Effects of β-Naphthoflavone on Ugt1a6 and Ugt1a7 Expression in Rat Brain

Yukiko Sakakibara, Miki Katoh,* Yuya Kondo, and Masayuki Nadai

Department of Pharmaceutics, Faculty of Pharmacy, Meijo University; 150 Yagotoyama, Tenpaku-ku, Nagoya 468–8503, Japan.

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Uridine 5′-diphosphate-glucuronosyltransferase (UGT) catalyzes a major phase II reaction in a drug-metabolizing enzyme system. Although the UGT1A subfamily is expressed mainly in the liver, it is also expressed in the brain. The purpose of the present study was to elucidate the effect of β-naphthoflavone (BNF), one of the major inducers of drug-metabolizing enzymes, on Ugt1a6 and Ugt1a7 mRNA expression and their glucuronidation in the rat brain. Eight-week-old male Sprague-Dawley rats were treated intraperitoneally with BNF (80 mg/kg), once daily for 7 d. Ugt1a6 and Ugt1a7 mRNA expression increased in the cerebellum and hippocampus (Ugt1a6: 2.1- and 2.3-fold, respectively; Ugt1a7: 1.7- and 2.8-fold, respectively); acetaminophen glucuronidation also increased in the same regions by 4.1- and 2.7-fold, respectively. BNF induced Ugt1a6 and Ugt1a7 mRNA expression and their glucuronidation, and the degree of induction differed among 9 regions. BNF also upregulated CYP1A1, CYP1A2, and CYP1B1 mRNAs in the rat brain. Since the aryl hydrocarbon receptor signaling pathway was activated by BNF, it is indicated that Ugt1a6 and Ugt1a7 were induced via AhR in the rat brain. This study clarified that Ugt1a6 and Ugt1a7 mRNA expression and their enzyme activities were altered by BNF, suggesting that these changes may lead to alteration in the pharmacokinetics of UGT substrate in rat brain.

Key words uridine 5′-diphosphate-glucuronosyltransferase; brain; induction; β-naphthoflavone (BNF); aryl hydrocarbon receptor

Uridine 5′-diphosphate-glucuronosyltransferase (UGT) catalyzes the conjugation of glucuronic acid to a broad spectrum of endogenous and exogenous substrates. The UGT1A subfamily is involved in the metabolism of endogenous compounds and various drugs. UGT is mainly expressed in the liver, but has also been detected in extrahepatic tissues, such as the intestine and kidney. Therefore, it is expected that UGT1As would be involved in the metabolism of several drugs that have pharmacological effects in the brain, such as acetaminophen, amitriptyline, and valproic acid. UGT1As also have potential to catalyze the glucuronidation of neurotransmitters and neurosteroids, such as serotonin, dopamine, and estradiol. A previous study reported that the induction of CYP 3A in the brain by anti-epileptic drugs increased the local testosterone metabolism, which led to the reduction in the testosterone levels in the brain independent of the plasma levels of testosterone (Ferguson and Tyndale, 2011), suggesting that CYP in the brain could contribute to local metabolism. Thus, it is possible that the changes in the expression levels of UGT1As in the brain might alter the efficacy of UGT substrates in the brain.

The expression levels of UGT are regulated by nuclear receptors that activate target gene transcription. The aryl hydrocarbon receptor (AhR), one of the nuclear receptors, regulates the expression of CYP and conjugating enzymes. When the ligand binds to AhR, the AhR protein is translocated from the cytoplasm into the nucleus as the ligand–AhR complex, which is the heterodimerized with AhR nuclear translocator. The heterodimer binds to xenobiotic responsive elements to enhance the transcription of AhR target genes. Many compounds are known as AhR ligands, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene (MC), and β-naphthoflavone (BNF). A previous study showed that hepatic Ugt1a6 and Ugt1a7 mRNA expression were upregulated after treatment with single dose of TCDD (3.9 µg/kg) or BNF (100 mg/kg/d) for 4 d in rats. The result indicated that Ugt1a6 and Ugt1a7 were target genes of AhR. The AhR mRNA was detected in various areas of the rat brain. Therefore, transcriptional activation of Ugt1a6 and Ugt1a7 in rat brain could occur via AhR signaling pathway in rat brain. It is known that some AhR ligands also lead to transcriptional activation of CYP expression in the brain, such as the induction of CYP1A1 by TCDD. Another study showed that mRNA expression of CYP1A1 and CYP1A2 increased in rat astrocytes after BNF exposure. These results indicated that the expression levels of Ugt1a6 and Ugt1a7 could be induced via AhR and affect the xenobiotic metabolism leading to toxicity and disorder homeostasis in the brain. However, it is still unclear whether Ugt1a isoforms in the brain are induced by xenobiotics.

The purpose of this study was to clarify the induction potency of Ugt1a6 and Ugt1a7 mRNA expression and their enzyme activities in the rat brain after treatment with BNF as a ligand of AhR.

MATERIALS AND METHODS

Materials Acetaminophen (APAP) was purchased from Nacalai Tesque (Kyoto, Japan). APAP β-α-glucuronide, alamethicin, BNF, 7-ethoxyresorufin, resorufin and uridine 5′-diphosphoglucuronic acid trisodium salt were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals

* To whom correspondence should be addressed. e-mail: mkatoh@meijo-u.ac.jp
and solvents were of the highest commercially available grade.

**Animal Treatment** The present study was approved by the Institutional Animal Care and Use Committee of Meijo University. Eight-week-old male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) were treated intraperitoneally once daily for 7 d with BNF at a dose of 80 mg/kg/d (n=15) or with corn oil as the control (n=15). The cerebellum, frontal cortex, parietal cortex, piriform cortex, hippocampus, medulla oblongata, olfactory bulb, striatum, and thalamus were excised from the brain.

**RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR)** Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, U.S.A.) and then treated with DNase I (Promega, Madison, WI, U.S.A.) according to the manufacturer’s instruction. Total RNA was used for the reverse transcription reactions with ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). PCR was performed using the Thermal Cycler Dice from TaKaRa BIO (Shiga, Japan) or ABI PRISM 7700 (Applied Biosystems, Foster City, CA, U.S.A.) with SYBR Premix Ex Taq II (TaKaRa Bio) or TaqMan Gene Expression Master Mix (Life Technologies), respectively. The primers used in the present study are described in Table 1 and were commercially synthesized by Greiner Bio-one (Tokyo, Japan). The PCR amplification conditions were as follows: initial denaturation step at 95°C for 30 s; 40 cycles of denaturation at 95°C for 5 s; annealing and extension steps at 60°C for 30 s. CYP1A1 amplification conditions were as follows: initial denaturation step at 95°C for 30 s; 40 cycles of denaturation at 95°C for 5 s; annealing and extension steps at 60°C for 30 s. CYP1A1 was measured using TaqMan® Gene Expression Assay. The relative expression level of each gene was calculated using the method described by Kessler et al.22 with ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). The PCR amplification conditions were as follows: initial denaturation step at 95°C for 30 s; 40 cycles of denaturation at 95°C for 5 s; and annealing and extension steps at 60°C for 30 s. CYP1A1 was measured using TaqMan® Gene Expression Assay. The relative expression level of each gene was calculated using the standard curve method. All data were normalized to the expression level of β-actin. Data are shown as the mean values for 5 rats in each group.

**Microsome Preparation** Pooled rat brain microsome were prepared from 10 rats as described previously21) and stored at −80°C until analysis. Protein concentration was determined using Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, U.S.A.).

**APAP Glucuronidation** APAP glucuronidation was determined using the method described by Kessler et al.22) with slight modifications. In a preliminary study, we confirmed the linearity of the protein concentrations, the substrate concentration, and incubation times. The incubation mixture contained 1 mM APAP, 0.5 mg/mL brain microsomes, 50 mM Tris–HCl buffer (pH 7.4), 10 mM MgCl2 and 25 μg/mL alamethicin. The reaction mixture was incubated at 37°C for 60 min, and the reaction was terminated with boiling for 3 min. The mixture was centrifuged at 14000×g for 10 min, and 10 μL of the supernatant was subjected to liquid chromatography-tandem mass spectrometry. Liquid chromatography was performed with a Prominen apparatus (Shimadzu, Kyoto, Japan) and an Inertsil ODS-3 column (3.0×150 mm; GL Sciences, Tokyo, Japan). The column temperature was 40°C, and the flow rate was 0.2 mL/min. The mobile phase consisted of methanol–0.1% formic acid (40:60, v/v). API4000 tandem mass spectrometer (Applied Biosystems) operated in the negative electrospray ionization mode. The turbo gas was maintained at a temperature of 300°C. Mass/charge (m/z) ion transitions were recorded with m/z 326.0 and 150.0 for APAP β-ν-glucuronide in the multiple reaction-monitoring mode. The limit of detection for APAP β-ν-glucuronide was 0.3 pmol, and the limit of quantification in the reaction mixture was 30 nm with a CV of <10%. Data are presented as the mean of 3 independent determinations using pooled brain microsomes from 10 rats.

**Ethoxyresorufin-O-Deethylase (EROD) Activity** EROD activity was determined using the method described by Sakakibara et al.23) In a preliminary study, we confirmed the linearity of the protein concentrations, the substrate concentration, and incubation times. The reaction mixture containing 0.2 mg/mL brain microsomes and 2 μM 7-ethoxyresorufin was incubated at 37°C for 30 min. Data are presented as the mean of 3 independent determinations using pooled brain microsomes from 10 rats.

**Statistical Analysis** Statistical analyses were performed using Student’s paired t-test with the Kaleida-Graph computer program (Synergy Software, PA, U.S.A.).

## RESULTS

**Changes in Ugt1a mRNA Expression by BNF in Rat Brain** Following the treatment with BNF, Ugt1a6 mRNA expression increased in the cerebellum, hippocampus, and medulla oblongata by 2.1-, 2.3-, and 2.1-fold, respectively (Fig. 1A). Ugt1a7 mRNA expression increased in the cerebellum and hippocampus by 1.7- and 2.8-fold, respectively (Fig. 1B).

**Changes in APAP Glucuronidation by BNF in Rat Brain** Changes in Ugt1a6 and Ugt1a7 enzymatic activities following

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**Table 1. Primer Sequence Used in the Present Study**
BNF treatment in rat brains were assessed using APAP as a probe, since APAP glucuronidation is primarily catalyzed by Ugt1a6 and Ugt1a7 (Fig. 2). APAP glucuronidation significantly increased after BNF treatment in the cerebellum, hippocampus, and olfactory bulb by 4.1-, 2.7-, and 1.5-fold, respectively.

Expression of AhR in Rat Brain The expression of AhR mRNA increased only in the hippocampus (Fig. 3). The expression level of AhR mRNA was not related to the Ugt1a6 and Ugt1a7 mRNA expression levels in rat brain.

Changes in CYP1A1, CYP1A2, and CYP1B1 mRNA Expression and EROD Activity The mRNA expression of CYP1A1 (Fig. 4A) and CYP1B1 (Fig. 4C), which is regulated by AhR, increased in all 9 regions of the rat brains following BNF treatment. CYP1A2 mRNA also increased in the cerebellum, parietal cortex, hippocampus, medulla oblongata, olfactory bulb, and thalamus in response to BNF treatment (Fig. 4B). EROD activity was not detected in the brains of the control rats, except for olfactory bulb (Fig. 5). However, EROD activity was detected after BNF treatment in all 9 regions of the rat brain.

Expression of Nrf2 in the Rat Brain The expression of Nrf2 mRNA differed among 9 regions (Fig. 6). The expression level of Nrf2 mRNA was not related to the Ugt1a6 and Ugt1a7 mRNA expression levels in the rat brain.

DISCUSSION

In the present study, we found that the expression of Ugt1a6 and Ugt1a7 mRNAs and their enzyme activities in the rat brains were increased by BNF. The degree of induction differed among 9 regions. Ugt1a6 mRNA expression significantly increased in the cerebellum, hippocampus, and medulla oblongata. Ugt1a7 mRNA expression significantly increased in the cerebellum and hippocampus. APAP glucuronidation also increased in those regions, presumably resulting in elevated Ugt1a6 and Ugt1a7 mRNA expression levels. Changes in the expression of Ugt1a6 and Ugt1a7 mRNAs and their enzyme activities could alter the pharmacokinetics of UGT substrates in rat brains locally. However, the increase of APAP glucuronidation was not correlated with the increase of Ugt1a6 and Ugt1a7 mRNA in the medulla oblongata and olfactory bulb.
bulb. It has been reported previously that UGT1A mRNA and protein levels do not always correlate with each other.\(^{24-26}\) Similar to the reports, an unexplained epigenetic mechanism of post-transcriptional regulation of Ugt1a6 and Ugt1a7 may also exist in rat brains.

In the previous study, Ugt1a6 and Ugt1a7 mRNA expression in rat liver increased by 5- and 10-fold, respectively, following treatment with an AhR ligand, BNF (100mg/kg) for 4d.\(^{27}\) Another study reported that APAP glucuronidation in the rat

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**Fig. 4. Changes in CYP1A1 (A), CYP1A2 (B), and CYP1B1 (C) mRNA Expression by BNF in Rat Brains**

Each column represents the relative expression level in the rat brain, which is shown as the mean±S.D. (n=5). The expression level of mRNA was normalized to that of β-actin. **p<0.01, ***p<0.001 compared with the control. N.D., not detected.

**Fig. 5. Changes in EROD Activity by BNF in Rat Brains**

Each column represents the mean±S.D. of 3 independent measurements. ***p<0.001 compared with the control. N.D., not detected.

**Fig. 6. Nrf2 mRNA Expression in Rat Brains**

Each column represents the mean±S.D. in 3 independent determinations. The expression level of mRNA was normalized to that of β-actin.
liver significantly increased in response to BNF.22) Therefore, elevated expression levels of Ugt1a6 and Ugt1a7 mRNAs in the present study might be attributed to transcriptional activation via the AhR signaling pathway. AhR mRNA was expressed constitutively in all 9 regions (Fig. 3). Since CYP1A1, CYP1A2, and CYP1B1, which are AhR-regulated genes, were induced in rat brains (Figs. 4, 5), it was suggested that the AhR signaling pathway was activated by BNF in the rat brain. Although CYP1A1 mRNA expression and EROD activity increased in all 9 regions, Ugt1a6 and Ugt1a7 mRNA expression did not increase in all 9 regions. These results indicated that the response to the transcriptional activation via the AhR signaling pathway differed between Ugt1a6 or Ugt1a7 and CYP1A1 or CYP1B1. The molecular mechanism concerning induction of Ugt1a6 and Ugt1a7 mRNAs may be slightly different from that of CYP1A1 or CYP1B1 mRNA.

The previous study suggested that AhR is involved in the activation of another nuclear receptor, NF-E2-related factor-2 (Nrf2).28,29) Nrf2 is activated by oxidative/electrophile activation of another nuclear receptor, NF-E2-related factor different from that of CYP1A1 or CYP1B1 mRNA. induction of Ugt1a6 and Ugt1a7 mRNAs may be slightly different from that of CYP1A1 or CYP1B1 mRNA.

REFERENCES
