DIFFERENTIAL RESPONSE TO ARYLHYDROCARBON RECEPTOR LIGANDS IN CELL TYPES

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Abstract
The aryl hydrocarbon receptor (AhR) mediates a variety of biological responses to ubiquitous environmental pollutants. Flavonoids, which are polyphenolic antioxidants naturally present in vegetables, fruits, and beverages exhibit multiple activities and interact with several cellular receptors, including AhR. In this study, we investigated the AhR agonist/antagonist effects of α-naphthoflavone (ANF) and β-naphthoflavone (BNF) on the expression of CYP1A1 using several human cell lines. BNF, a typical agonist for AhR, significantly elevated the CYP1A1 mRNA expression in all cells. In contrast, 5 μM ANF induced the CYP1A1 mRNA expression in Hep3B and HepG2, but not in LAN5, HUAEC, and HEK293 cells. We also investigated the xenobiotic response element (XRE)-mediated activities of ANF and indigo in Hep3B and HEK293 cells which were transiently transfected with reporter gene plasmids. The plasmid was constructed with a luciferase reporter gene linked to the 800b-promoter region of the AhR gene including the XRE. The induction profile of HEK293 cells was similar to that of Hep3B cells. These results suggest no direct participation of XRE involved in the 800b-promoter region in cell-specific responses against AhR ligands.

Introduction
The aryl hydrocarbon receptor (AhR) is a ligand-activated basic helix-loop-helix transcription factor (bHLH) that plays a pivotal role in mediating a broad range of distinct toxic responses induced by polyhalogenated and polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds[1,2]. Unliganded AhR is located in the cytoplasm associated with heat shock protein 90 (hsp90) and a 38-kDa, immunophilin-related protein. Upon ligand binding, hsp90 is released from the complex and the receptor translocates into the nucleus and dimerizes with the ARNT protein[3]. The heterodimer binds to the xenobiotic response element (XRE)[4] and alters the expression of genes controlled by enhancer XREs. XREs, with the conserved core sequences "GCGTG", are found in the promoter regions of several genes involved in the metabolism of xenobiotics, including cytochrome P450s (CYP1A1, CYP1A2, and CYP1B1) and NAD (P) H-quinone oxidoreductase. Studies of the regulation of these genes, especially the regulation of CYP1A1, have provided a basis for understanding the mode of action of TCDD and related compounds[5]. It has been reported that flavonoids, which are ubiquitously expressed in plant foods, act as an AhR agonist. However, some studies suggest that flavonoids act as an AhR antagonist in both *in vitro* and *in vivo* models[6,7]. For example, resveratrol antagonistically suppresses the expression of CYP1A1 induced by TCDD[8]. The present study aimed to investigate the effects of flavonoids on the inducibility of the CYP1A1 mRNA expression in several human cell lines. We used flavonoid compounds that have been reported to act either as a typical agonist, β-naphthoflavone (BNF), or as partial/complete antagonist of AhR, α-naphthoflavone (ANF), in order to investigate the effects of the AhR agonists/antagonists on various cell lines.

Materials and Methods
Cell culture. Hep3B, HepG2, and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% of fetal bovine serum, and incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. LAN5 cells were maintained in RPMI 1640 medium (Sigma) containing 10% of fetal bovine serum. HUAEC cells were maintained in EBM-2 medium (Sanko Junyaku Co., Ltd., Tokyo, Japan). Before treatment with chemicals, the cells were cultured in serum-reduced (0.1 %) medium for 24 h and then test chemicals were added to the medium. Twelve hours later, the cells were harvested and washed 2 times with PBS(-).
Detection of CYP1A1 mRNA induction. The total RNA of the cells were isolated by Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The total RNA was reverse-transcribed into single-stranded cDNA by using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and 1 μg of total RNA. The reverse-transcription was performed sequentially at 75°C for 5 min, 25°C for 15

min, 42°C for 60 min, and 72°C for 10 min. The amplification was carried out in a final volume of 15 μl in a reaction mixture containing 1 μl of cDNA, 1.25 units of GoTaq (Promega, Madison, WI), and 7.5 pmol of each primer. The primers for the polymerase chain reaction (PCR) were 5'-GGAGACCTTCCGACACTCTT-3' (forward) and 5'- CCTTGTCGATAGCACCATCA-3' (reverse) for CYP1A1; and 5'-CAAGAGATGGCCACGGCTGCT-3' (forward) and 5'-TCCTTCTGCATCCTGTCGGCA-3' (reverse) for β-actin. These were designed on the basis of the published sequences of human CYP1A1 (accession no. NM_000499) and β-actin (accession no. NM_001101.2). The PCR was performed first at 94°C for 2 min and then the amplification cycles were performed at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The amplification cycles were 30 cycles for CYP1A1 and 18 cycles for β-actin. The amplified cDNA fragments generated were resolved in 2% agarose gels and visualized using a digital imaging system.

Immunoblotting. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were performed as described previously[9] using anti-AhR antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) or anti-β-actin rabbit serum generated in our laboratory.

Cloning. The CYP1A1 promoter fragment (-800 to +73) was amplified using a High Fidelity PCR System (Applied Biosystems, Foster City, CA) from the genomic DNA of Hep3B cells. The primers for PCR were designed on the basis of the published sequences of the 5'-flanking region of human CYP1A1 (accession no. AF253322). The primers were 5'-AAGGTACCTTCTGTGCTCTGCCAATCAA-3' (forward), which includes a *Kpn*I site (underline) and 5'-AGCCTCGAGGCTGGGAAGGGTGGACTCT-3' (reverse), which includes a *Nhe* site (double underline). The PCR was performed first at 94°C for 2 min and then the 30 amplification cycles were performed at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec. The amplified DNA fragment was subcloned into the pGEM-T Easy Vector (Promega). The constructed plasmid including 874 bp of the CYP1A1 promoter region was inserted into the pGL3 luciferase reporter plasmid (Promega) at the *Kpn*I and *Xho*I sites. Ligation products were transformed into DH5α competent *E. coli* cells. High quality plasmids for transfection were prepared using a QIAGEN Plasmid Midi Kit (QIAGEN).

Transient transfection and luciferase activity assay. Each cell was seeded at 70% confluent in 35-mm plates 1 day before transfection. Two micrograms of test plasmid, 0.1 μg of phRL-TK vector were cotransfected into the cells using the GenePORTER2 Transfection Reagent (San Diego, CA). After incubation for 24 h, the culture medium was replaced with fresh serum-reduced medium. After incubation for 12 h, test chemicals were added to the medium and incubated for 12 h. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega).

Results and Discussion

The effect of flavonoids on CYP1A1 induction was examined using Hep3B and HepG2, human hepatic carcinoma cell lines; LAN5, human neuroblastoma cells; HUAEC, human umbilical artery endothelial cells; and HEK293, human embryonic kidney cells. When the cells were exposed to 5 μM BNF, the CYP1A1 mRNA expression was induced significantly in all cell lines (Figure 1). The highest inductive effect was found in HepG2 cells, which have an approximately 20-fold induction. In contrast, the lowest induction was found in LAN5 cells, which have an approximately 2-fold induction. Previous studies showed that ANF was an AhR antagonist for the induction of AhR-mediated CYP1A1[10]. However, in this study, exposure to 5 μM ANF resulted in the significant induction of CYP1A1 mRNA in the Hep3B and HepG2 cells, whereas no induction was observed in the LAN5, HUAEC, and HEK293 cells. These data demonstrate that the AhR antagonist activity of ANF is variable among cell lines, and that their fold inducibility is also dependent on cell context.

A previous report showed the higher affinity of the AhR for BNF compared to ANF[11]. It was assumed that the response for ANF may be due to the abundance of AhR, and we therefore examined the amount of AhR protein in the cell. Unexpectedly, however, there were no correlations between the inductive effect by ANF and the AhR expression among the tested cells (Figure 2). In addition to the AhR, the ARNT mRNA expression level also had no relationship to the response for ANF (data not shown). A luciferase reporter plasmid containing the 800b-promoter region of the AhR gene was prepared. The construct including XRE was transfected into Hep3B or HEK293 cells, which were incubated with ANF or BNF for 12 h. Treatment with ANF enhanced the AhR promoter activity 1.7-fold and 1.8-fold in the Hep3B and HEK293 cells, respectively (Fig.3). When the cells were treated with BNF, direct inhibition of luciferase activity was observed. When indigo, an endogenous AhR ligand[12], was used for the AhR agonist, the promoter activity was increased 3.0-fold and 3.6-fold in the Hep3B

and HEK293 cells, respectively.
These results suggested that the agonistic effect of ANF in hepatic cells may be attributed to a cell-specific factor and/or regulation element. In order to understand the differences in the action of ANF on hepatocytes or extrahepatocytes, further studies on the promoter region of AhR are needed.

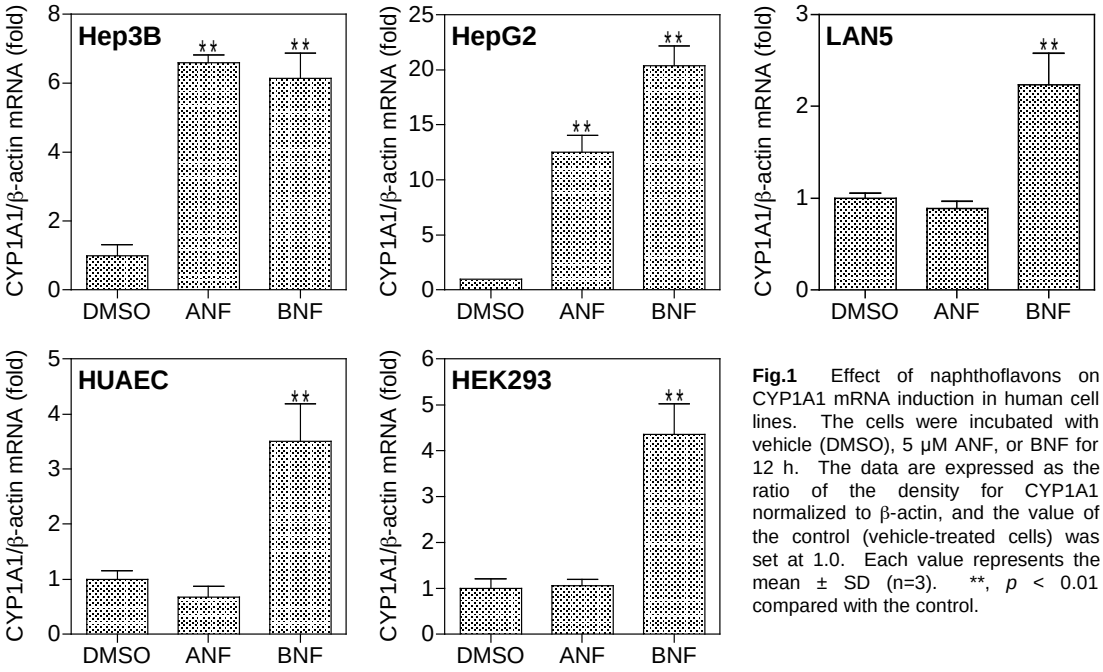

Fig.1 Effect of naphthoflavons on CYP1A1 mRNA induction in human cell lines. The cells were incubated with vehicle (DMSO), 5 μM ANF, or BNF for 12 h. The data are expressed as the ratio of the density for CYP1A1 normalized to β-actin, and the value of the control (vehicle-treated cells) was set at 1.0. Each value represents the mean ± SD (n=3). **, $p < 0.01$ compared with the control.

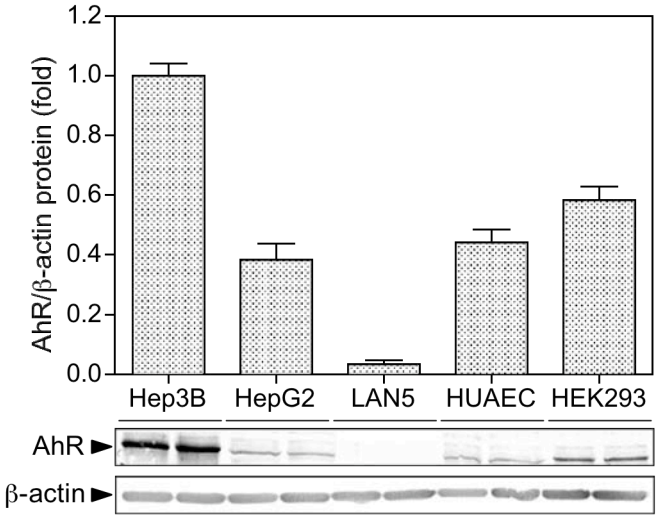

Fig.2 Analysis of AhR protein in human cell lines. The data are expressed as the ratio of the density for AhR normalized to β-actin, and the value of the Hep3B was set at 1.0. Each value represents the mean ± SD (n=3).

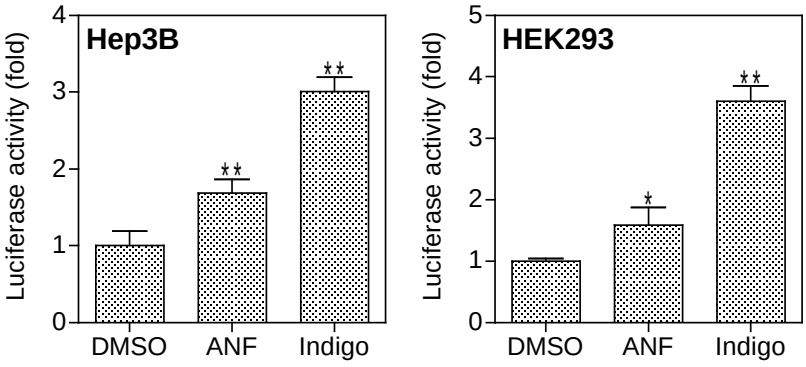

Fig.3 Induction of *CYP1A1* promoter-luciferase activity by ANF or indigo. Luciferase activity was measured in the Hep3B or HEK293 cells transiently transfected with the *CYP1A1* promoter-luciferase construct. A dual luciferase reporter assay was performed with the lysates obtained from the cells after exposure to ANF (5 μM) or indigo (5 μM) for 12 h. Activation of the reporter gene was calculated as a relative change to the *Renilla* luciferase activity. Each value represents the mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$ compared with the control. Control (vehicle-treated cells) was set at 1.0.

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