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| journal or publication title | FEBS Open Bio  |
| volume                       | 4  |
| number                       | 1  |
| page range                   | 796-803  |
| year                         | 2014-01  |
| 出版者                          | Elsevier   |
| 関連リンク                        | <a href="https://doi.org/10.1016/j.fob.2014.09.003">https://doi.org/10.1016/j.fob.2014.09.003</a> ( <a href="https://doi.org/10.1016/j.fob.2014.09.003">https://doi.org/10.1016/j.fob.2014.09.003</a> )  |
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| URL                          | <a href="http://hdl.handle.net/10295/00006196">http://hdl.handle.net/10295/00006196</a>  |

doi: 10.1016/j.fob.2014.09.003

journal homepage: [www.elsevier.com/locate/febsopenbio](http://www.elsevier.com/locate/febsopenbio)

# The activation mechanism of the aryl hydrocarbon receptor (AhR) by molecular chaperone HSP90

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## ARTICLE INFO

### Article history:

Received 5 August 2014

Revised 9 September 2014

Accepted 10 September 2014

### Keywords:

Aryl hydrocarbon receptor  
AhR  
Dioxin receptor  
Molecular chaperone  
HSP90

## ABSTRACT

The aryl hydrocarbon receptor is a member of the nuclear receptor superfamily that associates with the molecular chaperone HSP90 in the cytoplasm. The activation mechanism of the AhR is not yet fully understood. It has been proposed that after binding of ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene (3-MC), or  $\beta$ -naphthoflavone ( $\beta$ -NF), the AhR dissociates from HSP90 and translocates to the nucleus. It has also been hypothesized that the AhR translocates to the nucleus and forms a complex with HSP90 and other co-chaperones. There are a few reports about the direct association or dissociation of AhR and HSP90 due to difficulties in purifying AhR. We constructed and purified the PAS domain from AhR. Binding of the AhR-PAS domain to  $\beta$ -NF affinity resin suggested that it possesses ligand-binding affinity. We demonstrated that the AhR-PAS domain binds to HSP90 and the association is not affected by ligand binding. The ligand 17-DMAG inhibited binding of HSP90 to GST-PAS. In an immunoprecipitation assay, HSP90 was co-immunoprecipitated with AhR both in the presence or absence of ligand. Endogenous AhR decreased in the cytoplasm and increased in the nucleus of HeLa cells 15 min after treatment with ligand. These results suggested that the ligand-bound AhR is translocated to nucleus while in complex with HSP90.

We used an *in situ* proximity ligation assay to confirm whether AhR was translocated to the nucleus alone or together with HSP90. HSP90 was co-localized with AhR after the nuclear translocation. It has been suggested that the ligand-bound AhR was translocated to the nucleus with HSP90. Activated AhR acts as a transcription factor, as shown by the transcription induction of the gene CYP1A1 8 h after treatment with  $\beta$ -NF.

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**Abbreviations:** 17-DMAG, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin, dimethylsulfoxide; 3-MC, 3-methylcholanthrene; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; bHLH, basic helix-loop-helix; CYP1A1, cytochrome P450 1A1; DAPI, 4',6-diamidino-2-phenylindole; DEPC, dihydrochloride, diethylpyrocarbonated; GST, glutathione, glutathione S-transferase; HSP90, 90-kDa of heat shock protein; IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside; IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside; NLS, nuclear localization signal; PAS, per-*arnt*-*sim*; PLA, proximity ligation assay; RT-PCR, reverse transcription-polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XAP2, hepatitis B virus X-associated protein; XRE, xenobiotic responsible element;  $\beta$ -NF,  $\beta$ -naphthoflavone

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## 1. Introduction

Molecular chaperone HSP90 is an abundant and essential protein in eukaryotic cells. HSP90 regulates the physiological functions of more than 300 proteins including steroid hormone receptors in the cells [1–3]. The aryl hydrocarbon receptor (AhR) is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of nuclear receptors [4,5]. AhR is the ligand-dependent transcriptional regulator that mediates the toxic effects of chemicals, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene (3-MC) or  $\beta$ -naphthoflavone ( $\beta$ -NF) [5]. AhR forms a complex with the molecular chaperone HSP90, co-chaperone p23, and the hepatitis B virus X-associated protein XAP2 in the

<http://dx.doi.org/10.1016/j.fob.2014.09.003>

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cytoplasm [5,6]. After binding of a ligand, AhR is translocated into a nucleus and AhR forms a complex with the partner molecules of Arnt (AhR nuclear translocator). The AhR/Arnt heterodimer binds to the xenobiotic responsible element (XRE) in order to react with the xenobiotic in the promoter area of the cytochrome P450 1A1 (CYP1A1), one of the xenobiotic metabolizing enzymes. TCDD causes toxicological effects like a suppressed immune response, impaired reproduction, and promotion of carcinogenesis [5–9].

The AhR has several functional domains. A (bHLH) motif is involved in the DNA binding near the NH<sub>2</sub>-terminal region. The per-Arnt-Sim homology (PAS) domain is near the COOH-terminus of the bHLH region. The PAS domain is made from PAS-A and PAS-B. It has been reported that HSP90 binds to the PAS B domain, and ligand binding to the AhR protein most probably changes the conformation of the HSP90/AhR complex to expose the nuclear localization signal (NLS) of AhR, leading to nuclear translocation of the complex [10]. The glutamine (Q) rich region is near the carboxyl-terminal of AhR [11,12].

HSP90 may control the activation of AhR [13]. It has been reported that HSP90 is released from the ligand-bound AhR when co-incubated with the cell extracts containing ARNT, but not the ARNT-deficient mouse hepatoma cells [14]. Purification of AhR, is difficult and so there are few reports regarding the direct interaction between HSP90 and AhR. The activation mechanism of AhR has not yet been fully understood and it is not known whether the ligand-bound AhR is translocated to the nucleus by itself in complex with HSP90.

## 2. Results

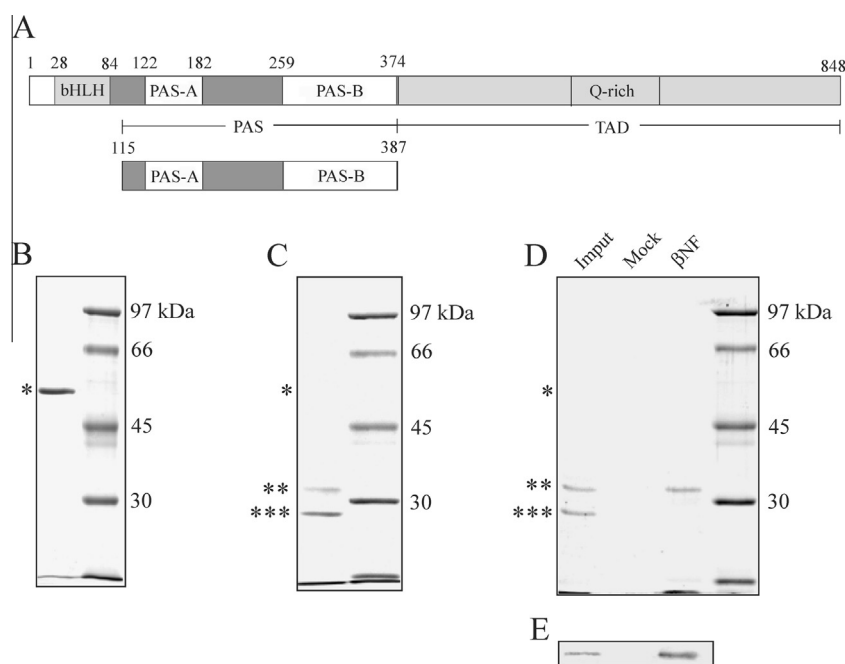
### 2.1. Association between HSP90 and AhR

In the present study, we constructed the human AhR PAS domain [15] as shown in Fig. 1A. The PAS domain contains two subdomains, PAS A and PAS B. The GST-AhR PAS domain was expressed in *Escherichia coli* and purified (Fig. 1B). We confirmed

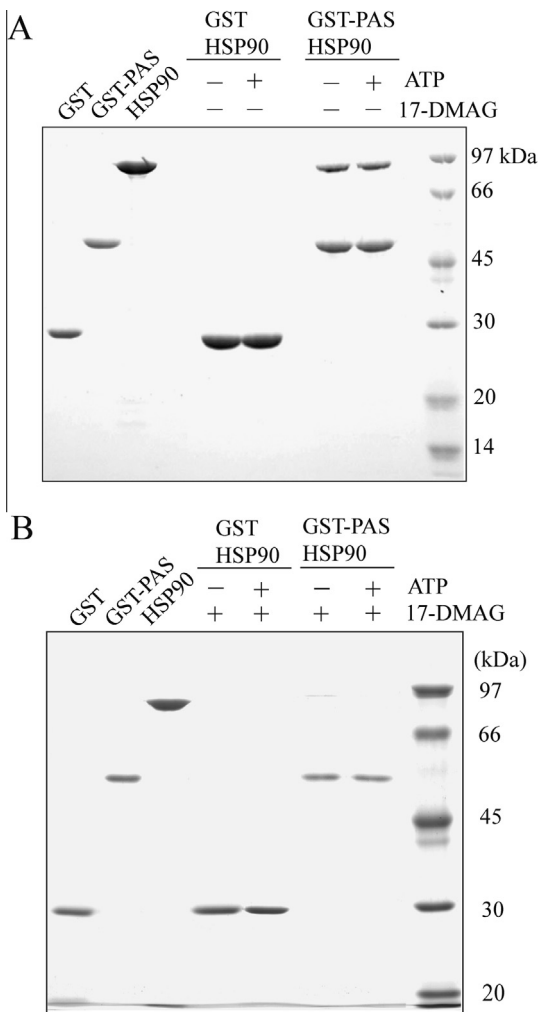
that the purified AhR PAS domain has a ligand-binding activity using a  $\beta$ -NF affinity column. The purified AhR PAS domain was digested in PAS and GST using a Factor Xa (Fig. 1C). Although no proteins bound to mock resin, 32-kDa protein was able to bind to  $\beta$ -NF affinity resin (Fig. 1D). We could confirm that the  $\beta$ -NF binding protein was AhR PAS by immunoblotting (Fig. 1E). Thus, the purified AhR PAS domain possesses ligand-binding ability.

We first analyzed whether the purified proteins make a complex under ligand-free conditions. The GST-AhR PAS domain or GST only was incubated with HSP90, and then reacted with GST resin. As shown in Fig. 2A, no HSP90 was detected in the lane of GST alone in the presence or absence of ATP. On the other hand, HSP90 was detected in the lane of the GST-AhR PAS domain both in the presence or absence of ATP. ATP has no effect on the interaction between the GST-PAS domain and HSP90 under the ligand-free conditions. These results suggested that the purified proteins had a proper interaction between HSP90 and AhR in the absence of the ligand. We investigated the influence of 17-DMAG on the association between HSP90 and AhR. In the presence of 17-DMAG, we could detect HSP90 as very faint proteins band (Fig. 2B). These results suggested that 17-DMAG inhibited binding of HSP90 to GST-PAS.

Next, we elucidated whether the ligand has an effect on the interaction between the AhR and HSP90. We performed a ligand treatment after the AhR PAS domain and HSP90 formed a complex. The AhR PAS domain and HSP90 were reacted as well as the above-mentioned experiments, then finally  $\beta$ -NF or DMSO was added to the complex and incubated. After incubation, the sample was reacted with the GST resin. As shown in Fig. 3, the lane of the GST-AhR PAS domain could detect HSP90 in the presence or absence of ATP and  $\beta$ -NF. However, the lane of GST detected only GST, while no HSP90 was detected. These results were the same as in Fig. 1B in the absence of ligand. The ligand did not cause dissociation of the AhR PAS domain from the HSP90 complex. These results suggested that the ligand is able to bind to the AhR PAS domain while in complex with HSP90.



**Fig. 1.** AhR PAS domain binds to HSP90. (A) Domain structure of human AhR. bHLH, PAS, and TAD indicate basic helix-loop-helix, PER-ARNT-SIM, and transcriptional activation binding domain, respectively. In the present study, we constructed and purified AhR-PAS including PAS-A and PAS-B (115–387). (B) Purified GST-PAS domain was analyzed by SDS-PAGE (9% gel). (C) Digested GST-PAS by factor Xa were separated by SDS-PAGE (9% gel). (D) The factor Xa digested AhR-PAS domains (input) were added to Mock resin (Mock) or  $\beta$ -naphthoflavone ( $\beta$ -NF) affinity resin. The bound proteins were separated by SDS-PAGE (9% gel) (D) or by immunoblotting using an anti-AhR antibody (E). In panels B and D, asterisk, double asterisks, and triple asterisks indicate uncut GST-PAS domain, cut PAS domain, and cut GST, respectively.



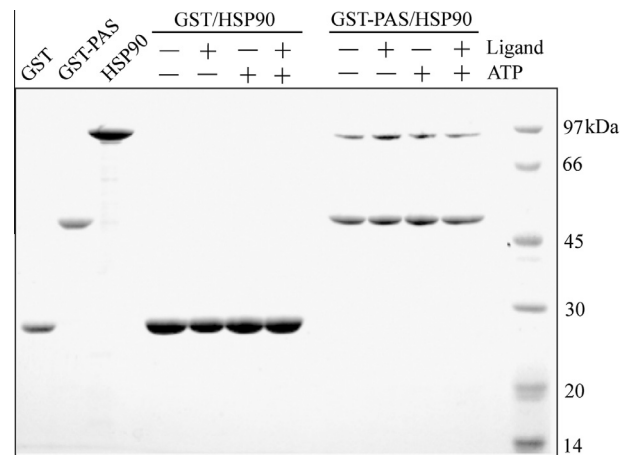
**Fig. 2.** GST pull-down assay confirming the interaction of HSP90 with AhR PAS domain in the absence or presence of 17-DMAG. The purified GST, GST-PAS, and HSP90 were incubated with GST resin in the absence (A) or presence of 50  $\mu$ M 17-DMAG (B). The elutants from the glutathione column were analyzed by SDS-PAGE (A: 11% gel; B: 9% gel). Lanes 1–3 of gels were input from purified GST (28 kDa), GST-AhR PAS (57 kDa), and HSP90 (90 kDa) as a control, respectively. Pull-down assays were performed using purified GST or GST-AhR PAS domain and purified HSP90 in the absence (–) or presence (+) of ATP.

We also considered the case when the ligand is bound to AhR alone prior to making a complex between AhR and HSP90. The GST-AhR PAS domain alone was treated with  $\beta$ -NF or DMSO, then the reaction solution was incubated with HSP90 and reacted with the GST resin. In all the lanes of the GST-AhR PAS domain, HSP90 was detected in the presence or absence of ATP and  $\beta$ -NF (Fig. 4). In contrast, the lane where GST only was applied, no HSP90 was detected. The GST-AhR PAS domain is still able to bind to HSP90 after binding of the ligands.

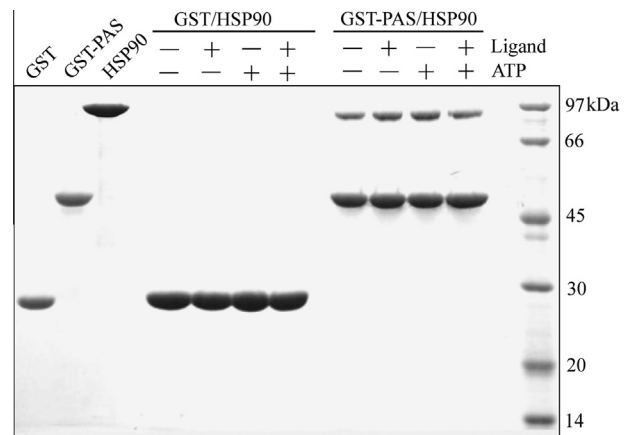
These results suggested that the ligand bound AhR PAS domain could bind to HSP90, also the ligand could bind to the complexed form of the AhR PAS domain with HSP90, and the AhR PAS domain did not dissociate from HSP90 under the same conditions. The association between HSP90 and AhR was not affected by the timing of the ligand binding.

## 2.2. HSP90 is co-localized with AhR after nuclear translocation

We investigated the amounts of AhR in the cytosol after treatment of ligand for 0, 15, 30, 60, and 120 min. As shown in Fig. 5A, total amounts of AhR in the cytosol decreased about

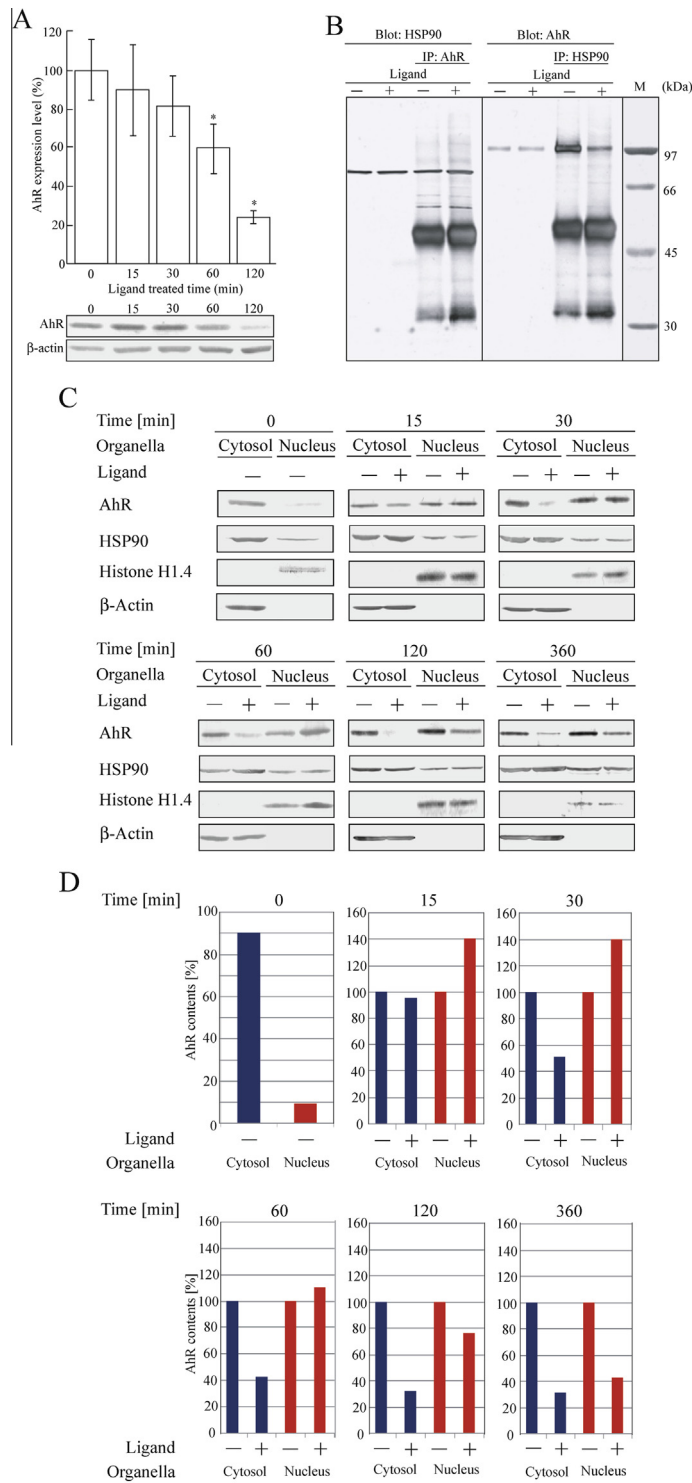


**Fig. 3.** Influence of ligand on the complex between AhR-PAS domain and HSP90. To perform pull-down analysis, GST or GST-AhR PAS domain and HSP90 were incubated with GST resin in the absence (–) or presence (+) of ATP. After incubation, 10  $\mu$ M  $\beta$ -NF was added as a ligand and used for pull-down assay. DMSO was used as the control. The elutants from the glutathione resin were analyzed by SDS-PAGE (11% gel). Lanes 1, 2, and 3 indicate GST, GST-PAS, and HSP90, respectively.



**Fig. 4.** Influence of ligand on AhR-PAS domain and the ability of making a complex with HSP90. The GST or GST-AhR PAS domain was incubated with glutathione resin in the presence of 10  $\mu$ M  $\beta$ -NF.  $\beta$ -NF was added to GST or GST-PAS domain before making a complex with HSP90. After incubation, HSP90 was added in the presence or absence of ATP. DMSO was used as the control. Lanes 1, 2, and 3, indicates GST, GST-PAS, and HSP90, respectively. The elutants from the glutathione resin were analyzed by SDS-PAGE (11% gel).

10%, 20%, 40%, and 80% at 15, 30, 60, and 120 min, respectively. It has been suggested that ligand-bound AhR is translocated from cytosol to nucleus within a short time of about 30 min. To confirm the interaction between HSP90 and the AhR, we performed an immunoprecipitation assay. HeLa cell whole-cell extracts were then immunoprecipitated with an anti-AhR antibody followed by immunoblotting with an anti-HSP90 or an anti-AhR antibody. The data clearly showed that HSP90 co-immunoprecipitated with AhR (Fig. 5B). In the presence of the ligand, the amount of co-precipitating AhR is significantly lower than in the absence of ligand in the cytoplasm (Fig. 5B). We investigated the amounts of AhR in the cytosol after treatment of  $\beta$ -NF for 0, 15, 30, 60, 120, and 360 min. As shown in Fig. 5C and D, total amounts of AhR in the cytosol decreased about 10%, 50%, 60%, 70%, and 70% at 15, 30, 60, 120, and 360 min, respectively. On the contrary, total amounts of AhR in the nucleus increased about 40%, 40%, and 10% at 15, 30, and 60 min, respectively. The quantity of AhR in the nucleus decreased about 20% and 60% at 120 and 360 min, respectively. It has been suggested that ligand-bound AhR is translocated from cytosol to



**Fig. 5.** Total amounts of endogenous AhR in the cytosol and nucleus of HeLa cells. (A) HeLa cells were treated with 0.05% DMSO (–) or 1  $\mu$ M  $\beta$ -NF (+) for 0, 15, 30, 60, and 120 min at 37 °C. The lysate was collected and centrifuged for 60 min at 105,000g, then the supernatant was collected. AhR in the cytosol were analyzed by immunoblotting with an anti-AhR antibody or an anti-  $\beta$ -actin antibody ( $n = 4$ , \* $p < 0.05$ ). The AhR/ $\beta$ -actin ratio was quantified using Image J software (Drop of Wisdom). (B) HeLa cells were treated with 0.05% DMSO (–) or 3  $\mu$ M  $\beta$ -NF (+) for 30 min at 37 °C. The lysate was collected and centrifuged, then the supernatant was collected. Immunoprecipitation was performed using an anti-AhR antibody or anti-HSP90 antibody followed by immunoblotting with an anti-HSP90 antibody or an anti-AhR antibody. In panel, the HeLa cell lysate (left two lanes) or immunoprecipitated samples (right two lanes) were separated by SDS–PAGE followed by immunoblotting. The subunit molecular weight of HSP90 and AhR are 90 and 110 kDa, respectively. The 50- and 32-kDa protein bands were the IgG heavy- and light chain, respectively. M, molecular marker proteins. C, HeLa cells were treated with 0.05% DMSO (–) or 1  $\mu$ M  $\beta$ -NF (+) for 0, 15, 30, 60, 120, and 360 min at 37 °C. The lysate was collected and centrifuged for 60 min at 105,000g, then the supernatant was collected as a cytosol fraction. Or the lysate in 2.2-M sucrose solution was centrifuged for 60 min at 30,000g, then the precipitate was collected as a nuclear fraction. AhR, HSP90 in the cytosol or nucleus were analyzed by immunoblotting with an anti-AhR antibody and anti-HSP90 antibody. The  $\beta$ -actin was analyzed as a cytosol marker protein using an anti-  $\beta$ -actin antibody. The Histone H1.4 was analyzed as a nucleus marker protein using a Histone H1.4 antibody. The AhR/ $\beta$ -actin ratio in the cytosol and the AhR/Histone H1.4 ratio in the nucleus were quantified using Image J software (Drop of Wisdom) (D).



nucleus within a short time of about 15 min. On immunoblotting, we could not detect significant changes of HSP90 quantity in the cytosol after treatment with  $\beta$ -NF.

To confirm the interaction between HSP90 and the AhR, we tried to detect endogenous HSP90/AhR complex in the HeLa cells. HeLa cells were treated with 5  $\mu$ M  $\beta$ -NF for 2 h. In the current study, we used *in situ* proximity ligation assays (PLA). The PLA technique displayed a high sensitivity in detecting the specific HSP90/AhR-complexes *in situ*. As shown in Fig. 6, *in situ* PLA signals were detected in the HeLa cells as red dots. The red signals were shown in the cytoplasm, but not in the nucleus in the absence of  $\beta$ -NF. On the contrary, we could detect red dots in the nucleus and around nucleus in the presence of  $\beta$ -NF. It has been suggested that HSP90 and AhR present in the cytoplasm make a complex in the absence of a ligand. After binding of the ligand, AhR is translocated into the nucleus still in association with HSP90.

### 2.3. $\beta$ -NF-induced expression of CYP1A1

Cytochrome P450 1A1 (CYP1A1) is one of the xenobiotic metabolizing enzymes (XMEs) that is induced by polycyclic aromatic hydrocarbons (PAHs). We analyzed the mRNA of CYP1A1 by RT-PCR (Fig. 7). CYP1A1 mRNA was induced at 2 h after the addition of  $\beta$ -NF. We quantified the induction rate of CYP1A1 mRNA based on the results of Fig. 7A and B shows the CYP1A1 mRNA level. The CYP1A1 mRNA increase was about double at 2 h and 3 times at 8 h.

Based on these results, we confirmed that HSP90 is co-localized with AhR after nuclear translocation. It has been suggested that HSP90 and AhR present in the cytoplasm make a complex in the absence of a ligand. After binding of the ligand, AhR is translocated into the nucleus while maintaining the formation in an HSP90 complex.

## 3. Discussion

The association between AhR and HSP90 has been demonstrated using a sucrose density gradient [16], an immunoprecipitation assay [17], an *in vitro*-translated AhR [14,18], and [ $^{35}$ S]-methionine labeled AhR [15,19]. The influence of HSP90 inhibitor on HSP90 and CYP1A1 protein expression has also been reported using an immunoblot analysis [20], and using cultured lung AD cells [21]. However, there are few reports concerning the direct interaction between the purified AhR and HSP90, because of the difficulty of expressing and purifying AhR. The detailed nuclear localization mechanism of AhR has not yet been fully understood.

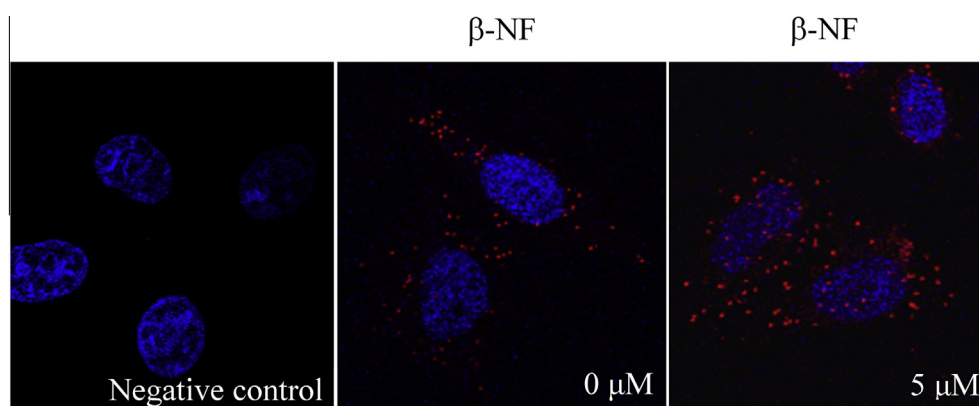
We constructed and purified the human AhR-PAS domain as a GST fusion protein. Although we tried to express and purify the His-tagged AhR-PAS domain or AhR-PAS domain alone, we could only purify the GST-AhR PAS domain. AhR is a protein that is very unstable and hard to purify. In the present study, we confirmed the direct interaction between the AhR PAS-domain and HSP90 using purified proteins.

We ascertained whether or not the purified AhR PAS-domain can bind to a ligand using an affinity resin.  $\beta$ -NF has the functional group C=O. In the present study, we tried to make a  $\beta$ -NF affinity resin using various affinity resins. We selected an Epoxy-activated Sepharose 6B.

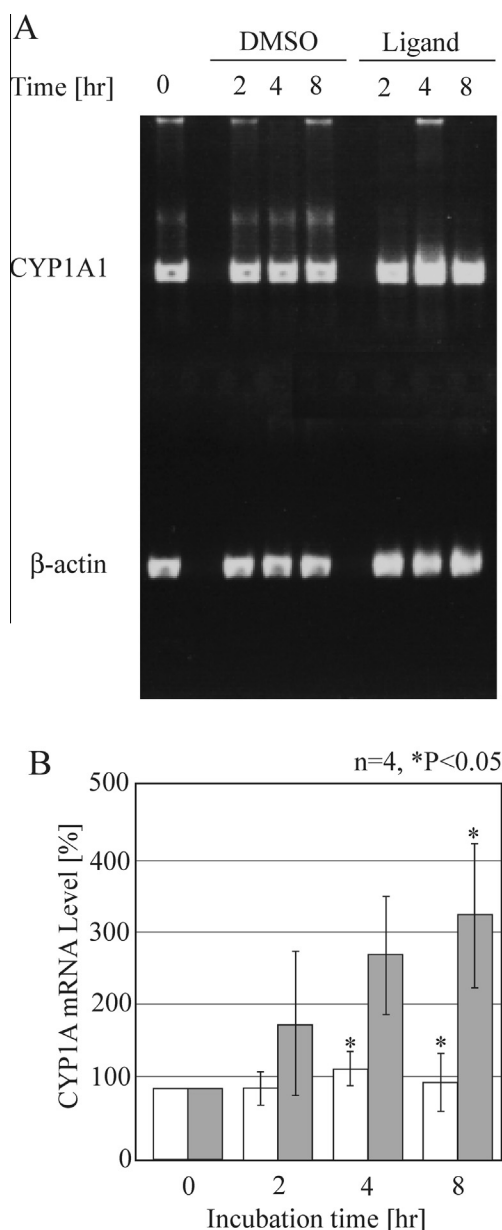
This is particularly useful for the coupling of the small molecule ligand. The AhR PAS-domain could bind to the affinity resin, but not to the Mock resin. This means that the purified AhR PAS-domain forms a peculiar highly advanced structure and has a ligand-binding ability. We investigated association between GST-PAS and HSP90. In the absence of HSP90 inhibitor, we could detect the association. On the contrary, the interactions were almost disappeared in the presence of 17-DMAG. 17-DMAG binds to N-domain of HSP90 and resulted in the dissociation of GST-PAS. 17-DMAG is more effective than 17-AAG. It has been shown that 17-DMAG significantly reduced proteins levels of AhR in human lung cancer adenocarcinoma H1355 cells [21]. These results suggested that 17-DMAG-induced dissociation of HSP90 may result in AhR degradation.

We investigated the influence on the timing of the ligand binding. If the activated AhR was translocated into the nucleus alone, the ligand bound AhR was dissociated from HSP90. Fig. 3 shows the result of the AhR and HSP90 complex formation first, and then the ligand binding to the complex. On the contrary, Fig. 4 is the result of ligand binding to AhR first, and after that ligand bound AhR make a complex with HSP90. These *in vitro* results suggested that AhR-HSP90 complex remains intact after binding of the ligand, and that the activated AhR may be translocated to the nucleus with HSP90.

We also analyzed whether or not the ligand-bound AhR makes a complex with HSP90 using *in situ* proximity ligation assays (PLA). *In situ* PLA signals of HSP90/AhR-complexes were detected in the HeLa cells as red dots. It has been suggested that the ligand-bound AhR, which was translocated in a nucleus with HSP90, substitution of HSP90 and Arnt, and activated AhR worked as a transcription factor, and as a result, CYP1A1 was induced. Based on the *in vitro* and *in vivo* results, we concluded that AhR and HSP90 were translocated into the nucleus as a complex.



**Fig. 6.**  $\beta$ -NF induced AhR and HSP90 nuclear translocation in HeLa cells. HeLa cells were treated with  $\beta$ -NF (5  $\mu$ M) or DMSO (0.1%) for 120 min. Complex formation between HSP90 and AhR was measured by *in situ* PLA using rabbit anti-AhR antibodies, mouse anti-HSP90 antibodies, and corresponding reagents. *In situ* PLA is indicated by red signals. Blue staining indicates DAPI staining of cell nuclei. Control was stained with non-immune rabbit- and mouse serum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 7.** Analysis of CYP1A1 in the 3MC treated HeLa cells using RT-PCR. (A) HeLa cells were treated with  $\beta$ -NF (3  $\mu$ M) or DMSO (0.1%) for 0, 2, 4, and 8 h. CYP1A1 or  $\beta$ -actin was amplified using RT-PCR, then detected by agarose gel electrophoresis as described under "Section 4". (B) The CYP1A1/ $\beta$ -actin ratio based on (A) was quantified using Image J software. The white bars indicate DMSO control and gray bars indicate  $\beta$ -NF treated cells.

Regarding the interaction between AhR and HSP90, there are published reports, but few give details at the biochemical protein level. We showed directly the interaction of AhR and HSP90 using purified protein in the present study. AhR is translocated into nucleus with HSP90. 17-DMAG induces a dissociation of HSP90 from AhR. The AhR–HSP90 complex is not affected by the timing of ligand binding. We also showed the AhR–HSP90 complex in nucleus after treatment with ligand by PLA.

#### 4. Materials and methods

##### 4.1. Materials

The rabbit polyclonal anti-HSP90 was previously described [22].  $\beta$ -NF as the ligand of AhR was purchased from Sigma–Aldrich

Japan, and the ligand stock solution was initially dissolved in dimethylsulfoxide (DMSO) which was purchased from Sigma–Aldrich Japan. 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17 DMAG) as the inhibitor of HSP90 was purchased from Invitrogen (San Diego, U.S.A.). Isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) was purchased from Nakarai Tesque. 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) was purchased from Life Technologies.

The total RNA was isolated from the HeLa cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). The amount and purity of the total RNA was estimated by spectrophotometric analysis at A260 and A280. The RNA quality was determined by agarose gel electrophoresis following ethidium bromide staining. Aliquots of the total RNA were diluted in diethylpyrocarbonated (DEPC)-treated water and stored at  $-80^{\circ}\text{C}$ . RNA (4  $\mu$ g) was used to synthesize the first strand complementary DNA (cDNA) with Super Script III First-Strand (Invitrogen) under the following general conditions: denaturation at  $94^{\circ}\text{C}$  for 15 s, annealing at  $55^{\circ}\text{C}$  for 30 s, and extension at  $68^{\circ}\text{C}$  for 30 s for up to 40 cycles using an iCycler (BioRad). The cDNAs were PCR-amplified by iCycler (BioRad) with the primers of AhR. The AhR-PAS domain [1326] was amplified by PCR (iCycler, BioRad) using the forward primer 5'-GTGCACATGGGAGAATTCT-TATTACAGGCTCTGA-3' and reverse primer 5'-CGCGCCGCTCATCCA TTTTATAAAGCAGG-3. The resulting PCR products were inserted into the Sall/NotI sites of the pGEX-5X-3 vector (Takara Bio, Japan). The pGEX-5X-3-AhR-PAS constructs were checked by a DNA sequencer (PRISM 3100, ABI). A full length cDNA of HSP90 $\alpha$  was kindly provided by Dr. Yokoyama (RIKEN, Japan). To generate the HSP90 recombinant protein expression, the HSP90 cDNA was amplified by PCR (iCycler, BioRad) using the forward primers 5'-GGATCCATGCCTGAGGAAACCCAGACC-3' and reverse primer 5'-TCTAGATTAGTCTAGTCTTCCATGCG-3. The resulting PCR products were inserted into the BamHI/XbaI sites of the pColdI vector (Takara Bio, Japan). The generated pColdI-HSP90 constructs were confirmed by DNA sequencing (PRISM 3100, ABI).

##### 4.2. Recombinant protein expression and purification

The AhR PAS domain was expressed in an *E. coli* Arctic Express Competent Cell (Stratagene). The cells were grown at  $30^{\circ}\text{C}$  in LB BROTH medium (Invitrogen) supplemented with 100  $\mu$ g/ml ampicillin for 3 h, then cultured at  $10^{\circ}\text{C}$  for 30 min. The cells were next induced by the addition of 0.5 mM IPTG and the cultures were incubated at  $10^{\circ}\text{C}$  for an additional 24 h. The cells were harvested by centrifugation at 20,000g for 15 min at  $4^{\circ}\text{C}$ , and the cell pellets were suspended in 10 mM Tris–HCl, pH 7.4. The cells were sonicated, centrifuged at 20,000g for 10 min at  $4^{\circ}\text{C}$  and the formed pellets collected. The collected pellets were suspended in buffer (1 M Arginine, 10 mM Tris–HCl, pH 7.4), then dialyzed with 10 mM Tris–HCl, pH 7.4, overnight to remove the Arginine. After dialysis, the lysates were cleared by centrifugation at 20,000g for 10 min at  $4^{\circ}\text{C}$ . The supernatant was applied to glutathione columns (Glutathione Sepharose 4B, GE Healthcare Life Science), washed with 10 mM Tris–HCl, pH 7.4, and then eluted with elution buffer (20 mM Glutathione, 10 mM Tris–HCl, pH 7.4). The HSP90 was expressed in *E. coli* BL21 (Promega). The cells were grown in LB BROTH medium (Invitrogen) supplemented with 100  $\mu$ g/ml ampicillin until the OD600 reached 0.5 at  $37^{\circ}\text{C}$ . The culture medium was cooled for 30 min to  $15^{\circ}\text{C}$ . The cells were then induced by the addition of 0.5 mM IPTG, and the culture was incubated at  $15^{\circ}\text{C}$  for an additional 24 h. The cells were sonicated for two cycles, centrifuged at 20,000g for 10 min at  $4^{\circ}\text{C}$ , then the supernatants were collected. Proteins were applied to the Q Sepharose column (GE Healthcare Life Science), then washed with wash buffer (0.3 M NaCl in 10 mM Tris–HCl, pH 7.4.). After washing, the proteins were eluted with a linear gradient of 0.3–0.6 M NaCl in

10 mM Tris–HCl, pH 7.4. The HSP90 fractions were concentrated by ultrafiltration, mixed in an equal amount of Ni column applied buffer (40 mM Imidazole in 10 mM Tris–HCl, pH 7.4), and applied to the Ni–NTA affinity column (GE Healthcare Life Science) equilibrated with the Ni column equilibration buffer (20 mM Imidazole, 300 mM NaCl in 10 mM Tris–HCl, pH 7.4). After washing with the Ni column wash buffer (50 mM Imidazole, 300 mM NaCl in 10 mM Tris–HCl, pH 7.4), the proteins were eluted with a linear gradient of 0.1–0.5 M imidazole in 0.3 M NaCl, 10 mM Tris–HCl, pH 7.4). The HSP90 fractions were concentrated by ultrafiltration. The protein concentration was then measured using the standard BCA assay (Thermo Scientific).

#### 4.3. GST pull-down assay

For the GST pull-down assay, 2.5  $\mu$ M GST-AhR PAS or 2.5  $\mu$ M GST protein was added to a solution of 2.5  $\mu$ M HSP90, 50  $\mu$ M 17-DMAG, 1 mM ATP, and 150  $\mu$ l buffer A (0.1 M KCl, 10 mM MgCl<sub>2</sub>, 20 mM Na<sub>2</sub>MoO<sub>4</sub>, 0.6 M NaCl, 5% Glycerol, 0.1% NP-40 in 25 mM Hepes–KOH, pH 7.4). The total volume of the sample was 300  $\mu$ l by adding buffer B (5% Glycerol, 0.1% NP-40 in 25 mM Hepes–KOH pH 7.4) and incubated using a rotator (LD-79, Labinco) with gentle rotation for 15 min at 37 °C. The samples were loaded onto a GST resin (GE Healthcare Life Science) equilibrated with buffer C (50 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM Na<sub>2</sub>MoO<sub>4</sub>, 0.3 M NaCl, 5% Glycerol, 0.1% NP-40 in 25 mM Hepes–KOH, pH 7.4) and incubated at 4 °C for 15 min with gentle rotation followed by spinning at 2100g for 10 s at 4 °C to remove the supernatant. The beads were washed three times with buffer C and eluted by boiling at 100 °C for 5 min in SDS sample buffer. For the ligand treatment, the proteins were similarly reacted with the GST resin. An aliquots of 10  $\mu$ M  $\beta$ -NF or DMSO in 300  $\mu$ l of buffer C was then added to the sample, incubated at RT for 30 min with gentle rotation followed by spinning at 2100g for 10 s at 4 °C to remove the supernatant. The beads were washed three times with buffer C and eluted by boiling at 100 °C for 5 min in SDS sample buffer. For the ligand treatment before the addition of HSP90, the GST-AhR PAS or GST protein was reacted with  $\beta$ -NF or DMSO and 150  $\mu$ l buffer A. The sample total was 300  $\mu$ l by adding buffer B. The samples were then incubated for 30 min at RT with gentle rotation, added to 2.5  $\mu$ M HSP90 and 1 mM ATP, and incubated for 15 min at 37 °C with gentle rotation. The samples were then similarly treated as previously described. All the GST pull-down samples were separated by SDS–PAGE (9% gel) and detected by Coomassie Brilliant Blue R-250 staining.

#### 4.4. Affinity chromatography

$\beta$ -NF-Sepharose was prepared using  $\beta$ -NF (Sigma–Aldrich Japan) and Epoxy-activated Sepharose 6B (GE Healthcare Life Science) according to the manufacturer's instructions. The purified GST-PAS (200  $\mu$ g) was digested using Factor Xa (GE Healthcare Life Science) according to the manufacturer's instructions. The digested samples were added to  $\beta$ -NF-Sepharose or Mock (without  $\beta$ -NF)-Sepharose equilibrated with 25 mM Hepes (pH 7.4) buffer and incubated with gentle rotation using a rotator (LD-79, Labinco) for 30 min at 4 °C. After washing with the same buffer three times, the bound proteins were separated by SDS–PAGE or by immunoblotting using an anti-AhR antibody.

#### 4.5. Cell culture and transfection

The human cervical cancer cell line, HeLa cell, was maintained in Dullbecco's modified Eagle's medium (DMEM; SIGMA) containing 10% fetal bovine serum (Equitech-Bio, Inc, Kerrville, TX), 20 units/ml penicillin and 20  $\mu$ g/ml streptomycin (Invitrogen,

Carlsbad, CA) under 5% CO<sub>2</sub> at 37 °C. Before transfection, the culture medium in the plate was replaced with DMEM containing 10% fetal bovine serum. For transfection, the expression plasmids (pcDNA3-Flag-HA-AhR or pcDNA3) were introduced into the HeLa cells using a FuGENE HD (Promega, Madison, WI) according to the manufacturer's protocol. After transfection, the cells were cultured in DMEM before being used for analysis.

#### 4.6. Immunoprecipitation assay

The HeLa cells were treated with 1  $\mu$ M  $\beta$ -NF or 0.05% DMSO for 30 min at 37 °C. The cells were then washed with PBS three times and lysed in TNE buffer (Tris–HCl 10 mM pH 7.8, NaCl 0.15 M, Nonidet P-40 1%, EDTA 1 mM, PMSF 1 mM). The lysate was collected and centrifuged at 20,000g for 10 min at 4 °C. After centrifugation, the supernatant and precipitate were collected. The AhR antibody (Thermo, MA1-513) were added to the supernatant or precipitate and incubated for 2 h at 4 °C. A 100  $\mu$ l aliquot of Protein G Sepharose (GE Healthcare Life Science) was added, then incubated for 1 h at 4 °C. The samples were washed with TNE buffer three times. After washing, the samples were separated by SDS–PAGE or by immunoblotting using an anti-AhR or anti-HSP90 antibody. The precipitate of the HeLa cells, treated with 1  $\mu$ M  $\beta$ -NF or 0.05% DMSO for 30 min at 37 °C, were separated by immunoblotting using an anti-AhR antibody.

#### 4.7. Subcellular fractionations of HeLa cells

The HeLa cells were treated with 1  $\mu$ M  $\beta$ -NF or 0.05% DMSO for 30 min at 37 °C. The cells were then washed with PBS three times and lysed in TNE buffer (10 mM Tris–HCl, pH 7.8, NaCl 0.15 M, Nonidet P-40 1%, EDTA 1 mM, PMSF 1 mM, 0.25 M sucrose). All operations were carried out at 0–4 °C. After centrifugation at 600g for 5 min, the precipitate was discarded. The 600 g supernatant was further centrifuged at 7000g for 10 min, and the supernatant (S1) and precipitate (P1) were treated by further centrifugation. The supernatant (S1) was centrifuged at 54,000g for 60 min, and the supernatant was further centrifuged at 105,000g for 60 min. The 105,000g supernatant was used as the cytoplasm. Nuclear extract preparation was carried out according to Wilson et al. [23]. The samples were separated by SDS–PAGE or by immunoblotting using an anti-AhR or anti-HSP90 antibody. The  $\beta$ -actin and Histone H1.4 were used as a cytosol and nuclear fraction marker, respectively. The bands of AhR for 0 min express the subcellular localization in which endogenous AhR is expressed. Cytosol and nucleus AhR expression level are normalized against  $\beta$ -actin and histone H1.4, respectively. The bar graphs indicate AhR band level relative to the DMSO-treated control band for each fractionation.

#### 4.8. Cell treatment and in situ proximity ligation assay (in situ PLA)

The HeLa cells were grown on coverslips. Twenty-four hours after culture, the cells were treated with 1  $\mu$ M  $\beta$ -NF dissolved in DMSO or DMSO only and incubated for 8 h. After incubation, the cells were washed twice with cold phosphate-buffered saline (PBS), then fixed in cold methanol at –20 °C for 10 min and cold acetone at –20 °C for 1 min. The fixed cells were washed with PBS twice and blocking buffer (1% BSA in PBS) for 10 min at room temperature.

The PLA assay was performed using detection kit (Olink Bioscience). HeLa cells seeded on coverslips were fixed in 95% ethanol for 10 min at 37 °C and incubated with 3% BSA in PBS for 1 h at room temperature. The primary antibodies specific for AhR (anti-rabbit AhR: Santa Cruz) and HSP90 (anti-mouse HSP90: Acris antibodies) were added, and the slides were incubated overnight at 4 °C. After



washing threefold with TBS, the PLA Probe PLUS (anti-rabbit antibody) and MINUS (anti-mouse antibody), which were secondary antibodies, were applied and the cells were stored for 2 h at 37 °C. The slides were washed twice with TBS; the hybridization solution was added for 15 min at 37 °C. The slides were washed once with TBS and incubated with ligation solution for 15 min at 37 °C. After twice washing with TBS, the cells were incubated with the amplification solution for 90 min at 37 °C and washed twice in TBS. The detection solution was then applied for 1 h at 37 °C with shading. Finally, the cells were washed with SSC; coverslips were mounted and visualized by confocal fluorescence microscopy.

#### 4.9. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was isolated from the HeLa cells treated with DMSO or  $\beta$ -NF for 0, 2, 4, and 8 h using the RNeasy Mini Kit (Qiagen, Valencia, CA). The amount and purity of the total RNA for each sample were estimated by spectrophotometric analysis at A260 and A280. The RNA quality was determined by agarose gel electrophoresis following ethidium bromide staining. Aliquots of the total RNA were diluted in diethylpyrocarbonated (DEPC)-treated water and stored at  $-80^{\circ}\text{C}$ . RNA (4  $\mu\text{g}$ ) was used to synthesize the first strand complementary DNA (cDNA) with Super Script III First-Strand (Invitrogen) under the following general conditions: denaturation at  $94^{\circ}\text{C}$  for 15 s, annealing at  $55^{\circ}\text{C}$  for 30 s, and extension at  $68^{\circ}\text{C}$  for 30 s for up to 40 cycles using iCycler (BioRad). The cDNAs were PCR-amplified by iCycler (BioRad) with the primers of CYP1A1 (forward, 5'-ACCACCAAGAAGCTTAGCC-3'; reverse, 5'-GAAGAGTGTCGGAAG-3'),  $\beta$ -actin (forward, 5'-GCTCGTCCGCAACGGCTC-3'; reverse, 5'-CAAACATGATCTGGTTCATCTTCTC-3'). The PCR products were separated in 1% agarose gels and stained with ethidium bromide. The CYP1A1/ $\beta$ -actin ratio was quantified using Image J software (Drop of Wisdom).

#### Author contribution

MO and HI in the research design; NT, KF, YN, HO, AH, SH, SY, TO, MH, KS, KO, SY conducted the experiment. EG and HI wrote or contributed to the writing of the paper.

#### Conflict of interest statement

The authors declare that they have no conflicts of interest.

#### Acknowledgments

H.I. was supported by a Grant-in-Aid for Scientific Research (Exploratory Research: 16651056) from the Japanese Ministry of Education, Science, Sports and Culture.

#### References

- [1] Li, J. and Buchner, J. (2013) Structure, function and regulation of the hsp90 machinery. *Biomed. J.* 36, 106–117.

- [2] Röhl, A., Rohrberg, J. and Buchner, J. (2013) The chaperone Hsp90: changing partners for demanding clients. *Trends Biochem. Sci.* 38, 253–262.
- [3] Erlejman, A.G., Lagadari, M., Toneatto, J., Piwien-Pilipuk, G. and Galigniana, M.D. (2014) Regulatory role of the 90-kDa-heat-shock protein (Hsp90) and associated factors on gene expression. *Biochim. Biophys. Acta* 1839, 71–87.
- [4] Kewley, R.J., Whitelaw, M.L. and Chapman-Smith, A. (2004) The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int. J. Biochem. Cell Biol.* 36, 189–204.
- [5] Mimura, J. and Fujii-Kuriyama, Y. (2003) Functional role of AhR in the expression of toxic effects by TCDD. *Biochim. et Biophys. Acta* 1619, 263–268.
- [6] Nebert, D.W. and Gonzalez, F.J. (1987) P450 genes: structure, evolution, and regulation. *Annu. Rev. Biochem.* 56, 945–993.
- [7] Beischlag, T.V., Luis Morales, J., Hollingshead, B.D. and Perdew, G.H. (2008) The aryl hydrocarbon receptor complex and the control of gene expression. *Crit. Rev. Eukaryot. Gene Expr.* 18, 207–250.
- [8] Knutson, J.C. and Poland, A. (1982) Response of murine epidermis to 2,3,7,8-tetrachloro-dibenzo-p-dioxin: interaction of the ah and hr loci. *Cell* 30, 225–234.
- [9] Poland, A. and Knutson, J.C. (1982) 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* 22, 517–554.
- [10] Lees, M.J. and Whitelaw, M.L. (1999) Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein Arnt. *Mol. Cell. Biol.* 19, 5811–5822.
- [11] Taylor, B.L. and Zhulin, I.B. (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.* 63, 479–506.
- [12] Gu, Y.Z., Hogenesch, J.B. and Bradfield, C.A. (2000) The PAS superfamily: sensors of environmental and developmental signals. *Annu. Rev. Pharmacol. Toxicol.* 40, 519–61.
- [13] Pongratz, I., Mason, G.G. and Poellinger, L. (1992) Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. Evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity. *J. Biol. Chem.* 267, 13728–13734.
- [14] McGuire, J., Whitelaw, M.L., Pongratz, I., Gustafsson, J.A. and Poellinger, L. (1994) A cellular factor stimulates ligand-dependent release of hsp90 from the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J. Biol. Chem.* 269, 2438–2446.
- [15] Lindbro, M.C., Poellinger, L. and Whitelaw, M.L. (1995) Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the HLH/PAS dioxin receptor-Arnt transcription factor complex. *EMBO J.* 14, 3528–3539.
- [16] Heid, S.E., Pollenz, R.S. and Swanson, H.I. (2000) Role of heat shock protein 90 dissociation in mediating agonist-induced activation of the aryl hydrocarbon receptor. *Mol. Pharmacol.* 57, 82–92. Phelan, D.M., Brackney, W.R. and Denison, M.S. (1998) The Ah receptor can bind ligand in the absence of receptor-associated heat-shock protein 90. *Arch. Biochem. Biophys.* 353, 47–54.
- [17] Soshilov, A. and Denison, M.S. (2011) Ligand displaces heat shock protein 90 from overlapping binding sites within the aryl hydrocarbon receptor ligand-binding domain. *J. Biol. Chem.* 286, 35275–35282.
- [18] Hogenesch, J.B., Chan, W.K., Jackiw, V.H., Brown, R.C., Gu, Y.Z., Pray-Grant, M., Perdew, G.H. and Bradfield, C.A. (1997) Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J. Biol. Chem.* 272, 8581–8593.
- [19] Fukunaga, B.N., Probst, M.R., Reisz-Porszasz, S. and Hankinson, O. (1995) Identification of functional domains of the aryl hydrocarbon receptor. *J. Biol. Chem.* 270, 29270–29278.
- [20] Wiseman, S.B. and Vijayan, M.M. (2007) Aryl hydrocarbon receptor signaling in rainbow trout hepatocytes: role of hsp90 and the proteasome. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* 146, 484–491.
- [21] Chen, P.H., Chang, J.T., Li, L.A., Tsai, H.T., Shen, M.Y. and Lin, P. (2013) Aryl hydrocarbon receptor is a target of 17-allylamino-17-demethoxygeldanamycin and enhances its anticancer activity in lung adenocarcinoma cells. *Mol. Pharmacol.* 83, 605–612.
- [22] Itoh, H., Ogura, M., Komatsuda, A., Wakui, H., Miura, A.B. and Tashima, Y. (1999) A novel chaperone-activity-reducing mechanism of the 90-kDa molecular chaperone HSP90. *Biochem. J.* 343, 697–703.
- [23] Wilson, S.R., Joshi, A.D. and Elferink, C.J. (2013) The tumor suppressor Kruppel-like factor 6 is a novel aryl hydrocarbon receptor DNA binding partner. *J. Pharmacol. Exp. Ther.* 345, 419–429.