Regulation of Cytochrome P450 2E1 under Hypertonic Environment through TonEBP in Human Hepatocytes

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ABSTRACT

Whereas the liver as well as the other organs are continually exposed to the change of osmotic status, it has never been investigated whether activities and gene expressions of drug-metabolizing enzymes, including cytochromes P450, are dependent on osmotic change in the liver. In the present study, we determined that CYP2E1 is induced under hypertonic environments at a transcriptional level in human primary hepatocytes, as assessed by cDNA microarray and real time–reverse transcription-polymerase chain reaction analyses. Both a protein level and the catalytic activity of CYP2E1 were consistently increased in response to hypertonic conditions. In promoter-reporter assay, it was demonstrated that −586 to −566 in the CYP2E1 5′-flanking region was necessary for 2E1 promoter activation by hypertonic stimulation. It is noteworthy that tonic-ity-response element (TonE) consensus sequence was found at −578 to −568 in human CYP2E1 5′-flanking region, and electrophoretic mobility shift assay demonstrated the interaction of TonE binding protein (TonEBP) with TonE motif of CYP2E1 promoter. Furthermore, cotransfection of a CYP2E1 promoter construct with wild-type TonEBP expression vector enhanced promoter activity under both isotonic and hypertonic conditions, whereas dominant-negative TonEBP suppressed an induction of CYP2E1 promoter activity. These results indicate that the level of CYP2E1 is induced by hypertonic condition via TonEBP transactivation. The present study suggests that osmotic status may influence individual responses to the substrate of CYP2E1.

The liver is the main organ that metabolizes and detoxifies xenobiotics, such as pharmaceutical drugs and chemical toxicants, and it abounds in drug-metabolizing enzymes, including cytochromes P450 (P450s) superfamily proteins (Gonzalez, 1988, 2005). Within the P450 superfamily, the CYP1, CYP2, and CYP3 families in particular play an important role in metabolism of xenobiotics. It is well known that the genes of these enzymes are regulated by xenobiotics, hormones, and pathological conditions (Gonzalez, 1988), leading to changes in the catalytic capacity of drug metabolism. Whereas the mechanisms of the chemically induced transcriptional activation of P450 genes have been revealed, that of CYP2E1 is complex and not well understood. In addition to the regulation by xenobiotics, the level of CYP2E1 is influenced by pathophysiological conditions; e.g., CYP2E1 expression is induced under obesity, diabetes, alcoholic, and nonalcoholic steatohepatitis (Caro and Cederbaum, 2004). Furthermore, the increased CYP2E1 level was also observed even under normal physiological conditions, such as in overfed rats (Raucy et al., 1991), in rats fed a high-fat diet (Yoo et al., 1991), and in fasted rats (Hong et al., 1987; Johansson et al., 1988). Although several studies demonstrated that hormones, such as insulin (De Waziers et al., 1995), leptin (Leclercq et al., 2000) and thyroid (Peng and Coon, 1998) and growth hormones (Chen et al., 1999), could mediate the regulation of CYP2E1 expression, the molecular mechanisms governing the CYP2E1 expression have remained to be elucidated.

On the other hand, cellular osmolality can be changed by

ABBREVIATIONS: P450, cytochrome P450; TonEBP, tonicity-response element binding protein; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; SSC, standard saline citrate; RT, reverse transcription; TonE, tonicity-response element; SV40, simian virus 40; CMV, cytomegalovirus; dnTonEBP, dominant-negative TonEBP; HEK, human embryonic kidney; UGT, UDP glucuronosyltransferase; NAT, N-acetyl-transferase; SULT, sulfotransferase; GST, glutathione transferase; NFκB, nuclear factor κB; NFAT, nuclear factor of activated T cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; TauT, taurine transporter.

various physiological or pathophysiological conditions, such as food or water intake, nutrition state, various hormones, and oxidative stress (Häussinger et al., 1993), suggesting the possibility that the activity of P450s may be influenced by osmotic conditions. Osmotic change in the tissues leads to disturbance of normal ion movement and cell swelling, resulting in cell injury and death (Dmitrieva et al., 2001). In addition, hypertonic stress activates some signaling pathways associated with the impairment of cell viability, such as c-Jun N-terminal kinase and CD95/epidermal growth factor receptor pathways (Reinehr et al., 2002, 2003). On the other hand, it activates the signaling molecules responsible for adapting against hypertonic environment, such as transcriptional factor TonEBP (tonicity-response element binding protein), also called NFAT5, which belongs to Rel/nuclear factor-κB/NFAT family (Woo et al., 2002a; Ho, 2006). TonEBP is activated under hypertonic environment and regulates osmoprotective genes, such as osmolyte transporters [e.g., taurine transporter (TauT) (Ito et al., 2004), sodium/myoinositol transporter, betaine/GABA transporter-1 (BGT-1) (Burg et al., 1997)] and molecular chaperones [e.g., 70-kDa heat shock protein (Hsp70) (Woo et al., 2002b) and osmotic stress protein (Osp94) (Kojima et al., 2004)], which confer the resistance to cells against hypertonic environments.

In the present study, we first screened the influence of hypertonicity on gene regulation of P450s and the other drug-metabolizing enzymes in human hepatocytes and found that the mRNA expression of CYP2E1 is induced under hypertonic environments. Furthermore, we demonstrated the molecular mechanism involved in the CYP2E1 up-regulation in response to hypertonic stimulation by analyzing promoter function. This article provides a new perspective: that osmotic environment controls the capacity of drug metabolism and chemical detoxification in the liver.

Materials and Methods

Cell Culture. Preserved primary human hepatocytes were purchased from XenoTech LLC (Lenexa, KS) and BD Biosciences (San Jose, CA). Cells were seeded at 1 to 2.5×10^5 cells/cm^2 on plates coated with collagen type 1 and cultured in Lanford medium (Charles River Japan, Yokohama, Japan) for 2 days. Then, cells were exposed to isotonic or hypertonic media. Hypertonic media were prepared by adding 20 to 50 mM sodium chloride (NaCl + 20, NaCl + 50) or 50 to 100 mM sucrose (Suc50, Suc100) into the medium, as described previously (Ito et al., 2004). To confirm that the experimental results did not depend on the reagents, we generated two kinds of hypertonic conditions by using both permeant (sodium chloride) and impermeant agents (sucrose).

Human hepatocarcinoma cell line HepG2 cells were cultured in minimum essential medium containing 10% fetal bovine serum and were then exposed to hypertonic stress for 24 h, as described above.

Human embryonic kidney cell line, HEK293 cells, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were transfected with the expression plasmids by using FuGene6 according to the manufacturer's protocol (Roche) and were then harvested for EMSA.

cDNA Microarray for Drug-Metabolic Enzymes. Total RNA was prepared from cells using QIAzol, according to the manufacture's instructions (QIAGEN, Hilden, Germany). Total RNA (10 μg) were reverse-transcribed in the presence of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), Cy3-dUTP (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), oligo(dT)$_{12-18}$primer (Invitrogen, Carlsbad, CA), and RNase Inhibi-

tor (Toyobo, Osaka, Japan) in buffer consisting of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol, and deoxynucleoside 5'-triphosphates. Reactions were each carried out at 42°C for 80 min, with an addition of SuperScript II Reverse Transcriptase 40 min after start. The resulting Cy3-labeled cDNA probes were purified with MinElute PCR Purification Kit (QIAGEN) according to the manufacturer's protocol.

Kurabo Multiple Assay DNA array for Human (MAPH-01, Kurabo) was used in gene expression screening. The microarray slides were first pretreated with blocking solution consisting of 4× standard saline citrate (SSC), 0.5% SDS, and 1% bovine serum albumin at 42°C for 45 min. The labeled cDNA in hybridization buffer [consisting of 2× SSC, 4× Denhardt's solution (Sigma-Aldrich, St. Louis, MO), and salmon sperm DNA (Invitrogen)] were denatured at 95°C for 2 min and cooled to room temperature. Then, cDNA were applied to each individual array window and hybridized at 65°C for 16 h. After hybridization, the solutions of labeled cDNA on the microarray slides were flushed away by a solution containing 2× SSC and 0.1% SDS, and then the microarray slide was immediately washed in following solutions; 2× SSC and 0.1% SDS at room temperature for 5 min, 0.2× SSC and 0.1% SDS at room temperature for 5 min, 0.2× SSC and 0.1% SDS at 55°C for 5 min, 0.2× SSC at room temperature for 1 min, and 0.05× SSC at room temperature for 2 min.

The images for the hybridized array was captured by GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA), and quantified by using the Genepix Pro 6.0 software (Molecular Devices). The adjusted intensity equals the intensity of each gene minus the background value. The genes with an adjusted intensity of less than 2-fold the background value were not detected. The normalized intensity to GAPDH gene were calculated by the following formula and compared with other treatment: normalized intensity = $(X - Z)/(Y - Z) \times 10^3$, where X is the adjusted intensity of target gene, Y is the adjusted intensity of GAPDH gene, and Z is the median of adjusted intensities of the negative controls.

Real-Time Quantitative Reverse Transcription-PCR. Total RNA (1 μg) was subjected to the reverse transcription with ReverTra Ace (Toyobo), using oligo(dT)$_{12-18}$ primer (Invitrogen) at 42°C for 60 min, followed by PCR. Quantitative RT-PCR analyses were performed by using an ABI7700 (Applied Biosystems, Foster City, CA) with SYBR green and *Taq*-Gold DNA polymerase (Applied Biosystems). The PCR primers used are shown in Table 1. PCR parameters were as follows: initial denaturation at 95°C for 5 min to activate Tag DNA polymerase followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. GAPDH was used as an internal control.

Western Blot Analyses. Western blot was performed as described previously (Ito et al., 2004). Anti-CYP2E1 (Calbiochem, San Diego, CA) and anti-GAPDH antibody (Chemicon, Temecula, CA) were used.

Measurement of CYP2E1 Activity. CYP2E1 activity was determined according to previous reports (Dicker et al., 1990; Rodríguez-Antona et al., 2002; Thasler et al., 2006). In brief, cultured primary hepatocytes were washed twice, incubated with 0.5 mM *p*-nitrophenol in Krebs-Henseleit buffer containing 200 mg/L glucose at 37°C for 60 min, and then the reaction was terminated by adding trichloroacetic acid to a final concentration of 5% (w/v). Cells were harvested and centrifuged at 10,000*g* for 10 min, and the supernatants were assayed for 4-nitrocatechol by adding 10 M NaOH (1:10) and immediately determining the absorbance at 546 nm.

Plasmids. A DNA fragment of CYP2E1 promoter region positioning from −1361 to +32 was amplified by PCR using human genomic DNA as a template and the PCR primers −1361F and +32R, which is conjugated with a HinDIII site (Table 2), and a XhoI/HinDIII fragment that contained CYP2E1 promoter region from positions −1342 to +32 was cloned into pGL3-basic (Promega, Madison, WI) (p2E1–1342). Then this fragment was used as the template for the preparation of different lengths of CYP2E1 promoter region. Primer sequences are shown in Table 2. Different lengths of CYP2E1 pro-

moter region from positions −586 to +32 and −566 to +32 were prepared by PCR and inserted into firefly luciferase plasmids pGL3-basic (p2E1–586 and p2E1–566). Reporter plasmid containing −230 to +32 (p2E1–230) was generated by self-ligation of the NheI-cut fragment of p2E1–586. Mutation of the tonicity-responsive element (TonE) site was generated in p2E1–586 by PCR using the primer −586mutF shown in Table 2. This PCR product was inserted into pGL3 to create p2E1–586mut. The plasmids were verified by sequencing. The reporter plasmid p4 × 2E1TonE-SV40-Luc was generated by insertion of four copies of the double-stranded TonE motif of CYP2E1 promoter region, 5′-CTAGCGGATCCCATGGAATTTTC-CAGTTCATGGAATTTTCCAGTTCATGGAATTTTCCAGTT-3′ into the multicloning site pGL3-promoter vector containing SV40 promoter (Promega). The expression vectors carrying TonEBP (pCMV-TonEBP) and dominant-negative TonEBP (pCMV-dnTonEBP) were generated previously (Ko et al., 2000; Ito et al., 2004).

Luciferase Assay. Transient transfection into HepG2 cells was performed by using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. Assay was performed using the Dual Luciferase assay system (Promega) as described previously (Ito et al., 2004). Control plasmid (pRL-TK; Promega) was cotransfected for used as an internal standard.

Immunofluorescence Microscopic Examination. Immunofluorescence microscopic examination was performed as described pre-viously (Ito et al., 2007). Immunostaining was performed using anti-TonEBP(1439-1455) antibody (1:100; Chemicon International, Temecula, CA) and Alexa Fluor 488 secondary antibody (Invitrogen). Cells were examined with the use of an inverted tissue culture microscope (IX70; Olympus, Tokyo, Japan).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared form HEK293 or HepG2 cells cells, and EMSA was performed as described previously (Ito et al., 2007). To prepare DNA probes for EMSA, single-strand oligonucleotides (2E1-TonE: sense, AACTG-GAAAATTCCATG; antisense, CATGGAATTTTCCAGTT) were end-labeled by [γ-^{32}P]ATP and then were annealed at room temperature. Nuclear extracts were incubated with ^{32}P-labeled DNA probe (10 nM) and poly(dI-dC) at 30°C for 20 min. To perform the competition assay, excess concentration (500 nM) of wild-type or mutant 2E1-TonE, wild TauT-TonE oligonucleotides (2E1-TonEmut: sense, AACTCGATCAT-TCCATG; antisense, CATGGAATGATCGAGTT; TauT-TonE: sense, AGCTGGTATTTTTCCACCCAG; antisense, CTGGGTGGAAAAATAC-CAGCT; underlining indicates mutated sites) (Ito et al., 2007) was preincubated for 5 min, followed by the incubation with radiolabeled probe. For supershift assay, 2 μg of antibodies [anti-TonEBP (NFAT5) antibody (Chemicon) or control IgG (Santa Cruz)] was added after 20 min from adding radiolabeled oligoprobe. The DNA-protein complex was fractionated by 4% polyacrylamide gel. The gels were dried and processed for autoradiography.

TABLE 1

PCR primers and conditions used in RT-PCR

Gene	Sequence	Annealing Temperature	Amplicon Size
		°C	*bp*
CYP1A1			
Forward	5′-GATCCTTGTGATCCCAGGCTC-3′	60	122
Reverse	5′-GAAACTCCGTGGCCGACAT-3′		
CYP1A2			
Forward	5′-CTTTGACAAGAACAGTGTCCG-3′	60	226
Reverse	5′-AGTGTCCAGCTCCTTCTGGAT-3′		
CYP2B6			
Forward	5′-GGCCATACGGGAGGCCCTTG-3′	60	236
Reverse	5′-AGGGCCCCCTTGGATTTCCG-3′		
CYP2C9			
Forward	5′-ACATTGACCTTCTCCCCACCAGCC-3′	62	357
Reverse	5′-CAAATCCATTGACAACTGGAGTGG-3′		
CYP2D6			
Forward	5′-GGCTGCTGGACCTAGCTCAG-3′	60	264
Reverse	5′-GGTTCTCATCATTGAAGCTG-3′		
CYP2E1			
Forward	5′-TCAATCTCTGGACCCCAACTGT-3′	60	115
Reverse	5′-AGTCACGGTGATACCGTCCATT-3′		
CYP3A4			
Forward	5′-GCAGGAGGAAAATTGATGCAGTT-3′	60	76
Reverse	5′-GTCAAGATACTCCATCTGTAGCACAGT-3′		
UGT2B4			
Forward	5′-GGCTGTACAAGTGGATACCCCAG-3′	60	147
Reverse	5′-CAGGTTGATCTGCAAACAATGGA-3′		
TauT			
Forward	5′-CGCTCTCTGCCTTCTTTTAGTCTG-3′	60	109
Reverse	5′-CATGGCGAATGGAAAAGTGG-3′		
GAPDH			
Forward	5′- CAATGGAAATCCCATCACCATC-3′	60	101
Reverse	5′-TGAAGACGCCAGTGGACTCC-3′		

TABLE 2

PCR primers used to generate promoter-reporter constructs

Restriction enzyme sites or mutated sites are indicated by underlines or lower-case letters, respectively

primer	Sequence
2E1–1361F	5′-CATTGTCAGTTCTCACCTC-3′
2E1–586F	5′-TTCTCGAGTTCTTCCATGGAATTTTCCAGT-3′
2E1–586mutF	5′-TTCTCGAGTTCTTCCATGGAATgctagcGTTAACTTGAG-3′
2E1–566	5′-TTCTCGAGGTTAACTTAACTTGAGAAAGTGGATCG-3′
2E1+32R	5′-TTAAGCTTTGGACACCAGCAGGAGGAAG-3′

Statistical Analysis. Each value was expressed as the mean ± S.E. Statistical significance was determined by Student's *t* test. Differences were considered statistically significant when the calculated *P* value was less than 0.05.

Results

The Effects of Hypertonic Environment on the Expressions of Drug Metabolism-Related Genes in Human Hepatocytes. To identify the drug metabolism-related genes regulated by hypertonic stimulation, preliminary DNA microarray analysis was carried out using RNA prepared from two lines of human primary hepatocytes cultured under isotonic or hypertonic media prepared by adding NaCl or sucrose. Based on the analysis, CYP2E1, CYP1A1, and UGT2B4 were up-regulated more than 2-fold by treatment with both NaCl and sucrose in both lines of hepatocytes, whereas CYP1A2 was down-regulated less than 0.5-fold (Table 3). These results indicate that these genes were regulated by hypertonic stimulation but not the unique influences of NaCl or sucrose. No other drug metabolism-related genes, including *P450s*, *UGTs*, *SULTs*, *NATs*, and *GSTs* filled the criteria (more than 2-fold or less than 0.5-fold by treatment with both NaCl and sucrose in both lines of cells).

Quantitative RT-PCR validated the change of each gene in primary hepatocytes exposed to hypertonic conditions (Fig. 1A). In addition, TauT mRNA, which has been reported to be induced by hypertonicity in many type of cells (Uchida et al., 1992; Satsu et al., 1999; Ito et al., 2004), was also up-regulated in primary hepatocytes exposed to hypertonic conditions, also supporting the idea that the up-regulations of CYP2E1, CYP1A1, and UGT2B4 depend on osmotic changes. Furthermore, to confirm the effect of hypertonic stimulation to the other P450s, the levels of CYP2B6, -2C9, -2D6, and -3A4 mRNA were quantified by quantitative RT-PCR. Whereas the expression level of CYP2D6 mRNA was also significantly reduced by treatment with both NaCl and sucrose, CYP2B6 was decreased by sucrose but not NaCl (Fig. 1B). In addition, the levels of CYP2C9 and -3A4 were not changed.

CYP2E1 Is Up-Regulated and Activated under Hypertonic Conditions in Human Primary Hepatocytes. To clarify the effect of hypertonic stimulation on CYP2E1 expression, temporal changes of CYP2E1 mRNA was measured (Fig. 2A). The level of CYP2E1 mRNA was up-regulated after 24 to 48 h, but not 3 or 8 h, of the exposure to hypertonic condition. Furthermore, we investigated the effect of hypertonic stresses on CYP2E1 protein level by Western blot analyses and catalytic activity of *p*-nitrophenol as CYP2E1 activity. Consistent with the mRNA level, CYP2E1 protein was also induced in cells exposed to hypertonic con-

ditions for 24 to 48 h (Fig. 2B). CYP2E1 activity was concomitantly increased by the exposure of hepatocytes to hypertonic conditions for 24 to 48 h (Fig. 2C).

Identification of Tonicity Response Element in Promoter Region Upstream of Human *CYP2E1* Gene. It is well known that the level of CYP2E1, as well as that of the other P450s in the HepG2 cell line, is very low (Thasler et al., 2006) but is detectable at mRNA level (Sumida et al., 2000). We also ascertained that the ratio of CYP2E1 mRNA to GAPDH mRNA in HepG2 cells was less than 1000 times compared with that in primary hepatocytes (Data not shown). However, the up-regulation of CYP2E1 mRNA in response to hypertonic environments was observed in HepG2 cells (Fig. 3A), indicating that HepG2 cells conserve the signaling pathways governing the CYP2E1 up-regulation in response to hypertonic stimulations. Thus, HepG2 cells are available to analyze the molecular mechanisms for hypertonicity-induced CYP2E1 up-regulation. In addition, consistent with the data from primary hepatocytes, the increases of CYP1A1 and UGT2B4 and the decrease of CYP1A2 mRNA were also observed in HepG2 cells (Data not shown).

To determine the promoter region regulating the CYP2E1 expression under hypertonic stimulation, promoter gene assay was performed by using promoter-reporter plasmids containing different lengths of promoter region (Fig. 3B). Hypertonic stimulations activated promoter activity in cells transfected with p2E1–1342 or p2E1–586 but not p2E1–566 or p2E1–230. Thus, promoter sequence from −586 to −566 was necessary for the promoter activation by hypertonic stimulations. It is noteworthy that we identified tonicity response element (TonE) motif (TGGAAANNC/TNC/T) in the CYP2E1 promoter region in human genome sequence (GenBank accession no. NT_017795) (Fig. 3C, underlined sequences). As expected, mutagenesis at this motif (p2E1–586mut) eliminated the promoter activation induced by hypertonic stimulations (Fig. 3C).

Furthermore, promoter activity driven by reporter plasmid containing four repeats of TonE motif (p4 × 2E1TonE-SV40) was activated by hypertonicity, whereas that driven by reporter plasmid containing no TonE motif was not (Fig. 4). These results indicate that this TonE-consensus motif is crucial for regulation of 2E1 promoter activity in response to hypertonicity.

Regulation of CYP2E1 Promoter Activity by TonEBP. It is well established that activated TonEBP is translocated into nucleus under hypertonic conditions in many types of mammalian cells (Ko et al., 2000; López-Rodríguez et al., 2001). Nuclear translocation of TonEBP was also ascertained in HepG2 cells cultured in hypertonic media for 24 h (Fig. 5A). We investigated whether TonE locating in

TABLE 3

Influence of hypertonic stimulation on gene expression of drug-metabolizing enzymes in human primary hepatocytes (cDNA microarray analysis)

Criteria: change >2- or <0.5-fold in response to both NaCl (+20 or +50) and sucrose (Suc50 or Suc100) exposure compared with isotonic medium in both lines of primary hepatocyte culture (line 1 and line 2).

| Gene	Line 1				Line 2			
	NaCl + 20	NaCl + 50	Suc50	Suc100	NaCl + 20	NaCl + 50	Suc50	Suc100
	-fold change							
CYP1A1	1.4	4.3	1.7	3.2	2.5	2.0	3.1	1.3
CYP1A2	0.3	2.0	0.1	0.9	1.4	0.2	0.7	0.5
CYP2E1	1.3	3.2	0.6	2.4	6.0	6.6	8.5	3.5
UGT2B4	1.7	2.5	3.3	2.3	1.7	3.0	2.6	2.7

2E1 promoter region interacts with TonEBP protein. In nuclear extract from HEK293 cells transfected with expression vector carrying TonEBP, protein-DNA complex was detected, as assessed by EMSA (Fig. 5B). This complex was competed by unlabeled probe or unlabeled TauT-TonE oligonucleotides that correspond to the TonEBP consensus sequence in the

TauT gene promoter but not by unlabeled oligonucleotides with the consensus sequence mutated. Furthermore, DNA-protein complex formation was shifted by anti-TonEBP antibody but not control IgG, indicating that this DNA-protein complex consists of TonEBP. DNA-TonEBP complex was also consistently detected in nuclear extracts obtained from HepG2 cells, and this complex was increased by hypertonic stimulation, as assessed by EMSA (Fig. 5C), indicating that hypertonic stimulations enhance TonEBP transactivation and binding to TonE motif in CYP2E1 promoter.

Next, we confirmed whether TonEBP regulated CYP2E1 promoter activity through TonE motif. Luciferase activity driven on TonE-containing promoter-reporter plasmids (p2E1–1342, p2E1–586), but not mutated plasmid (p2E1–586mut), was increased by cotransfection of TonEBP-expressing vector even under isotonic conditions (Fig. 6, A and B). TonEBP overexpression resulted in enhancement of hypertonicity-induced activation of CYP2E1 promoter. Furthermore, promoter activation driven on p2E1–1342 were suppressed by cotransfection of expression vector carrying dnTonEBP under both isotonic and hypertonic conditions (Fig. 6A). Thus, TonEBP is a crucial regulator of basal and hypertonicity-induced expression of CYP2E1.

Discussion

Activities and expressions of P450s are altered under a wide variety of conditions, such as nutrition intake, fasting, and pathophysiological conditions. In the present study, we investigated the hypothesis that drug-metabolizing enzymes were regulated under hypertonic environments and demonstrated the up-regulation of CYP2E1, CYP1A1, and UGT2B4 and the down-regulation of CYP1A2 and CYP2D6 in response to hypertonic conditions in human hepatocytes. Furthermore, we demonstrated that TonEBP is involved in hypertonicity-induced CYP2E1 up-regulation through the TonE motif of its promoter region. Our results are the first demon-
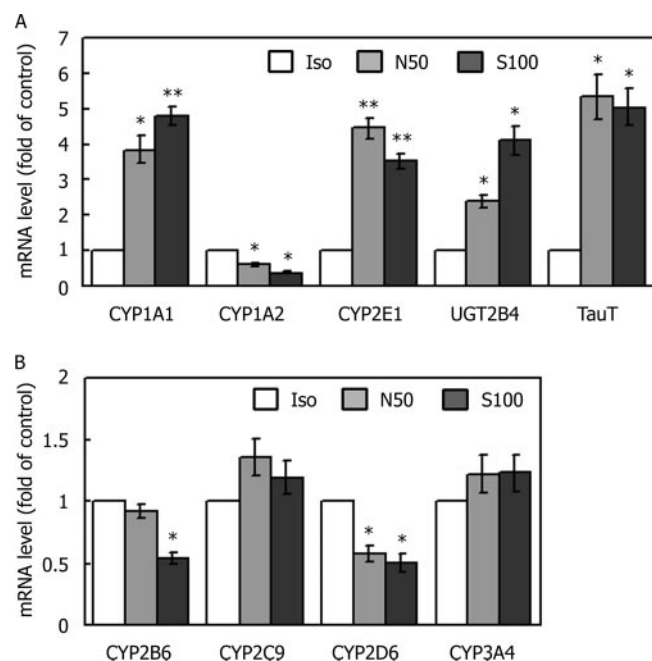
Fig. 1. Regulation of drug-metabolizing enzymes in human primary hepatocyte culture. Real-time RT-PCR was carried out by using primers specific for CYP2E1, CYP2A1, CYP1A2, UGT2B4, and TauT (A), CYP2B6, CYP2C9, CYP2D6, and CYP3A4 (B), as shown in Table 1, with total RNA prepared from human primary hepatocytes cultured in isotonic (Iso) or hypertonic media [NaCl + 50 mM (N50) or 100 mM sucrose (S100)] for 24 h. Data are shown in bar graphs and represent the mean ± S.E., $n = 5$. Data were obtained from five independent lines of primary hepatocytes. *, $p < 0.05$; **, $p < 0.01$ versus isotonic condition.
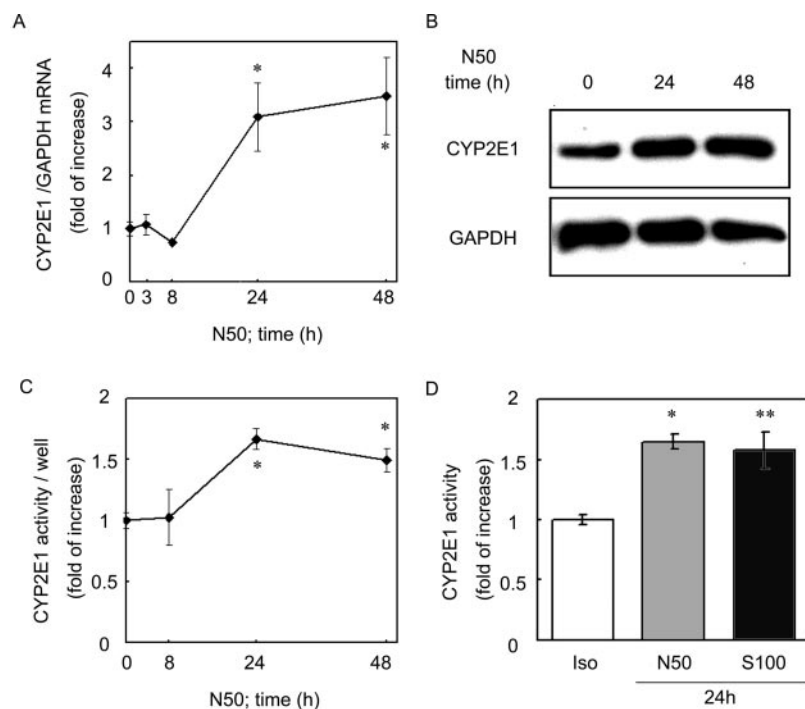

Fig. 2. Induction of CYP2E1 at mRNA and protein level under hypertonic conditions in human primary hepatocyte culture. Real-time RT-PCR (A) and Western blotting (B) were carried out to determine the level of CYP2E1 mRNA and protein of human primary hepatocytes cultured in hypertonic medium [NaCl + 50 mM (N50)] for the indicated time. C and D, CYP2E1 activity of human primary hepatocytes cultured in isotonic or hypertonic media [N50 or 100 mM sucrose (S100)] for the indicated times were measured by using *p*-nitrophenol as CYP2E1 substrate. Data represent the mean ± S.E., $n = 3$. *, $p < 0.05$; **, $p < 0.01$ versus isotonic condition. Similar results were obtained from at least two independent cell preparations.

stration that physiological osmotic state controls the activity and expression of drug-metabolizing enzymes.

Previous reports show that there are some putative transcriptional factor-binding motifs, such as signal transducer and activator of transcription, activator protein-1, NFAT, NFκB, CCAAT/enhancer-binding protein, at −671 to −544 of CYP2E1 5′-flanking region (Abdel-Razzak et al., 2004), whereas TonE motif is found at −578 to −568. Our results presented here revealed that this region is necessary for hypertonicity-induced activation of CYP2E1 promoter. Because putative binding sequences of other NFAT family proteins and NFκB are overlapped with TonE at −578 to −568, not only TonEBP but also other NFAT family proteins and NFκB were predicted to be involved in the regulation of CYP2E1 promoter in response to hypertonicity. However, other NFATs and NFκB have been demonstrated to be not activated by hypertonic stimulation, whereas TonEBP is activated (López-Rodríguez et al., 2001). Although TonEBP (NFAT5) is a member of NFAT family of proteins, only TonEBP is distinct from other NFATs (NFAT1–4), because it has no calcineurin-regulated domains and is not regulated by Ca^{2+}/calcineurin pathway, suggesting its particular function (Ho, 2003). Thus, it makes no sense that either NFAT1–4 or NFκB is involved in the up-regulation of CYP2E1 in response

to hypertonic condition. We consistently demonstrated that dnTonEBP overexpression suppressed hypertonicity-induced activation of CYP2E1 promoter. It has been reported that this deletion mutant does not influence the transcriptional activity of the other NFAT, NFκB despite interfering TonEBP dimerization (López-Rodríguez et al., 1999; Trama et al., 2002), indicating that the regulation of CYP2E1 promoter under hypertonic environment is associated with TonEBP transactivation.

In the present study, whereas the reporter activity driven by p4 × 2E1TonE-SV40, which contains four repeats of TonE motif, increases only 2- to 2.5-fold in response to hypertonicity; the 2E1–586 construct, which contained only one of the TonEBP sites, showed increased activity of more than 4-fold. These results suggest that other promoter regions of CYP2E1, in addition to the TonE motif, may be necessary for full activation of CYP2E1 transcription in response to hypertonic stress. Although molecular mechanisms of TonEBP transactivation remain to be elucidated, recent studies demonstrated that TonEBP interacts with some proteins, including the 90-kDa heat shock protein and poly(ADP-ribose) polymerase-1, and these proteins modulate TonEBP activity (Chen et al., 2007), indicating that interaction with some proteins regulates TonEBP function. Thus, some proteins
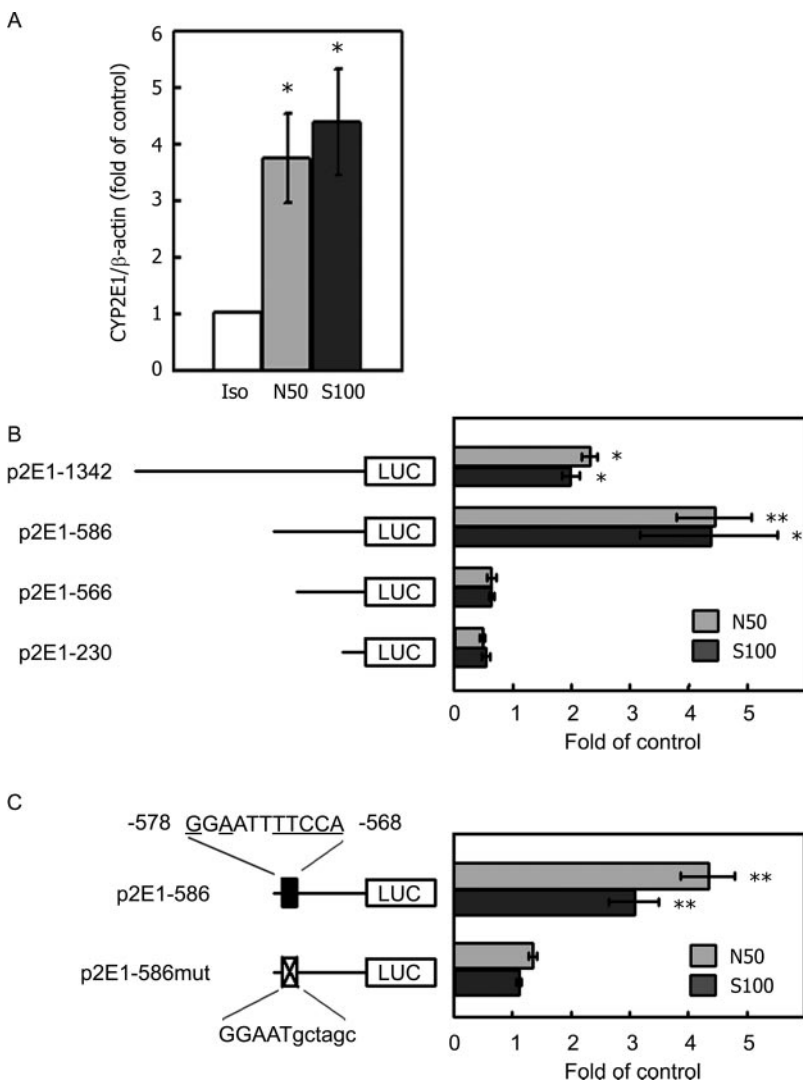

Fig. 3. 5′-Flanking region necessary for transcriptional activation of *CYP2E1* gene by hypertonic stimulation. A, quantitative RT-PCR was carried out with total RNA prepared from HepG2 cells cultured in isotonic (I) or hypertonic media [NaCl + 50 mM (N50) and 100 mM sucrose (S100)] for 24 h. Data obtained from three independent cell preparations were shown in bar graphs and represent the mean ± S.E., $n = 3$. B and C, luciferase assay driven on CYP2E1 promoter-reporter constructs [p2E1–1342, p2E1–586, p2E1–566 or p2E1–230 (B) or p2E1–586 or p-586mut (C)]. HepG2 cells were cotransfected with each promoter-reporter construct and pRL-TK and then cultured in isotonic or hypertonic media for 24 h. Then, luciferase activities were measured. Underlines indicate consensus sequence of TonE. Data are shown as -fold of corresponding control cells cultured in isotonic medium and represent the mean ± S.E., $n = 4$. ∗, $p < 0.05$; ∗∗, $p < 0.01$ versus isotonic condition. Each experiment was repeated at least three times with independent cell preparations.

may interact with TonEBP, which bind to DNA in the 5′-flanking region of CYP2E1 and cooperate TonEBP transactivation.

CYP2E1 catalyzes the metabolism of a wide range of exogenous and endogenous low-molecular-weight toxicants, such
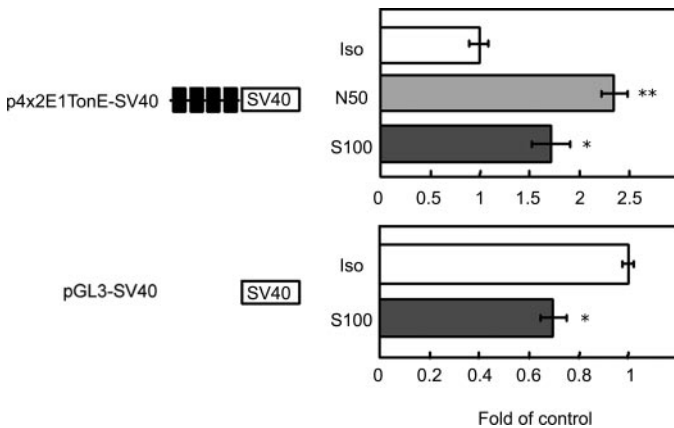

Fig. 4. Identification of tonicity response element (TonE) in CYP2E1 promoter. Promoter constructs, p4 × 2E1TonE-SV40 or empty pGL3-SV40, were cotransfected with pRL-TK into HepG2 cells and cultured in isotonic (Iso) or hypertonic media [NaCl + 50 mM (N50) and 100 mM sucrose (S100)] for 24 h. Then, luciferase activities were measured. Data are shown as -fold of corresponding control cells cultured in isotonic medium and represent the mean ± S.E., $n = 3$. *, $p < 0.05$; **, $p < 0.01$ versus isotonic condition. Each experiment was repeated at least three times with independent cell preparations.
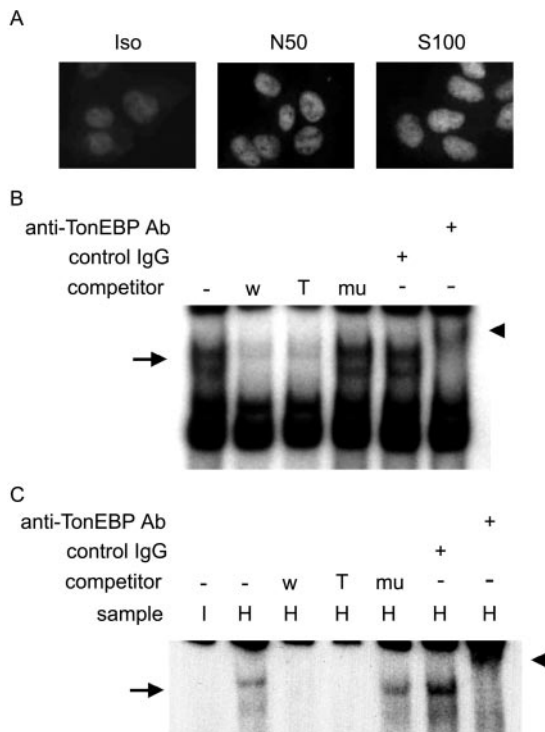
Fig. 5. TonEBP binds with TonE motif in CYP2E1 promoter. A, HepG2 cells were exposed to hypertonic conditions for 24 h and were then assessed by immunocytochemistry using anti-TonEBP antibody. B and C, EMSA was performed with nuclear extract from HEK293 cells transfected with pFLAG-TonEBP (B) or HepG2 cells cultured in isotonic (I) or hypertonic (Suc100) (H) media (C) and ^{32}P-labeled 2E1-TonE oligonucleotide. w, wild-type oligonucleotide; T, TonE motif encoded in 5′-flanking region of TauT gene (Ito et al., 2004); mu, mutant oligonucleotide. Arrows and arrowheads indicate TonEBP/DNA complex and supershifted bands, respectively.

as alcohol, acetaminophen, and lipids (Caro and Cederbaum, 2004). CYP2E1, as well as the other P450 family proteins, is critical for body's defense against xenobiotics exposure. This study demonstrated CYP2E1 is involved in adaptive response against hypertonic environment via TonEBP activation. Although an essential role of TonEBP is not well understood, a number of evidences support that TonEBP plays cytoprotective roles against hypertonic stimulation in mammalian tissues. For example, inhibition of TonEBP by dominant-negative TonEBP resulted in the impairment of cell viability and the increase in the susceptibility against hypertonic stress (Trama et al., 2002; Wang et al., 2005; Ito et al., 2007). The present study provides a new insight that TonEBP regulates the metabolism of pharmaceutical drugs and exogenous toxicants in the liver. On the other hand, previous reports illustrated that the oxidative stress caused by CYP2E1 is likely to be involved in hepatic pathogenesis, such as alcohol- or acetaminophen-induced hepatic toxicity (Lee et al., 1996; Zaher et al., 1998; Cederbaum et al., 2001; Caro and Cederbaum, 2004; Gonzalez, 2005), implying that TonEBP might contribute to hepatic pathogenesis through CYP2E1 up-regulation.

Our investigation revealed novel evidence that CYP1A1 and UGT2B4 are also up-regulated by hypertonic stimulations in human hepatocytes. The motif-search analyses failed
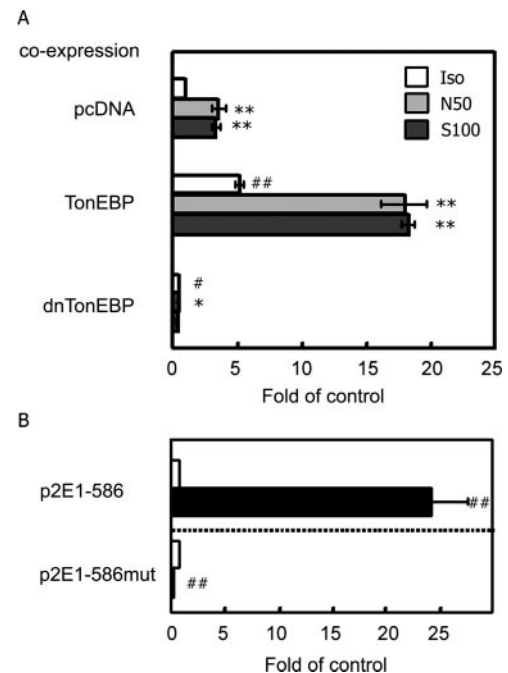
Fig. 6. Involvement of TonEBP on hypertonicity-induced activation of CYP2E1 promoter. Effect of expression vector carrying wild-type or dominant-negative TonEBP on promoter activity driven on CYP2E1 promoter-reporter constructs. A, HepG2 cells were cotransfected with p2E1–1342 and pRL-TK, and each expression vector [pcDNA (100 ng/well), pCMV-TonEBP (1 ng/well), and pCMV-dnTonEBP (100 ng/well)] and cultured in isotonic (white bar) or hypertonic media (NaCl + 50 mM, light gray bar; 100 mM sucrose, dark gray bar) for 24 h. B, HepG2 cells were transfected with reporter plasmids (p2E1–586 or p2E1–586mut) and expression vectors [pcDNA (100 ng/well), white bar; pCMV-TonEBP (100 ng/well), black bar] and cultured in isotonic medium for 24 h. Then, luciferase activities were measured. Data are shown as -fold of corresponding control cells cultured in isotonic medium and represent the mean ± S.E., $n = 4$. *, $p < 0.05$; **, $p < 0.01$ versus isotonic condition; ##, $p < 0.01$ versus pcDNA. Each experiment was repeated three times with independent cell preparations.

to detect any TonE consensus sequences within 5000 base pairs of 5′-flanking region of each gene in human genome sequence [CYP1A1 (GenBank accession no NT_010194) and UGT2B4 (GenBank accession no. NT_077444)]. Because the genome structures of drug-metabolizing enzymes, including P450 and UGT superfamilies, are commonly complex, the regulation of these genes may be involved with *cis*-elements far from each transcript start site or controlled by other TonEBP-independent pathways.

In addition, we identified the reduction of CYP1A2 and CYP2D6 mRNAs in response to hypertonic environments. Although the regulatory mechanisms of the transcription of these enzymes are well investigated, we did not find any putative transcript factors involved in the down-regulation of CYP1A2 and CYP2D6 by hypertonic stress. On the other hand, hypertonic stress has been reported to enhance mRNA decay (Teixeira et al., 2005). Thus, it is also possible that the mRNA decay pathway may target these genes, leading to lower stability of these mRNAs. Furthermore, CYP2B6 mRNA was down-regulated by the exposure to sucrose, but not NaCl, indicating that the induction of CYP2B6 is independent of the effect of hypertonic stress. Because these P450s are responsible for the metabolism of a large number of drugs and changes and may have clinical consequences, further studies will be required to clarify the molecular mechanism and the clinical significance of these findings.

In the present study, we performed cDNA microarray for only 2 lines (lots) of primary hepatocytes. Based on the results obtained from DNA microarray, the levels of no other genes were altered by hypertonicity among the P450, UGT, NAT, and SULT families. It is well known that the expression levels of these genes are widely different among individuals, so the data of each gene widely differ among different lines of hepatocytes. Furthermore, we could not determine the change of the genes expressed at low level because of the scattered data and/or the limitation of detection. Taken together, it can be barely concluded that we detected all of genes susceptible for hypertonic environments by using microarray. Indeed, further experiments by quantitative RT-PCR analysis have identified reduction in CYP2B6 and CYP2D6 mRNAs despite their being undetectable by DNA microarray technique.

In summary, we demonstrated that the level of CYP2E1 was up-regulated under hypertonic conditions via TonEBP transactivation. Because tissue and plasma osmolality is altered even by common events in human life, such as nutrients, hormones, and dehydration (Häussinger et al., 1993), the metabolizing capacity of CYP2E1 is likely to be changed in daily life. In addition, pathophysiological changes of osmolality may affect to the activity and expression of CYP2E1 in the liver; e.g., the up-regulation of CYP2E1 in diabetes could be associated with an increase in osmolality caused by acidosis (Owens et al., 1998). This unique response of CYP2E1 to hypertonicity may contribute to the wide variety of individual responses to drug therapy, and further studies will be required to determine the clinical importance of TonEBP/CYP2E1 pathway in drug metabolism.

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