The graph shows the number of Tacr3-positive cells/unit length (mm) of the subgranular zone of bilateral hemispheres. Abbreviations: EphA5, Ephrin type A receptor 5; MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil, Tacr3, Tachykinin receptor 3.

ampal formation: Immunohistochemical localization of EphA5 and Tacr3 in the hippocampal formation was examined at PND 20 and PNW 11.

On PND 20, EphA5 showed weak immunoreactivity in the pyramidal neurons throughout the hippocampal formation in the untreated controls. This immunoreactivity was unchanged by exposure to anti-thyroid agents. On the other hand, very sparse distribution of strongly immunoreactive cells for EphA5 was observed in the region of the CA1 pyramidal cell layer and stratum oriens in the untreated control animals, but immunoreactive cells were significantly increased showing scattered distribution by PTU at both 3 and 12 ppm (Fig. 3A). MMI-exposed animals also showed a small increase in the number of strongly positive cells with EphA5. Increased intensity in immunoreactivity of EphA5 was also observed in the gray matter consisting of neuropil at the stratum oriens of the CA1 region (Fig. 3A), and also in the molecular layer of the dentate gyrus at PND 20 after exposure to anti-thyroid agents, especially in PTU-exposed groups (data not shown).

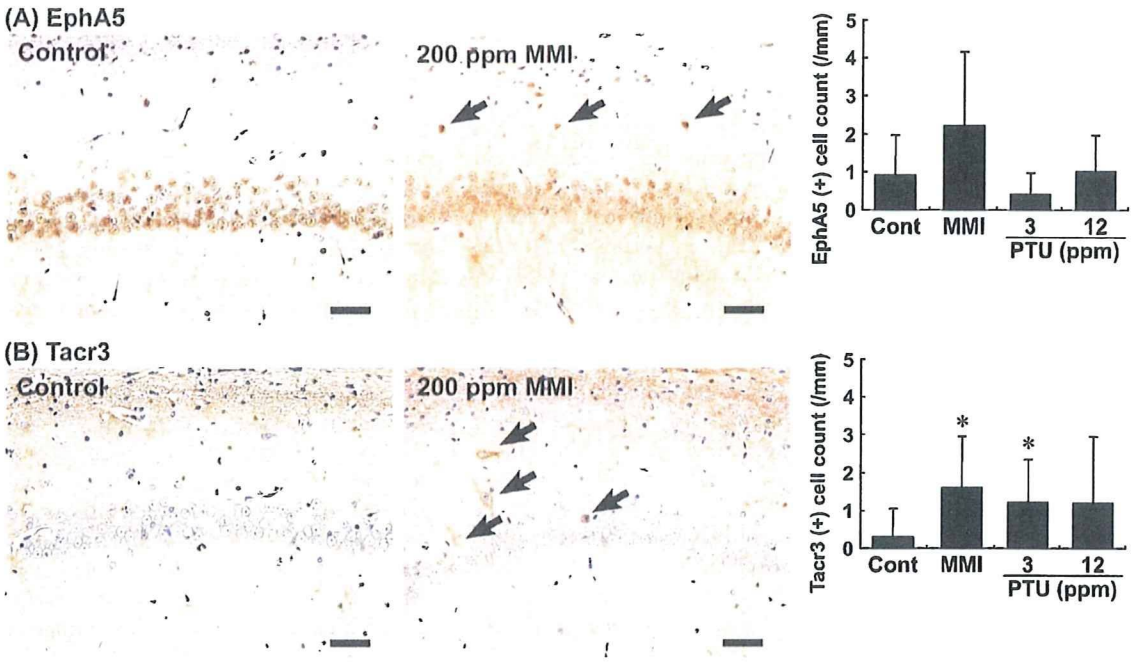

Fig. 4. Distribution of immunoreactive cells for EphA5 and Tacr3 in the hippocampal formation at PNW 11 of rats exposed maternally to anti-thyroid agents. (A) EphA5-immunoreactive cells with moderate staining intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region. EphA5-positive cells in a case exposed to 200 ppm MMI (Right) as compared with the control animal (Left). The arrows show positive cells. Bar =50 μm. The graph shows the number of EphA5-positive cells/unit length (mm) of the CA1 region of the bilateral hemispheres. (B) Tacr3-immunoreactive cells with weak to moderate staining intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Immunoreactivity is rather faint as compared with that observed at PND 20. Note the higher number of Tacr3-positive cells in a case exposed to 200 ppm MMI (Right) as compared with the control animal (Left). Bar =50 μm. The graph shows the number of Tacr3-positive cells/unit length (mm) of the CA1 region of bilateral hemispheres. * $P<0.05$ versus untreated controls (Student's *t*-test). Abbreviations: EphA5, Ephrin type A receptor 5; MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil; Tacr3, Tachykinin receptor 3.

With regards to Tacr3, the number of positive cells was increased with a scattered distribution showing strong intensity in the CA1 region similarly to that of EphA5 in the animals exposed to MMI or PTU on PND 20, but they were mostly absent in the untreated controls (Fig. 3B). Similarly, Tacr3-immunoreactive cells were sparse in the subgranular zone of the dentate gyrus in the MMI and PTU-exposed animals and in the untreated controls, but there were no differences in the number of positive cells as compared with the untreated controls (Fig. 3C). In addition, increased intensity in neuropil-immunoreactivity of Tacr3 was also observed in the strata oriens and radiatum of the CA1 region in all exposure groups of anti-thyroid agents (Fig. 3B).

On PNW 11, EphA5 showed weak immunoreactivity in the pyramidal neurons throughout the hippocampal formation in the untreated controls. This immunoreactivity was unchanged by exposure to anti-thyroid agents. EphA5-immunoreactive cells with moderate staining intensity were very sparsely observed in the region of the CA1 pyramidal cell layer and stratum oriens in the untreated control animals. There was no statistically significant increase in the number of these immunoreactive cells after exposure to PTU, while animals exposed to MMI showed a tendency for an increased number of immunoreactive cells (Fig. 4A). Increased neuropil-immunoreactivity of EphA5 as observed at PND 20 in exposure groups of anti-thyroid agents was mostly disappeared at PNW 11 (data not shown).

As well as at PND 20, Tacr3-immunoreactive cells were mostly absent in the untreated controls at PNW 11; however, a few immunoreactive cells with weak to moderate intensity were observed in the stratum oriens of the CA1 region in the animals exposed to anti-thyroid agents. There was a statistically significant difference in the animals treated with MMI or 3 ppm PTU compared with the untreated controls (Fig. 4B). Although the change was non-significant and lacked dose-dependence, 12 ppm PTU also showed an increasing tendency in the number of Tacr3-immunoreactive cells. In addition, increased neuropil-immunoreactivity of Tacr3 as observed at PND 20 in exposure groups of anti-thyroid agents was mostly disappeared at PNW 11 (data not shown).

DISCUSSION

In our recent study using rats [24], after maternal exposure to MMI or PTU, we detected typical hypothyroidism-related changes in the thyroid-related hormone levels, and hippocampal CA1 pyramidal neurons due to neuronal mismigration, as previously reported [8]. We also observed white matter changes, which seem to be due to impaired oligodendroglial development [6, 21]. To visualize molecules related to impaired neuronal development, microdissected CA1 region-specific global gene expression profiling was performed in the present study using the same animals that were used in our previous study. Two recently published studies have used microarrays to examine the expression profiles in the cerebral cortex and hippocampus of genes linked to developmental hypothyroidism caused by maternal PTU-exposure [7, 19]. In accordance with these studies, the genes that were significantly down-regulated in the present study included those that play roles in myelination, such as *Mobp* and myelin-associated glycoprotein, suggestive of the reflection of suppressed myelination by developmental hypothyroidism [21]. However, the genes that were found to be up-regulated on microdissected CA1 pyramidal cell layer, including *Efna5* and *Tacr3*, in the present study, have not been identified in previous studies. This difference may be related to the target tissues collected and the methods used, including microdissection of CA1 pyramidal cell layer from paraffin-embedded sections in the present study versus manual dissection of the cortical tissues from unfixed tissues in the previous studies.

EphA5 is a tyrosine kinase receptor that is almost exclusively expressed in the nervous system [15]. EphA5 and its ligand are important in mediating axon guidance, topographic projection, development, cell migration and the plasticity of limbic structures [15]. In addition, the transient expression of EphA5 during development is correlated with early neurogenesis and the migration of differentiated cells in the midbrain [3]. Thus, although expression of EphA5 was mostly weak in the euthyroid CA1 pyramidal neurons at PND 20, the increased number of EphA5-expressing cells with strong intensity in the CA1 region during developmental hypothyroidism in the present study reflects the neuronal mismigration caused by anti-thyroid agents. However, this increase was recovered after cessation of developmental hypothyroidism. Ephrins and their receptors are recently identified molecules and functional relationship between subfamily proteins is largely unknown; however, we, in the present study, found down-regulation of Epha7, another subfamily ephrin receptor, in all exposure groups of anti-thyroid agents (Table 1).

Tacr3, a member of the mammalian tachykinin peptide neurotransmitter/neuromodulator receptor family, is predominantly expressed in neurons in both the peripheral and central nervous systems, including the hippocampus [25]. There is increasing evidence of the role of Tacr3 on the survival and function of dopaminergic neurons. The survival of mesencephalic dopaminergic neurons during development largely depends on excitatory inputs, and tachykinins, through their receptors, are reported to play role in excitation [20]. On the other hand, senktide, a Tacr3 agonist, activates dopaminergic neurons to stimulate the release of dopamine and serotonin, and hyperlocomotion in gerbils [14]. Abnormal excitatory action of D_2-like receptor, one of the major subtypes of dopaminergic receptors, was observed on glutamatergic transmission in the CA1 synapses in the adult stage of rats after developmental hypothyroidism, suggesting a permanent disruption of synaptic integration in the CA1 neural networks [16]. While the role of Tacr3 in the hippocampal CA1 region during development is not clear, the increase in Tacr3-positive cells with strong intensity in this region during developmental hypothyroidism suggests a cell survival effect of tachykinin-3. Although the magnitude of the change was decreased, as compared with that at the end of the developmental hypothyroidism, the increased number of Tacr3-positive cells in the CA1 region of MMI and 3 ppm PTU-exposed animals may be an outcome of permanent disruption of synaptic integration, as described by Oh-Nishi *et al.* [16]. However, sparse distribution of Tacr3-positive cells may reflect that impairment sustained in a small population of aberrantly migrated neurons.

In conclusion, in this study, we have shown gene expression profiles showing altered expression in response to developmental hypothyroidism by analysis on microdissected hippocampal CA1 pyramidal cell layer in rats. Immunohistochemical analysis of the two candidate molecules revealed that developmental hypothyroidism until weaning is associated with the persistence of Tacr3-expressing neurons until the adult stage in the CA1 region, suggestive of the reflection of permanent disruption of synaptic integration. These findings probably reflect a mechanism to facilitate cell survival of aberrantly developed neurons due to mismigration.

ACKNOWLEDGMENT(S). We thank Miss Tomomi Morikawa for her technical assistance in conducting the animal study. We also thank Mrs. Shigeko Suzuki and Miss Ayako Kaneko for their technical assistance in preparing the histological specimens. This work was supported in part by Health and Labour Sciences Research Grants (Research on Risk of Chemical Substances) from the Ministry of Health, Labour and Welfare of Japan. All of the authors disclose that there are no conflicts of interest that could inappropriately influence the outcomes of the present study.

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J. Biochem. 2010;147(3):353–360 doi:10.1093/jb/mvp169

JB THE JOURNAL OF BIOCHEMISTRY

Suppression of AhR signaling pathway is associated with the down-regulation of UDP-glucuronosyltransferases during BBN-induced urinary bladder carcinogenesis in mice

Received August 18, 2009; accepted October 15, 2009; published online October 29, 2009

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Down-regulation of carcinogen detoxifying enzymes might be a critical factor in tumour formation by increasing the carcinogen concentration in the target organ. Previous reports revealed that the expression of *UGT1A* mRNA is either lost or decreased in certain human cancer tissues, including urinary bladder cancer. To elucidate this down-regulation mechanism, we used an *N*-nitrosobutyl (4-hydroxybutyl) amine (BBN)-induced mouse urinary bladder carcinogenesis model. Similar to human cancer, the expressions of *Ugt1a6*, *Ugt1a9* and total *Ugt1a* mRNA in the BBN-induced bladder cancer were markedly decreased compared with those of normal mice. BBN down-regulated the basal *Ugt1a* mRNA expression in a time-dependent manner and this was reversible in the first 2 weeks of BBN treatment. However, after 4 weeks of BBN treatment the repression became persistent after the cessation of BBN treatment. Aryl hydrocarbon receptor (AhR) regulates the constitutive and inducible expression of *Ugt1a* mRNA. We found that the constitutive *Ugt1a* mRNA expression is decreased in the bladder of AhR knockout (KO) mice. Furthermore, BBN-induced *Ugt1a* down-regulation was lost in AhR KO mice, and the canonical AhR target gene *Cyp1a1* was similarly down-regulated by BBN in the bladder. These results demonstrate that BBN repressed *Ugt1a* mRNA expression *via* suppression of AhR signaling pathway during BBN-induced carcinogenesis.

Keywords: UDP-glucuronosyltransferase/urinary bladder carcinogenesis/AhR.

Abbreviations: AhR, Aryl hydrocarbon receptor; BBN, *N*-nitrosobutyl (4-hydroxybutyl) amine; Cyp1a1, cytochrome P450, family 1, subfamily a, polypeptide 1; Gstp1, Glutathione *S*-transferase, placental isoform 1; 3-MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; UGT, UDP-glucuronosyltransferase.

Down-regulation of carcinogen detoxifying enzymes in target organs is likely to play a critical role in chemically induced carcinogenesis by leading to an increased local concentration of carcinogens (*1–5*). UDP-glucuronosyltransferases (UGTs) contribute to cellular detoxification through their glucuronidation of potentially toxic carcinogens and xenobiotics (*6–8*) and are thereby key players in the defense mechanism against chemical-induced carcinogenesis (*9*) and teratogenesis (*10*). Carcinogens, such as aromatic amines and polycyclic aromatic hydrocarbons (PAHs), are detoxified by conjugation with glutathione or UDP-glucuronic acid (*11, 12*). The 19 human *UGT* cDNAs identified so far include nine *UGT1A* genes encoded by a single *UGT1A* locus on chromosome 2 and 10 individually encoded *UGT2* genes on chromosome 4 (*13*). *UGT1A* genes mainly catalyse the glucuronidation of aromatic amines and PAHs (*13*) and down-regulation of *UGT1A* gene expression has been associated with liver, digestive tract and urinary bladder tumors in human (*1–4*).

Each *Ugt1a* gene consists of a gene specific protein-encoding first exon and 2–5 common exons. Gene-specific promoters 5′ of the first exons control the specific expression of individual *Ugt1a* genes. Basal *Ugt1a* mRNA expression is regulated tissue-specifically by several transcription factors, such as hepatocyte nuclear factor 1 and CAAT-enhancer binding protein (*14, 15*). On the other hand, the inducible expression of *Ugt1a* mRNA is regulated by several xenobiotic receptors including pregnenolone X receptor, constitutive androstane receptors, peroxisome proliferators-activated receptors, liver X receptor, aryl hydrocarbon receptor (AhR) and NF-E2-related factor 2 (Nrf2) (*16–20*).

The AhR regulates inducible expression of both phase 1 and phase 2 drug metabolizing enzyme genes (*21, 22*). AhR is usually sequestered in the cytoplasm in association with Hsp90, p23 and XAP2/ARA9. Upon binding to halogenated or polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene (3-MC),

respectively, AhR translocates into the nucleus, and dimerizes with its partner molecule, Arnt (AhR nuclear translocator), and then binds to its cognate enhancer sequences called XREs in the regulatory region of *Ugt1a*, *Cyp1a1* and glutathione S-transferase a1 (*Gsta1*) genes (*23–25*). Chen *et al.* (*26*) recently showed that the AhR controls *UGT1A* gene expression more profoundly than was previously anticipated from transgenic mouse studies with the human *UGT1A* locus. Although a distribution of XREs occurs immediately upstream of the *UGT1A1*, *UGT1A6* and *UGT1A9* first exons, TCDD treatment activated the expression of all the human *UGT1A* genes in small and large intestines, suggesting that AhR regulates the transcriptional activity of the whole *UGT1A* locus (*26*). Nrf2 also plays important role in *Ugt1a* gene expression. Nrf2 is activated by electrophiles, such as oltipraz and sulforaphane, and coordinately regulates expression of phase 2 drug metabolizing enzymes including Ugt1a6 and Gsts (*27*, *28*).

Oral administration of *N*-nitrosobutyl(4-hydroxybutyl)amine (BBN) to rodents induced cancer specifically in urinary bladder (*29*). BBN itself is either metabolized by alcohol/aldehyde dehydrogenase-mediated oxidation to yield *N*-nitrosobutyl (3-carboxypropyl)amine (BCPN) or by UGT to form BBN-glucuronide conjugate which is easily excreted from bladder (*30*). If BBN or BCPN are metabolized through the α-hydroxylation pathway and chemically cleaved, their corresponding reactive species of alkylcarbonium ion are generated. Carbonium ion binds covalently to DNA and enhances carcinogenesis in uroepithelial cells (*31*, *32*). We previously demonstrated that *Ugt1a* mRNA expression is specifically down-regulated in the mouse urinary bladder after BBN exposure (*27*). This may reduce the local glucuronidation activity against carcinogens, allowing their accumulation and consequent promotion of DNA mutations. In this study, we used a BBN-induced urinary bladder carcinogenesis model to elucidate the mechanism of *Ugt1a* mRNA down-regulation during carcinogenesis.

Materials and methods

Animals and reagents

BBN was purchased from Tokyo Kasei (Tokyo, Japan) and 3-MC was bought from Sigma Chemical Co. (St Louis, MO, USA). Nrf2-deficient mice of an ICR/129SV background (*28*) were backcrossed for nine generations with C57BL/6J mice acquired from CLEA Japan (Tokyo, Japan). The *Ahr*$^{-/-}$ mice (*33*) have been backcrossed to C57BL/6J mice for seven generations. Mice were housed in stainless steel cages in an animal room maintained at 24 ± 2°C and with a 12 h light/dark cycle. Mice were fed a purified AIN-76A diet (Oriental MF; Oriental Yeast Co., Tokyo) and given water *ad libitum*. BBN was dissolved in tap water to the set concentrations and supplied *ad libitum* in dark bottles. 3-MC was dissolved in corn oil to a concentration of 4 mg/ml. Mice were treated with a single injection of 80 mg/kg of 3-MC intraperitoneally.

RNA blot analysis

After the experimental period, mice were analysed by autopsy. Total RNAs from whole urinary bladders or cancer lesions were extracted with Isogen (Nippon Gene, Toyama) according to the manufacturer's instructions. Total RNAs (10 μg) were separated by 1.5% agarose gel electrophoresis containing 2.2 M formaldehyde and transferred to a nylon membrane. Membranes were hybridized with the ^{32}P-labelled gene-specific cDNA probes and washed with the stringent washing conditions (final wash was done by 0.1% SSC, 0.1% SDS solution for 30 min at 55°C). cDNA probes for *Ugt1a6*, *Ugt1a9* and total *Ugt1a* have been described (*27*) and a cDNA probe for *Gstp1* was kindly provided by Dr Kimihiko Satoh.

Immunoblot analysis of mAhR

Total proteins from whole mouse bladders were homogenized on ice in 500 μl of RIPA lysis buffer [PBS (pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM sodium ortho-vanadate and 1 mM DTT]. After incubation for 30 min on ice, homogenates were centrifuged at maximum speed (15 000 r.p.m.) in a microcentrifuge for 30 min at 4°C. Protein concentrations of the supernatants were determined by Coomassie Plus Protein Assay Reagent (Pierce). Proteins were separated by 10% SDS–PAGE and electro-transferred onto an Immobile membrane (Millipore, Bedford, MA, USA). The membrane was incubated for 8 h at 4°C with anti-AhR antibody (SA-210; BIOMOL, PA, USA) diluted 1:200 in TBST (TBS plus 0.05% Tween-20). Immunoreactive proteins were detected using horseradish peroxidase-conjugated anti-IgG antibody and ECL (Amersham Biosciences).

Statistical analyses

Data were expressed as means ± SEM. The student's *t*-test was used to determine the statistical differences among groups. A $P<0.05$ was interpreted as statistically significant.

Results

Reduced Ugt1a mRNA expression in BBN-induced mouse urinary bladder cancer

We previously found that BBN dose-dependently down-regulated *Ugt1a* mRNA expression after 2 weeks of BBN exposure in a manner independent of Nrf2 (*27*). In addition, decreased *UGT1A* mRNA expression has been reported in several human cancers. To examine whether *Ugt1a* mRNA expression is decreased in BBN-induced urinary bladder cancer, we examined the mRNA expressions of *total Ugt1a* and its representative isoforms *Ugt1a6* and *Ugt1a9* in BBN-induced urinary bladder carcinoma. For this purpose, mice were treated with 0.05% BBN for 12 weeks and *Ugt1a* mRNA expressions in the urinary bladder were analysed 10 weeks after ceasing treatment. Unlike untreated mice, those administered BBN suffered from apparent urinary bladder cancer, with bladder lesions that were nodular rather than papillary in shape. The expressions of *Ugt1a6*, *Ugt1a9* and *total Ugt1a* mRNAs in the urinary bladders of BBN-treated mice were significantly decreased by 87.7, 98.2 and 80.0%, respectively, compared to those of control mice (Fig. 1). On the other hand, *Gstp* mRNA expression was increased by 290.3%, indicating that the down-regulation of *Ugt1a* mRNA expression in the cancerous urinary bladder is specific amongst phase 2 genes.

Persistent down-regulation of Ugt1a mRNA *after prolonged BBN exposure*

In order to examine the mechanism of *Ugt1a* suppression in more detail, we analysed *Ugt1a* mRNA expression after exposure to 0.05% BBN for 0, 3, 7 and 14 days (Fig. 2A and B). The expressions of *Ugt1a6*, *Ugt1a9* and *total Ugt1a* mRNAs were down-regulated

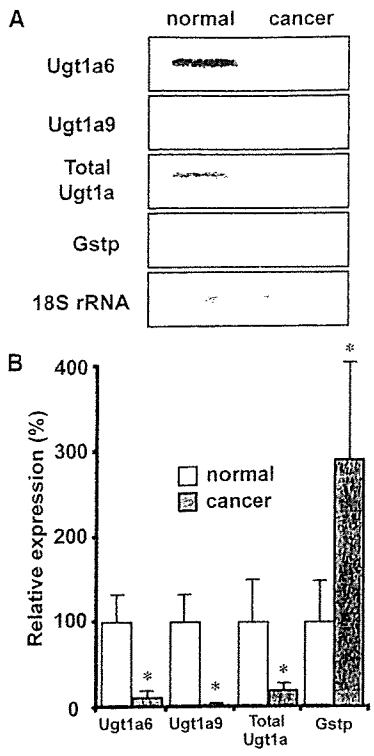

Fig. 1 The expressions of *Ugt1a6*, *Ugt1a9* and total *Ugt1a* mRNAs in BBN-induced urinary bladder cancer compared with those in normal urinary bladder. Mice were treated with vehicle (normal) or 0.05% BBN (cancer) for 12 weeks. Ten weeks after the cessation of BBN treatment, the expression of *Ugt1a* mRNA in the urinary bladder was analysed. The band intensities of the RNA blot (**A**) were quantified by densitometric analysis and the mRNA levels of phase 2 genes were normalized by *18S rRNA* levels. The expression level of each phase 2 gene in vehicle-treated mice was arbitrarily set to 100 and that of the BBN-treated mice is shown as a percentage of this control (**B**). The means from four mice are presented with the SEM ($n=4$). $^*P \leq 0.05$ compared with untreated wild-type mice.

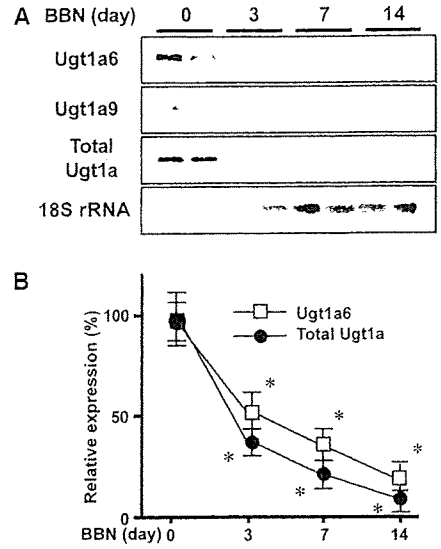

Fig. 2 Effect of BBN on *Ugt1a* mRNA expression in the urinary bladder. Mice were treated with 0.05% BBN or vehicle for 0, 3, 7 and 14 days, then urinary bladder RNA were extracted for RNA blot analysis. The band intensities of the RNA blot (**A**) were quantified by densitometric analysis and expression levels of phase 2 genes were normalized by *18S rRNA* levels. The mRNA level of each *Ugt1a* gene in untreated mice was arbitrarily set to 100 and that of BBN-treated mouse is shown as a percentage of this control (**B**). The means from four mice are presented with the SEM ($n=4$). $^*P \leq 0.05$ compared with untreated wild-type mice.

by BBN treatment as early as 3 days and decreased in a time-dependent manner until 14 days.

It is known that gene expression is altered irreversibly after cancerous transformation. Therefore, we examined whether the down-regulation of *Ugt1a* mRNA expression is reversible after the cessation of BBN treatment. For this purpose, mice were treated with 0.05% BBN for 2 weeks and *Ugt1a* mRNA expression in the urinary bladder was examined 1, 2, 4, 7 and 14 days after the cessation of BBN treatment. Suppression of *Ugt1a6*, *Ugt1a9 and total Ugt1a* mRNA levels was maintained up to 4 days after the interruption of BBN treatment (Fig. 3A). However, 7 days after ceasing BBN administration, *Ugt1a* mRNA expression increased to a level exceeding those of untreated mice, demonstrating the reversibility of the process after a short period (*i.e.* 2 weeks) of carcinogen treatment.

But what of longer term BBN exposure? Mice were treated with 0.05% BBN for 2, 4, 6, 9 and 12 weeks and the expression of *Ugt1a6 and* total *Ugt1a* mRNAs was examined 7 days after discontinuing BBN treatment at each time point (Fig. 3B). We found that the expression of *Ugt1a6 and* total *Ugt1a* mRNAs did not fully recover after the mice were treated with BBN for 4 weeks or longer. Thus, chronic BBN exposure of greater than 2 weeks resulted in the persistent down-regulation of *Ugt1a* mRNA expression.

Ugt1a down-regulation by BBN treatment is not observed in the AhR KO mouse bladder

Ugt1a mRNA expression is regulated by multiple transcription factors, such as Nrf2 and the AhR (*16*, *17*). To elucidate if the Nrf2 or AhR pathway is responsible for the down-regulation of *Ugt1a* mRNA expression by BBN, we analyzed *Ugt1a* mRNA expression in the urinary bladders of $Nrf2^{-/-}$ and $Ahr^{-/-}$ mice after exposure to 0.05% BBN for 2 weeks (Fig. 4A and B). Importantly, the basal expressions of *Ugt1a6*, *Ugt1a9* and *total Ugt1a* mRNAs in $Ahr^{-/-}$ mice were significantly decreased by 80.0%, 92.8% and 83.8%, respectively, compared with those of wild-type mice. On the other hand, *Ugt1a6*, *Ugt1a9* and *total Ugt1a* mRNA expressions in $Nrf2^{-/-}$ mice were not significantly altered compared with those of wild-type mice. After BBN treatment, *Ugt1a* mRNA expression was significantly decreased by >80% in both $Nrf2^{-/-}$ and wild-type mice. However, in $Ahr^{-/-}$ mice, the constitutively low *Ugt1a* mRNA expression was not further reduced upon BBN exposure.

Further confirmation that the AhR indeed regulates *Ugt1a* mRNA expression in the urinary bladder was provided by treating both wild-type and $Ahr^{-/-}$ mice with the AhR activator 3-MC. The expressions of *Ugt1a* and *Cyp1a1* mRNAs were measured 48 h post-intraperitoneal injection of 3-MC (Fig. 4C). The expressions of *Ugt1a6*, *Ugt1a9*, *total Ugt1a* and *Cyp1a1* mRNAs in wild-type mice were significantly

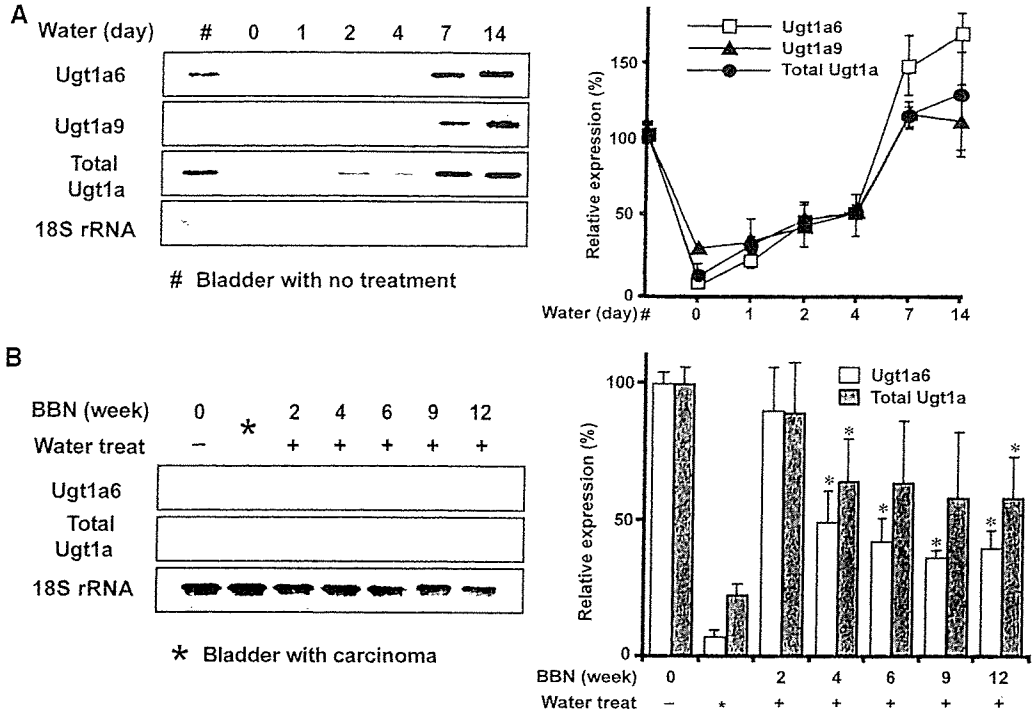

Fig. 3 Expression of *Ugt1a* mRNAs after the cessation of BBN treatment. (A) Mice were treated with 0.05% BBN for 2 weeks and total RNAs from the urinary bladders were examined for *Ugt1a* mRNA expression 1, 2, 4, 7 and 14 days after the cessation of BBN treatment. (**B**) Mice were treated with 0.05% BBN for 2, 4, 6, 9 and 12 weeks. Total RNAs from the urinary bladders were examined for *Ugt1a* mRNA expression 7 days after the cessation of BBN treatment at each time point. The band intensities of the RNA blots (left panels) were quantified by densitometry and the expression levels of *Ugt1a* mRNAs were normalized by *18S rRNA* levels. The expression level of *Ugt1a* mRNA in untreated mice was arbitrarily set to 100 and that of the BBN-treated mouse is shown as a percentage of this control (right panels). Values are presented as means ± SEM ($n=4$). $^*P \leq 0.05$ compared with untreated wild-type mice.

increased by 157.7, 143.8, 194.0 and 1509.0%, respectively, after 3-MC treatment. However, the constitutive expression of these genes was decreased in $Ahr^{-/-}$ mice and induction by 3-MC was lost. These results clearly demonstrate a similar regulation of mRNA expression under the influence of the AhR between *Ugt1a* and *Cyp1a1* genes in the urinary bladder.

Expression of AhR protein and the activity of the AhR pathway after BBN treatment

To examine the effect of BBN on AhR activity, we measured the expression of AhR protein and the AhR target gene expression in the urinary bladder after 2 weeks of BBN treatment. Immunoblot analysis using whole urinary bladder extracts demonstrated that BBN treatment significantly increased AhR protein in a dose-dependent manner (Fig. 5A). Because RNA blot analysis showed that the *Ahr* mRNA level was not increased by BBN treatment (data not shown), AhR protein translation rate or stability may be increased by BBN treatment. We evaluated the AhR target gene expression after BBN treatment by measuring *Cyp1a1* mRNA expression by RNA blot analysis. Mice were treated with 0.01, 0.05 and 0.1% BBN for 2 weeks and the expression of *Cyp1a1* mRNA in urinary bladder was examined. The results revealed that BBN treatment decreased *Cyp1a1* mRNA expression. These results indicated that AhR signaling is suppressed by BBN treatment although AhR protein itself is increased by BBN (Fig. 5B).

AhR activity is repressed in BBN-induced cancerous tissue

Because AhR protein is increased but its activity may be suppressed by BBN, we next analyzed AhR protein level and *Cyp1a1* expression in cancerous tissue. Mice were administered BBN for 12 and then with water for 10 weeks following the stoppage of BBN treatment, *Cyp1a1* mRNA expression in the cancerous tissues was examined. RNA blot analysis demonstrated a decrease in *Cyp1a1* mRNA expression similar to the decrease in *Ugt1a* mRNA expression, indicating the persistent repression of AhR signaling in cancerous urinary bladder (Fig. 5C). Consistent with the results of Fig. 5A, the expression of AhR protein in whole bladder extract remained elevated even 10 weeks after ending BBN treatment (Fig. 5D). Thus, BBN suppresses AhR signaling pathway in cells, hence repressing *Ugt1a* mRNA expression during BBN-induced carcinogenesis.

Discussion

Our study established that *Ugt1a* mRNA expression is markedly decreased in BBN-induced urinary bladder cancer, with the decreased expression commencing during the early phase of continuous BBN administration. In *Ahr* KO mice, although the expression of

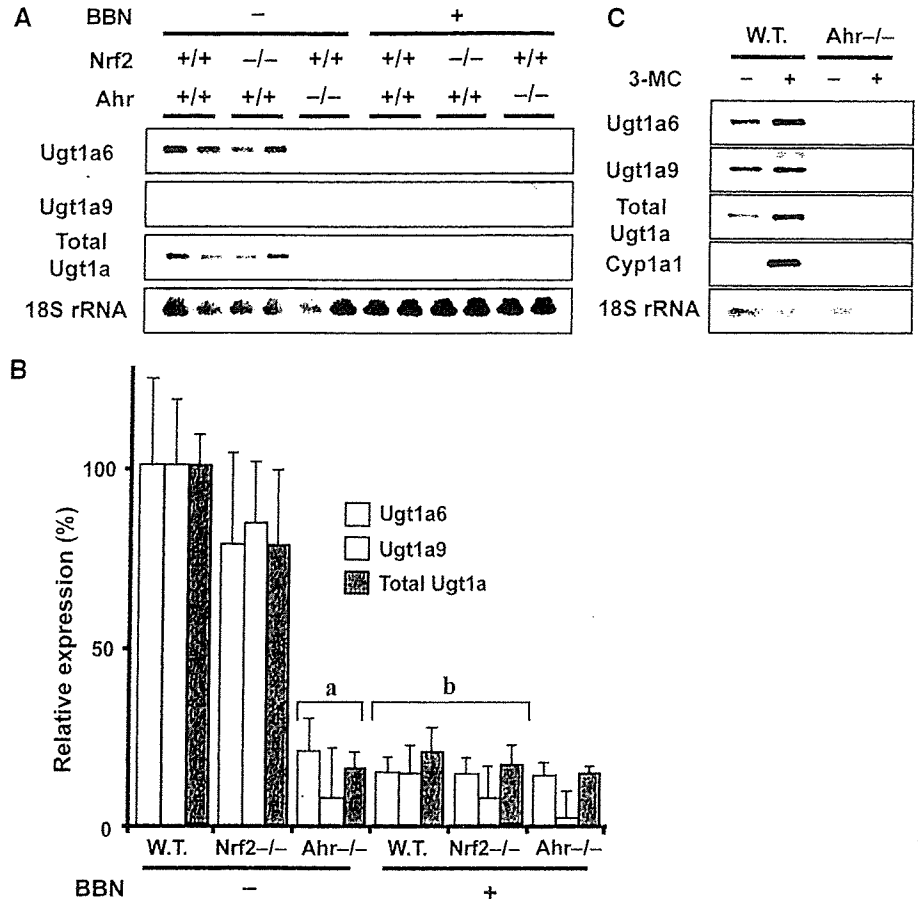

Fig. 4 Role of AhR on *Ugt1a* mRNA expression in the urinary bladder. (A) Effect of *Ahr* or *Nrf2* gene deletion on *Ugt1a* mRNA expression. Wild-type, $Nrf2^{-/-}$ and $Ahr^{-/-}$ mice were treated with 0.05% BBN for 2 weeks and the expression levels of *Ugt1a* mRNAs in the urinary bladders were examined. (B) The expression level of *Ugt1a* mRNA in untreated wild-type mice was arbitrarily set to 100 and that of each mouse as indicated in the figure is shown as a percentage of this control. (A) $P \leq 0.05$ compared with untreated wild-type mice. (B) $P \leq 0.05$ compared with untreated mice of the same genotype. (C) Effect of 3-MC on *Ugt1a* mRNA expression in the urinary bladder. Wild-type and $Ahr^{-/-}$ mice were injected intraperitoneally with 80 mg/kg of 3-MC. After 48h, the expressions of *Ugt1a* mRNAs and the *Cyp1a1* mRNA were examined by RNA blot analysis.

Ugt1a mRNA was constitutively low, BBN treatment did not further suppress *Ugt1a* mRNA expression. BBN appears to down-regulate *Ugt1a* mRNA expression *via* the suppression of AhR-dependent signaling pathway. These results indicate that the potent carcinogen BBN facilitates carcinogenesis by repressing the expression of AhR-dependent detoxification genes.

Our current hypothesis of *Ugt1a* mRNA down-regulation during BBN-induced urinary bladder carcinogenesis is summarized in Fig. 6. Under normal conditions, when the urinary bladder is continually exposed to urine, the basal expression of *Ugt1a* mRNA is up-regulated by the AhR that is activated by urinary ligands such as indigos (*34*). BBN-treatment increases AhR protein level in the cells, but the *Ugt1a* mRNA expression is suppressed by BBN-treatment. After long BBN exposure, the down-regulation of *Ugt1a* mRNA becomes persistent and this may allow for the accumulation of carcinogen and consequently predispose the urinary bladder to carcinogenesis.

It is known that BBN is metabolized by the conjugation of glucuronic acid in rat (*30*). However, UGT isozymes responsible for BBN-glucuronidation are not clear at present. We previously demonstrated that Nrf2 activator oltipraz induce BBN-glucuronidation in an Nrf2-dependent manner in mouse liver (*27*). As Nrf2 regulates a battery of *Ugt1a* as well as *Ugt2b* mRNA expression, we surmise that either of these Ugt family members probably catalyse BBN glucuronidation in mice (*35*, *36*).

Several studies have clarified the mechanism of gene silencing in cancer. For example, methylation of the 5′ CpG island is thought to play an important role in the inactivation of tumor suppressor genes in cancer. The *GSTP1* gene is the major GST isoform expressed in normal human prostate and is silenced in the majority of prostate tumors by the hypermethylation of CpG islands in the 5′ regulatory region (*5*). Similarly, expression of *UGT1A* mRNA is also down-regulated in the early stages of human liver and biliary carcinogenesis, but the mechanism has not been elucidated (*1*). To determine the mechanism of down-regulation of *Ugt1a* mRNA, we evaluated methylation of the *Ugt1a6* gene promoter in mouse cancerous urinary bladder. However, we failed to detect methylation of CpG in the promoter between −1.2 kb and 0.1 kb (data not shown).

It is important to note that BBN does not repress *Ugt1a* mRNA expression in the liver. Since BBN is metabolized to its reactive species only in the urinary bladder, it might be the reactive metabolites of BBN that are repressing the AhR. The repression mechanism was examined using primary culture of urinary bladder epithelial cells, but we found that neither BBN nor BCPN down-regulated basal *Ugt1a* mRNA expression. This might reflect the fact that the AhR does not contribute to basal *Ugt1a* mRNA expression *in vitro*, because of the absence of its urinary ligands. In contrast to the urinary bladder, transcription factors other than the AhR may contribute to the basal expression of *Ugt1a* mRNA in the liver. If this is the case, even if BBN represses liver AhR, it would not repress the expression of *Ugt1a mRNA*. Clarification of these possibilities requires further study.

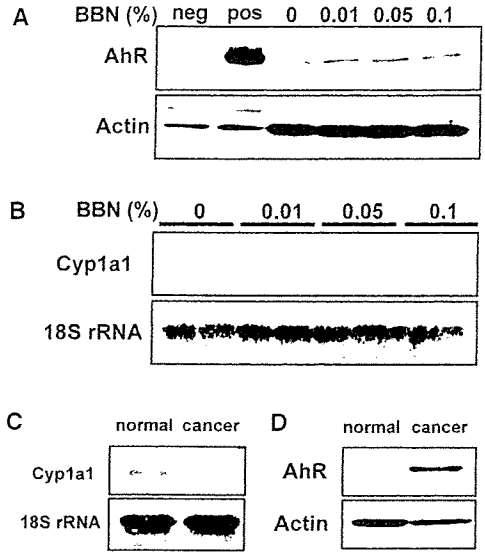

Fig. 5 The effect of BBN treatment on the AhR pathway. (**A**) Effect of BBN on the expression of the AhR in the urinary bladder. Mice were treated with vehicle, or 0.01%, 0.05%, 0.1% BBN for two weeks. Total cell extracts of the urinary bladders were examined by immunoblot analysis using anti-AhR antibody. neg, the total cell extract of COS-7 cells transfected with empty vector; posi, the total cell extract of COS-7 cells transfected with AhR expression vector. anti-β-actin antibody was used as a loading control. (**B**) Effect of BBN on *Cyp1a1* mRNA expression. Mice were treated with 0.01, 0.05 and 0.1% BBN or vehicle for two weeks and total RNAs from the urinary bladders were examined for the expression of *Cyp1a1* gene. (**C**) Down-regulation of *Cyp1a1* mRNA expression in urinary bladder cancer. Mice were treated with vehicle (normal) or 0.05% BBN (cancer) for 12 weeks and then with water for 10 weeks. *Cyp1a1* mRNA expression in the urinary bladder was examined by RNA blot analysis. (**D**) AhR protein expression in urinary bladder cancer. Mice were treated with vehicle (normal) or 0.05% BBN (cancer) for 12 weeks and then with water for 10 weeks. Total cell extracts of cancerous urinary bladders were examined by immunoblot analysis using anti-AhR antibody. Anti-β-actin antibody was used as a loading control.

It is known that ligand-coupled AhR is rapidly degraded by the ubiquitin–proteasome system (UPS) (*37*, *38*). Several studies reported that many transactivators possess rapid-turnover characteristics, indicating an association between transcriptional activation and protein degradation (*39*). Indeed, the proteolysis of some transcriptional activators by the UPS can stimulate transcription (*40*, *41*). However, since the proteasome inhibitors MG132 and lactacystin block AhR degradation by TCDD, but lead to an enhancement of AhR transcriptional activity, proteolysis *per se* may not be essential for the transactivation activity of the AhR (*37*, *38*). Recently, Chen *et al.* (*42*) demonstrated that MEK inhibitor U0126 stabilizes the AhR and increases its steady-state levels, but also diminishes the ability of the activated AhR to induce *Cyp1a1* in response to TCDD. In that paper, the authors speculated that Erk induces a conformational change that provokes both transcriptional activation and degradation of the AhR. Collectively, these studies argue that degradation of the cellular AhR is not necessarily a requirement for transcription, but a property of the ligand-activated form of the receptor. It is not clear whether the same phenomenon occurs in BBN-treated bladder, but an interesting possibility might be that BBN inhibits Erk in the urinary bladder.

The repression of *Ugt1a* mRNA expression becomes persistent after longer BBN exposure (i.e. after 4 weeks or longer exposure), and the inhibition of AhR signaling pathway and the repression of *Ugt1a* mRNA was also observed in the cancerous urinary bladder tissue, that is even 10 weeks after the cessation of BBN treatment (Fig. 5). Although it is not clear whether the inhibition mechanisms of AhR signaling pathway are identical in the non-cancerous and cancerous tissues, these results suggest that persistent

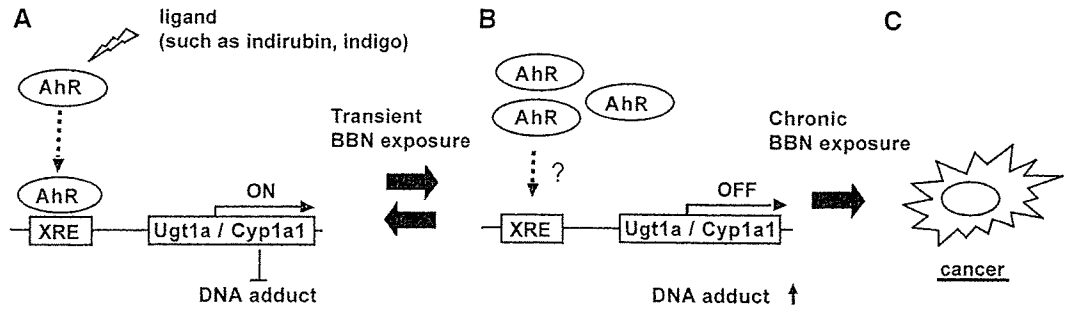

Fig. 6 The hypothetical mechanism of *Ugt1a* mRNA down-regulation during urinary bladder carcinogenesis. (**A**) Normally, the basal mRNA expressions of *Ugt1a* and *Cyp1a1* are mediated by the AhR that is constitutively activated by exposure to AhR ligands in the urine. (**B**) The AhR signaling pathway is repressed either by BBN or BBN metabolites by yet unidentified mechanisms, which down-regulates the mRNA expressions of *Ugt1a* and *Cyp1a1*. (**C**) If BBN exposure has been transient, the expression of *Ugt1a* mRNA swiftly recovers to normal levels. However, after a long exposure to BBN of >4 weeks, the down-regulation becomes persistent.

inhibition of *Ugt1a* expression *via* inhibition of AhR signaling pathway play an important role in carcinogenesis. Further studies will be required to find out how BBN or its metabolites inhibit AhR signaling pathway in the bladder.

This is the first report demonstrating that suppression of AhR signaling pathway is associated with the down-regulation of *Ugt1a* mRNA during urinary bladder carcinogenesis. Continuous exposure to carcinogen causes persistent repression of *Ugt1a* mRNA expression and may induce carcinogenesis. Conversely, we previously reported that Nrf2 activation antagonizes the BBN-induced repression of urinary *Ugt1a* mRNA expression (*27*). Thus, Nrf2 activation by dietary anticarcinogenic vegetables and fruits not only induces phase 2 expression, but also counteracts carcinogen-mediated repression of *Ugt1a* mRNA expression, thereby protecting the urinary bladder from carcinogenesis.

Acknowledgements

We thank Drs Atsushi Maruyama, Nobuhiko Harada and Tania O'Connor for their help and critical reading of the manuscript.

Funding

This work was supported in part by grants from JST-ERATO, JSPS, the Ministry of Education, Science, Sports and Technology, the Ministry of Health, Labor and Welfare, and the Naito Foundation.

Conflict of interest

None declared.

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BIOCHEMICAL PHARMACOLOGY 77 (2009) 474–484

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Review

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

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ARTICLE INFO

Article history:
Received 13 August 2008
Accepted 28 August 2008

Keywords:
AhR
Dioxin
Estrogen
Cullin 4B
Ubiquitin ligase

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, CUL4-B^{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.

Contents

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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.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.08.034

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 (ERα and ERβ) [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

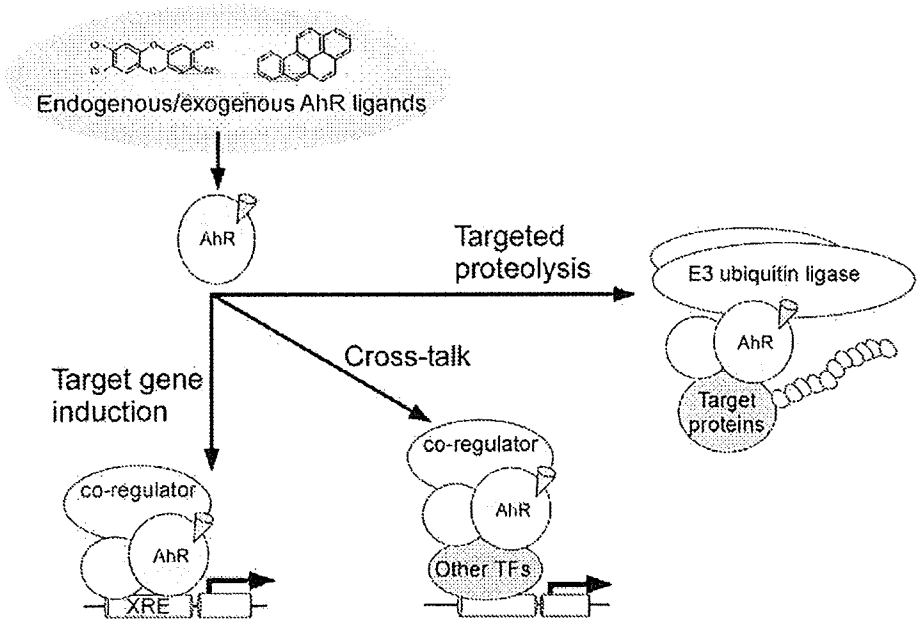

Fig. 1 – Different modes of the AhR signaling pathways. Molecular pathways for AhR-mediated biological actions. AhR may exhibit its biological actions through different modes of pathways as illustrated. Typically, AhR directly binds to its target gene promoters and induces expression of these genes. 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.

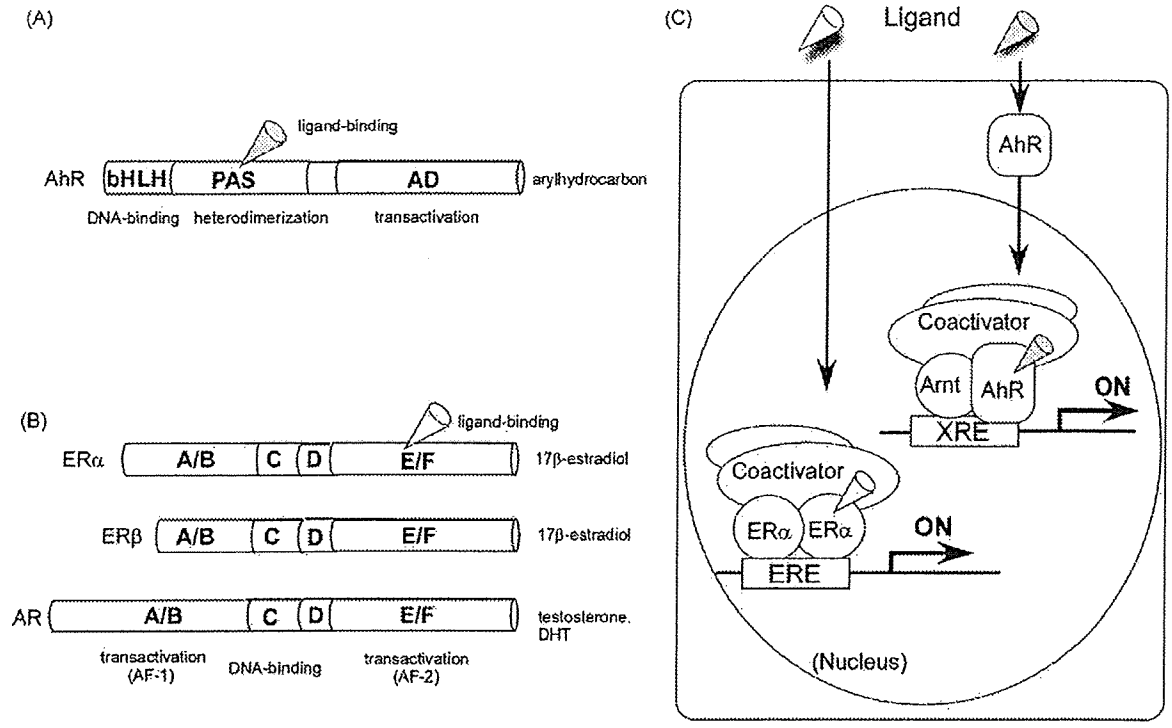

Fig. 2 – Structure and molecular mechanism of AhR and nuclear receptors. A and B domain structures of AhR (A) and nuclear receptors (B). Domain architectures and cognate ligands for these receptors are illustrated. bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim domain; AD, activation domain; AF, activation 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

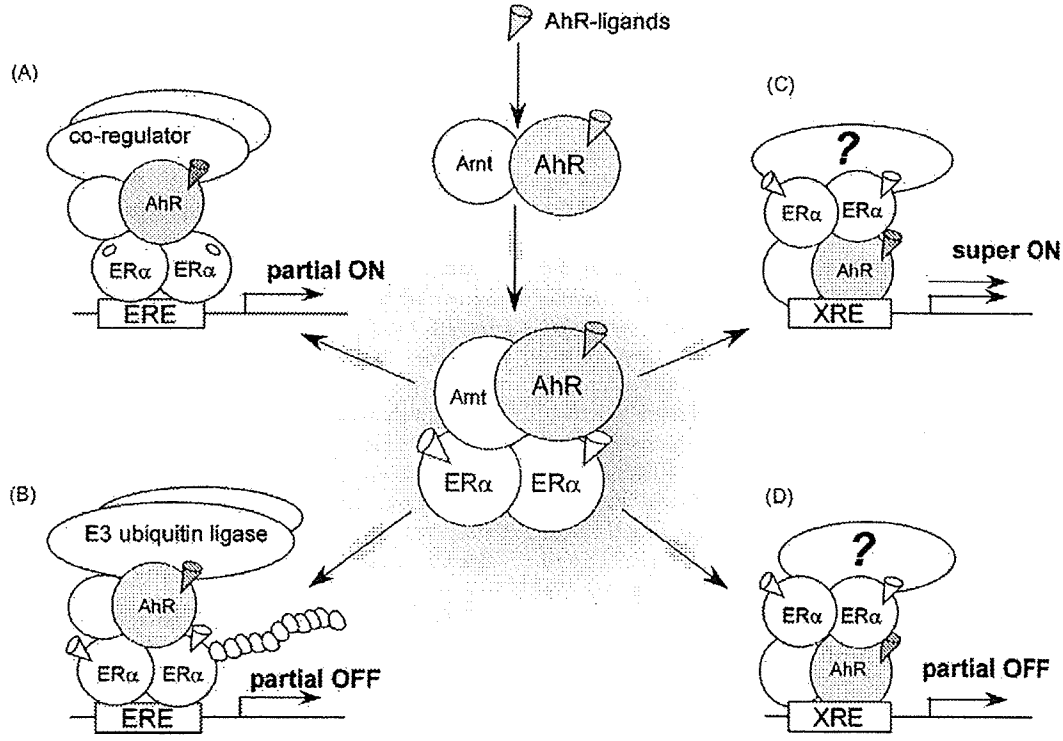

Fig. 3 – Cross-talk of AhR with ERα through direct association. Ligand-bound AhR directly associates with estrogen or androgen receptors (ERα, ERβ, or AR) in the nucleus. This association leads to different types of cross-talk between AhR and ERs/AR, as illustrated (see text for details). (A) Ligand-bound 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-

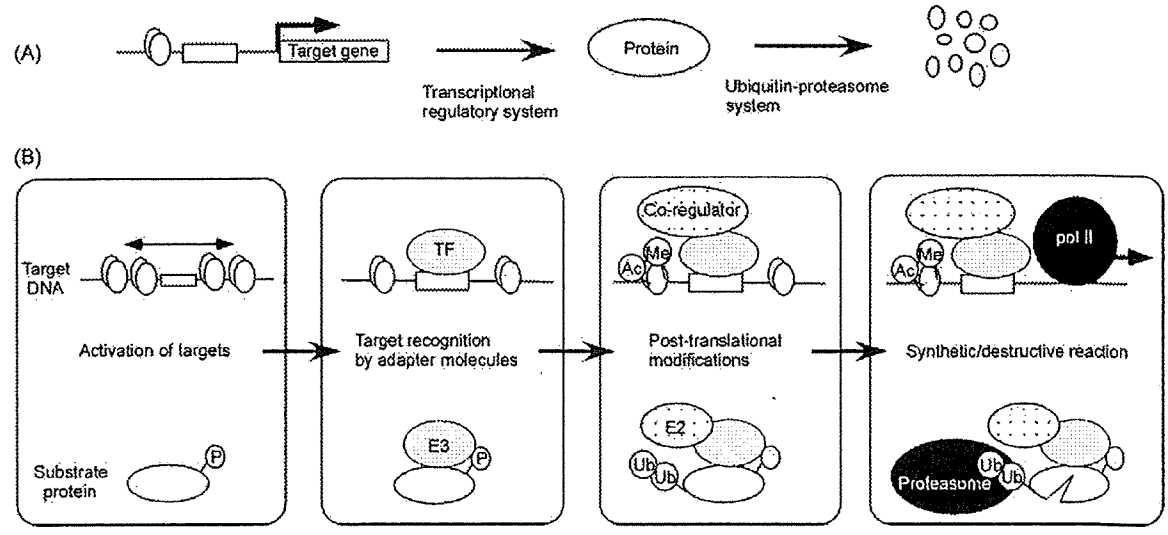

Fig. 4 – The ubiquitin–proteasome system. (A) The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. (B) The transcription factors and E3 ubiquitin ligases primarily serve as target-specifying adapters 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-

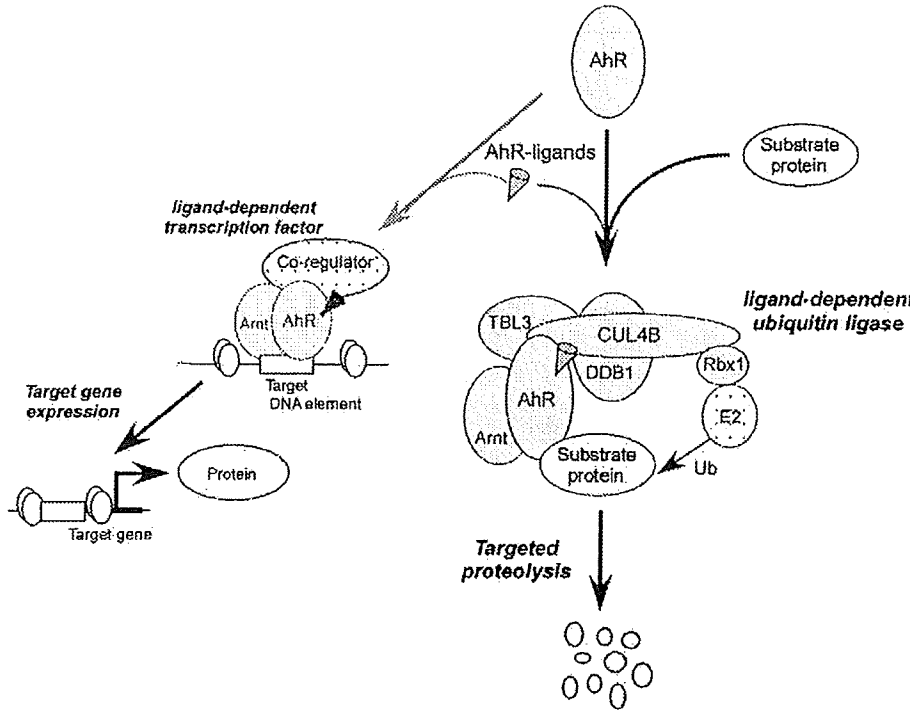

Fig. 5 – An E3 ubiquitin ligase activity of AhR. Ligand-bound AhR assembles a CUL4B-based atypical E3 ubiquitin ligase complex, CUL4B^{AhR}, to mediate a non-genomic signaling pathway of fat-soluble ligands. AhR serves as a ligand-dependent ubiquitin ligase, as well as a transcription factor (see text for details). DDB1, damaged-DNA-binding protein 1; TBL3, transducin-beta-like 3.

tion and degradation has been demonstrated in knock-down experiments. Degradation of ERα or AR in the uterus and prostate is inducible by treatment with AhR ligands. Such degradation of ERα or AR is not seen in AhR-deficient mice [10,33]. This confirms that the AhR has E3 ubiquitin ligase activity *in vivo*. The anti-estrogenic effects of AhR ligands on estrogen-dependent uterine cell proliferation [14] appear to be mediated by the E3 ubiquitin ligase activity of AhR.

3.3. *Perspectives on the E3 ubiquitin ligase activity of AhR in cross-talk pathways*

Although it is well established that AhR is a key factor in mediating the adverse effects of dioxin-type compounds [8–10], the underlying mechanisms for this remain elusive. The putative functions of the previously identified target genes for AhR appear unlikely to fully explain the diverse range of biological actions of AhR ligands [11] (Fig. 1). The discovery of CUL4B^{AhR} suggests that the adverse effects of AhR ligands in sex hormone signaling are, at least in part, attributable to the enhanced degradation of sex steroid receptors through E3 ubiquitin ligase activity of AhR [18,19] (Fig. 5). Target selectivity of the transcriptional regulatory system and the ubiquitin–proteasome system depends on specificity conferred by sequence-specific transcription factors and E3 ubiquitin ligases. To date, however, no single factor has been shown to function as a specificity factor in both target selection systems. Therefore, AhR is the first sequence-specific transcription factor identified that acts as an E3 ubiquitin ligase that also targets substrates for accelerated protein degradation. It is possible that other transcription factors, such as nuclear receptors, also function as E3 ubiquitin ligase components in some cellular contexts. Fat-soluble ligands for nuclear receptors are reported to have 'non-genomic' actions independent of transcriptional regulation-mediated effects. Considered together, ubiquitin ligase-based signaling mechanisms may possibly be involved in these non-genomic actions of various fat-soluble ligands.

From a mechanical point of view, AhR appears to be a unique and atypical type of substrate-specific component in cullin-based E3 complexes. AhR does not bear the reported signature motifs such as F-box [39], but directly associates with CUL4B. Substrate recognition by the other substrate-specific components in ubiquitin ligase complexes is usually evoked by substrate modifications such as phosphorylation [38–41] and hydroxylation [43,44,58]. However, recognition and subsequent ubiquitination of sex steroid receptors by AhR requires dioxin-type ligands, and does not occur following normal modifications of sex steroid receptors. Thus, it is plausible that activation of atypical E3 complexes may be a strategy of sensors for environmental stresses to respond to these stresses (Fig. 6). Supporting this, Hsp70 acts as an atypical substrate-specific adapter within the CHIP E3 complex in response to heat shock stress [63]. Hsp70 interacts with misfolded proteins and promotes their degradation. It later undergoes auto-catalytic degradation through CHIP [63]. In response to DNA damage, an atypical E3 complex alters the stability of TIP60, which in turn regulates ataxia-telangiectasia

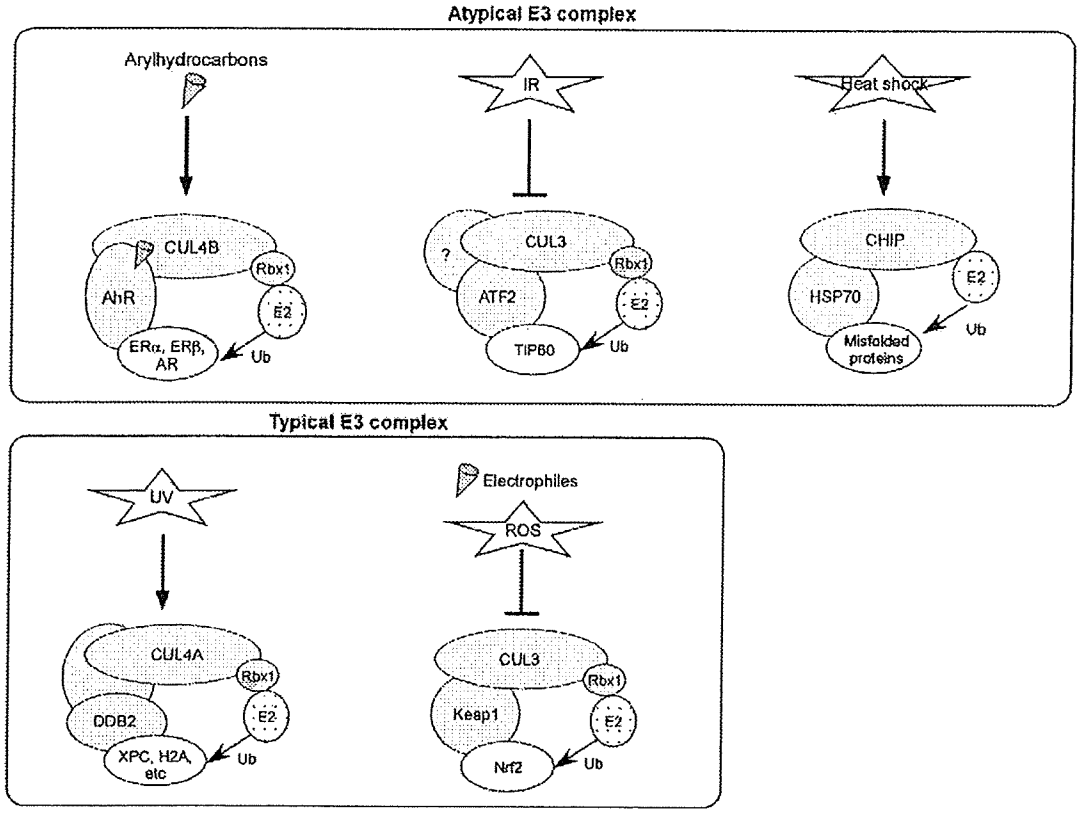

Fig. 6 – Atypical E3 complexes as sensors for environmental stresses. Several examples of E3 ubiquitin ligase-based perception of environmental stresses are illustrated. In the top panel, signal-responsive factors serve as atypical components of E3 complexes. In the bottom panel, canonical E3 components with conserved signature motif act as signal-responsive factors. ATF2, activating transcription factor-2; TIP60, tat interactive protein 60; CHIP, C-terminus of Hsp70 interacting protein; Hsp70, heat shock protein 70; XPC, xeroderma pigmentosum group C; H2A, histone H2A; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NF-E2-related factor 2; IR, ionizing radiation; ROS, reactive oxygen species.

mutated (ATM) activation in DNA repair [64]. Activating transcription factor-2 (ATF2) promotes the degradation of TIP60 by assembling a CUL3-based complex under non-stressed conditions. ATF2 dissociates from TIP60 in response to ionizing radiation (IR), resulting in enhanced TIP60 stability and activity [64]. Functional regulation of E3 components is also seen with the CUL3-based component Keap1 in the oxidative stress response [65], and CUL4A-based components DDB2 and CSA in the DNA damage response [61]. Considered together, E3 components that respond to environmental stress may be more diverse than initially believed (Fig. 6). It is possible that $CUL4B^{AhR}$ may cross-talk with these stress-responsive E3 ligases to modulate their functions. As WDXR/DWD motif containing components, including DDB2 and CSA, also bind to CUL4B [46], it is possible that AhR may associate or interfere with these CRL subunits.

The E3 ubiquitin ligase activity of AhR and the transcriptional activity of AhR appear to be responsible for a distinct set of biological events induced by AhR ligands (Fig. 5). As substrate-specific adapters of ubiquitin ligase complexes are capable of recognizing a number of proteins, identification of other $CUL4B^{AhR}$ substrate proteins may reveal new molecular links between AhR-mediated signaling and other signaling pathways and cellular events. In this regard, it is of interest that AhR interacts with various transcription factors [11], such as Rb/E2F1 [66], SF1/Ad4BP [33], and NF-κB [67], to modulate their functions. AhR has recently been shown to regulate the differentiation of Th17 and T_{reg} cells [68–70]. This may be mediated by a functional interaction with STAT1 [70]. In addition, although the underlying mechanisms remain unknown, AhR also modulates the function of transcription factors [71] such as GR and RAR [72,73]. Considering the evolutionary conservation of AhR, it is likely that the intrinsic function of AhR is to mediate the signal transduction of endogenous ligands in cross-talk pathways. A current area of interest is the identification of candidate degradation substrates for AhR which are abnormally stabilized in AhR-deficient mice. In summary, several lines of recent evidence define a novel role for AhR as a ligand-dependent E3 ubiquitin ligase to regulate target-specific protein destruction. The ubiquitin ligase activity of AhR, together with the cross-talk of AhR with nuclear receptors through direct association, provides an additional layer of complexity for AhR biology. Characterization of these new molecular aspects of AhR function may lead to a greater understanding of the diverse biological actions induced by endogenous and exogenous AhR ligands.