Correlation of dysfunction of nonmuscle myosin IIA with increased induction of Cyp1a1 in Hepa-1 cells

Masayuki Ebina a,b, Masahiko Shibazaki c, Kyoko Kudo a,b, Shuya Kasai a,b, Hideaki Kikuchi a,*

a Science of Bioresources, United Graduate School of Agricultural Science, Hiwase University, Morioka 020-8551, Japan
b Department of Biochemistry and Biotechnology, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan
c Department of Molecular Genetics, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan

Abstract

The aryl hydrocarbon receptor (AhR) is one of the best known ligand-activated transcription factors and it induces Cyp1a1 transcription by binding with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Recent focus has been on the relationship of AhR with signaling pathways that modulate cell shape and migration. In nonmuscle cells, nonmuscle myosin II is one of the key determinants of cell morphology, but it has not been investigated whether its function is related to Cyp1a1 induction. In this study, we observed that (+)-blebbistatin, which is a specific inhibitor of nonmuscle myosin II, increased the level of CYP1A1-mRNA in Hepa-1 cells. Comparison of (−)-blebbistatin with (+)-blebbistatin, which is an inactive enantiomer, indicated that the increase of CYP1A1-mRNA was due to nonmuscle myosin II inhibition. Subsequent knockdown experiments observed that reduction of nonmuscle myosin IIA, which is only an isoform of nonmuscle myosin II expressed in Hepa-1 cells, was related to the enhancement of TCDD-dependent Cyp1a1 induction. Moreover, chromatin immunoprecipitation assay indicated that the increase of Cyp1a1 induction was the result of transcriptional activation due to increased binding of AhR and RNA polymerase II to the enhancer and proximal promoter regions of Cyp1a1, respectively. These findings provide a new insight into the correlation between the function of nonmuscle myosin II and gene induction.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Halogenated or polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo[a]pyrene (BaP), are widespread environmental pollutants. The toxic effects of these compounds in experimental animals include carcinogenicity, immunotoxicity and the development of malformations such as cleft palate [1–3]. The aryl hydrocarbon receptor (AhR) belongs to the basic helix-loop–helix/Per-Arnt-Sim (bHLH/PAS) superfamily, and functions as a transcription factor after binding with ligands such as TCDD and BaP. Unliganded AhR forms a cytosolic complex with heat shock protein 90 (Hsp90), hepatitis B virus X-associated protein 2 (XAP2) [4], and a co-chaperone protein called p23 [5]. Upon ligand binding, AhR undergoes a conformational change, dissociates from the cytosolic complex, and then translocates to the nucleus. Inside the nucleus, AhR dimerizes with AhR nuclear translocator (ARNT), and the AhR/ARNT heterodimer recognizes its target sequence, the xenobiotic response element (XRE), in target genes such as Cyp1a1, Cyp1a2, and Nqo1, and then recruits several transcriptional cofactors for induction of their target genes [6–8]. AhR null mice display non-inducible carcinogenicity, a small liver, and eye and kidney anomalies [9,10]. Therefore, it is thought that, in addition to being the central component in TCDD toxicity, AhR plays an important role in development. In relation to the developmental function of AhR, a ligand-independent signaling pathway and an endogenous ligand for AhR activation have been proposed [11–13]. Recently, several reports have indicated the involvement of AhR in cell physiology [14]. Diéry et al. have reported that TCDD-activated AhR modulates the morphology and plasticity of MCF-7 cells through a mechanism that depends on c-Jun N-terminal kinase (JNK) [15], and more recently, Bui et al. have reported that the upregulation of Hef-1 by TCDD-activated AhR is involved in cell migration and plasticity [16]. Furthermore, Carvajal-Gonzalez et al. have reported that AhR regulates cell shape and adhesion by modulating Vav3-dependent signaling [17].

Myosin II is a bipolar, hexameric protein complex that is composed of two heavy chains, two essential light chains, and two regulatory light chains. Hydrolysis of ATP by the myosin II motor domain alters...
the position of the domain relative to bound actin filaments (F-actin), which generates a contractile force that is thought to be important for cell motility, adhesion, and shape [18–21]. In vertebrates, the myosin II family consists of at least 15 different isoforms, and includes skeletal, cardiac and smooth muscle, and nonmuscle myosin heavy chains. Moreover, nonmuscle myosin II comprises three isoforms: nonmuscle myosin IIA, IIB, and IIC (NMIIA, IIB, and IIC). The amino acid sequence of these three isoforms is well conserved throughout the entire protein and they share certain cellular functions, but differences in expression pattern in embryonic and adult tissues [22,23], intracellular localization [24], and function [25] suggest that each isoform has a distinct role. The discovery of (−)-blebbistatin, which is a specific inhibitor of nonmuscle myosin II, has allowed the function of nonmuscle myosin II to be investigated in more detail [26]. Zhang and Rao have indicated that nonmuscle myosin II plays a role in the maintenance of cell morphology [27]. In another study, Ivanov et al. have shown by siRNA-mediated downregulation that NMIIA has a unique role in maintaining cell morphology and intercellular junctions [28]. The functions of nonmuscle myosin II have been elucidated gradually over time, but their correlation with gene regulation remains unclear.

In this study, we used (−)-blebbistatin and siRNA-mediated downregulation to examine the role of nonmuscle myosin II in the Cyp1a1 induction in Hepa-1 cells.

2. Materials and methods

2.1. Cell culture

The NIH 3T3 cell line was kindly provided by Prof. Ken Itoh (Hiroasaki University, Japan). Hepa-1c1c7 (Hepa-1) and NIH 3T3 cells were maintained in Dulbecco’s Modified Eagle’s Medium (Nissui, Tokyo, Japan) that contained 5% fetal bovine serum (Biosource International, Inc., Camarillo, CA, USA), 100,000 U/l penicillin, 100 mg/l streptomycin, and 3.7 g/l NaHCO3 in humidified 95% air and 5% CO2 at 37 °C.

2.2. Chemicals

TCDD was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). (−)- and (+)-blebbistatin (≥98% purity) was from Cayman Chemical (Ann Arbor, MI, USA). These were diluted in DMSO. The toxicities of these chemicals were tested by examining their effect on the growth rate of Hepa-1 cells using 0.3% trypan blue in calcium-magnesium-free-PBS, and the chemicals were used within the non-toxic dose range (in terms of cell viability).

2.3. RNA extraction, cDNA synthesis, RT-PCR and quantitative real-time PCR (qPCR)

RNA extraction was performed as described previously [29]. cDNA was synthesized by using M-MulV reverse transcriptase (Fermentas, Hanover, MD, USA). To confirm the expression of mRNA for members of the myosin II family, PCR reactions were performed on cDNA derived from Hepa-1 and NIH 3T3 cells and mouse lung tissue, using Phusion (Finnzymes, Keilaranta, Espoo, Finland) and the primer sets published previously [22]. The following primers for smooth muscle myosin were designed with Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/): 5′-GGAAACGAGGCTTCATTTGTTCC-3′ and 5′-ACCCCTTGTGACGGCGCTTGG3′. PCR amplification was as follows: an initial step at 98 °C for 30 s, then 35 cycles at 98 °C for 10 s, 60 °C for 20 s, and 72 °C for 15 s, with a final step at 7 min at 72 °C. The resulting PCR products were separated by electrophoresis on 2% agarose gels that included ethidium bromide. qPCR was performed in a reaction mixture that contained Thunderbird™ SYBR qPCR Mix (Toyobo, Osaka, Japan), and 6 μM for each primer set. Amplification was performed in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany), where the reaction mixture was heated to 95 °C for 30 s, and then underwent 40 cycles of denaturing at 95 °C for 5 s, annealing at 55 °C for 10 s, and elongation at 72 °C for 15 s. Primer sets for qPCR were as described previously: Cyp1a1 and Nqo1 [30], Cyp1a2 and Gapdh [31]. Gapdh was also amplified in each sample as an internal control for all the qPCR reactions.

2.4. Preparation of total cell extract, cytosolic fraction and nuclear fraction

Cells were suspended in a cell lysis buffer [15 mM HEPES pH 7.9, 0.35 M KCl, 1 mM MgCl2, 0.15 mM EDTA, 10% glycerol, 0.35% NP-40, 3 mM 2-mercaptoethanol, and 1× Complete™ Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany)], kept on ice for 10 min, and centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant was used as the total cell extract. Cytosolic fraction and nuclear fraction were prepared by our previously described method [32]. The protein concentrations of the total cell extract, cytosolic fraction and nuclear fraction were measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), to give a final concentration of 5 nM. After

2.5. SDS-PAGE and western blotting

The protein samples were resolved by 6 or 9% SDS-PAGE, and proteins were transferred to PVDF membranes (GE Healthcare, Little Chalfont, Bucks, UK). After Ponceau S staining to confirm an even transfer, the membranes were blocked with a blocking buffer [0.1% (w/v) Tween20-TBS (T-TBS) that contained 5% skimmed milk]. The membranes were incubated with primary antibody, washed twice in T-TBS, and then incubated with secondary antibody followed by two washes in T-TBS. Finally, immunoreactive bands were visualized using an enhanced chemiluminescence kit (GE Healthcare) and an X-ray film (Fujiﬁlm Co., Tokyo, Japan). The following primary antibodies were used: anti-AhR (M20), anti-GAPDH (FL-335) and anti-Lamin B (M-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-LDH (Chemicon Inc., USA); a Myosin II Isotype Antibody Sampler Kit (Cell Signaling Technology, Inc., Danvers, MA, USA); anti-smooth muscle myosin heavy chain (G4; Santa Cruz Biotechnology); anti-actin (ACTN 05; Thermo Scientiﬁc, San Jose, CA, USA); anti-vinculin (h-VIN1; Sigma–Aldrich Japan, Tokyo, Japan); and anti-RNAPII (05-623; Upstate Biotechnology, Charlottesville, VA, USA). Secondary antibodies were: horseradish-peroxidase-conjugated goat anti-rabbit, rabbit anti-mouse, and rabbit anti-goat IgG (DAKO, Cambridge, UK).

2.6. Preparation and transfection of siRNA

For the RNA interference (RNAi) experiments, siRNA duplexes were synthesized by using a Silencer siRNA Construction Kit (Ambion Inc., Austin, TX, USA) in accordance with the manufacturer’s protocol. Oligonucleotides for mouse control siRNA (sICont) and siRNA against NMIIA were as described previously by Vasscto et al. [33]. We chose si1 and si2 from that study, and named these as siNMIIA-1 and siNMIIA-2. Hepa-1 cells (3 × 105) were plated in culture dishes (6 cm diameter) and transfected by using Lipofectamine™ RNAiMAX (Invitrogen Corporation, Carlsbad, CA, USA), to give a final concentration of 5 nM. After
transfection, the cells were incubated for 2–4 days, and harvested for western blotting or other experiments.

2.7. Immunofluorescence labeling and image analysis

Phase-contrast images were obtained with an Olympus IMT-2 fluorescence microscope (Olympus Corporation, Tokyo, Japan). Chemical-treated or siRNA-transfected cells on coverslips coated with type I collagen (Iwaki Co., Tokyo, Japan) were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. To monitor focal adhesion and F-actin, the cells were first immunostained with antivinculin and Alexa 488-conjugated secondary antibody ( Molecular Probes) and then labeled with rhodamine–phalloidin ( Cytoskeleton Inc., Denver, CO, USA). Nuclei were stained with Hoechst 33258 ( Sigma). Fluorescence microscopy images were obtained using a laser scanning confocal microscope ( FV-1000; Olympus Co., Tokyo, Japan).

2.8. Chromatin immunoprecipitation ( ChIP) assay and qPCR analysis

The ChIP assay was performed as described by Schnekenburger et al. [30], with some modifications. After chemical treatment or siRNA transfection, Hepa-1 cells were incubated for 10 min at room temperature with 1% formaldehyde, and then incubated for a further 10 min with 0.125 M glycine. After harvesting by centrifugation, cells were resuspended in a cell burst buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, and 1× Protease Inhibitor Cocktail), and aliquots were removed as total input.

Chromatin was diluted 1:10 in a ChIP dilution buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% Tween-20, and 1× Protease Inhibitor Cocktail), and aliquots were removed as total input. The diluted chromatin was incubated overnight on a rotator at 4 °C, with 1 μg of antibodies specific for the protein of interest, or 1 μg of normal goat or rabbit IgG ( Santa Cruz Biotechnology) and Dynabeads Protein G ( Invitrogen). Procedures for ChIP assay after antibody reactions were as described by Shibazaki et al. [34]. DNA was dissolved in TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA) and subjected to qPCR with ChIP primer sets for the Cyp1a1 enhancer and proximal promoter regions, which corresponded to the −0.8 kb and −0.1 kb primer sets, respectively, that were described previously [30].

2.9. Statistics

Numerical values from individual experiments were pooled and expressed throughout as the mean ± standard deviation of the mean. The numbers obtained were compared by two-tailed Student’s t test, with p < 0.05 considered to be statistically significant.

3. Results

3.1. Effect of (−)-blebbistatin on Cyp1a1 induction in Hepa-1 cells

We used (−)-blebbistatin to examine whether inhibition of nonmuscle myosin II functions affects the Cyp1a1 induction in Hepa-1 cells. Hepa-1 cells have been used widely by both us and others to examine in detail the mechanism of Cyp1a1 transcription by TCDD [78,30,34]. (−)-Blebbistatin increased Cyp1a1 induction in both the presence and absence of TCDD (Fig. 1A). In the presence of 40 μM (−)-blebbistatin, (−)-blebbistatin itself increased the basal

![Fig. 1. Effects of nonmuscle myosin II inhibitor on CYP1A1 mRNA induction.](image-url)
level of CYP1A1 mRNA, to a level that was approximately 40% of that detected in the presence of TCDD in (−)-blebbistatin-untreated cells. In addition, the level of CYP1A1 mRNA by TCDD was also increased additively. To clarify whether the increase of CYP1A1-mRNA was due to the inhibition of nonmuscle myosin II, we compared the effect of (−)-blebbistatin and its enantiomer, (+)-blebbistatin, on the induction of Cyp1a1. Although a faint increase of CYP1A1-mRNA was observed by (+)-blebbistatin treatment, clear increase of Cyp1a1 induction was observed both in the presence and absence of TCDD by (−)-blebbistatin treatment. Previously, we have reported that tyrosine kinase inhibitors suppress TCDD-triggered Cyp1a1 induction through promoting the degradation of total AhR or inhibiting AhR nuclear localization [32]. Therefore, there is a possibility that (−)-blebbistatin treatment increases the amount of total AhR or its nuclear localization, which we examined by western blotting. Fig. 1C (upper panel) and D indicates that the increased induction of Cyp1a1 by TCDD in (−)-blebbistatin-treated cells was not due to an increased level of either total AhR or nuclear-localized AhR. Moreover, in the presence of (−)-blebbistatin the total amount of AhR decreased gradually from 4 h, although after 24 h the level had stabilized (lower panel in Fig. 1C). It is well known that several genes are induced by TCDD-activated AhR, and these genes include phase I and phase II genes of xenobiotic metabolizing enzymes. To examine the effect of (−)- and (+)-blebbistatin on the induction of other AhR target genes, we chose Cyp1a2 and Nqo1 as representative phase I and II genes, respectively, and compared them with Cyp1a1. As shown in Fig. 1E, whereas increased induction of Cyp1a2 by (−)-blebbistatin was similar to that of Cyp1a1, Nqo1 induction increased less in both the presence and absence of TCDD. These results indicate that the inhibition of nonmuscle myosin II functions is related to the increased induction of AhR target genes.

3.2. NMIIA is the only isoform expressed in Hepa-1 cells

In addition to being a highly selective inhibitor of nonmuscle myosin II, (−)-blebbistatin also inhibits smooth muscle myosin at high dose (IC50 = 80 μM) [35]. To determine the target for (−)-blebbistatin, we investigated which isoform of nonmuscle myosin II was expressed in Hepa-1 cells. Fig. 2A shows that mainly NMIIA mRNA was detected, and smooth muscle and NMIIIC mRNA were detected to a lesser extent, by RT-PCR. However, Fig. 2B shows that, at the protein level, only NMIIA was detected in Hepa-1 cells. Moreover, immunoprecipitation experiments showed that the interaction of NMIIA and actin was disrupted by treatment with 40 μM (−)-blebbistatin, but not (+)-blebbistatin (Fig. 2C). These results suggest that NMIIA is the only target of (−)-blebbistatin in Hepa-1 cells.

3.3. Knockdown of NMIIA enhances CYP1A1-mRNA induction by TCDD

We used RNAi (siRNA) to reduce the level of endogenous NMIIA and to examine whether knockdown of NMIIA altered the induction of CYP1A1 mRNA in a similar manner to (−)-blebbistatin treatment. As shown in Fig. 3A, the level of NMIIA was reduced substantially at 4 days after transfection with siNMIIA, whereas siNMIIA-2 had a smaller effect. In accordance with this, the induction of Cyp1a1 by TCDD was enhanced more by siNMIIA-1 than by siNMIIA-2 (Fig. 3B). Although (−)-or (+)-blebbistatin enhanced the basal level of Cyp1a1 induction, knockdown by siNMIIA did not enhance it. In a similar way, the enhancement of TCDD-triggered induction was observed for Cyp1a2 and Nqo1 (Fig. 3D). Fig. 3C shows that the enhancement of AhR target gene induction by siNMIIA was not due to an increased localization of AhR to the nucleus. These results indicate that the reduction of NMIIA by knockdown of the protein enhances the induction of AhR target genes by TCDD.

3.4. (−)-Blebbistatin and knockdown of NMIIA elicit the same morphological change in Hepa-1 cells

It is known that nonmuscle myosin II forms actomyosin through its interaction with F-actin, and plays a fundamental role in determining cell morphology. It has been reported that the inhibition of nonmuscle myosin II by (−)-blebbistatin treatment or the knockdown by siRNA results in changes in cell morphology, with the reorganization of vinculin-containing focal adhesions and the F-actin cytoskeleton [27,28]. However, the effects of the inhibition and knockdown of NMIIA have not been compared in the same cell line. Hence, we performed immunostaining of Hepa-1 cells in order to compare the effects of (−)-blebbistatin and siNMIIA in terms of cell morphology. Phase contrast images showed that (−)-blebbistatin induced the formation of fibroblast-like or dendritic extensions in Hepa-1 cells (Fig. 4A, upper panel). To analyze cytoskeletal remodeling, we performed immunocytochemistry to monitor the status of vinculin-containing focal adhesions and the F-actin cytoskeleton. The (−)-blebbistatin-treated cells contained fewer focal adhesions and F-actin than the DMSO- or (+)-blebbistatin-treated cells, although the levels of vinculin and actin in the (−)-blebbistatin-treated cells did not differ from those in other cells (middle and lower panel in Fig. 4A and B). These changes were not affected by TCDD treatment (Supplemental Fig. 3). Fig. 4C and D shows that the morphological changes and cytoskeletal reorganization that were induced by the knockdown of NMIIA were similar to those induced by (−)-blebbistatin treatment, and occurred in the absence of changes in the levels of vinculin and actin. Furthermore, they were not affected by TCDD treatment (data not shown). These results suggest that the inhibition and knockdown of NMIIA have similar effects on cell morphology and cytoskeletal organization in Hepa-1 cells.
Fig. 3. Effect of reduction of NMIIA on the induction of Cyp1a1 transcription by TCDD in Hepa-1 cells. (A) Hepa-1 cells were transfected with control siRNA (siCont) or siRNA against NMIIA (siNMIIA-1 and -2) at a final concentration of 5 nM. After culture for 2–4 days, cells were harvested and total cell extracts were prepared. Equal amounts of total cell extract (5 μg) were subjected to western blotting with each antibody. (B) Nine days after siRNA transfection, Hepa-1 cells were treated for 9 h with DMSO or 4 nM TCDD. Cells were harvested and total RNA was isolated followed by reverse transcription. The amount of CYP1A1 and GAPDH mRNA was determined by qPCR. The values plotted from three independent experiments (means ± SD) represent the relative induction and correspond to the ratio of expression of CYP1A1/GAPDH mRNA given relative to the mean value obtained after TCDD treatment of siCont-transfected cells, which was set arbitrarily at 100. * indicates significant difference (p<0.05) from the value obtained after TCDD treatment of siCont-transfected cells. (C) After transfection with siCont or siNMIIA for 4 days, Hepa-1 cells were treated for a further 1.5 h with DMSO (DM) or 4 nM TCDD (TC) and cytosolic and nuclear fractions were prepared. Aliquots of each fraction (10 μg) were used to examine the extent of nuclear localization of AhR by western blotting, as described in the legend for Fig. 1D. (D) qPCR was performed to quantify the relative induction of Cyp1a2 and Nqo1, using reverse-transcribed cDNA samples derived from siCont- and siNMIIA-1-transfected cells. □ and ■ indicate DMSO and TCDD treatments, respectively. The values plotted correspond to those plotted in (A).

3.5. Binding of AhR and RNA polymerase II to each target region of Cyp1a1 is increased by NMIIA dysfunction

It is well known that the induction of transcription of Cyp1a1 by ligand-activated AhR is accompanied by dynamic events on the genomic promoter, which includes the binding of AhR to the enhancer region, which contains an XRE, and RNA polymerase II (RNAPII) to the proximal region, which contains a TATA box [30]. The results of the ChIP assay showed that TCDD-triggered binding of AhR and RNAPII was increased by (−)-blebbistatin treatment (Fig. 5A and B, TC and (+)-Bleb + TC vs. (−)-Bleb + TC). The ChIP assay also showed a similar increase in siNMIIA-1-transfected cells (Fig. 5C and D, siCont + TC vs. siNMIIA-1 + TC). Basal binding of AhR and RNAPII was increased by blebbistatin treatments (Fig. 5A and B, DM vs. (−)-Bleb + DM and (+)-Bleb + DM), but not by siNMIIA-1 transfection (Fig. 5C and D, siCont + DM vs. siNMIIA-1 + DM). These results indicate that the increase of Cyp1a1 induction by the inhibition or knockdown of NMIIA is due to the increased binding of AhR and RNAPII to the enhancer and proximal regions of Cyp1a1, respectively.

4. Discussion

It is well known that nonmuscle myosin II plays an important role in cell motility, migration, and shape [20], although it has not been investigated thoroughly whether the function of the protein is related to gene induction. The inhibitor (−)-blebbistatin can be used to investigate the function of nonmuscle myosin II due to its cell permeability and selectivity for the protein [26]. In this study, we examined the correlation between the function of nonmuscle myosin II and the induction of AhR target genes. We observed that (−)-blebbistatin treatment increased the CYP1A1-mRNA expression both in the presence and absence of TCDD, and then confirmed that this occurred through the inhibition of NMIIA, which was only an isof orm that was expressed in Hepa-1 cells.

Phase contrast imaging and immunofluorescence showed that treatment of Hepa-1 cells with (−)-blebbistatin and transfection with siNMIIA resulted in the same morphological changes and cytoskeletal reorganization, which included the formation of dendritic extensions and loss of vinculin-containing focal adhesions and F-actin. More recently, Liu et al. have reported that treatment with (−)-blebbistatin suppresses the contraction and accelerates the migration of mouse hepatic stellate cells (HSCs), and this is accompanied by fewer vinculin-containing focal adhesions and F-actin [36]. Although we did not examine cell contraction and migration in the present study, we expect that Hepa-1 cells in which NMIIA function is inhibited or knocked down would have similar characteristics to HSCs treated with (−)-blebbistatin.

We noticed that the amount of AhR in Hepa-1 cells was decreased slightly by (−)-blebbistatin treatment or siNMIIA transfection (Figs. 1C and 3A). It has been suggested that unliganded AhR interacts with F-actin via binding to XAP2 [37]; therefore, AhR might become sensitive to degradation after the disruption of F-actin as a result of the inhibition or knockdown of NMIIA.

Furthermore, in spite of the reduction in the amount of total AhR after the inhibition or knockdown of NMIIA, it was clear that the amount of nuclear-localized AhR by TCDD in these cells was almost the same as that in the control cells (Figs. 1D and 3C). This might indicate that the reduced amount of AhR in cells that are dysfunctional for NMIIA is sufficient to translocate into the nucleus and induce the target genes of AhR. Therefore, the reduction of AhR might not affect the amount of nuclear AhR.

Increase of Cyp1a1 induction by TCDD was not due to an increased level of either total AhR or nuclear-localized AhR. Moreover, the kinetics of AhR nuclear localization by TCDD was similar between DMSO- and (−)-blebbistatin-treated cells, and between siCont and siNMIIA-1-transfected cells (Supplemental Fig. 1, middle and lower panel). As a consequence, we focused on the binding of AhR and RNAPII to the enhancer and proximal promoter regions of the Cyp1a1 gene at 1.5 h after TCDD treatment, which corresponds to the peak binding of AhR and RNAPII to each target region [30,32,34,38]. The ChIP assay indicated that the increase of Cyp1a1 induction by TCDD occurred via an increase in the binding of AhR and RNAPII to their target regions (Fig. 5). It has been proposed that the induction of Cyp1a1 by ligand-bound AhR involves the formation of a chromatin loop due to interaction between...
the enhancer and proximal promoter region [39]. The results of our ChIP assay imply that dysfunction of NMIIA results in the formation of a tighter chromatin loop, which increases the accessibility of transcriptional cofactors. Notably, treatment with (−)- or (+)-blebbistatin alone increased the binding of AhR and RNAPII to their target regions (Fig. 5A and B), which could explain the increase of basal Cyp1a1 induction in (−)- and (+)-blebbistatin-treated cells (Fig. 1B). The low level of AhR that was detected in the nuclear fraction of (−)- or (+)-blebbistatin-treated cells in the absence of TCDD (Fig. 1Da and Supplemental Fig. 1, upper panel), and the results of the reporter gene assay that used mutant F318A, which has no ligand binding ability [40], in the Gal-4 AhR fusion system [41] (Supplemental Fig. 2) implied that (−)- and (+)-blebbistatin bind to AhR as a weak ligand. The findings also suggest that (−)-blebbistatin has two mechanisms of action: firstly it acts as a weak ligand of AhR, and secondly it enhances gene induction by inhibiting NMIIA.

Several members of the myosin superfamily have been identified in the nucleus [42]. Among these, nuclear myosin I interacts with RNAPII [43], and forms interchromatin networks [44]. Li and Sarna have reported that the assembly of the RNAPII pre-initiation complex on the promoter of the intercellular adhesion molecule-1 (ICAM-1) gene is regulated by nuclear myosin II (smooth muscle and nonmuscle) [45]. In our study, we did not clarify the mechanism for the increase of AhR and RNAPII binding to their target regions. If NMIIA functions in the nucleus in a similar manner to the nuclear myosins, we can speculate that it suppresses Cyp1a1 induction by recruiting a co-repressor, such as histone deacetylase 1 (HDAC1), to the proximal promoter region.

The cytoskeleton contains microtubules, as well as actomyosin, and a crosstalk between actomyosin and microtubules is involved in maintaining cell motility and shape [46,47]. Dvorak et al. have implied that TCDD-triggered Cyp1a1 induction is suppressed by colchicines, which are known disruptors of microtubules [48]. From our results, we speculate that the enhancement/suppression of Cyp1a1 induction by TCDD, which occurs through the disruption of actomyosin/microtubules, involves the crosstalk between the two cytoskeletal components.
NMIIA has been investigated as a potential clinical cause of MYH9-related diseases such as May–Hegglin anomaly, and is not inhibited by alpha-naphthoflavone, Arch. Biochem. Biophys. 358 (1998) 351–358.


[26] M. Shibazaki, T. Takeuchi, S. Ahmed, H. Kikuchi, Suppression by p38 MAP kinase activation of the dioxin/aryl hydrocarbon receptor (AhR) target genes by TCDD was observed in cells transfected with siNMIIA; therefore this type of abnormal gene induction in medial epithelial cells might contribute to TCDD-induced cleft palate.

In conclusion, we demonstrated that the function of NMIIA is related to the induction of AhR target genes in Hepa-1 cells. Although further investigation is needed to establish how dysfunction of NMIIA enhances the binding of AhR and RNAPII to their target regions, our study sheds new light on the relationship between the function of NMIIA and gene induction.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbagrm.2011.01.002.

Acknowledgments

The authors express their appreciation to Prof. Sei-ichi Ishiguro and Dr. Taku Ozaki for helpful advice.

We thank the staff of the Gene Research Center and Center for Instrumental Analysis in Hirosaki University.

This work was supported by Grants-in-aid for Science Research (B) (No. 15310032) and (C) (No.21510065) from the Ministry of Education, Culture, Sports, Science and Technology (Monbu Kagakusho), and the Global Environment Research Fund from the Ministry of the Environment, Japan (C-0803).

References


