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4S Polycyclic Aromatic Hydrocarbon Receptor (Glycine *N*-Methyltransferase) and the Aryl Hydrocarbon Receptor Nuclear Translocator (Hypoxia Inducible Factor-1β) Interaction in Chinese Hamster Ovary and Rat Hepatoma Cells: 4S PAH-R/ARNT Hetero-Oligomers?

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ABSTRACT

Rat *CYP1A1* promoter-luciferase, transiently transfected wild-type and 4S PAH receptor (glycine *N*-methyl transferase, GNMT)-transformed Chinese hamster ovary (CHO) cells were exposed to benzo[*a*]pyrene and assayed for luciferase activity as an indicator of *CYP1A1* promoter activity. CHO cells transformed with the rat 4S PAH receptor/GNMT expression vector had twice the induction level of luciferase activity with respect to wild-type CHO cells in concert with previously published reports that the 4S PAH receptor/GNMT mediates benzo[*a*]pyrene induction of CYP1A1 gene expression. Lysates of GNMT-transformed CHO cells and wild-type H4IIE rat hepatoma cells exposed to benzo[*a*]pyrene were immuno-precipitated with anti-GNMT antibodies, separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membrane for Western blot analysis with anti-aryl hydrocarbon receptor nuclear translocator (ARNT, HIF-1β) antibodies. Results of this analysis indicated that the 4S PAH receptor/GNMT forms a hetero-oligomer (dimer?) with ARNT/HIF-1β which dissociates in the presence of B[*a*]P. These observations further indicate the role of GNMT (which has been shown to be multifunctional) and B[*a*]P in the induction of CYP1A1 and also a potential role of GNMT in the modulation of hypoxia inducible factor-1 function with respect to the HIF-1β subunit (ARNT). J. Cell. Biochem. 112: 2015–2018, 2011.

KEY WORDS: BENZO[*a*]PYRENE (B[*a*]P); POLYCYCLIC AROMATIC HYDROCARBON (PAH); CYTOCHROME P4501A1 (CYP1A1); ARYL-HYDROCARBON RECEPTOR (AHR; 8S RECEPTOR; DIOXIN RECEPTOR); 4S-PAH RECEPTOR (4S PAH-R); GLYCINE *N*-METHYLTRANSFERASE (GNMT); ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR (ARNT); HYPOXIA INDUCIBLE FACTOR-1BETA (HIF-1β); XENOBIOTIC RESPONSE ELEMENT (XRE); POLYCYCLIC AROMATIC HYDROCARBON RESPONSE ELEMENT (PRE)

The cytochrome P450-dependent monooxygenases represent a large family of isozymes that catalyze the metabolic activation of protoxins, procarcinogens, and the detoxification of a multitude of environmental substrates [Conney, 1967, 1982; Nebert and Gonzalez, 1987]. The CYP1 subfamily includes CYP1A1, CYP1A2, and CYP1B1 [Morville et al., 1983; Thomas et al., 1983; Sutter et al., 1991]. Normally CYP1A1 expression is not constitutive in adult human tissues, however, its expression is highly induced by exposure to polyaromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons found in tobacco smoke, environmental contaminants, and dietary constituents [Whitlock, 1986]. Cytochrome P4501A1 (CYP1A1) expression is primarily under transcriptional control and involves the interaction of both positively and negatively acting transcription factors [Whitlock, 1986; Hines et al., 1988; Boucher et al., 1995; Sterling and Bresnick, 1996].

The 4S polyaromatic hydrocarbon receptor (glycine *N*-methyltransferase [GNMT]) binds benzo[*a*]pyrene (B[*a*]P) an environmental pre-carcinogen as a dimer that is subsequently trans-located to the nucleus were it binds to the PAH-4S receptor consensus sequence (PRE) of the CYP1A1 gene [Bhat and Bresnick, 1997].

GNMT was identified as a homo-tetramer with a M_r of 132,000 and a monomer with a M_r of 31,500 from rat liver [Cook and Wagner, 1984]. Glycine *N*-methyltransferase (*S*-adenosyl-L-methionine:glycine methyltransferase, EC 2.1.1.20) catalyzes the synthesis of sarcosine from S-adenosylmethionine and glycine and also functions as a folate binding protein [Cook and Wagner, 1984]. Raha et al. [1994, 1995] subsequently demonstrated that GNMT was also

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the PAH-4S receptor. Chinese hamster ovary cells were subsequently utilized to establish that the expression of GNMT in the absence of expression of AHR mediated the induction of expression of CYP1A1 by B[*a*]P and B[*e*]P [Bhat and Bresnick, 1997].

The aryl-hydrocarbon receptor (AHR) also referred to as the 8S receptor or Dioxin (TCDD) receptor has been studied extensively and forms a complex with the chaperone molecule, heat shock protein 90 (HSP90) [Okey, 2007]. TCDD diffuses through the cell membrane into the cytosol where it binds AHR with concomitant dissociation of HSP90 and replacement by aryl-hydrocarbon receptor nuclear translocator (ARNT), the TCDD/AHR/ARNT complex enters the nucleus and binds to the xenobiotic response element (XRE) of transcriptional regulatory regions of genes containing the XRE, notably CYP1A1 [Okey, 2007].

We report in this study that the 4S PAH-R (GNMT) also complexes with ARNT (HIF-1β) and in the presence of B[*a*]P, the amount of ARNT comprising this complex is attenuated.

MATERIALS AND METHODS

CELL CULTURE

CHO D422 cells, were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 in minimal essential medium, supplemented with 40 mg/L proline containing 50 mg/ml gentamycin and 10% fetal bovine calf serum [Bhat and Bresnick, 1997]. H4IIE, rat hepatoma cells were cultured as described [Sterling et al., 1993].

STABILE TRANSFORMATION

The plasmid construct, pMAMneo/GNMT was stably transfected into CHO cells by the Lipofectin method (CHO-GNMT cells) [Bhat and Bresnick, 1997] or by electroporation using the GIBCO/BRL Cell Porator in HBS buffer solution [21 mM HEPES (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose] at 800 μf, low voltage, and fast charge rate.

TRANSIENT TRANSFECTION

Transfection of wild-type or pMAMneo/GNMT stably transformed CHO cells with pMCLUC-1 which contains −3,015 to +2,545 of the rat CYP1A1 gene fused to the luciferase reporter gene [Sterling et al., 1994] was carried out as described [Sterling and Bresnick, 1996].

LUCIFERASE ASSAY

Luciferase activity was determined as described [Sterling and Bresnick, 1996].

IMMUNOPRECIPITATION

Immunoprecipitation with either rabbit anti-GNMT (4S PAH-R) (a gift from Dr. Conrad Wagner) or rabbit anti-ARNT (HIF-1β) (a gift from Dr. William Greenlee) was carried out as described [Sterling and Bresnick, 1996].

WESTERN BLOTTING

Western blotting was carried out as described [Sterling and Cutroneo, 2004] using either rabbit anti-GNMT or rabbit anti-ARNT.

RESULTS

pMCLUC-1, transiently transfected wild-type (WT) and pMCLUC-1, transiently-transfected, pMAMneo-GNMT, stably transformed CHO cells (KCL-2) were exposed to 4 μM B[*a*]P in DMSO for 24 h. Wild-type CHO cells had a 5-fold induction of luciferase activity (CYP1A1 promoter activity) compared to DMSO vehicle-treated wild-type cells while KCL-2 cells had a 10-fold induction of luciferase activity compared to DMSO vehicle-treated KCL-2 cells (Fig. 1). CHO-GNMT and H4IIE cells were exposed to DMSO (vehicle) or 4 μM B[*a*]P for 24 h, lysed and immunoprecipitated with rabbit anti-rat GNMT overnight. The immunoprecipitate was separated by SDS–PAGE and transferred to PVDF membrane and probed with rabbit anti-ARNT. ARNT (HIF-1β) co-precipitated with GNMT indicating that these two proteins are in an oligomeric (dimeric?) form in the cytosol of these

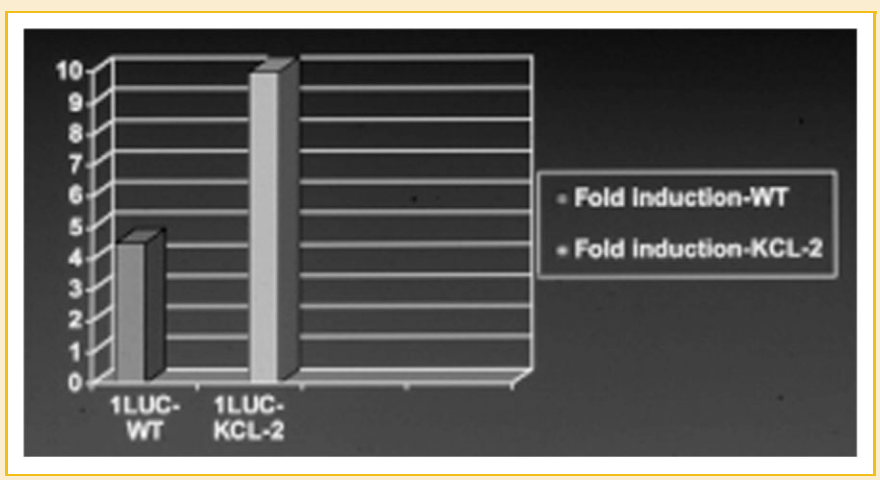

Fig. 1. Fold induction of CYP1A1 promoter-lucifierase activity in pMCLUC-1, transiently transfected, wild-type (WT) and GNMT stably transformed (KCL-2) CHO cells by 4 μM B[*a*]P.

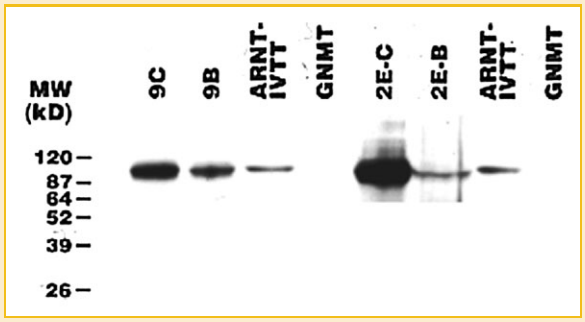

Fig. 2. Co-immunoprecipitation of ARNT (HIF-1β) by anti-GNMT antibodies from lysates of GNMT stably transformed CHO cells [clone 9, Bhat and Bresnick, 1997] and wild-type H4IIE rat hepatoma cells exposed to DMSO and 4 μM B[*a*]P for 24 h. Lane 9C, vehicle-treated stably transformed CHO cell lysate (50 μg), lane 9B, 4 μM B[*a*]P-treated stably transformed CHO cell lysate (50 μg), lane 2EC, vehicle-treated H4IIE cell lysate (50 μg), lane 2EB, 4 μM B[*a*]P-treated H4IIE cell lysate (50 μg), lanes ARNT-IVTT and GNMT, 20 μl in vitro transcribed and translated ARNT (HIF-1β) and 4 μg purified rat liver GNMT respectively. The immunoprecipitated cell lysates and ARNT and GNMT controls were separated by SDS–PAGE and transferred to PVDF membrane and subsequently all were reacted against anti-ARNT antibodies.

cell lines in the absence and presence of B[*a*]P (Fig. 2). Furthermore the amount of ARNT (HIF-1β) in the complex is significantly reduced after treatment with B[*a*]P (Fig. 2). Anti-ARNT antibodies did not cross-react with purified rat liver GNMT (Fig. 2). KCL-2 cells were also immunoprecipitated with anti-GNMT antibodies (Fig. 3, lane e) or anti-ARNT antibodies (Fig. 3, lane f) overnight and subsequently separated by SDS–PAGE and blotted to PVDF membrane along with 2, 4, 6, and 8 μg of purified rat liver GNMT (Fig. 3, lanes a–d) and in vitro transcribed and translated (IVTT) ARNT (Fig. 3, lane g). Anti-GNMT antibodies immunoprecipitated GNMT from KCL-2 cells (Fig. 3, lane e) and anti-ARNT antibodies co-immunoprecipitaed GNMT from KCL-2 cells (Fig. 3, lane f). Anti-GNMT antibodies did not cross react with IVTT ARNT (Fig. 3, lane g) further indicating the existence of GNMT/ARNT(HIF-1β) complexes in GNMT-transformed CHO cells and wild-type H4IIE rat hepatoma cells.

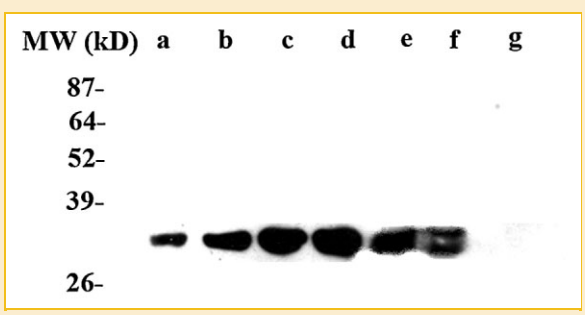

Fig. 3. Lanes a, b, c, d contained 2, 4, 6, and 8 μg of purified rat liver GNMT respectively. Lane e, GNMT stably transformed CHO (KCL-2) cell lysate immunoprecipitated with anti-GNMT. Lane f, GNMT stably transformed CHO (KCL-2) cell lysate immunoprecipitated with anti-ARNT (HIF-1β). Lane g, 20 μl IVTT-ARNT. Immunoprecipitated cell lysates, purified GNMT and in vitro translated ARNT (HIF-1β) were separated by SDS–PAGE and transferred to PVDF membrane and subsequently reacted with anti-GNMT antibodies.

DISCUSSION

Figure 1 shows a fivefold induction of a rat cytochrome P4501A1 promoter-lucifierase reporter gene activity in transiently transfected wild-type CHO cells by 4 μM B[*a*]P. This result was unexpected since RT-PCR showed little or no expression of GNMT messenger RNA and no detectable GNMT protein by Western blot analysis of wild-type CHO cells [Bhat and Bresnick, 1997]. However, wild-type CHO cells stably transformed with pMAMneo-GNMT (KCL-2 cells) showed twice the induction of the CYP1A1 promoter-luciferase reporter gene activity as compared to wild-type CHO cells (Fig. 1) which is consistent with the findings of Bhat and Bresnick [1997]. More importantly the 4S-PAH binding protein (GNMT) forms a hetero-oligomer (dimer?) with aryl-hydrocarbon receptor nuclear translocator (ARNT, HIF-1β) (Figs. 3 and 4). ARNT, HIF-1β was first shown to dimerize with the aryl-hydrocarbon receptor (AHR) [Hoffman et al., 1991; Reyes et al., 1992]. The discovery of ARNT led to significant advances in understanding AHR function and subsequently hypoxic signaling where ARNT (HIF-1β) plays a vital role [Fryer and Simon, 2006]. ARNT function is also involved in vascular tumorigenesis [Rankin et al., 2005] and type 2 diabetes [Gunton et al., 2005]. ARNT and AHR are members of a family of regulatory proteins with bHLH/PAS domains [Gu et al., 2000].

This report indicates that GNMT, which is multi-functional [Cook and Wagner, 1984; Raha et al., 1994; Bhat and Bresnick, 1997] has another function which remains to be fully understood with respect to hetero-oligomer formation with HIF-1β (ARNT) which in of itself is an important factor in cancer development [Chang et al., 2009]. HIF-1β (ARNT) has also been shown to interact with several other transcription factors including *c-jun* [Chang et al., 2009].

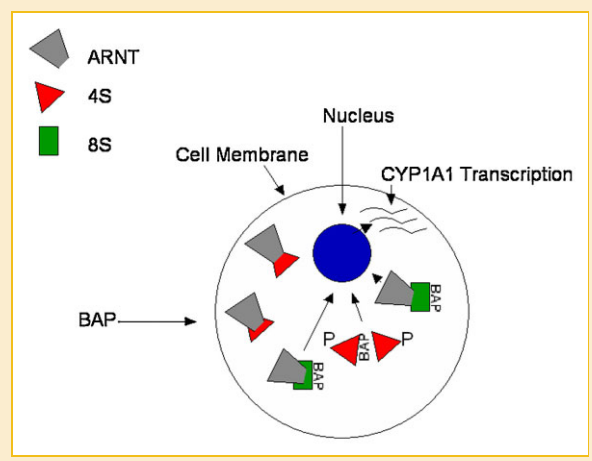

Fig. 4. Hypothetical model of the possible interaction of B[*a*]P with the 4S receptor (PAH-receptor, GNMT), the 8S receptor (AHR, Dioxin receptor), and the aryl-hydrocarbon receptor nuclear translocator (ARNT, HIF-1β). "P" indicates a phosphorylated 4S receptor.

The amount of ARNT (HIF-1β) co-immunoprecipitated with anti-GNMT antibodies is significantly reduced upon exposure to B[*a*]P suggesting that upon dimer formation of GNMT with bound B[*a*]P [Bhat and Bresnick, 1997], ARNT (HIF-1β) dissociates from GNMT (Fig. 4). Further experimental analysis is necessary to completely understand the biological importance of the 4S-PAH receptor (GNMT) and ARNT (HIF-1β) interaction in normal physiology and cancer development and progression.

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