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The aryl hydrocarbon receptor and glucocorticoid receptor interact to activate human metallothionein 2A

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ABSTRACT

Although the aryl hydrocarbon receptor (AHR) and glucocorticoid receptor (GR) play essential roles in mammalian development, stress responses, and other physiological events, crosstalk between these receptors has been the subject of much debate. Metallothioneins are classic glucocorticoid-inducible genes that were reported to increase upon treatment with AHR agonists in rodent tissues and cultured human cells. In this study, the mechanism of human metallothionein 2A (*MT2A*) gene transcription activation by AHR was investigated. Cotreatment with 3-methylcholanthrene and dexamethasone, agonists of AHR and GR respectively, synergistically increased *MT2A* mRNA levels in HepG2 cells. *MT2A* induction was suppressed by RNA interference against AHR or GR. Coimmunoprecipitation experiments revealed a physical interaction between AHR and GR proteins. Moreover, chromatin immunoprecipitation assays indicated that AHR was recruited to the glucocorticoid response element in the *MT2A* promoter. Thus, we provide a novel mechanism whereby AHR modulates expression of human *MT2A* via the glucocorticoid response element and protein–protein interactions with GR.

Introduction

The aryl hydrocarbon receptor (AHR), a member of the basic helix–loop–helix/Per–Arnt–Sim (bHLH/PAS) family, is a ligand-activated transcription factor responsible for the development and adaptive response to environmental changes elicited by toxic agents, hypoxia, and the light and dark cycle (Gu et al., 2000). AHR plays a key role in xenobiotic responses as a receptor for environmental contaminants such as dioxins, coplanar polychlorinated biphenyls, and benzo[*a*]pyrene. In the absence of ligand, AHR exists in the cytoplasm in a complex with XAP2, two HSP90 chaperone proteins, and the co-chaperone protein p23. Upon binding with a ligand, AHR translocates to the nucleus, where it dimerizes with its heterodimer partner, AHR nuclear translocator (ARNT) (Denison et al., 2011). This AHR-ARNT heterodimer binds a DNA sequence termed the xenobiotic response element (XRE) and subsequently activates transcription of target genes represented by the phase I xenobiotic-metabolizing enzymes of cytochrome P450 (CYP) 1A1, CYP1A2, and CYP1B1 and by the phase II drug metabolizing enzymes of glutathione S-transferase and UDP-glucuronosyltransferase, as well as NAD(P)H quinone oxidoreductase 1 (Nebert et al., 2004; Omiecinski et al., 2011). Induction of these enzymes results in the metabolism and detoxification of xenobiotics, including some AHR ligands. However, increased metabolism can also cause adverse effects that result from oxidative stress and metabolic activation of certain xenobiotic chemicals. In addition to ligand-induced xenobiotic responses, AHR is related to various physiological events, such as cell proliferation and differentiation, development, reproduction, and immune system regulation.

While XRE-dependent mechanism is important for AHR transcriptional regulation, recent studies have indicated that AHR modulates gene expression without directly binding DNA. Instead, AHR interacts with other transcription factors, such as the RelA subunit of nuclear factor-kappa B (NFκB) (Tian et al., 1999; Kim et al., 2000) and estrogen receptor α (Beischlag and Perdew, 2005), and modulates the activity of these molecules in an XRE-independent manner. Thus, protein–protein interactions appear to be important for the various biological functions of AHR.

Glucocorticoids, including cortisol and corticosterone, are steroid hormones secreted from the adrenal glands that play a role in stress

Abbreviations: 3-MC, 3-methylcholanthrene; AHR, aryl hydrocarbon receptor; bHLH/PAS, basic helix–loop–helix/Per–Arnt–Sim; ChIP, chromatin immunoprecipitation; Dex, dexamethasone; GR, glucocorticoid receptor; GRE, glucocorticoid response element; MT, metallothionein; MT2A, metallothionein 2A; XRE, xenobiotic response element.

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response through the regulation of carbohydrate metabolism and inflammation. The glucocorticoids are ligands of glucocorticoid receptor (GR), a member of the nuclear receptor superfamily. Glucocorticoid intracellular signaling is governed by GR. In the absence of ligand, GR exists in the cytoplasm in a complex with HSP90. After activation by ligand binding, GR enters the nucleus. GR forms a homodimer, and the GR dimer binds with a glucocorticoid response element (GRE) and regulates transcription of its target genes (Bamberger et al., 1996). On the other hand, protein–protein interactions between GR and other transcription factors are essential for GR-mediated transcriptional suppression. For example, synthetic glucocorticoids are widely used as therapeutic anti-inflammatory agents. In this mechanism, GR binds to NFκB and selectively represses transcription of proinflammatory cytokines such as IL-8 (Nissen and Yamamoto, 2000; Luecke and Yamamoto, 2005).

Both AHR and GR play essential roles in development, reproduction, immune system regulation, and stress responses, and there are apparent relationships between the activities of these two receptors. Cleft palate is a malformation that can be induced in the laboratory by excessive activation of AHR. Administration of dioxin to a pregnant mammal induces cleft palate in the fetus through activation of AHR (Mimura et al., 1997). Excess glucocorticoids also induce cleft palate. A synergistic effect was observed in embryonic cleft palate induced by cotreatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and hydrocortisol, AHR and GR agonists, respectively (Pratt, 1985; Abbott, 1995; Abbott et al., 1999). Using a reporter gene assay with a GRE-driven reporter construct, Dvořák1 et al. show that TCDD enhanced GR transactivation induced by dexamethasone (Dex), a synthetic GR agonist, in HepG2 cells (Dvořák et al., 2008). Wang et al. showed that AHR ligands such as polyaromatic hydrocarbons synergistically enhance Dex-induced transactivation of GR in ovarian granulosa HO23 cells (Vrzal et al., 2009). This synergistic effect was independent of the individual expression levels of AHR or GR. Others performed a comprehensive analysis to identify latent transcription factors associated with AHR-signaling (Frericks et al., 2008). A computational search for over-represented elements in the promoter region of TCDD-affected genes in a thymic epithelial cell line identified 37-transcriptional binding sites concluding with a GR binding sequence. These data imply that AHR and GR are closely associated with each other. However, the molecular mechanism of their interaction has not yet been clarified.

Mammalian metallothioneins (MTs) are classic glucocorticoid-inducible genes (Karin et al., 1980; Hager and Palmiter, 1981; Karin and Richards, 1982; Kelly et al., 1997). MTs are low molecular weight, cysteine-rich metal binding proteins. MTs are believed to play an important role in homeostasis of essential metals and biological protection against environmental toxicity represented by heavy metals or reactive oxygen species. In our previous study, DNA microarray analyses revealed that the mRNA levels of MT isoforms *Mt1* and *Mt2* were elevated in the liver of mice that were administered low doses of TCDD (Sato et al., 2008). Other groups also reported elevated mRNA or protein levels of MTs in rat (Nishimura et al., 2001; Fletcher et al., 2005) and mouse tissues (Kurachi et al., 2002; Boverhof et al., 2005) following acute exposure to high doses of TCDD, as well as in HepG2 cells treated with TCDD (Frueh et al., 2001). However, a functional XRE was not identified in the promoter region of MT genes, and the mechanism of transcriptional activation mediated by AHR is still unclear.

In this study, we identified human metallothionein 2A (*MT2A*) as an intrinsic gene whose transcription is regulated by AHR–GR interactions. In cultured cells, expression of human *MT2A* was cooperatively increased by cotreatment with Dex and 3-methylcholanthrene (3-MC), an AHR agonist, in a receptor-dependent manner. Our chromatin immunoprecipitation (ChIP) assay results indicate that AHR is recruited to the *MT2A* promoter. Moreover, coimmunoprecipitation experiments reveal a physical interaction between AHR and GR. Thus, we conclude that AHR modulates *MT2A* gene expression via the glucocorticoid response element and a novel protein–protein interaction with GR.

Materials and methods

Materials. TCDD, 3-MC, Dex, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). HepG2 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. HeLa and COS7 cells were kindly provided by Dr. Yoshida, Scientific University of Tokyo.

Animal experiments. TCDD-treatment of mice and total RNA isolation from the liver were performed as described previously (Sato et al., 2008). Briefly, C57BL/6N wild-type and *Ahr*-null male mice, aged 6–7 weeks, were orally administered doses of 5, 50, or 500 ng TCDD/kg body weight (bw) in corn oil by gavage once a day for 18 days. Animals in the control groups were administered the same volume of corn oil without TCDD. Throughout the experimental period, the animals had free access to AIN93G standard diets (Reeves et al., 1993) and desalted water. On the 19th day, experimental animals were sacrificed and hepatic RNA was isolated using a phenol/guanidine-isothiocyanate-based reagent (Isogen; Nippon Gene Co., Tokyo, Japan).

The Animal Research-Animal Care Committee of the Graduate School of Agricultural Science, Tohoku University approved the experimental plan of the present study. All experiments were performed under the guidelines framed by this committee in accordance with Japanese governmental legislation (1980). The same committee supervised the care and use of mice in this study.

Northern hybridization analyses. Total RNA (20 μg) from the liver was denatured in an RNA gel-loading buffer at 65 °C for 5 min and loaded onto 1.2% agarose gels containing formaldehyde (Sambrook et al., 1989). After electrophoresis, the RNA was transferred to Hybond N + nylon membranes (GE Healthcare, Tokyo, Japan) using the capillary method. DNA fragments encoding mouse metallothionein-1, rat metallothionein-2, and α-tubulin were labeled with [^{32}P] dCTP (MP Biomedicals, Irvine, CA). The RNA-blotted filters and labeled cDNA were incubated at 68 °C for 1.5 h in ExpressHyb Hybridization Solution (Clontech Laboratories, Palo Alto, CA). After hybridization, the filters were washed twice with 2× SSC (150 mM sodium chloride, 150 mM sodium citrate) for 30 min and with 0.1× SSC for 40 min. The filters were then exposed on a Fuji imaging plate (Fuji Photo Film, Tokyo, Japan) for an adequate period of time and analyzed using a BioImage analyzer FLA-2000 (Fuji Photo Film). The relative mRNA expression levels were normalized to the amount of α-tubulin mRNA.

Cell culture. HepG2, HeLa, and COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). HepG2 cells were plated and cultured for 16 h. The media was replaced with additive-free DMEM (without serum and antibiotics) and incubated for 24 h. Next, TCDD, 3-MC and/or Dex, or vehicle (DMSO) control was added at 0.1% v/v to the media and cells were incubated for 9 h. Cells were washed twice with phosphate-buffered saline (PBS), and total RNA was isolated. Total RNA (4 μg) was denatured at 65 °C for 5 min with 2.5 μM oligo-dT primer (GE Healthcare) and 0.5 mM dNTP (GE Healthcare). The RNA was incubated in 20 μl of RT buffer [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol] containing 50 U SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and 20 U RNaseOUT RNase inhibitor (Invitrogen) for 60 min at 50 °C. An aliquot of synthesized cDNA was used as the template for quantitative PCR using an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA). The target cDNAs were amplified using gene-specific primers (Table 1) and SYBR Premix Ex Taq (Takara Bio, Otsu, Japan) solution. The relative mRNA expression levels were normalized to the amount of eukaryotic translation elongation factor 1α1 (*EEF1A1*) mRNA.