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Transcriptional regulation of human carboxylesterase 1A1 by nuclear factor-erythroid 2 related factor 2 (Nrf2)☆

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ABSTRACT

Human carboxylesterase (CES) 1A, which is predominantly expressed in liver and lung, plays an important role in the hydrolysis of endogenous compounds and xenobiotics. CES1A is reported to be induced in human hepatocytes by butylated hydroxyanisole, ticlopidine and diclofenac, and the induction is assumed to be caused by oxidative stress. However, the molecular mechanism remains to be determined. In this study, we sought to investigate whether CES1A is regulated by nuclear factor-erythroid 2 related factor 2 (Nrf2), which is a transcriptional factor activated by oxidative stress, and clarify the molecular mechanism. Real-time reverse transcription-PCR assays revealed that CES1A1 mRNA was significantly induced by *tert*-butylhydroquinone (tBHQ) and sulforaphane (SFN), which are representative activators of Nrf2 in HepG2, Caco-2 and HeLa cells. The induction was completely suppressed with small interfering RNA for Nrf2. In HepG2 cells, the CES1A protein level and imidapril hydrolase activity, which is specifically catalyzed by CES1A, were also significantly induced by tBHQ and SFN. Luciferase assays revealed that the antioxidant response element (ARE) at −2025 in the *CES1A1* gene was responsible for the transactivation by Nrf2. In addition, electrophoretic mobility shift assays and chromatin immunoprecipitation assays revealed that Nrf2 binds to the ARE in the *CES1A1* gene. These findings clearly demonstrated that human CES1A1 is induced by Nrf2. This is the first study to demonstrate the molecular mechanism of the inducible regulation of human CES1A1.

1. Introduction

Human carboxylesterase (CES) is a member of serine hydrolase superfamily and involved in the hydrolysis of endogenous compounds and xenobiotics. In human, the CES1A and CES2 families play important roles in drug metabolism. The CES1A mainly hydrolyzes substrates with a small alcohol group and large acyl group, such as imidapril [1] and osertamivir [2]. In contrast, the CES2 prefers substrates with a large alcohol group and small acyl group, such as CPT-11 [3] and heroin [4]. Since CES1A has been reported to play roles as triglyceride hydrolase (TGH) [5], acyl coenzyme A:cholesterol acyltransferase (ACAT) [6] and cholesteryl ester hydrolase (CEH) [7], it seems probable that CES1A is important for the lipid metabolism as well as the drug metabolism. CES1A is predominantly expressed in liver and lung, whereas CES2 is expressed in colon and liver [8]. Human CES1A is classified into two isoforms, CES1A1 and CES1A2, with high homology at the mRNA level (99.3%). It has been accepted that the *CES1A2* gene is inverted and duplicated with the *CES1A1* gene [9]. However, we recently demonstrated that the *CES1A2* gene is a variant of the *CES1A3* pseudogene [10]. The sequence identity in the 5′-flanking region between the *CES1A1* and *CES1A2* genes is approximately 90% and the sequences downstream intron 1 of them are identical. Since only the N-terminal signal peptide sequences in exon 1 of CES1A1 and CES1A2 are different, mature proteins produced from both mRNA are identical. Our previous study revealed that the levels of CES1A1 mRNA transcribed from the *CES1A1* gene were substantially higher in liver than those of CES1A2 mRNA transcribed from the *CES1A2* gene [10]. Therefore, it is plausible that the level of CES1A1 mRNA rather than that of CES1A2 mRNA affects the level of mature protein and enzyme activity.

Cells are protected against oxidative stress by increasing the transcription of a group of genes coding antioxidant proteins and phase II enzymes, such as NAD(P)H:quinone oxidoreductase1 (NQO1) [11], heme oxigenase1 (HO-1) [12], and UDP-glucuronosyltransferase (UGT) 1A1 [13]. The most important regulator of the up-regulation is the transcription factor nuclear factor-erythroid 2 (NF-E2) related factor 2 (Nrf2). Nrf2 is a member of the cap'n'collar (CNC) family of transcriptional factors and contains a C-terminal basic leucine zipper structure that facilitates dimerization and DNA binding [14]. Nrf2 is highly expressed in detoxification tissues such as liver and kidney, and tissues that are exposed to the

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external environment, such as skin, lung and gastrointestinal tract [15]. In the absence of cellular stress, Nrf2 is localized in cytosol by binding with Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1), which acts as a substrate adopter for Cullin-dependent E3 ubiquitin ligase complex. Under the condition of oxidative stress, Nrf2 is released from Keap1 and translocates into the nucleus. After heterodimerization with small Maf protein, Nrf2 stimulates the transcription of the downstream genes by binding to antioxidant response element (ARE), a *cis*-acting enhancer with a consensus sequence defined as 5′-TMAnnRTGABnnnGCAwwww-3′, in which the core nucleotide is underlined [16,17]. *tert*-Butylhydroquinone (tBHQ) and sulforaphane (SFN) are known to be representative activators of Nrf2 [18].

There are few reports of the regulatory mechanism of human CES1A. Hosokawa et al. [19] demonstrated that Sp1 and C/EBP are involved in the basal expression of CES1A1. In addition, Nishimura et al. [20] reported that, in human hepatocytes, CES1A mRNA was not induced by rifampicin and omeprazole, which are potent ligands of pregnane X receptor and aryl hydrocarbon receptor (AhR), respectively. Thus, the inducible regulation of CES1A has not been reported until now. Recently, Takakusa et al. [21] reported that CES1A was induced by butylated hydroxyanisole, ticlopidine and diclofenac in human hepatocytes, and that the induction would be stimulated by oxidative stress. As described above, it is well known that Nrf2 is involved in the induction by oxidative stress. In addition, a computer-assisted homology search revealed putative AREs within −3200 bp of the *CES1A1* gene. Thus, it is plausible that Nrf2 is involved in the transcriptional regulation of CES1A. These lines of evidence prompted us to investigate whether the human *CES1A1* gene is regulated by Nrf2.

2. Materials and methods

2.1. Chemicals and reagents

tBHQ was purchased from Wako Pure Chemical Industries (Osaka, Japan). L-SFN was purchased from Alexis (San Diego, CA). Imidapril and imidaprilat were kindly supplied by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). Anti-human Nrf2 antibodies (C-20 and H-300), which recognize the C- and N-terminus of Nrf2 protein, respectively, were from Santa Cruz Biotechnology (Santa Cruz, CA). Stealth Select RNAi for Nrf2 (HSS107130) (5′-aaucacugaggccaaguagugugguc-3′) and Stealth RNAi negative control, Medium GC Duplex #2 were from Invitrogen (Carlsbad, CA). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All other reagents were of the highest grade commercially available.

2.2. Cells and culture conditions

The human hepatoma cell line HepG2 and human colon carcinoma cell line Caco-2 were obtained from American Type Culture Collection (Manassas, VA). Human adenocarcinoma of the cervix of uterus cell line HeLa was obtained from RIKEN BioResource Center (Ibaraki, Japan). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). HepG2 and Caco-2 cells were cultured in DMEM supplemented with 10% FBS and 0.1 mM nonessential amino acids (Invitrogen). The cells were maintained at 37 °C under an atmosphere of 5% CO_2.

2.3. Real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis

Total RNA was extracted using RNAiso (Takara Bio, Shiga, Japan) and cDNA was synthesized from total RNA using ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Human GAPDH mRNA was quantified by real-time RT-PCR using the Smart Cycler (Cepheid, Sunnyvale, CA) as described previously [22]. CES1A1, CES2 and HO-1 mRNA levels were quantified under the same condition. PCR was performed with the following primer sets: CES1A1, CES1A1 S and CES1A1 AS; CES2, CES2 S and CES2 AS; HO-1, HO-1 S and HO-1 AS (Table 1).

Table 1
Sequences of oligonueleotide used in the present study.

Oligonucleotide	Sequence
For real-time RT-PCR	
CES1A1 S[a]	5′-atgtggctccgtgcct-3′
CES1A1 AS[a]	5′-tcttcacaaagctccatggt-3′
CES2 S[b]	5′-aacctgtctgcctgtgaccaagt-3′
CES2 AS[b]	5′-acatcagcagcgttaacattttctg-3′
HO-1 S[c]	5′-atagagcgaaacaagcaga-3′
HO-1 AS[c]	5′-tagagctgtttgaacttgg-3′
GAPDH S[d]	5′-ccagggctgcttttaactc-3′
GAPDH AS[d]	5′-gctcccccctgcaaatga-3′
For SOE-PCR	
pGL3 S	5′-tagcaaaataggctgtcccc-3′
pGL3 AS	5′-tcgatatgtgcatctgtaaaa-3′
ARE2 mt-A	5′-atctaaggcaaattTTCTTGTCAgctcatt-3′
ARE2 mt-B	5′-aatgagcTGACAAGAAaatttgccttagat-3′
ARE3 mt-A	5′-acagcaactcaatgTTAAAGTCAgaaccag-3′
ARE3 mt-B	5′-ctggttcTGACTTTAAcattgagttgctgt-3′
ARE4 mt-A	5′-cccgtgagattaatTTTGTCTCAcgatctt-3′
ARE4 mt-B	5′-aagatcgTGAGACAAAattaatctcacggg-3′
For ChIP assays	
CES1A1 −2178 S	5′-gaccttaggcaatccctcct-3′
CES1A1 −1855 AS	5′-tggctgtaatcttgtcagtgtttc-3′
CES1A1 −1274 S	5′-tctttgtgtacaagcttttgtg-3[r]
CES1A1 −956 AS	5′-cacaaggaagtcactcaaag-3′
CES1A1 −958 S	5′-gtgtccccagcagcttgtaa-3′
CES1A1 −666 AS	5′-aaaatgaactccgtccccc-3′
For electrophoresis mobility shift assays	
cARE[e]	5′-gatcttttatgctgagtcatggttt-3′
CES1A1 ARE4	5′-atttaagatcgTGAGACAGCattaatctc-3′
CES1A1 ARE4mt	5′-atttaagatcgTGAGACAAAattaatctc-3′
CES1A1 ARE6	5′-taggggaattGCTGGGTCAtatggaaactc-3′
CES1A1 ARE6mt	5′-taggggaattTTTGGGTCAtggaaactc-3′

The core ARE is indicated by capital letters and mutated nucleotides are underlined.
[a] From Fukumi et al. [10].
[b] From Sanghani et al. [23].
[c] From Nakamura et al. [24].
[d] From Tsuchiya et al. [22].
[e] From Balogun et al. [12].

2.4. Immunoblot analysis

The expression of CES1A protein was measured by SDS-polyacrylamide gel electrophoresis and immunoblot analysis according to Laemmli [25]. For the preparation of cell homogenates, HepG2 cells were suspended in TGE buffer (10 mM Tris–HCl (pH 7.4), 20% glycerol, 1 mM EDTA) and disrupted by freeze–thawing three times. The protein concentrations were determined according to Bradford [26]. Cell homogenates (30 μg) were separated on 7.5% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P (Millipore, Billerica, MA). The membranes were probed with polyclonal rabbit anti-human CES1A (Abcam, Cambridge, MA), and IRDye680-labeled anti-rabbit IgG and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) were used for detection. The relative expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare, Buckinghamshire, UK).

2.5. Imidapril hydrolase activity

The imidapril hydrolase activity was determined according to the method described previously with a slight modification [27]. A

typical standard reaction mixture (total volume, 0.2 ml) contained HepG2 cell homogenates (1 mg/ml), 100 mM Tris–HCl buffer (pH 7.4) and 100 μM imidapril. The final concentration of the organic solvent in the reaction mixture was <1.0%. The reaction was initiated by the addition of imidapril after 2 min of preincubation at 37 °C. After 30 min of incubation, the reaction was terminated by adding 100 μl of ice-cold acetonitrile. After the removal of protein by centrifugation at 9500 × *g* for 5 min, a 10 μl portion of the supernatant was subjected to liquid chromatography-tandem mass spectrometry with an Inertsil ODS-3 analytical column (2.1 mm × 100 mm; GL Science, Tokyo, Japan). The mobile phase consisted of acetonitrile/10 mM ammonium formate (20:80). The mass/charge (*m*/*z*) ion transitions were recorded in the multiple reaction monitoring mode: *m*/*z* 378.2 and 206.3 for imidaprilat.

2.6. Construction of reporter plasmid

A pGL3 plasmid containing two copies of the consensus ARE (2× cARE) in the human *NQO-1* gene (5′-gatcagtcacagtgactcagcagaatct-3′), which was previously constructed in our laboratory [24], was used as a positive control. The PCR fragments of the 5′-flanking region (−3207 to −1 bp) in the *CES1A1* gene amplified using genomic DNA samples were cloned into the pGL3-basic vector (Promega, Madison, MI) after treatment with Klenow Fragment (Takara) and digestion with *Xho* I. This plasmid (−3207 to −1 bp) was used for construction of the other reporter plasmids (−1531 to −1, and −833 to −1 bp) by digestion and subcloning. Throughout this article, base A in the initiation codon ATG is denoted +1 and the base before A is numbered −1. Three reporter plasmids containing ARE(s), the −3041 to −2891 bp plasmid that contained ARE1, the −2365 to −1989 bp plasmid that contained ARE2, ARE3 and ARE4, and the −1310 to −1170 bp plasmid that contained ARE5 and ARE6 were constructed by PCR and subcloning. The plasmids mutated at the AREs (ARE2 mt, ARE3 mt and ARE4 mt) were constructed by splicing with overlap extension (SOE)–PCR [28]. The regions around the nucleotides to be mutated were amplified using primer pairs pGL3 S and mt-A (PCR 1), or mt-B and pGL3 AS (PCR 2) (Table 1). The following SOE–PCR was performed with the primer pair pGL3 S and pGL3 AS, combining the PCR products 1 and 2. The SOE–PCR products were digested with the appropriate restriction enzymes and subcloned into the pGL3-tk vector. The nucleotide sequences were confirmed by DNA sequence analysis (Long-Read Tower DNA sequencer; GE Healthcare).

2.7. Transfection and luciferase assay

For siRNA transfection, HepG2, Caco-2 and HeLa cells were transfected with 30 pmol siRNA by using lipofectamine RNAiMAX (Invitrogen). After incubation for 24 h, the cells were treated with 80 μM tBHQ, 10 μM SFN or 0.1% dimethyl sulfoxide (DMSO) for 24 h. For the luciferase assays, HepG2 cells were seeded into 24-well plates at 1×10^5 cells/well. Transfection was performed using Tfx-20 reagent (Promega). The transfection mixtures consisted of 200 ng of pGL3 plasmid, 10 ng of phRL-TK plasmid (Promega), and 100 ng of human Nrf2 expression plasmid [24] or pTARGET empty plasmid as a control. The cells were harvested 48 h after transfection and lysed to measure the luciferase activity using a Dual Luciferase Reporter Assay System (Promega). The relative luciferase activities were normalized with the *Renilla* luciferase activities.

2.8. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (TOYOBO) and purified by Microspin G-50 columns (GE Healthcare). The oligonucleotide sequences are shown in Table 1. The labeled probe (40 fmol, ~20,000 cpm) was applied to each binding reaction in 25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA (pH 8.0), 10% glycerol, 50 mM KCl, 0.5 mM dithiothreitol, 0.5 mM (*p*-amidinophenyl)methanesulfonyl fluoride, 1 μg of poly(dI-dC), 5 μg of salmon sperm DNA, and 8 μg of the nuclear extracts from 0.1% DMSO- or 10 μM SFN-treated HepG2 cells prepared using NE-PER Nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) 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 5-, 25-, and 50-fold excesses of unlabeled competitors. For supershift experiments, 2 μg of anti-Nrf2 antibodies (C-20 and H-300) or normal rabbit IgG were preincubated 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 film for 24 h. The DNA–protein complexes were detected with a Fuji Bio-Imaging Analyzer BAS 1800 (Fuji Film, Tokyo, Japan).

2.9. Chromatin immunoprecipitation (ChIP) assay

When HepG2 cells reached 60% confluence in 60-mm dishes, they were treated with 0.1% DMSO or 10 μM SFN. After 24 h incubation, ChIP assays were performed using a ChIP assay kit (Upstate, Lake Placid, NY) according to the manufacturer's protocol. Rabbit anti-Nrf2 antibodies (C-20 and H-300) and normal rabbit IgG were used for immunoprecipitation of the protein–DNA complexes. PCR was performed with the following primer sets: region 1, CES1A1 −2178 S and CES1A1 −1855 AS; region 2, CES1A1 −1274 S and CES1A1 −956 AS; region 3, CES1A1 −956 S and CES1A1 −666 AS (Table 1). The PCR conditions were as follows: after initial denaturation at 94 °C for 3 min, the amplification was performed by denaturation at 94 °C for 25 s, annealing at 58 °C (region 1), 54 °C (region 2) or 60 °C (region 3) for 25 s, and extension at 72 °C for 30 s for 30 cycle. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide.

2.10. Statistical analysis

Data are expressed as mean ± SD. Statistical significance between two groups was determined by two-tailed Student's *t*-test. Statistical significance between multiple groups was determined by ANOVA followed by Dunnett or Tukey test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Induction of CES1A in cultured cells

The effects of tBHQ and SFN on the expression of CES1A1 mRNA in HepG2, Caco-2 and HeLa cells were examined (Fig. 1). By treatment with 80 μM tBHQ and 10 μM SFN, the expression of CES1A1 mRNA was significantly increased in HepG2 (2.6- and 3.4-fold, respectively), Caco-2 (3.4- and 4.4-fold) and HeLa cells (3.4- and 3.0-fold). Under the same condition, the level of HO-1 mRNA, which is already known to be induced by Nrf2, was also significantly induced in all cell lines. In contrast, the level of CES2 mRNA was not changed in any of cell lines. The CES1A protein expression and enzyme activity were measured to examine whether they were increased by the induction of CES1A1 mRNA expression by tBHQ and SFN in HepG2 cells. Immunoblot analysis revealed that the expression of CES1A protein was clearly increased up to 1.73- and 2.12-fold in HepG2 cells by tBHQ and SFN, respectively (Fig. 1B). In Caco-2 and HeLa cells, it was difficult to detect CES1A protein by immunoblot analysis due to the

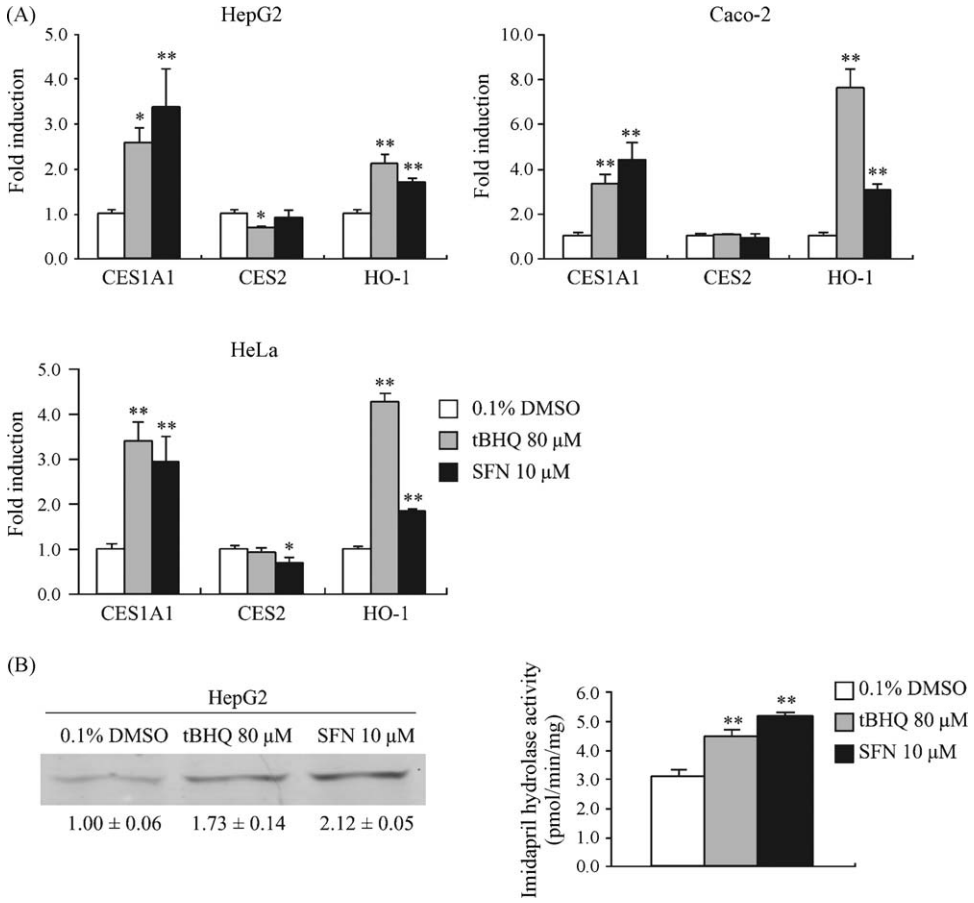


Fig. 1. (A) Effects of tBHQ and SFN on the CES1A1, CES2 and HO-1 mRNA expression in HepG2, Caco-2, and HeLa cells. The cells were treated with 80 μM tBHQ, 10 μM SFN or DMSO vehicle (0.1%) for 24 h. The expressions of CES1A1, CES2 and HO-1 mRNA were determined by real-time RT-PCR and normalized with the GAPDH mRNA levels. Effects of tBHQ and SFN on the expression of CES1A protein (B) and the imidapril hydrolase activity (C) in HepG2 cells. The cells were treated with 80 μM tBHQ, 10 μM SFN or DMSO vehicle (0.1%) for 24 h. The expression of CES1A protein was determined by immunoblot analysis. Each column represents the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control (vehicle).

low expression level. Imidapril hydrolase activity, which is specifically catalyzed by CES1A, was also significantly increased in HepG2 cells by tBHQ and SFN (from 3.4 ± 0.23 to 4.13 ± 0.23 and 5.31 ± 0.15 pmol/min/mg protein, respectively) (Fig. 1C). These results indicate that the induction of CES1A1 mRNA expression by tBHQ and SFN led to increases in CES1A protein expression and enzyme activity.

3.2. Effect of Nrf2 knockdown on the induction of CES1A1 by tBHQ and SFN

To investigate whether Nrf2 is responsible for the induction of CES1A1 by tBHQ and SFN, the endogenous Nrf2 was knockdowned in HepG2, Caco-2 and HeLa cells (Fig. 2). It was confirmed that the Nrf2 mRNA expression was decreased 79%, 82%, and 88% by siRNA-Nrf2 transfection in HepG2, Caco-2, and HeLa cells, respectively. In all cell lines, tBHQ and SFN mediated induction of CES1A1 mRNA was completely suppressed by the knockdown of Nrf2. In HepG2 and Caco-2 cells, the basal expression level of CES1A1 mRNA was not changed by siRNA-Nrf2 transfection, whereas it was significantly decreased in HeLa cells. These results suggested that Nrf2 plays a critical role in the induction of CES1A1 by tBHQ and SFN.

3.3. Identification of Nrf2 binding site in the 5′-flanking region of the CES1A1 gene

A computer-assisted homology search identified six putative AREs within −3200 bp of the *CES1A1* gene (Fig. 3). These AREs at −2971, −2323, −2203, −2025, −1283, and −1218 bp were termed ARE1, ARE2, ARE3, ARE4, ARE5 and ARE6, respectively. To investigate whether AREs in the *CES1A1* gene were functional in Nrf2-dependent transactivation, luciferase assays were performed using reporter plasmids containing the 5′-flanking region of the *CES1A1* gene (−3207 to −1, −1531 to −1, and −833 to −1 bp) in HepG2 cells (Fig. 4A). The transcriptional activity of 2× cARE used as positive control was increased up to 2.2-fold by the overexpression of Nrf2. Unexpectedly, the overexpression of Nrf2 did not activate the transcriptional activities of any plasmids. Then, we constructed three reporter plasmids containing ARE-neighboring regions and luciferase assays were performed (Fig. 4B). The transcriptional activities of the −2365 to −1989 bp plasmid that contained ARE2, ARE3 and ARE4 were increased up to 2.0-fold by the overexpression of Nrf2. In contrast, Nrf2 did not activate the transcriptional activities of the −3041 to −2891 bp plasmid that contained ARE1 and the −1310 to −1170 bp plasmid that contained ARE5 and ARE6. To further confirm the functional ARE, a mutation was introduced into each ARE of the −2365 to −1989 bp plasmid. The Nrf2-dependent transcriptional activation with the −2365 to −1989 bp plasmid was completely abolished by introducing the mutation in ARE4 (Fig. 4C). These results suggest that the ARE4 works as a functional Nrf2 response element in the *CES1A1* gene.

3.4. Nrf2 directly binds to the ARE at −2025 of the CES1A1 gene

To examine whether Nrf2 directly binds to ARE4, EMSA was performed using the nuclear extract prepared from DMSO- or SFN-treated HepG2 cell and ^{32}P-labeled ARE4 as a probe (Fig. 5). When

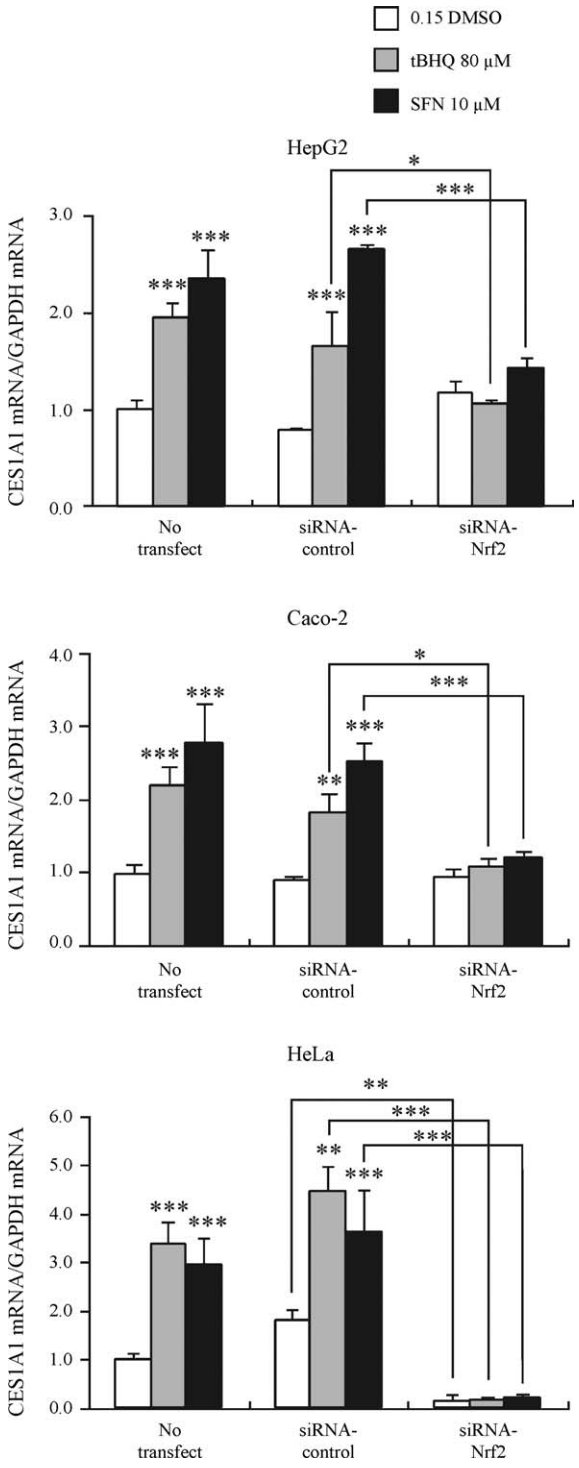
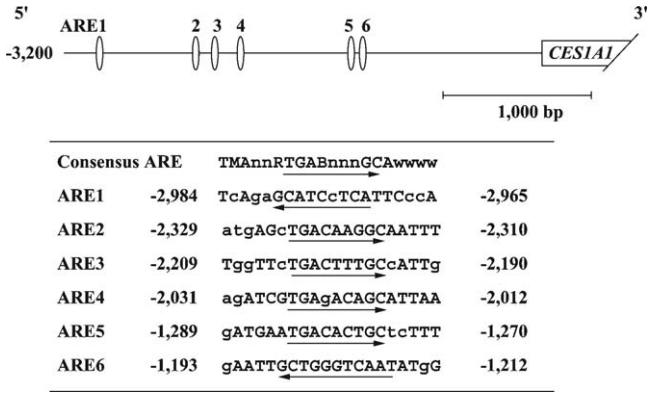


Fig. 2. Effect of Nrf2 knockdown on CES1A1 mRNA induction in HepG2, Caco-2 and HeLa cells. The expression of CES1A1 mRNA was determined by real-time RT-PCR. After 24 h transfection with siRNA, the cells were treated with 80 μM tBHQ, 10 μM SFN or DMSO vehicle (0.1%) for 24 h. To normalize RNA loading and PCR variations, the CES1A1 mRNA levels were corrected with the GAPDH mRNA levels. Each column represents the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control (vehicle).

the probe was incubated with the nuclear extracts from the SFN-treated HepG2 cells, the shifted band was clearly observed. The band density was diminished with both anti-Nrf2 antibodies (C-20 and H-300), although the supershifted band was observed only with the anti-Nrf2 antibody (C-20). These results indicate that the shifted band contained Nrf2 complexes. Moreover, the shifted



Consensus ARE		TMAnnRTGABnnnGCAwwww	
ARE1	-2,984	TcAgaGCATCcTCATTCccA	-2,965
ARE2	-2,329	atgAGcTGACAAGGCAATTT	-2,310
ARE3	-2,209	TggTTcTGACTTTGCcATTg	-2,190
ARE4	-2,031	agATCGTGAgACAGCATTAA	-2,012
ARE5	-1,289	gATGAATGACACTGCtcTTT	-1,270
ARE6	-1,193	gAATTGCTGGGTCAATATgG	-1,212

Fig. 3. Schematic diagram of putative AREs in the 5′-flanking region of the human *CES1A1* gene. 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 indicated by an arrow. The nucleotides that are consistent with the consensus ARE are shown with capital letters.

band was competed out by excess amounts of unlabeled cARE or ARE4, but not by the mutant ARE, ARE4 mt. These results suggested that Nrf2 binds to the ARE located at −2025 in the 5′-flanking region of the *CES1A1* gene.

To further examine whether Nrf2 binds to ARE4 in intact cells, ChIP assays were performed using DMSO- or SFN-treated HepG2 cells (Fig. 6). PCR was performed with primers designed to amplify the −2178 to −1855 bp (region 1) containing ARE4, the −1274 to −956 bp (region 2) containing ARE6 and the −958 to −666 bp (region 3) containing no AREs. Normal rabbit IgG was used as a negative control of immunoprecipitation for ChIP assays. As shown in Fig. 6, the immunoprecipitants of SFN-treated HepG2 cells obtained with anti-Nrf2 antibody (C-20) generated a distinct PCR product for region 1, but those obtained with anti-Nrf2 antibody (H-300) or normal rabbit IgG did not. Using primer sets for region 2 and region 3, no PCR products were obtained with any antibodies. These results suggest that Nrf2 binds to the ARE located at −2025 in the 5′-flanking region of the *CES1A1* gene in intact cells.

4. Discussion

In this study, we demonstrated that human CES1A1 is induced by Nrf2. The human *CES1A1* gene and *CES1A3* pseudogene are inverted and duplicated genes. Our recent study demonstrated that the *CES1A2* gene is a variant of the *CES1A3* pseudogene [10]. In the present study, we demonstrated that the ARE4 in the *CES1A1* gene was the key element of Nrf2-mediated induction, but the sequence of the region corresponding to ARE4 in the *CES1A1* gene exists in the *CES1A2* gene. Therefore, it is conceivable that CES1A2 mRNA derived from the *CES1A2* gene is also induced by Nrf2. The copy numbers of the *CES1A2* gene and *CES1A3* pseudogene were investigated for HepG2, Caco-2 and HeLa cells according to Fukami et al. [10] (data not shown). HepG2 and Caco-2 cells had two copies of the *CES1A3* gene, but HeLa cells had one copy each of the *CES1A3* and *CES1A2* genes. However, in HeLa cells, CES1A2 mRNA was not detected (data not shown), thus the induction of CES1A2 by Nrf2 could not be further investigated. Since the expression of CES1A2 mRNA is much lower than that of CES1A1 mRNA in human liver [10], the Nrf2-mediated induction of CES1A2 would have a minor effect on the CES1A enzyme activity.

NQO1, HO-1, and UGT1A1 are well known to be induced by tBHQ and SFN via Nrf2 [11–13]. Recently, these inductions have attracted attention as biomarkers of the electrophilic stress caused by the formation of reactive metabolites because they serve as a cellular defense against electrophiles and oxidative stress products [29]. It is plausible that CES1A1 is also a possible biomarker of

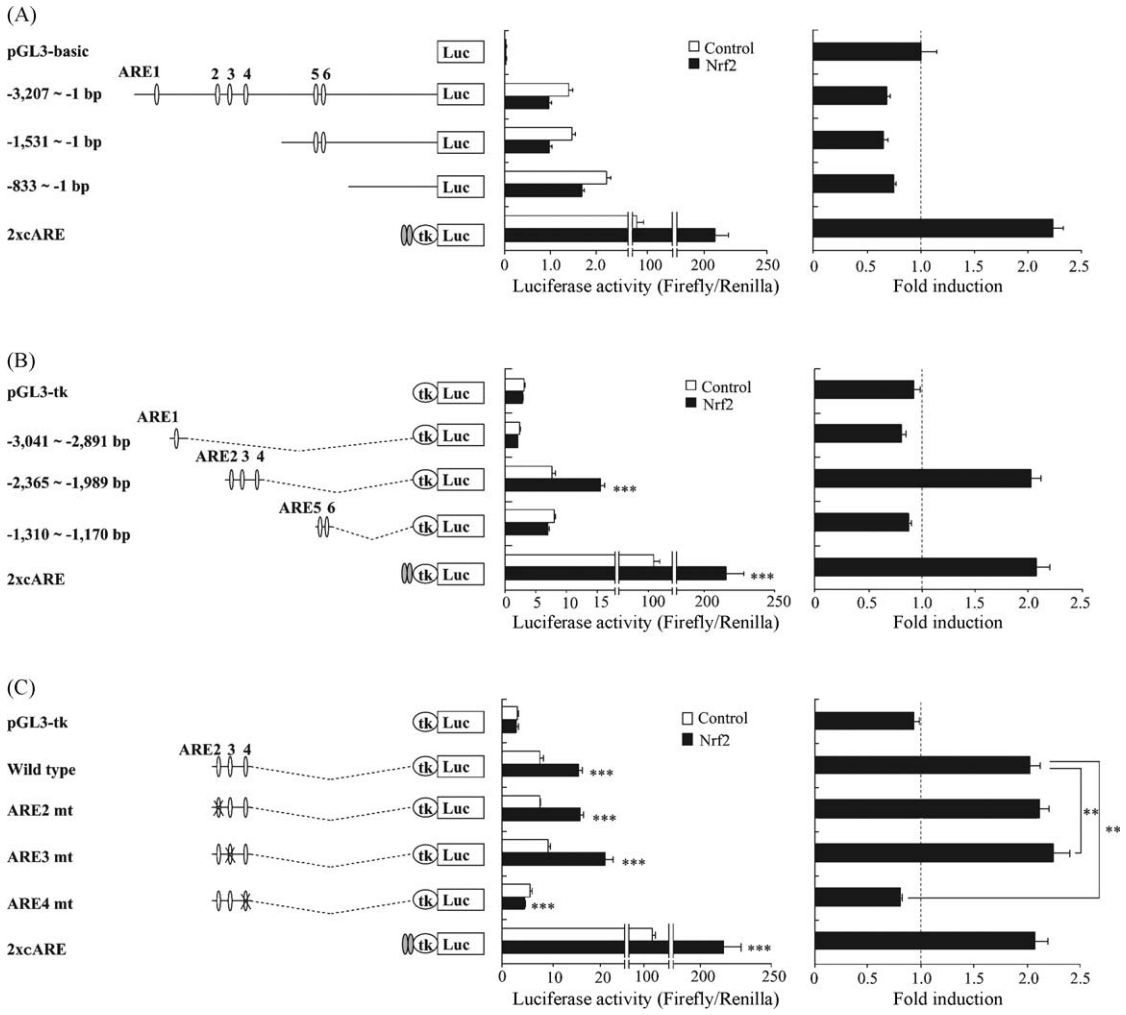


Fig. 4. Relative promoter activity of constructs containing putative ARE(s) in the 5′-flanking region of the *CES1A1* gene and the effect of the overexpression of Nrf2 in HepG2 cells. After 48 h transfection, the cells were harvested and assayed for the luciferase activities according to the manufacturer's protocol. The relative luciferase activities were normalized with the *Renilla* luciferase activities. The right part of the figure shows the fold induction of the transcriptional activity by the overexpression of Nrf2. Each column represents the mean ± SD of three independent experiments. 2×cARE indicates the plasmid containing two copies of the consensus ARE in the human NQO-1 gene. ARE2 mt, AER3 mt, ARE4 mt indicate the plasmids mutated at the ARE2, ARE3, and ARE4, respectively. Control, pTARGET empty plasmid. $^{**}P < 0.01$ and $^{***}P < 0.001$.

electrophilic stress because it was demonstrated in this study that CES1A1 is induced by Nrf2. tBHQ and SFN are also known as ligands of AhR [30,31]. NQO1, HO-1, and UGT1A1 are induced by AhR as well as by Nrf2 [32–34]. In the present study, CES1A1 induction by tBHQ and SFN was completely suppressed by knockdown of Nrf2. In addition, it was reported that CES1A mRNA is not induced by omeprazole, a potent ligand of AhR, in human hepatocytes [20]. These results indicate that only Nrf2 but not AhR would be involved in the CES1A1 induction by tBHQ and SFN. Interestingly, in HeLa cells, the basal expression level of CES1A1 mRNA was significantly decreased by the knockdown of Nrf2. This result indicates that Nrf2 regulates the basal expression of CES1A1 in HeLa cells. Similarly, it was reported that the basal expression level of carboxylesterase in lung and small intestine is decreased in Nrf2 knockout mice [35,36]. In addition to Sp1 and C/EBP [19], Nrf2 might also regulate the basal expression level of CES1A1.

Generally, the genes induced by Nrf2 are involved in detoxification [18]. Because CES1A expression was induced by Nrf2, it was conceivable that CES1A is also involved in detoxification. It was reported that carboxylesterase in human lung microsomes, in which CES1A is highly expressed, is involved in the detoxification of vinyl carbamate [37]. To our knowledge, this is the only report suggesting the involvement of CES1A in the detoxification of reactive intermediates and carcinogens. It appears that CES1A is associated with lipid elimination in the liver [4–6]. Oxidative stress is one of the causal factors for hepatic steatosis, which leads to liver injury [38]. Although there were no reports about the relevance of CES1A to hepatic steatosis, CES1A may be involved in the defense of the cells against hepatic steatosis.

As shown in Fig. 4B, the transcriptional activities using the plasmid containing sequence −2365 to −1989 in the 5′-flanking region of the *CES1A1* gene were increased up to 2.0-fold by overexpression of Nrf2, and it was demonstrated that the ARE4 works as a functional Nrf2 response element in the *CES1A1* gene. Although we analyzed the transcriptional activity using plasmids containing the sequences −3207 to −1, −1531 to −1, and −833 to −1 bp in the 5′-flanking region of the *CES1A1* gene, the activity was not increased by the overexpression of Nrf2 (Fig. 4A). This result was similar to that in our previous study in which a significant increase of the transcriptional activity by Nrf2 overexpression was not observed using plasmids containing the sequences −2191 or −1408 to −7 in the 5′-flanking region of the *UGT2B7* gene [24]. It is assumed that the plasmid containing the long sequence results in a complicated conformation, which makes it difficult for Nrf2 to bind to the target ARE.

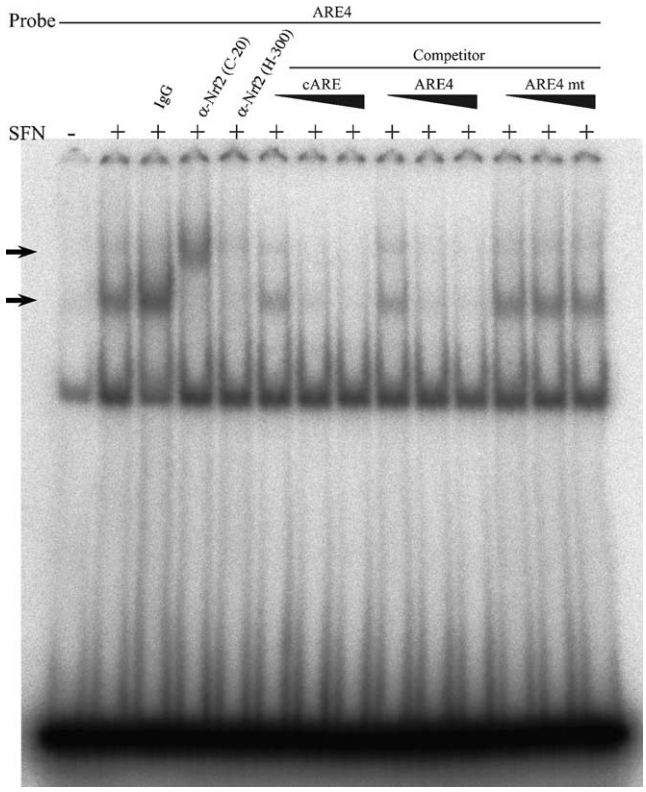


Fig. 5. Electrophoretic mobility shift assays of Nrf2 binding to ARE4 in the *CES1A1* gene. Oligonucleotide probes of CES1A1 ARE4 labeled with ^{32}P were incubated with nuclear extracts prepared from HepG2 cells treated with 10 μM SFN or DMSO vehicle (0.1%). The sequences of the oligonucleotides are shown in Table 1. Cold oligonucleotides were used as a competitor at 5-, 25-, and 50-fold molar excess. For supershift analyses, 2 μg of anti-Nrf2 antibodies (α-Nrf2) or normal rabbit IgG (IgG) were preincubated with the nuclear extracts on ice for 30 min. The *lower* arrow indicates the position of the Nrf2-dependent shifted band, and the *upper* one indicates the supershifted complex by anti-Nrf2 antibodies.

EMSA and ChIP assays revealed that Nrf2 binds to the 5′-flanking region in the *CES1A1* gene (Figs. 5 and 6). In EMSA, the supershifted band was observed only with the anti-Nrf2 antibody (C-20) but not with the anti-Nrf2 antibody (H-300). This result is consistent with our previous study [24]. In ChIP assays, the immunoprecipitants of SFN-treated HepG2 cells obtained with anti-Nrf2 antibody (C-20) generated PCR products for region 1 containing functional ARE, but those obtained with anti-Nrf2 antibody (H-300) or normal rabbit IgG did not. Anti-Nrf2 antibody (C-20) recognizes the C-terminal of Nrf2, whereas anti-Nrf2 antibody (H-300) recognizes the N-terminal. This difference in the recognition sites between anti-Nrf2 antibodies (C-20 and H-300) may affect the results in EMSA and the ChIP assays.

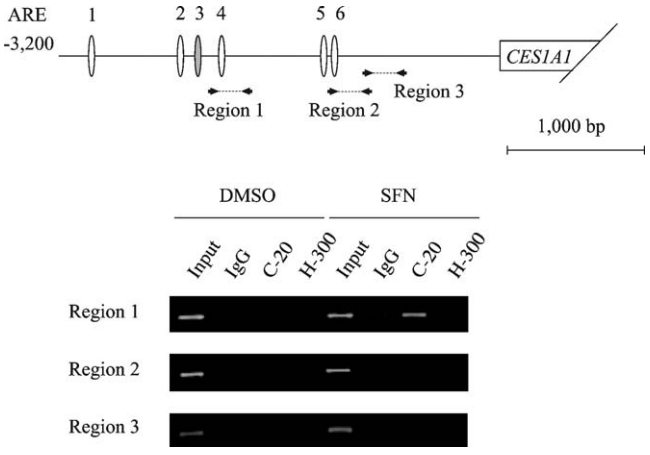


Fig. 6. ChIP assays of Nrf2 binding to the *CES1A1* gene in HepG2 cells. Schematic diagram of the *CES1A1* gene is shown at the top. ChIP assays were performed as described in Section 2. HepG2 cells were treated with 10 μM SFN or DMSO vehicle (0.1%) for 24 h. Rabbit anti-Nrf2 antibodies (C-20 and H-300) and normal rabbit IgG were used for immunoprecipitation of protein–DNA complexes. DNA fragments amplified by PCR were analyzed on 2% agarose gel.

Deng et al. [39] and Wu et al. [40] reported that the cholesterol-lowering drug probucol induces HO-1 via oxidative stress. Aburaya et al. [41] reported that Nrf2 is activated by non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, diclofenac, ibuprofen and aspirin. Moreover, isothiocyanates such as SFN and phenethyl isothiocyanate are found broadly distributed among cruciferous vegetables, e.g., cabbage and broccoli. It was reported that the intake of cruciferous vegetables for 2 weeks lowers serum bilirubin concentrations (from 15.73 to 14.02 μmol/L) by up-regulating UGT1A1 activity [42]. In human skin, NQO1 activity is induced up to 1.5-fold by sulforaphane-containing broccoli sprouts [43]. Because CES1A is involved in the metabolism of a number of clinically used drugs and prodrugs, the combined use of drugs or dietary foods that activate Nrf2 might affect the drug response.

In conclusion, we found that Nrf2 transcriptionally activates CES1A1 through binding to the ARE in the 5′-flanking region. This is the first study to demonstrate the molecular mechanism of the inducible regulation of human CES1A1.

Acknowledgement

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