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Characterization of human *CYP1A1/1A2* induction by DNA microarray and α-naphthoflavone

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Abstract

DNA microarrays and real time PCR were used to analyze the mechanism of gene induction by CYP1A1 inducers, β-naphthoflavone, and omeprazole, in the human hepatocellular carcinoma HepG2 cells. Reproducible and significant inductions were observed in a limited number of genes including *CYP1A1* and *CYP1A2*. Genes induced by omeprazole included several protein tyrosine kinase targets. This result confirmed that omeprazole could modulate gene expressions through protein tyrosine kinase-mediated pathway. Induction ratios were considerably different from *CYP1A1* and *CYP1A2* (>10-fold) to other induced genes (<5-fold). α-Naphthoflavone, which is known as an antagonist to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, inhibited the inductions of heme oxygenase 1, glutamate-cysteine ligase (modifier unit), and thioredoxin reductase by β-naphthoflavone but not those of *CYP1A1* and *CYP1A2*. It unexpectedly enhanced the β-naphthoflavone-mediated *CYP1A1* and *CYP1A2* induction. These results suggest that the *CYP1A1* and *CYP1A2* genes, which share their 5′ enhancer regions, are regulated differently from the other genes. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: DNA microarray; Expression profiling; *CYP1A1*; *CYP1A2*; Omeprazole; α-Naphthoflavone; β-Naphthoflavone; HepG2; Protein tyrosine kinase

CYP1A1 is one of the cytochrome P450s that convert their substrates into water-soluble derivatives as an initial step of metabolic pathways. Regulatory mechanisms for *CYP1A1* gene expression have been intensively studied over the years because chemicals that are good substrates for CYP1A1 enzyme are also good inducers for *CYP1A1* gene expression and also because many chemicals show an increase in toxic or carcinogenic activities after being metabolized by CYP1A1 (reviewed in [1]). For example, polycyclic aromatic hydrocarbons such as benzo[a]pyrene are oxygenated by CYP1A1 to generate arene oxides, which are chemically reactive electrophiles and act as carcinogens. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent inducer of CYP1A1. Mechanistic studies on CYP1A1 induction by TCDD revealed the existence of two regulators, aromatic hydrocarbon receptor (AhR) and Ah receptor nuclear translocater (Arnt). Both are transcription factors that contain basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) motifs. Once TCDD reaches the cytosol, cytosolic AhR binds to TCDD, releasing Hsp90, and enters the nucleus. In the nucleus, the AhR forms a heterodimer with Arnt and binds to the defined DNA sequence known as a xenobiotic responsive element (XREs; 5′-TNGCGTG-3′). A number of XREs have been found in the upstream region of *CYP1A1* [2], demonstrating that the gene is a special target of the AhR–Arnt pathway. Other CYP1A1 inducers such as 3-methyl cholanthrene or β-naphthoflavone have been thought to act in the same way as TCDD. Besides this induction mechanism, an alternative mechanism has also been postulated for benzimidazoles including omeprazole, an inhibitor of H^+/K^+-ATPase, since these

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compounds do not bind AhR directly [3]. According to a report by Kikuchi et al. [4], omeprazole is able to activate AhR through a protein tyrosine kinase-mediated pathway.

In this study, we attempted to characterize these two pathways, AhR-binding (induced by β-naphthoflavone) and protein tyrosine kinase-mediated (induced by omeprazole), in human hepatocellular carcinoma cell line HepG2 using DNA microarray analysis and α-naphthoflavone, which was known to antagonize TCDD on AhR [5]. We obtained the following results: (1) induction of *CYP1A1* and *CYP1A2* by β-naphthoflavone and omeprazole was prominent; (2) α-naphthoflavone unexpectedly induced *CYP1A1* and *CYP1A2* synergistically with β-naphthoflavone; (3) plasminogen activator inhibitor 1 and galectin 3 were suggested as potential targets of CYP1A1 inducers through the protein tyrosine kinase pathway.

Materials and methods

Cells and chemicals. HepG2 cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin under 5% CO_2 at 37 °C. α-Naphthoflavone, β-naphthoflavone, and omeprazole were purchased from Wako Pure Chemicals and dissolved in DMSO.

Treatment of cells with chemicals. α-Naphthoflavone (10 μM), β-naphthoflavone (1 μM), omeprazole (200 μM) or DMSO (vehicle) was added to HepG2 cells (70%–80% confluent). The cells were cultured for 9 h and harvested for RNA preparation. Each chemical treatment was performed in two independent cultures.

RNA preparation. Total RNA was prepared with an RNeasy Mini Total RNA Extraction Kit (Qiagen) according to manufacturer's instructions. Total RNA for real time PCR was treated with RNase-free DNase I (Qiagen) during the RNeasy procedure.

DNA microarray analysis. Converting total RNA (10 μg) to the targets for Affymetrix GeneChip DNA microarray hybridization was done according to manufacturer's instructions. The targets were hybridized to human U95A GeneChip DNA microarrays (Affymetrix) for 16–24 h at 45 °C. After the hybridization, the DNA microarrays were washed and stained on a Fluidics Station (Affymetrix) according to the protocol provided by Affymetrix. The DNA microarrays were then scanned and the images obtained were analyzed by GeneChip Expression Analysis Software (version 3.2) (Affymetrix). DNA microarray analysis was done in duplicate for each sample. The results of the DNA microarray analysis are available upon request (e-mail to ishida@nihs.go.jp).

Selection of genes induced by CYP1A1 inducers. The data calculated by the comparison analysis using GeneChip Expression Analysis Software were used to identify genes upregulated by CYP1A1 inducers. The criteria for deciding inducibility were established, based on a previously reported analysis [6]. Genes fulfilling the following criteria were considered as "induced genes" in a given DNA microarray analysis: (1) "Difference Call" was either "Induced" or "Marginally Induced"; (2) the induction ("Fold Change") was greater than or equal to twofold; and (3) the "Average Difference" value of chemically treated samples was greater than or equal to 50. Two independent replicates of the chemical treatment of HepG2 cells were performed and DNA microarray analysis was done in duplicate for each sample. Each gene was counted as induced or uninduced in each of the four microarray analyses. Genes that were induced on all of the microarray analyses were selected as "reproducibly induced genes."

Real time PCR measurement of gene expression. cDNA for real time PCR was prepared using MultiScribe Reverse Transcriptase from TaqMan Reverse Transcription Reagents (Applied Biosystems). Gene expression was analyzed by Prism 7700 real time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix reagents with the following primers : CYP1A1 forward, 5′-GCTGACTTCATCCCTATTCTTCG-3′; CYP1A1 reverse, 5′-TTTTGTAGTGCTCCTTGACCATCT-3′; CYP1A2 forward, 5′-GGGCACTTCGACCCTTACAA-3′; CYP1A2 reverse, 5′-GCACATGGCACCAATGACG-3′; heme oxygenase 1 forward, 5′-TTCTCCGATGGGTCCTTACACT-3′; heme oxygenase 1 reverse, 5′-GGCATAAAGCCCTACAGCAACT-3′; glutamate-cysteine ligase (modifier subunit) forward, 5′-GGCACAGGTAAAACCAAATAGTAAC-3′; glutamate-cysteine ligase (modifier subunit) reverse, 5′-CAAATTGTTTAGCAAATGCAGTCA-3′; thioredoxin reductase 1 forward, 5′-TCCTATGTCGCTTTGGAGTGC-3′; thioredoxin reductase 1 reverse, 5′-GGACCTAACCATAACAGTGACGC-3′; heat shock protein (Hsp) 70 forward, 5′-GCCGAGAAGGACGAGTTTGA-3′; Hsp70 reverse, 5′-TCCGCTGATGATGGGGTTAC-3′. All primers were synthesized by GENSET KK. The sequence of each PCR amplified fragment was verified by sequencing.

Samples were prepared in triplicate and real time PCR measurement for each sample was done in duplicate. The expression level of each gene was normalized according to the expression level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and an induction ratio (treated/control) was obtained. The average of duplicate real time PCR measurements was used to calculate the mean induction ratio ± SD for each gene.

Results

Genes induced by CYP1A1 inducers

HepG2 cells were incubated with or without 1 μM β-naphthoflavone or 200 μM omeprazole for 9 h, after which the total RNA was prepared. Incubation time was set for 9 h because preliminary time course experiment showed that the induction of *CYP1A1* was much higher at 9 h than at 24 h. The expression of 12,626 genes was analyzed using Affymetrix human U95A GeneChip. No effect of the DMSO vehicle was detected by the DNA microarray analysis under the condition used (data not shown). Two separate experiments with β-naphthoflavone or omeprazole were conducted and DNA microarray analyses were repeated twice for each experiment. Using the data from these four analyses, we selected genes that were reproducibly induced by either β-naphthoflavone or omeprazole applying the criteria described in Materials and methods. Out of 12,626 genes, only four genes, *CYP1A1*, heme oxygenase 1, glutamate-cysteine ligase (modifier subunit), and thioredoxin reductase 1 were reproducibly "induced" by β-naphthoflavone (Table 1A). Omeprazole "induced" additional six genes (Table 1A). As described before [6], repeats of DNA microarray analyses are important to get reproducible data. For example, in the case of β-naphthoflavone, 41–71 genes were judged as "induced" in each analysis. However, after combining data from the four chips, the number of "induced" genes was

Table 1
Genes induced by *CYP1A1* inducers

Accession No.	Gene description	Mean induction (fold) ± SD ($n = 4$)	
		β-Naphthoflavone	Omeprazole
(A) Genes induced by β-naphthoflavone or omeprazole			
K03191	CYP1A1	61.9 ± 17.6 [4][a]	88.9 ± 51.8 [4]
X02612	CYP1A1	39.5 ± 17 [4]	60.2 ± 33.5 [4]
X02612	CYP1A1	24.3 ± 6.6 [4]	29.8 ± 7.2 [4]
Z82244	Heme oxygenase 1	4.6 ± 0.9 [4]	3.7 ± 0.2 [4]
L35546	Glutamate-cysteine ligase (modifier subunit)	4.9 ± 1.1 [4]	3.5 ± 1.1 [4]
X91247	Thioredoxin reductase 1	2.9 ± 0.5 [4]	2.5 ± 0.4 [4]
U79745	Monocarboxylic acid transporter 6	1.6 ± 0.2 [0][b]	4.9 ± 1.2 [4]
J03764	Plasminogen activator inhibitor 1	2.9 ± 1.5 [2][b]	4.2 ± 2.1 [4]
AB006780	Galectin 3	1.8 ± 0.2 [0][b]	3 ± 0.6 [4]
AF003837	Jagged 1	2.2 ± 0.2 [2][b]	2.8 ± 0.2 [4]
M11717	HSP70	0.5 ± 1.2 [0][b]	2.7 ± 0.4 [4]
M13485	Metallothionein I-B	1.4 ± 0.2 [0][b]	2.3 ± 0.3 [4]
(B) Genes induced by α-naphthoflavone			
Accession No.	Gene description	Mean induction (fold) ± SD ($n = 4$)	
		α-Naphthoflavone	
K03191	CYP1A1	29.8 ± 6.8	
X02612	CYP1A1	18.4 ± 2.8	
X02612	CYP1A1	21.5 ± 2.1	

[a] Times judged as "induced gene."
[b] Induction was not reproducible.

reduced to four. Other six genes were not judged as being reproducibly "induced" by β-naphthoflavone because they did not meet the criteria, although the average induction ratios of some genes were greater than twofold. Complete results as well as Table 1 with less reproducibly induced genes are available upon request (e-mail to ishida@nihs.go.jp). There are three *CYP1A1* probes on the human U95A GeneChip array and these were selected from different parts of the *CYP1A1* gene. In cases of β-naphthoflavone and omeprazole treatments, all the *CYP1A1* probes showed induction ratios of more than 20-fold. This activation was in striking contrast to those of other genes, which showed an induction of less than fivefold. Characterization of genes induced by either chemicals will be discussed below. Expression of *CYP1A2* could not be detected by the present DNA microarray analysis because no probe is available on the human U95A GeneChip.

Verification of induction of the selected genes

The reproducibility of inducing effects was confirmed by real time PCR for the five genes (*CYP1A1*, heme oxygenase 1, glutamate-cysteine ligase (modifier subunit), thioredoxin reductase 1, and Hsp70) in Table 1. For this purpose, HepG2 cells were exposed to β-naphthoflavone or omeprazole for 9 h and total RNA was prepared. Experiments were set up in triplicate for each treatment. As shown in Fig. 1, β-naphthoflavone induced *CYP1A1*, heme oxygenase 1, glutamate-cysteine ligase (modifier subunit), and thioredoxin reductase 1, but not Hsp70. Omeprazole induced all the five genes tested. Thus, the results of real time PCR were virtually the same as those from the DNA microarray analysis.

The behavior of *CYP1A2* is also interesting, since this gene product is a dominant form of the CYP1A in a human liver and is induced by either β-naphthoflavone or omeprazole in primary human hepatocyte and in human liver [7–10]. *CYP1A2* primer set was designed and the induction of *CYP1A2* gene was determined by real time PCR. Although the expression of *CYP1A2* was about 20-fold less than that of *CYP1A1* (data not shown), *CYP1A2* was also induced by both chemicals with high induction ratios similar to those of *CYP1A1*, as shown in Fig. 1.

Effect of α-naphthoflavone on gene induction by β-naphthoflavone or omeprazole

α-Naphthoflavone acts as an agonist for AhR when it was used alone, but it can also act as an antagonist for AhR when it is used with TCDD [5]. Therefore, the effects of α-naphthoflavone on gene induction by β-naphthoflavone or omeprazole were also investigated by real time PCR (Fig. 1). As previously reported [4], α-naphthoflavone could not antagonize the induction by omeprazole. The inductions of heme oxygenase 1, glutamate-cysteine ligase (modifier subunit), and thior-

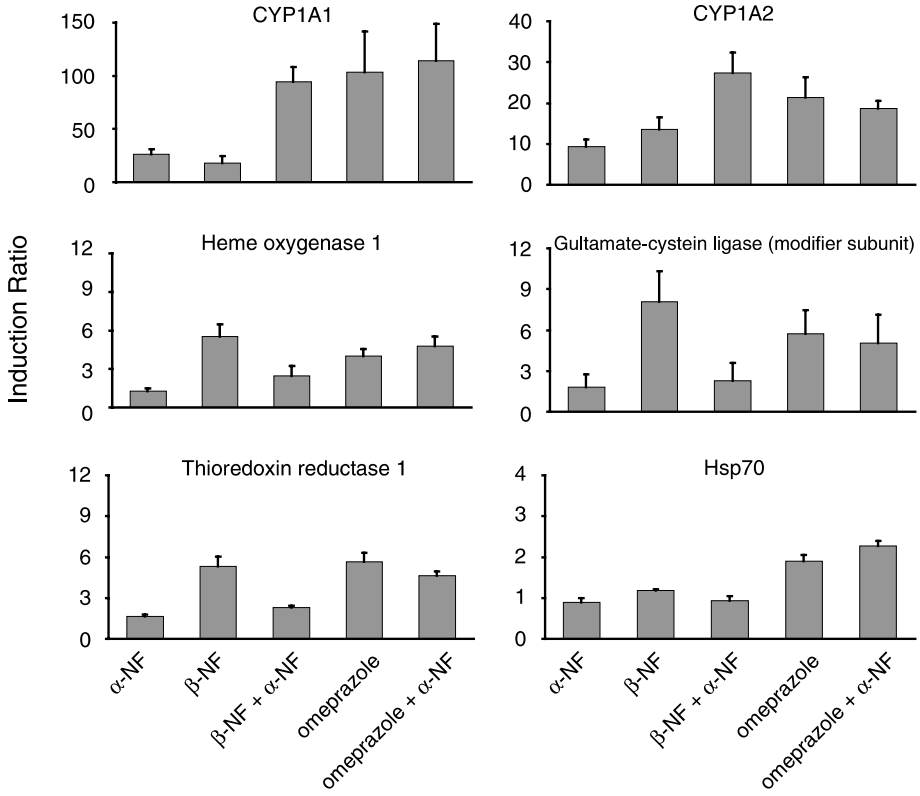

Fig. 1. Effects of naphthoflavones and omeprazole on gene induction measured by real time PCR. Expression levels of the genes selected from Table 1 and GAPDH were determined by real time PCR. Induction ratios of each gene (vertical axis) by chemicals were calculated using expression level, normalized to the level of GAPDH expression. Treatment by each chemical was done in triplicate and error bars indicate standard deviation among the triplicate samples. NF: naphthoflavone.

edoxin reductase 1 by β-naphthoflavone were suppressed by α-naphthoflavone. In contrast to these results and those obtained for TCDD [5], the induction of both *CYP1A1* and *CYP1A2* by β-naphthoflavone was enhanced by α-naphthoflavone. This enhancement by α-naphthoflavone was also detected, even at the concentration of 0.5 μM, indicating that this effect was not due to the high concentration of α-naphthoflavone (data not shown).

Genes induced by α-naphthoflavone

The enhancement of *CYP1A1* and *CYP1A2* induction by α-naphthoflavone was unexpected. The real time PCR analyses indicated that this enhancement was observed only in *CYP1A1* and *CYP1A2* (Fig. 1). One possible explanation for these results was that α-naphthoflavone was able to induce *CYP1A1* and *CYP1A2* but not other genes. To check this possibility, effects of α-naphthoflavone alone on gene expression were analyzed by the human U95A GeneChip at the same concentration used in Fig. 1. Genes induced by α-naphthoflavone were selected by the same criteria used for β-naphthoflavone or omeprazole. The result is shown in Table 1B. Only the *CYP1A1* gene was judged to be "induced" by α-naphthoflavone. Similar to β-naphthoflavone and omeprazole, the induction ratio for *CYP1A1* was around 20-fold. *CYP1A2* was also induced by α-naphthoflavone in the real time PCR analysis (Fig. 1). The results of these two analyses indicate that α-naphthoflavone induces only *CYP1A1* and *CYP1A2*.

Discussion

Characteristics of CYP1A1 and CYP1A2 gene induction through AhR-binding pathway

Our analyses revealed that the induction modes of *CYP1A1* and *CYP1A2* in HepG2 cells was strikingly different from those of other genes in two points. First, the induction ratios of *CYP1A1* and *CYP1A2* were much higher (more than 10-fold) than those of other genes (around 3–5-fold) (Table 1 and Fig. 1). *CYP1A1* was also induced at ratios higher than other genes in the analyses for TCDD-induced gene expression by DNA microarray [11,12]. The basal expression level of *CYP1A1* measured by real time PCR was comparable to those of glutamate-cysteine ligase (modifier subunit) and thioredoxin reductase 1 (data not shown). This obser-

vation indicates that higher induction ratios for *CYP1A1* are not due to a strong repression of basal expression of this gene. Second, when HepG2 cells were treated with both α-naphthoflavone and β-naphthoflavone, the induction of *CYP1A1* and *CYP1A2* was enhanced by α-naphthoflavone. Conversely, α-naphthoflavone showed antagonism in other genes. Kikuchi et al. [4] proposed two pathways for α-naphthoflavone, one is an AhR-binding pathway and the other is a protein tyrosine kinase-mediated pathway. When a low dose of α-naphthoflavone (10 μM) was used, it acted as an antagonist in the former pathway, and when high doses (50–100 μM) were used, it acted as a weak inducer through the latter pathway. Since the concentration used here was 10 μM, α-naphthoflavone was expected to act as an antagonist to β-naphthoflavone on AhR. This was true for the induced genes other than *CYP1A1* and *CYP1A2*. However, in our study, α-naphthoflavone enhanced the induction of *CYP1A1* and *CYP1A2* expression by β-naphthoflavone. This synergistic induction of *CYP1A1* and *CYP1A2* by the two naphthoflavones suggests that the regulation of *CYP1A1* and *CYP1A2* induction is controlled by a unique mechanism.

One clue for understanding this regulatory mechanism is the fact that *CYP1A1* and *CYP1A2* are located adjacently on chromosome 15q22. No other open reading frame is found between these two genes. They are separated by 23 kb and are located in an opposite orientation sharing the same 5′ flanking region [2]. Corchero et al. found 12 consensus AhR-binding sequences (5′-TNGCGTG-3′) and one TCACGC [2,13] in this region. Only two AhR-binding sites were found in the 3.5 kb upstream sequences of heme oxyganase 1, glutamate-cysteine ligase (modifier subunit), thioredoxin reductase 1, and Hsp70. If AhR can bind to these 13 binding sequences, it is likely that the AhR complexes can bind the enhancer regions of *CYP1A1* and *CYP1A2* than those of other genes more efficiently and induce higher levels, resulting in unique control of both genes. The result of analysis of α-naphthoflavone induced genes might support this idea. Since relatively high concentration of α-naphthoflavone is required for the induction of gene expression through XRE in the reporter assay system [5], only the genes that have multiple XREs might be able to be induced by α-naphthoflavone effectively in the genome context. The result that only *CYP1A1* and *CYP1A2* were induced by α-naphthoflavone indicated that this is the case.

Multiple AhR-binding sites might also contribute to the synergistic effect of α-naphthoflavone and β-naphthoflavone, although a precise mechanism remains to be clarified. The cooperativity observed in this study is not an artifact of a high concentration of α-naphthoflavone (10 μM), since the synergism was observed at lower concentrations in a dose-dependent manner in *CYP1A1* and *CYP1A2* induction (data not shown). The antagonism of α-naphthoflavone to β-naphthoflavone in other genes was also dose-dependent (data not shown).

Involvement of protein tyrosine kinase-mediated pathway in CYP1A1 and CYP1A2 induction

According to the report of Kikuchi et al. [4] omeprazole induces *CYP1A1* gene through the protein tyrosine kinase-mediated pathway. If this is the case, there should be genes that were also regulated through the same pathway. To find such genes, previous reports of genes in Table 1 were extensively searched. As the result, the expressions of two genes, plasminogen activator inhibitor 1 and galectin 3, were found to be regulated through protein tyrosine kinase pathway [14,15]. Together with *CYP1A1*, the expressions of three genes were known to be controlled under the protein tyrosine kinase-mediated pathway. This result indicates that omeprazole regulates gene expression, at least in part, through this pathway.

Another fact was found while comparing our result to those of Puga et al. and Frueh et al. [11,12]. They studied the alteration of gene expression induced by TCDD in HepG2 cells by the glass slide-type DNA microarray. *CYP1A1* and plasminogen activator inhibitor 1 were also listed in both lists and galectin 3 was listed in the list of Frueh et al. In our study, plasminogen activator inhibitor 1 and galectin 3 were seemed to be induced selectively by omeprazole. However, our preliminary data obtained from an independent experiment showed these genes were also induced by β-naphthoflavone in some degree. So it is plausible that the selectivity shown here might depend on the concentration of chemicals or the conditions of cells, i.e., cell density or days after plating. Combining these facts together, CYP1A1, plasminogen activator inhibitor 1, and galectin 3 would be induced by TCDD, β-naphthoflavone, and omeprazole. Interestingly, inductions of plasminogen activator inhibitor 1 and galectin 3 by β-naphthoflavone were not anatagonized by α-naphthoflavone in the same preliminary experiment (data not shown). Most of the studies on CYP1A1 inducers were done employing *CYP1A1* as the target. But, as discussed in the first part of this section, the expression of *CYP1A1* is tightly regulated by AhR-binding pathway. This might make it difficult to study the gene induction mechanism of CYP1A1 inducers independent of AhR-binding pathway, especially when chemicals bind to AhR, like β-naphthoflavone. Our results suggest that plasminogen activator inhibitor 1 and galectin 3 are useful tools for studying the induction mechanism of CYP1A1 inducers other than AhR-binding pathway, especially protein tyrosine phosphorylation-mediated pathway.

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