Human CYP2A6 is regulated by nuclear factor-erythroid 2 related factor 2

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Human CYP2A6 is regulated by nuclear factor-erythroid 2 related factor 2

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Abstract

Human CYP2A6 is responsible for the metabolism of nicotine and coumarin as well as the metabolic activation of tobacco-related nitrosamines. Earlier studies revealed that CYP2A6 activity was increased by dietary cadmium or cruciferous vegetables, but the underlying mechanisms remain to be clarified. In the present study, we investigated the possibility that Nrf2 might be involved in the regulation of CYP2A6. Real-time RT-PCR analysis revealed that the CYP2A6 mRNA level in human hepatocytes was significantly ($P < 0.01$, 1.4 fold) induced by 10 μM sulforaphane (SFN), a typical activator of Nrf2. A computer-based search identified three putative antioxidant response elements (AREs) in the 5'-flanking region of the *CYP2A6* gene at positions -1212, -2444, and -3441, termed ARE1, ARE2, and ARE3, respectively. Electrophoretic mobility shift assays demonstrated that Nrf2 bound only to ARE1. Luciferase assays using HepG2 cells revealed that the overexpression of Nrf2 significantly increased the reporter activities of the constructs containing a 30-bp fragment that included ARE1. However, the activity of the construct containing the intact 5'-flanking region (-1 to -1395) including ARE1 was not increased by the overexpression of Nrf2. In contrast, when the reporter construct was injected into mice via the tail vein, the reporter activity in the liver was significantly ($P < 0.05$, 1.9 fold) increased by SFN (1 mg/head) administration. In conclusion, we found that human CYP2A6 is regulated via Nrf2, suggesting that CYP2A6 is induced under oxidative stress.

Key words: cytochrome P450; nuclear factor-erythroid 2 related factor 2; transcriptional regulation

1. Introduction

Human cytochrome P450 2A6 (CYP2A6), which was first purified as coumarin 7-hydroxylase [1], is a major enzyme responsible for the metabolism of nicotine [2] and cotinine [3]. CYP2A6 also metabolically activates tobacco-specific nitrosamines such as 4-methylnitrosoamino-1- (3-pyridyl)-1-butanone and *N*-nitrosonornicotine [4]. Many studies have suggested that the interindividual variability in CYP2A6 activity affects smoking behavior or cancer susceptibility [5-7]. Genetic polymorphisms are the major factor contributing to the interindividual differences in CYP2A6 activity and expression, but dietary or environmental factors as well as endogenous factors such as steroid hormones are also involved. To understand the regulators of CYP2A6 expression, we have studied transcriptional factors regulating CYP2A6 expression and found that pregnane X receptor [8] and estrogen receptor [9] are involved in the CYP2A6 regulation. In addition, a recent study reported the involvement of glucocorticoid receptor in the regulation of CYP2A6 [10].

It has been reported that cadmium ingestion increased the CYP2A6 expression based on the fact that the extent of urinary excretion of cadmium was positively correlated with the extent of urinary excretion of 7-hydroxycoumarin after the administration of coumarin [11]. Mouse Cyp2a5, an orthologue of human CYP2A6, has also been reported to be induced by the administration of cadmium. Abu-Bakar et al [12] suggested that the induction of Cyp2a5 would be mediated by nuclear factor-erythroid 2 related factor 2 (Nrf2) because the induction was not observed in Nrf2 knock-out mice. Nrf2 is a transcription factor which regulates the expression of antioxidative and cytoprotective genes. Under normal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1, which stimulates proteasomal degradation of Nrf2 [13]. On cellular stimulation by oxidative stress, Nrf2 is dissociated from Keap1 and accumulates in the nucleus to regulate the expression of antioxidative and cytoprotective genes. Sulforaphane, which is well known as an activator of Nrf2, is contained in cruciferous vegetables such as broccoli sprouts. Interestingly, it has been reported that CYP2A6 activity was significantly increased after the consumption of broccoli

(500 g/day for 6 days) by 1.4 - 5.5 fold [14]. This background prompted us to investigate whether Nrf2 might be involved in the regulation of human CYP2A6.

2. Materials and Methods

2.1. Chemicals and reagents

L-Sulforaphane (SFN) and *tert*-butylhydroquinone (tBHQ) were obtained from LKT Laboratory (St. Paul, MN) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Anti-human Nrf2 antibodies (C-20) and (H-300), which recognize the C-terminus and N-terminus of the Nrf2 protein, respectively, and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dual Luciferase Reporter Assay System, pGL3-basic, phRL-TK, and pGL4.74 plasmid were purchased from Promega (Madison, WI). QIAGEN Plasmid Midi kit was from QIAGEN (Valencia, CA). MiraCLEAN Endotoxin Removal Kit and *Trans*IT-QR Hydrodynamic Delivery Solution were from Mirus Bio (Madison, WI). Oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Restriction enzymes were purchased from Takara (Shiga, Japan), TOYOBO (Osaka, Japan), and New England Biolabs (Beverly, MA). All other reagents were of the highest grade commercially available.

2.2. Cell culture

Human cryopreserved hepatocytes, lot 82 (Hispanic, female, 23 years) were purchased from In Vitro Technologies (Baltimore, MD). The hepatocytes were seeded into collagen-coated 6-well plates at 0.9×10^5 cells/well and maintained in HCM hepatocyte culture medium (Cambrex, East Rutherford, NJ) at 37°C under 5% CO_2. After 24 h, the culture medium was changed to HCM medium (epidermal growth factor- and antibiotics-free) containing 10 μM SFN or 0.1% (v/v) DMSO vehicle. Hepatocytes were maintained for 12 h or 24 h until harvesting.

Human hepatoma cell line HepG2 was obtained from American Type Culture

Collection (Manassas, VA). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 0.1 mM nonessential amino acids (Invitrogen) at 37°C under 5% CO_2.

2.3. Real-time RT-PCR analyses

Total RNA was isolated from human hepatocytes or mouse liver using RNAiso (Takara) following the manufacturer's protocol, and cDNA was synthesized as described previously [15]. The primers for human CYP2A6 [15] and human GAPDH [16] were described previously. The forward and reverse primers for mouse NAD(P)H:quinone oxidoreductase 1 (NQO1) were 5'-CCCTGATTGTACTGGCCCATT-3' and 5'-CGTCCTTCCTTATATGCTAG-3', respectively. The forward and reverse primers for mouse GAPDH were 5'-AAATGGGGTGAGGCCGGT-3' and 5'-ATTGCTGACAATCTTGAGTGA-3', respectively. Real-time RT-PCR assays were performed using the Smart Cycler (Cepheid, Sunnyvale, CA) as described previously [17].

2.4. Electrophoretic mobility shift assays

Double-stranded oligonucleotides were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (TOYOBO) and purified by Microspin G-50 columns (GE Healthcare, Buckinghamshire, UK). The oligonucleotide sequences for ARE1 and consensus ARE (cARE) on *Mus musculus* heme oxygenase-1 (HO-1) promoter were 5'-GTAGTAGCCCCTGACAAAGCAGGAATCAT-3' and 5'-GATCTTTTATGCTGAGTCATGGTTT-3', respectively [18]. The labeled probe (80 fmol, ~13,000 cpm) was applied to each binding reaction in 25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA, 10% glycerol, 50 mM KCl, 0.5 mM dithiothreitol, 0.5 mM (*p*-amidinophenyl) methanesulfonyl fluoride, 1 μg of poly (dI-dC), 10 μg of salmon sperm DNA, and 8 μg of the nuclear extracts from 80 μM tBHQ-treated HepG2 cells with a final reaction volume of 15 μl. To determine the specificity of the binding to the oligonucleotides, competition experiments

were conducted by co-incubation with 10-, 50-, and 200-fold excesses of unlabeled competitors. For super-shift experiments, 2 μg of anti-Nrf2 antibodies or normal rabbit IgG were pre-incubated with the nuclear protein on ice for 30 min. The reactions were incubated on ice for 15 min and then loaded on 4% acrylamide gel in 0.5 × Tris-borate EDTA buffer. The gels were dried and exposed to imaging plate for 18 h. The DNA-protein complexes were detected with a Fuji Bio-Imaging Analyzer BAS 1000 (Fuji Film, Tokyo, Japan).

2.5. Human Nrf2 expression plasmid and reporter constructs

Human Nrf2 expression plasmid and the pGL3-cARE plasmid containing two copies of the cARE on the human *NQO-1* gene were previously constructed [17]. Double-stranded oligonucleotide ARE1 on the human *CYP2A6* gene (5'-GTAGTAGCCCCTGACAAAGCAGGAATCAT-3') was cloned into the pGL3-tk plasmid digested with *Sma* I, resulting in single (pGL3/ARE1) and double (pGL3/2×ARE1) insertions. The pGL3/-3046 plasmid containing the 5'-flanking region from -3,046 to -1 of the *CYP2A6* gene was previously constructed [9]. The pGL3/-1395 and pGL3/-185 plasmids were constructed by ligating the fragments from pGL3/-3046 plasmid digested with *BST1107* I/*Hind* III and *Pvu* II/*Hind* III, respectively, into the *Sma* I/*Hind* III-digested pGL3-basic plasmid. The pGL3/-1013 plasmid was constructed by ligating the fragments from pGL3/-3046 plasmid digested with *Bgl* II /*Hind* III into the *Bgl* II/*Hind* III-digested pGL3-basic plasmid. The plasmid DNA was purified by QIAGEN Plasmid Midi kit (QIAGEN). Nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analyses.

2.6. In vitro transfection and luciferase assay

HepG2 cells were seeded into 24-well plates at 1.0×10^5 cells/well and incubated for 24 h before transfection. Transfection was performed using Tfx-20 reagent (Promega). In brief, the transfection mixture consisted of 150 ng of pGL3 plasmids, 5 ng of phRL-TK plasmid, and 100 ng of Nrf2 expression plasmid (or control vector). Forty-eight hours after the

transfection, the cells were harvested and lysed to measure the luciferase activity using a Dual Luciferase Reporter Assay System. The relative luciferase activities were normalized with the *Renilla* luciferase activities.

2.7. In vivo transfection

Male ICR mice (3 weeks old, 10-13 g) were obtained from SLC Japan (Hamamatsu, Japan). Mice were housed in a controlled environment (temperature 25 ± 1°C, humidity 50 ± 10%, and 12 h light/12 h dark cycle) in the institutional animal facility with access to food and water *ad libitum*. Mice were acclimatized for a week before use for the experiments. For in vivo transfection, 18-22 g mice were injected via the tail vein with 10 μg of pGL3 plasmids and 1 μg of pGL4.74 plasmid, in volumes of 0.1 ml/g of body weight within 5-8 s using the *Trans*IT-QR Hydrodynamic Delivery Solution. Endotoxin in plasmid preparations was removed using MiraCLEAN Endotoxin Removal Kit. After 18 h, 1 mg/head SFN or saline was intraperitoneally administered. The dose was decided referring previous studies [19, 20]. Animals were sacrificed 24 h later and the liver, approximately 100 mg, was removed and homogenized in 1 ml of passive lysis buffer (Dual Luciferase Reporter Assay System). The liver homogenates were centrifuged at 15,000 rpm for 10 min at 4°C. Twenty microliters of the supernatant were used to measure the firefly and *Renilla* luciferase activities. For each construct, at least three mice were transfected, and three independent experiments were performed. Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, as approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan.

2.8. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed by an unpaired two-tailed Student's *t* test. A value of *P* less than 0.05 was considered statistically significant.

3. Results

3.1. SFN induces CYP2A6 mRNA expression in human hepatocytes

We first examined whether the CYP2A6 level in human hepatocytes was increased by SFN treatment (Fig. 1). When human hepatocytes were treated with 10 μM SFN for 12 h, a significant induction (1.4-fold, $P < 0.01$) of the CYP2A6 mRNA level was observed. With 24-h treatment, a similar induction (1.3-fold induction, $P < 0.05$) was observed. These results suggest that CYP2A6 mRNA is induced by SFN.

3.2. Nrf2 directly binds to the ARE on the CYP2A6 gene

To find potential binding sites of Nrf2 on the 5'-flanking region of *CYP2A6* gene, we investigated overlapping with the core sequence of consensus ARE 5'-TMAnnR<u>TGAY(C/T)nnnGC</u>Rwwww-3' (core sequence is underlined) using a computer program GENETYX-MAC for all probable nucleotide combination, and thereby we identified three putative AREs up to -4 kb of the 5'-flanking region of *CYP2A6* gene. These elements located at -1212, -2444, and -3441 were termed ARE1, ARE2, and ARE3, respectively (Fig. 2). We performed electrophoretic mobility shift assays to examine whether Nrf2 can bind to these AREs (Fig. 3). When the ^{32}P-labeled cARE was incubated with the nuclear extract prepared from the tBHQ-treated HepG2 cells, three bands were detected (Fig. 3, lane 1). The upper and lower bands were non-specific bands (NS). The middle band represented a shifted band, and its density was diminished with both anti-Nrf2 antibodies (C-20) and (H-300) (Fig. 3, lanes 2 and 3). Super-shifted band was observed only with the anti-Nrf2 antibody (C-20), consistent with our previous study on UGT2B7 [17]. When the ARE1 was used as a probe, a band the mobility of which was the same as that of the cARE-Nrf2 complex was observed (Fig. 3, lane 8). The band was clearly supershifted with the anti-Nrf2 antibody (C-20) (Fig. 3, lane 9) and was competed out by unlabeled cARE (Fig. 3, lanes 12, 13, and 14). These results indicated that Nrf2 specifically binds to ARE1 on the human *CYP2A6* gene. When ARE2 or ARE3 was used as a probe, no band was observed (data not shown).

3.3. ARE1 on CYP2A6 promoter is functional for transactivation via Nrf2

To examine whether ARE1 is functional for the transactivation via Nrf2, luciferase assays were performed using HepG2 cells. We first confirmed that the luciferase activity of the pGL3-cARE plasmid containing two copies of cARE used as a positive control, was significantly ($P < 0.001$) increased up to 2.7-fold by the overexpression of Nrf2 (Fig. 4A). The luciferase activities of the pGL3/ARE1 and pGL3/2×ARE1 plasmids containing one and two copies of ARE1 were significantly increased up to 1.3- and 2.0-fold, respectively, by the overexpression of Nrf2. Next we performed luciferase assay using a series of reporter plasmids containing the 5'-flanking region of *CYP2A6* gene (Fig. 4B). Contrary to our expectations, the luciferase activity of the pGL3/-1013 plasmid containing ARE1 was significantly decreased by the overexpression of Nrf2. The luciferase activities of the pGL3/-1395 and pGL3/-185 plasmids were also significantly decreased by the overexpression of Nrf2. These results suggest that the proximal promoter region possibly has a negative regulatory region responding to Nrf2, or HepG2 cells may lack transcriptional factors crucial for the transcriptional activity of CYP2A6.

3.4. Nrf2 activates CYP2A6 promoter activity in vivo

Next, we sought to determine the transactivity of the plasmids in mice in vivo, because mice liver contains sufficient levels of hepatic transcription factors, which is unlikely the case in cell lines. When the pGL3-cARE plasmid was injected into mice, the luciferase activity in the liver was significantly ($P < 0.05$) increased up to 2.1-fold by SFN treatment (Fig. 5A). It was confirmed that under this condition the endogenous mouse NQO1 mRNA level was significantly ($P < 0.001$) induced (2.4-fold) (Fig. 5B). The luciferase activity of the pGL3/-1395 plasmid containing ARE1 was significantly ($P < 0.05$) increased (1.9- fold) by SFN treatment, but that of the pGL3/-1013 plasmid was not (Fig. 5A). These results suggest that the CYP2A6 promoter containing ARE1 is transactivated by SFN in vivo.

4. Discussion

In the present study, we found that Nrf2 is involved in the regulation of human CYP2A6. Concerning the role of Nrf2 in the regulation of P450, mouse Cyp2a5 was the first reported case [12]. In addition, a recent study demonstrated that human CYP2J2 is regulated by Nrf2 [21]. We could provide evidence to put CYP2A6 into the short list of P450s that are regulated by Nrf2. It was clearly demonstrated that sulforaphane significantly increased the CYP2A6 mRNA level in human hepatocytes. Sulforaphane, an activator of Nrf2, is contained in cruciferous vegetables such as broccoli sprouts, horseradish, cabbage, and watercress. Interestingly, it has been reported that CYP2A6 activities were significantly increased after the consumption broccoli (500 g/day for 6 days) by 1.4 - 5.5-fold [14]. We believe that the present study may demonstrate the underlying molecular mechanism of the induction.

Electrophoretic mobility shift assays clearly demonstrated that Nrf2 directly bound to ARE1, but not ARE2 and ARE3, on the human *CYP2A6* gene. Previously, Abu-Bakar et al. [22] identified ARE (TGACagaGCA) at -2377 on the 5'-flanking region of the mouse *Cyp2a5* gene to which Nrf2 bound. Interestingly, the sequence of human ARE1 (TGACaaaGCA) has only one base difference with the mouse ARE. Although the core sequence of ARE2 (TGAC<u>ctg</u>GC<u>c</u>) is similar to that of ARE1, Nrf2 did not bind to ARE2. Thus, the differences in core sequence (underlined) might also be important for the binding of Nrf2. For the supershift assay, we used two kinds of anti-Nrf2 antibody (C-20 and H-300). The supershifted band was observed with the anti-Nrf2 antibody (C-20) but not with the anti-Nrf2 antibody (H-300). Anti-Nrf2 antibody (C-20) recognizes the C-terminal of Nrf2, whereas anti-Nrf2 antibody (H-300) recognizes the N-terminal. Since the N-terminal has a DNA-binding domain, the anti-Nrf2 antibody (H-300) seemed to interfere with the binding of Nrf2 to the DNA, not forming the antibody-Nrf2-DNA complex represented as a supershifted band.

In the luciferase assays, we first determined the effects of the treatment with SFN on the transactivity of the constructs. Unexpectedly, SFN treatment significantly decreased the firefly and *Renilla* luciferase activities derived from the pGL3-tk and phRL-TK plasmids,

respectively by approximately half (data not shown). Such a phenomenon was not observed when Nrf2 was overexpressed. It was assumed that SFN might affect the thymidine kinase promoter activities independently of Nrf2. By the overexpression of Nrf2, we found that ARE1 itself was functional for the transactivation. However, the luciferase activities of plasmids containing the intact 5'-flanking region of *CYP2A6* gene were not increased by the overexpression of Nrf2, even if it contained the ARE1. Similar results were obtained using HeLa cells (data not shown), suggesting that it was not a HepG2-specific phenomenon. It was considered that the sequences surrounding ARE may interfere the binding of Nr2, or these cell lines might lack some transcriptional factor(s) that are necessary for the transactivation of CYP2A6. It has been reported that the transactivity of the CYP2C8 promoter was successfully evaluated by the injection of the constructs in mouse in vivo, although such evaluation was unsuccessful in HepG2 cells [23]. Based on this report, we also performed the luciferase assay in mice in vivo and found that the SFN treatment significantly increased the transactivity of the pGL3/-1395 plasmid containing ARE1 (Fig. 5A). Thus, it was concluded that CYP2A6 is regulated by Nrf2 via ARE1.

CYP2A6 is responsible for nicotine metabolism [2]. Smokers adapt their smoking behavior to maintain their nicotine levels in the body [24]. Since the metabolism of nicotine by CYP2A6 is the principal pathway by which nicotine is removed from the circulation, an association between the CYP2A6 activity and cigarette consumption has been suggested [5, 6]. Cigarette smoking is known to cause oxidative stress, which activates Nrf2 [25, 26]. In addition, tobacco is a substantial source of cadmium, supported by the fact that the serum cadmium level of smokers is 3 folds higher than that of non-smokers [27]. Therefore, it is surmised that the CYP2A6 expression level might be higher in smokers than in non-smokers, although there is no report comparing the expression level of hepatic CYP2A6 protein in smokers versus that in non-smokers. In contrast, it has been reported that in vivo nicotine clearance [28] and in vivo coumarin metabolism [29] were lower in smokers than in non-smokers, suggesting the possibility that some constituents in tobacco smoke might have inhibitory effects on the CYP2A6 activity. Such inhibitory effects could possibly mask the

induction of CYP2A6, resulting in decreased in vivo metabolic potency. Thus, it would be of interest to compare the hepatic CYP2A6 expression levels in smokers and non-smokers, although it has been reported that the administration of nicotine itself downregulated CYP2A6-like enzyme expression in African green monkeys [30].

In conclusion, we found that Nrf2 regulates the human CYP2A6. This mechanism implies the possibility that the CYP2A6 expression may be increased by oxidative stress such as by cigarette smoking.

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Figure legends

Fig. 1. Effects of SFN treatment on the CYP2A6 mRNA level in human hepatocytes. Human hepatocytes were treated with 10 μM SFN or 0.1% DMSO for 12 h or 24 h. Total RNA was extracted and real-time RT-PCR was performed. To normalize the RNA loading, the CYP2A6 mRNA levels were corrected with the GAPDH mRNA levels. Each column represents the mean ± SD of three independent experiments. $*P < 0.05$ and $**P < 0.01$ compared with DMSO treatment.

Fig. 2. Schematic representation of the putative AREs on the *CYP2A6* genes and the sequences of the AREs. Numbers indicate the nucleotide position when the A in the initiation codon ATG is denoted + 1 and the base before A is numbered -1. The core ARE sequence is underlined. The nucleotides that are consistent with the consensus ARE are shown with bold letters.

Fig. 3. Electrophoretic mobility shift assays of the binding of Nrf2 to ARE of the *CYP2A6* gene. Oligonucleotides of the cARE in *Mus musculus* HO-1 promoter (*left*) and CYP2A6 ARE1 (*right*) were used as probes. Nuclear extracts were prepared from HepG2 cells treated with 80 μM tBHQ for 6 h. Cold oligonucleotides were used as a competitor in 10-, 50-, and 200-fold molar excess. For supershift analyses, 2 μg of anti-Nrf2 antibodies or normal rabbit IgG were preincubated with the nuclear extracts on ice for 30 min.

Fig. 4. Effects of overexpression of Nrf2 on CYP2A6 transactivation in HepG2 cells. Reporter plasmids containing ARE sequences (A) or the 5'-flanking region of *CYP2A6* gene with deletion from the 5' direction (B) were transiently transfected into HepG2 cells with Nrf2 expression plasmid (Nrf2) or pTARGET empty vector (control). The pGL3-cARE plasmid, which contains two copies of the cARE on the human *NQO-1* gene, was used as a positive control. The firefly luciferase activities were normalized with the *Renilla* luciferase

activities. Right panel shows the fold induction of the transcriptional activity by the overexpression of Nrf2. Each column represents the mean ± SD of three independent experiments. **$P < 0.01$ and ***$P < 0.001$ compared with control.

Fig. 5. Effects of SFN on CYP2A6 transactivation in *in vivo* mice liver transfections. Ten μg of pGL3 reporter plasmid and one μg of pGL4.74 plasmid were injected into the tail vein of male ICR mice. After 18 h, 1 mg/head SFN was intraperitoneally administered. After 6 h, the liver was removed and the homogenate and total RNA were prepared for the luciferase assay and real-time RT-PCR, respectively. (A) The firefly luciferase activities were normalized with the *Renilla* luciferase activities. Right panel shows the fold induction of the transcriptional activity by the treatment with SFN. Each column represents the mean ± SD (n = 3). *$P < 0.05$ compared with control. (B) NQO1 mRNA levels in mice injected with pGL3-cARE plasmid were determined by real-time RT-PCR. The NQO1 mRNA levels were normalized with the GAPDH mRNA levels. Each column represents the mean ± SD (n = 3). ***$P < 0.001$ compared with saline.

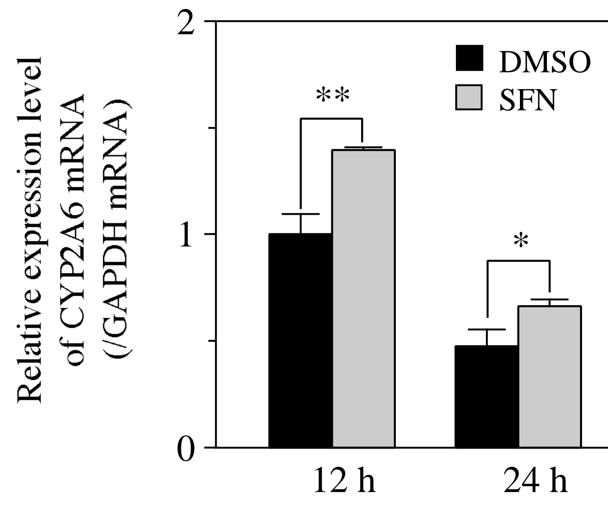
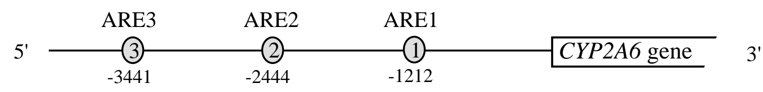

Relative expression level
of CYP2A6 mRNA
(/GAPDH mRNA)
2
1
0
DMSO
SFN
**
*
12 h
24 h

Fig. 2



Consensus ARE		TMAnnRTGAYnnnGCRwwww	
ARE1	-1212	AGCcccTGACaaaGCAGGAA	-1193
ARE2	-2425	TTGccATGACctgGCCAGGG	-2444
ARE3	-3441	TCAacATGAAcatGCTGAAA	-3422

Fig. 3

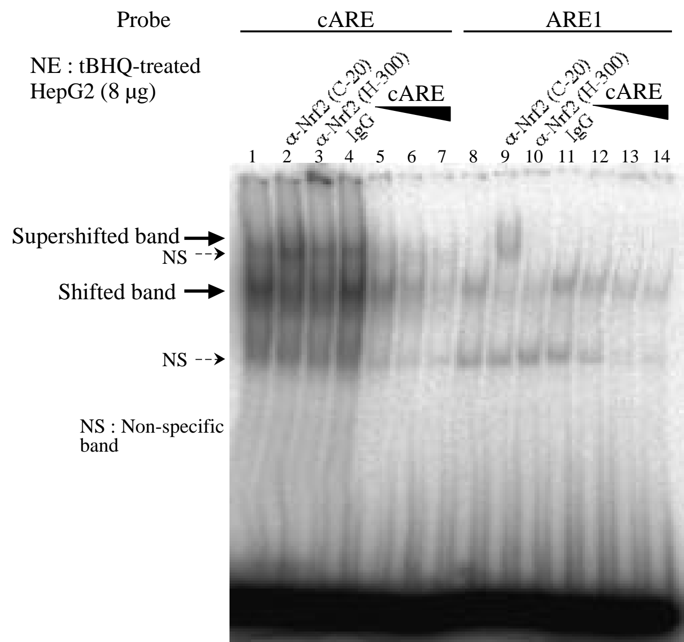


Fig. 4

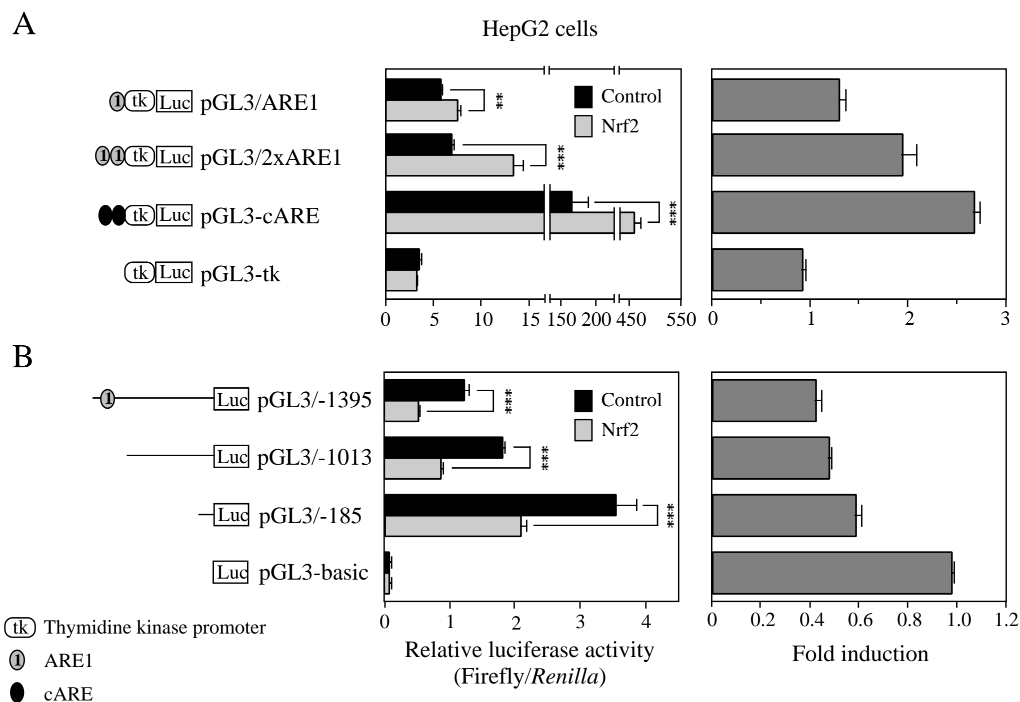
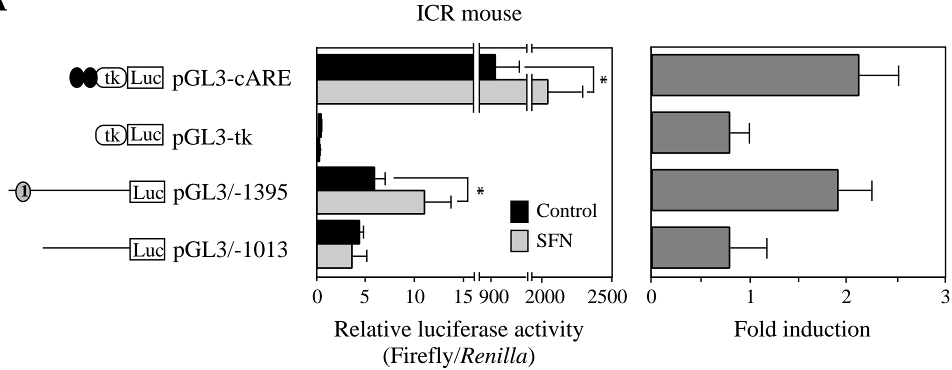


Fig. 5

A



B