EFFECTS OF LIGANDS FOR AH RECEPTOR ON HYPOXIC RESPONSE OF CELLS

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Abstract
The aryl hydrocarbon receptor (AhR) and hypoxia inducible factor-1α (HIF-1α) are basic helix-loop-helix PAS proteins that form heterodimers with Arnt. AhR and HIF-1α scramble for Arnt, suggesting that the activation of one pathway would inhibit the other due to competition for Arnt. The complex of AhR and Arnt binds to xenobiotic response element (XRE) and activates the transcription of genes such as *CYP1A1*, whose pathway mediates the toxicity of AhR ligands such as dioxin. The complex of HIF-1α and Arnt binds to hypoxia response element (HRE) and plays an important role in adaptation to low oxygen by activation of hypoxia response genes such as *erythropoietin (EPO)*. In this study, we investigated the cross-talk between the AhR and HIF-1 signaling pathways by using alpha-naphthoflavone (ANF), beta-naphthoflavone (BNF), 3,4',5-trimethoxybenzophenone (TMB), genistein and daidzein. All chemicals used in this study induced *CYP1A1* mRNA in Hep3B cells, and ANF but not BNF inhibited *EPO* mRNA induction under hypoxia. However, both ANF and BNF induced *EPO* mRNA under normoxia. We confirmed the activation of *CYP1A1* and *EPO* genes through XRE and HRE, respectively, with a luciferase reporter assay. These results suggested that a complex mechanism may be involved in *EPO* gene regulation by AhR ligands.

Introduction
The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-Arnt-Sim family of proteins.[1, 2] Ligand-free AhR in the cytosol is present as a complex associated with 90 kD of heat shock protein (HSP90).[3] The AhR mediates the toxic effects of several chemical carcinogens, including polycyclic and halogenated aromatic hydrocarbons. Ligand-elicited activation promotes the receptor to undergo a series of processes involving dissociation from HSP90, translocation to the nucleus, and formation of a heterodimer with the AhR nuclear translocation partner (Arnt). This heterodimer binds to the xenobiotic response element (XRE) in the promoter and enhancer regions of target genes such as the *CYP1A1* gene.[4, 5] Hypoxia-inducible factor-1 (HIF-1) is a central mediator of cellular responses to low oxygen and has recently become an important therapeutic target for solid tumor therapy.[6, 7] HIF-1 is a heterodimetric transcription factor composed of HIF-1α and HIF-1β (Arnt). Under normoxic conditions, the HIF-1α protein is hydroxylated by prolyl hydroxylases, which require oxygen for activity, and pVHL (an E3 ubiquitin ligase) recognizes hydroxylated HIF-1α, followed by degradation of ubiquitinated HIF-1α with 26S proteasomes.[8-10] Under hypoxia, the inhibition of prolyl hydroxylase causes the accumulation of HIF-1α, which is stabilized by binding with HSP90.[9, 10] HIF-1α forms a heterodimer with Arnt in the nucleus and binds to the hypoxia response element (HRE). This model implies that the activation of AhR may deprive Arnt of the Arnt/HIF-1α complex, preventing hypoxic response pathway.[11] Therefore, we studied the cross-talk between the AhR and HIF-1 signaling pathways. In this study, we investigated inhibition for the hypoxic response of cells using flavones, including antagonist and agonist for AhR, and analyzed the mechanism of this inhibition.

Materials and Methods
Cell culture The human hepatoma cell line Hep3B was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer of Tohoku University (Sendai, Japan). Hep3B cells were maintained in DMEM containing 10% FCS, and 1% penicillin and streptomycin at 37 ^{0}C in 5% CO_2.
Treatment: Hep3B cells were cultured in DMEM containing 10% FCS, and the FCS concentration was reduced to 0.1% at 24 h before the treatment with chemicals. For hypoxic treatment, the cells were incubated in 5% O_2, 5% CO_2 and 90% N_2 balanced with a modulator incubator chamber or were incubated in a sealed 2.5-L box with an Anero Pack for cells. [12] The Hep3B cells were incubated for 6-12 h under normoxia or hypoxia conditions in the presence of alpha-naphthoflavone (ANF), beta-naphthoflavone (BNF), 3,4',5-trimethoxybenzophenone

(TMB), daidzein, and genistein (5-50 μM). These chemicals were dissolved in dimethyl sulfoxide (DMSO).

RNA isolation, reverse transcription-PCR, and Western blotting: Total RNA was extracted from the Hep3B cells with Isogen. A reaction mixture containing 1 μg of RNA and 200 units of reverse transcriptase was reacted according to the manufacturer's protocol as follows: incubation for 10 min at 25 ^{0}C and 60 min at 42 ^{0}C, followed by heating for 10 min at 70 ^{0}C to stop the reaction. Polymerase chain reaction (PCR) was performed using a reaction mixture containing 10 pmol of each primer, 1.5 units of Ampli Taq, and 100 ng of cDNA according to the following protocol: 10 min at 96 ^{0}C and then 25 cycles (for *β-actin*) or 30 cycles (for *EPO* and *CYP1A1*) of 30 sec at 96 ^{0}C, 30 sec at 56 ^{0}C, and 1 min at 72 ^{0}C. The primers for *β-actin* were 5'-CAAGAGATGGCCACGGCTGCT-3' (sense) and 5'-TCCTTCTGCATCCTGTCGGCA-3' (antisense). The primers for *EPO* were 5'-GCCAGAGGAACTGTCCAGAG-3' (sense) and 5'-TTCTCCAGGTCATCCT GTCC-3' (antisense). The primers for *CYP1A1* were 5'-GGAGACCTTCCGACACTCTT-3' (sense) and 5'-CCTTGTCGATAGCACCATCA-3' (antisense)**.** For Western blotting, the treated cells were lyzed and subjected to SDS-polyacrylamide gel electrophoresis. The analyzed proteins were blotted to a nitrocellulose membrane and reacted with HIF-1α antibody. The HIF-1α proteins were visualized using horseradish peroxidase conjugated to goat anti-rabbit IgG and by 4-chloro-1-naphthol.

Luciferase reporter gene assay: Complementary oligonucleotides containing HRE of *EPO* were synthesized.[13] The promoter region (-800 to +73) of *CYP1A1* which harbors XRE was isolated by PCR. These DNA fragments were subcloned into pGL3 vector. The constructed plasmids were transfected into Hep3B cells with pRL-TK. The cells were cultured for 36 h and test chemicals were added to cells. After 6 to 12 h incubation, luciferase activity was measured with a Dual-Luciferase Reporter assay system.

Results and Discussion

ANF, BNF, TMB, daidzein, and genistein were added to the Hep3B cells under normoxia and hypoxia conditions. The expression of *CYP1A1* mRNA under normoxia and that of *EPO* mRNA under hypoxia were investigated by RT-PCR (Fig.1). All chemicals used in this study induced *CYP1A1*; ANF and BNF were most effective. In *EPO* induction under hypoxia, TMB strongly inhibited EPO induction and ANF also inhibited the induction. Genistein, a tyrosine kinase inhibitor, and daidzein, an inhibitor of casein kinase II, slightly induced *CYP1A1* mRNA as reported previously.[14] A high concentration of genistein inhibited *EPO* induction but it did not in this study (data not shown).

Both ANF and BNF could induce *CYP1A1* efficiently, and ANF but not BNF inhibited *EPO* induction. We further investigated the effects of these flavones on HIF-1 signaling (Fig. 2). Under normoxia, ANF increased *EPO* mRNA, although ANF inhibited *EPO* induction under hypoxia. The effects of BNF on the *EPO* expression were low. ANF and BNF stabilized the HIF-1α protein under normoxia but ANF but not BNF reduced the HIF-1α protein under hypoxia.

Luciferase reporter assays with constructed plasmids including XRE and HRE were performed. First, we confirmed the induction of *CYP1A1* mRNA by ANF, BNF, and indigo, an endogenous ligand for AhR[15] in Hep3B cell by RT-PCR (Fig. 3A). All chemicals efficiently induced *CYP1A1* mRNA. The XRE-mediated activities of ANF, BNF, and indigo were investigated using Hep3B, which was transiently transfected with reporter gene plasmids (Fig. 3B). A luciferase reporter construct containing the 800bp-promoter region of the *CYP1A1* gene which harbors XRE was transfected into Hep3B. Luciferase reporter activities were measured using the cells treated with ANF, BNF, and indigo. BNF directly inhibited luciferase activity (data not shown). ANF and indigo induced luciferase activity efficiently, indicating that the induction is mediated by XRE. Furthermore, the construct, including HRE of *EPO* for the luciferase reporter plasmid, was prepared and transfected to Hep3B cells (Figs. 3C and 3D). Under normoxia or hypoxia, ANF or indigo was added to the cells. Under normoxia, both ANF and indigo induced lucifarase activity mediated by HRE of *EPO*, although both inhibited the activity under hypoxia. Chan et al.[11] found that dioxin induces luciferase activity with HRE of *EPO* under normoxia. They concluded that the promoter region of *EPO* harbored the putative XREs and that *EPO* was induced by the AhR signaling pathway. In this study, we obtained similar results with luciferase reporter plasmids including XRE and HRE. However, we found that ANF and BNF stabilized the HIF-1α protein. These phenomena cannot be explained by a simple signaling pathway, and a complex mechanism may be involved in the induction and inhibition of EPO by AhR ligands .

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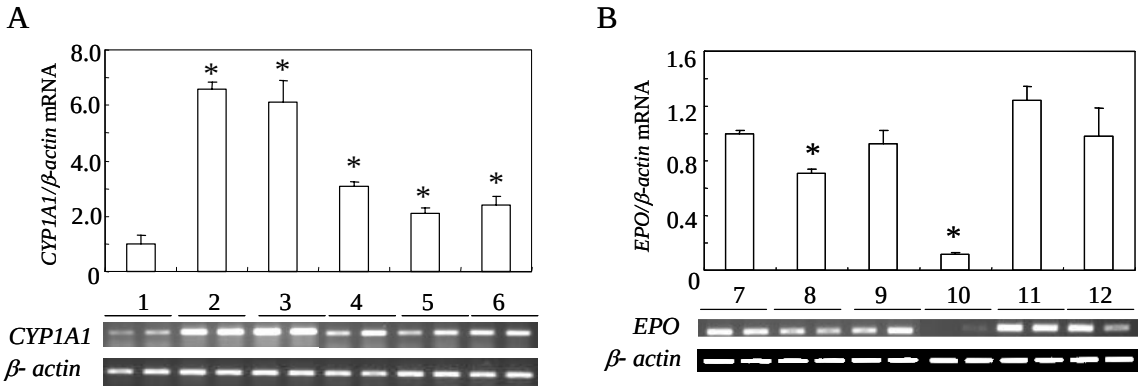

Fig. 1 Effects of flavones on AhR and HIF-1 signaling pathways. Each flavone (5 μM) dissolved in DMSO was added to Hep3B cells. RNA was isolated from the treated cells and RT-PCR was done with the RNA. The amplified DNA was analyzed by agarose gel electrophoresis and the intensity of bands was measured. Calculated values were normalized by values from *β-actin* and the control values were set at 1.0. (A) The expression of CYP1A1 was investigated by treatment with flavones under normoxia, indicating lane 1, control (normoxia); lane 2, ANF; lane 3, BNF; lane 4, TMB; lane 5, daidzein; and lane 6, genistein. (B) The expression of erythropoietin (EPO) was investigated by treatment with flavones under hypoxia, indicating lane 7, control (hypoxia), lane 8, ANF; lane 9, BNF; lane 10, TMB; lane11, daidzein; and lane 12, genistein. Four different plates were used in the experiment and the values are expressed as the mean ± SD.*, significantly different from each control, $p<0.01$.

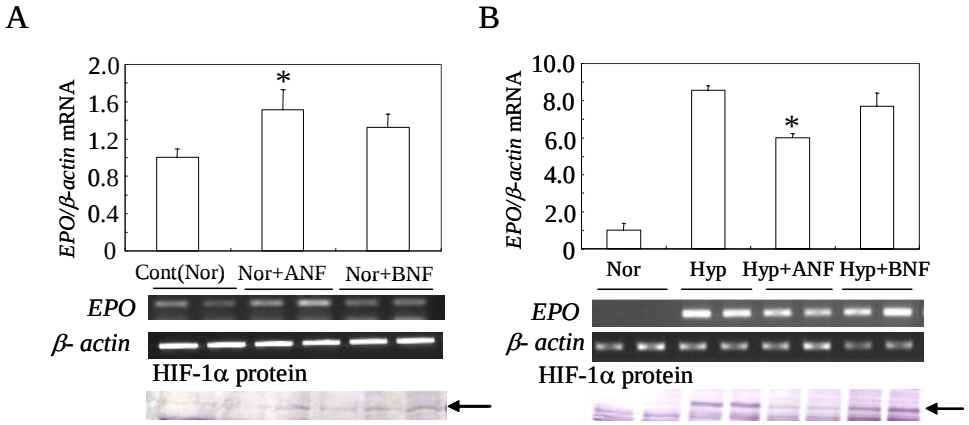

Fig. 2 Effects of ANF and BNF on HIF-1 signaling pathways. ANF and BNF (5 μM) were added to Hep3B cells. RNA was isolated from the treated cells and RT-PCR was done with the RNA. Amplified DNA was analyzed by agarose gel electrophoresis and the intensity of the bands was measured. The calculated values were normalized by values from *β-actin* and the control values were set at 1.0. In addition, the cells were homogenized and subjected to Western blotting with HIF-1α antibody. (A) The expression of *EPO* was investigated by treatment with ANF and BNF under normoxia. (B) The expression of *EPO* was investigated by treatment with ANF and BNF under hypoxia. Nor, normoxia; and Hyp, hypoxia Four different plates were used in the experiment and values are expressed as the mean ± SD.*, significantly different from each control, $p<0.01$.

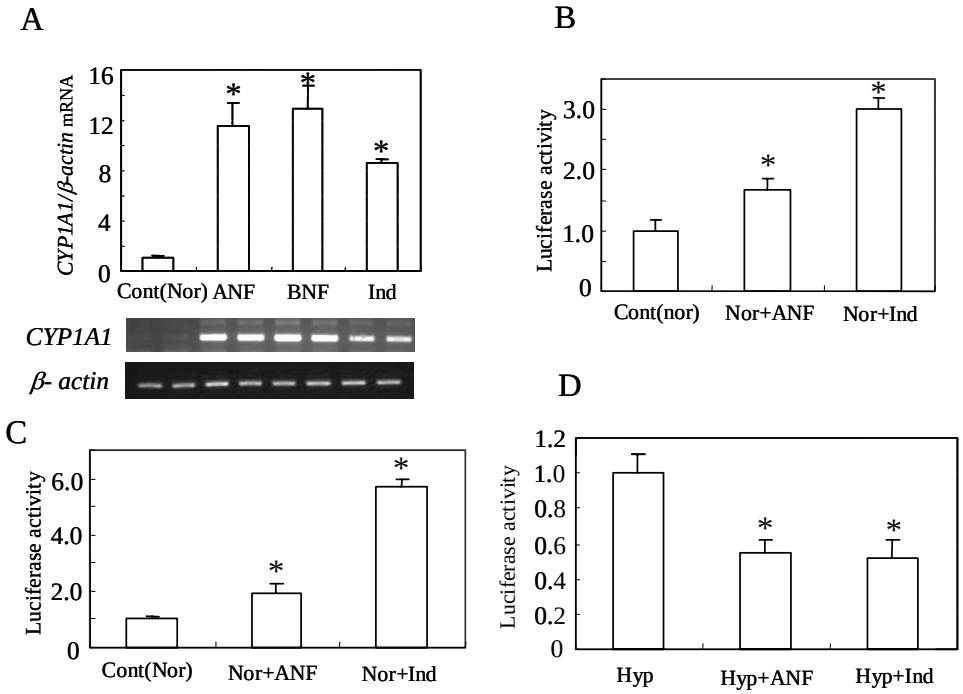

Fig. 3 Effects of ANF and Indigo on reporter activity including XRE or HRE. (A) Induction of *CYP1A1* by flavones and indigo (Ind) (B) Induction of luciferase activity with XRE (C) Induction of luciferase activity with HRE under normoxia (D) Induction of luciferase activity with HRE under hypoxia. The Hep3B cells were co-transfected with a reporter plasmid and a control plasmid (pRL-TK vector). At 36 h post-transfection, ANF or indigo were added to the transfected Hep3B, and the cells were incubated for 6 h under hypoxic conditions. The ratio (reporter/control luciferase activity) obtained from the cell lysates of each control experiment was set at 1.0. Luciferase activity is given as the mean ± SD of three separate experiments. *, significantly different from control, $p<0.01$.

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