Discovery of Osmosensitive Transcriptional Regulation of Human Cytochrome P450 3As by the Tonicity-Responsive Enhancer Binding Protein (Nuclear Factor of Activated T Cells 5)

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ABSTRACT

We report the discovery of an osmosensitive transcriptional control of human CYP3A4, CYP3A7, and CYP3A5. Ambient hypertonicity (350–450 mOsmol/kg) increased mRNA expressions of the CYP3A by ~10- to 20-fold in human-intestinal C2bbe1 cells, followed by an increase of CYP3A protein. Hypotonicity, on the other hand, suppressed CYP3A mRNA levels, indicating that physiological isotonic conditions may regulate the basal expression of CYP3A. Similar responses to ambient tonicity were observed in other human-derived cell lines (intestinal LS180 and hepatic HepG2) and human primary colonic cells. The 11-base pair tonicity-responsive enhancer (TonE) is an osmosensitive regulator that is activated by the transcription factor, the nuclear factor of activated T-cells 5 (NFAT5). Luciferase-based reporter assays of 13 consensus TonE motifs within ±10 kilobases (kb) from the transcription start sites of CYP3A showed that only the CYP3A7 intron 2 region (~5 kb downstream from the transcription start site), which contains two TonE motifs (+5076/+5086 and +5417/+5427), was responsive to hypertonicity stimuli. This observation was confirmed upon cotransfection with an NFAT5 expression vector, small interfering RNA, or dominant-negative NFAT5. Deletion and mutation analyses suggested that the TonE (+5417/+5427) is indispensable for the enhancer activity. NFAT5 binding to the CYP3A7 intron 2 TonE motif was demonstrated with electrophoretic mobility shift assay and in a native cell context by chromatin immunoprecipitation. We conclude that transcription of human CYP3A is influenced by ambient tonicity. The physiological significance of the tonic regulation of CYP3A enzymes remains to be determined.

The human cytochrome P450 3A (CYP3A) subfamily represents the most abundant cytochrome P450 drug-metabolizing enzymes in the liver and intestine. Together with membrane-bound transporters, they constitute a crucial component for drug elimination and excretion. Of the three major isoforms (CYP3A4, CYP3A5, and CYP3A7), CYP3A4 is the most abundant adult form, whereas CYP3A7 is the main fetal form. These isozymes collectively metabolize nearly half of all currently used medications.

The human CYP3A genes reside in a cluster on chromosome 7 (Nelson et al., 2004), and their expression is characterized by wide interindividual variations. Significant coex-
pression of the major CYP3A isoforms suggests the presence of common regulatory pathways. CYP3A induction by drugs and chemicals is known to be mediated by pregnane X receptor (PXR) (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998), constitutive androstane receptor (CAR) (Xie et al., 2000), the vitamin D receptor (VDR) (Schmiedlin-Ren et al., 1997; Thummel et al., 2001), and hepatocyte nuclear factor-4-α (Tirona et al., 2003). In contrast, the regulation of CYP3A basal expression in the absence of an inducing agent is poorly defined, although these nuclear factors and other regulatory proteins, such as CCAAT/enhancer-binding protein and hepatic nuclear factor-3-γ (Rodriguez-Antona et al., 2003), may also play a role. As we were examining the dietary effects on human CYP3A expression, we made an unexpected observation that osmotic environments seem to influence the expression of CYP3A.

In mammalian cells, ambient tonicity affects the function of a transcription factor called tonicity-responsive enhancer binding protein, which is also known as the nuclear factor of activated T-cells 5 (NFAT5) or the osmotic response element-binding protein. TonE binding protein/NFAT5/osmotic response element-binding protein (NFAT5 hereafter in this article) is a newly discovered fifth member of the NFAT family of transcription factors (López-Rodriguez et al., 1999; Miyakawa et al., 1999; Ko et al., 2000; Stroud et al., 2002), which forms an obligatory homodimer and transactivates its target genes via the tonicity-responsive enhancer (TonE). NFAT5 is the only known mammalian transcription factor that responds to changes in osmolality, in which an increase in ambient tonicity provokes NFAT5 translocation from the cytoplasm to the nuclear compartment (Woo et al., 2000). NFAT5 controls the expression of osmotic stress-response genes such as the sodium/myoinositol cotransporter (SMIT: SLC5A3) (Yamauchi et al., 1993), and aldose reductase (Ko et al., 1997). These proteins synthesize organic osmolytes or transport them into the cell, thereby counterbalancing the ambient hypertonic stimuli (Ho, 2006). Constitutive nuclear localization of NFAT5 has been shown, suggesting a regulatory role under isotonic conditions (Miyakawa et al., 1999; Woo et al., 2000).

NFAT5-mediated gene regulation has been examined extensively in kidney, which faces intense osmotic stresses. Its role in other tissues, except for immune cells, is virtually unknown. Gastrointestinal epithelia are exposed to elevated postprandial osmolality (Ladas et al., 1983; Houp, 1991; Kalantzzi et al., 2006). Moreover, the osmotic microenvironment in the liver is also not static but rather is active and dynamic (Go et al., 2004). Thus the NFAT5 regulation may play a role in other tissues, except for immune cells, in which a properly functioning NFAT5 is crucial to the cellular function and survival. In mammalian cells, ambient tonicity affects the function of a transcription factor called tonicity-responsive enhancer binding protein, which is also known as the nuclear factor of activated T-cells 5 (NFAT5) or the osmotic response element-binding protein. TonE binding protein/NFAT5/osmotic response element-binding protein (NFAT5 hereafter in this article) is a newly discovered fifth member of the NFAT family of transcription factors (López-Rodriguez et al., 1999; Miyakawa et al., 1999; Ko et al., 2000; Stroud et al., 2002), which forms an obligatory homodimer and transactivates its target genes via the tonicity-responsive enhancer (TonE). NFAT5 is the only known mammalian transcription factor that responds to changes in osmolality, in which an increase in ambient tonicity provokes NFAT5 translocation from the cytoplasm to the nuclear compartment (Woo et al., 2000). NFAT5 controls the expression of osmotic stress-response genes such as the sodium/myoinositol cotransporter (SMIT: SLC5A3) (Yamauchi et al., 1993), and aldose reductase (Ko et al., 1997). These proteins synthesize organic osmolytes or transport them into the cell, thereby counterbalancing the ambient hypertonic stimuli (Ho, 2006). Constitutive nuclear localization of NFAT5 has been shown, suggesting a regulatory role under isotonic conditions (Miyakawa et al., 1999; Woo et al., 2000).

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tane-Relat et al., 1995). The NFAT5 antibody (Affinity BioReagents, Golden, CO) was used at a 1000-fold dilution. The CYP3A4 antibody (Research Diagnostics, Flanders, NJ), which also recognizes CYP3A5 and CYP3A7, was used at a 500-fold dilution. β-Actin (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was used to ensure equal loading. For immunohistochemical analysis, after 4 h exposure to hypertonicity (NaCl-induced 400 mOsmol/kg), C
_bbe1 cells on glass coverslips were fixed in 4% paraformaldehyde with 0.2% X-100 in PBS and incubated with the above NFAT5 antibody at a 500-fold dilution in 5% donkey serum/PBS for 1 h at room temperature. Secondary antibody, Cy3-conjugated anti-rabbit IgG, was then used for 1 h at room temperature for visualization with fluorescent imaging microscopy.

Expression Plasmids, Small Interfering RNA, and Reporter Constructs. NFAT5 expression plasmid was made from KIAA0827 clone (a gift from Dr. Nagase, Kazusa Institute, Tokyo, Japan) by digestion with NotI and XhoI and ligated into pTARGET (Invitrogen). Human PXR expression plasmid (PEF-hPXR; Tiriona et al., 2003) was kindly provided by Dr. Kim (University of Western Ontario, London, ON, Canada). Dominant-negative NFAT5 [1-156], which lacks the first 156 amino acids, was derived by in-frame insertion of KIAA0827 cDNA corresponding to amino acid residues 157 to 581 into NotI and BamHI restriction sites of pFLAG-CMV-2 mammalian expression vector (Sigma, St. Louis, MO) at a concentration of 1 pmol of biotinylated probe, with or without the stated amount of competitor or mutant probes, or NFAT5 antibody, at a final volume of 50,000 cells/well. After 48 h, cells were transfected with 0.3 to 0.5 μg of the firefly luciferase reporter plasmids and 0.08 to 0.2 μg of pRL-TK plasmids (Promega, Madison, WI) containing a Renilla reniformis luciferase gene by Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen). In some experiments, cells were cotransfected similarly with NFAT5 expression vector, hPXR expression vector (PEF-hPXR; Tiriona et al., 2003), siRNA against NFAT5 (569R and inv569R; Na et al., 2003), a combination of four gene-specific siRNAs (SMARTpool NFAT5; Dharmacon), the dominant-negative NFAT5, or empty expression plasmids. At 24 to 48 h after transfection, cells were incubated in various experimental conditions for another 16 to 24 h unless otherwise stated. SMARTpool NFAT5 siRNA experiments were conducted as follows. Over-night seeded HepG2 cells at 50% confluence were transfected for 48 h with 32.5 nM siRNA against NFAT5 (siNFAT5) or equal molar mismatched siRNA controls. These siRNAs were earlier suspended in liposome carrier Dharmafect 1,2,3,4 transfection reagent (Dharmacon) at 0.1 μl/nM siRNA concentration in serum-free Opti-MEM (Invitrogen). Cells were then treated with different tonicity for 16 h. Luciferase activities of the cell extracts were determined using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated as observed relative light units from firefly luciferase normalized to R. reniformis luciferase values and expressed as ratios to its minimal promoter construct under isotonic conditions, unless otherwise stated. In some experiments, the ratios were further normalized to those of the respective reporter in a control isotonic condition. All experiments were done in triplicate and repeated at least three times.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from confluent C
_bbe1 cells treated for 4 h at NaCl-induced hyperosmolarity (400 mOsmol/kg) using Nuclear Extraction Kit (Panomics) according to the manufacturer’s instructions. Electrophoretic mobility shift assay (EMSA) was performed using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) with modifications. A 0.5-ml sample of the 27-5 DNA probe (+5490/+5435 from CYP3A7 transcriptional start site) was used to detect protein/DNA interaction with 100-, 200-, or 400-fold increase of competitor probes (ubiquitinylated) or mutant (taAAGAGA-aG, where capitalized letters represent base changes, and the dash represents a 1-bp deletion from the original “tggaaaggt”) NFAT5 antibody used for supershift was from Affinity BioReagents at a concentration of 2.5 μl per reaction. The binding reaction consists of 10 μg of nuclear extract, 1× binding buffer, 2 μg of poly (dIdC), 3 μg of random primers (Invitrogen), 5 mM MgCl₂, 0.05% Nonidet P-40, and 1 pmol of ubiquitinylated probe, with or without the stated amount of competitor or mutant probes, or NFAT5 antibody, at a final volume of 10 μl. Incubation was carried out for 40 min at room temperature for all reactions. The 6% polyacrylamide gel electrophoresis was allowed to run for 2 h before transfer to the nylon membrane followed by UV cross-linking (Ultraviolet Crosslinker; UVP, Upland, CA).
Detection by chemiluminescence was carried out according to manufacturer's instructions.

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation (ChIP) assay was done using the ChIP kit (Upstate Biotechnology, Lake Placid, NY). In brief, C2bbe1 cells were incubated under NaCl-induced hypertonic condition (400 mOsmol/kg) for 16 h, and proteins are cross-linked to DNA by 1% formaldehyde for 10 min at 37°C. The cells were then lysed in SDS and sonicated using a probe sonicator to obtain sheared DNA fragments ranging from 200 to 1000 bp. A 200-μl aliquot was taken for subsequent reverse-linking with 8 μl of 5 M NaCl, and the DNA phenol/chloroform was extracted and ethanol-precipitated. A 1% of fraction was used as the input control. Another aliquot of 200 μl was then diluted with the ChIP dilution buffer and incubated with salmon sperm DNA/protein A agarose beads for 1 h at 4°C to remove nonspecific DNA that initially binds to the beads. The supernatant was incubated with 2 μl (1:500) of rabbit polyclonal IgG NFAT5 antibody, CYP1A1 antibody (both from Santa Cruz Biotechnology) as irrelevant target antibody, or no antibody at 4°C overnight, and then fresh beads (60 μl) were added with agitation at 4°C for 1 h. The beads were washed twice according to the buffer systems supplied in the kit, and the protein/DNA complex is eluted with 250 μl of elution buffer (1% SDS and 0.1 M NaHCO3), and only 200 μl of supernatant is collected after 30 min of shaking in room temperature. This step was repeated twice to obtain 400 μl of eluted samples. The samples were reverse-cross-linked with 20 μl of 5 M NaCl for 4 h at 65°C and treated with proteinase K. DNA of the samples was then phenol/chloroform-extracted and ethanol-precipitated using standard techniques. DNA was resuspended in 100 μl of diethyl pyrocarbonate water for subsequent PCR reaction. All PCR reactions were performed in a 50-μl reaction mix using Mastercycler (Eppendorf, Boulder, CO) with 1% template (1 μl). All PCR conditions were as follows: 95°C for 2 min; 95°C for 45 s, then 60°C for 1 min, and 72°C for 30 s, for 40 cycles; 72°C for 2 min, except for the NFAT5-coding region (annealing temperature at 55°C, 1 min). The CYP3A7 intron 2 TonE regions were assessed as fragments A and B. Fragment A contains an antisense TonE motif +4688/+4698; and fragment B with a sense TonE +5417/+5427. The primers for fragment A (288 bp) are as follows: forward, 5'-gct att tgc acc tgt tga aa; and reverse, 5'-tgc atg tga tgc ctt tgt. Those for fragment B (271 bp) were as follows: forward, 5'-aac ggt ctc ctc ctt tgt ggt agc aa; and reverse, 5'-atg acttgt ctc ccttgt ctgt. For positive control, we detected a 194-bp PCR product of the SMIT promoter, which contains an active TonE sequence at ASPET Journals on December 23, 2017 molpharm.aspetjournals.org Downloaded from
CYP3A Expression Parallels Ambient Tonicity in Human-Derived Cells. NFAT5-mediated gene control is bidirectional (Woo et al., 2000). In other words, levels of target genes increase as ambient tonicity increases, whereas they decrease as ambient tonicity decreases. Changes of CYP3A expression in C_bbe1 cells followed this pattern (Fig. 2, left), paralleling ambient tonicity changes from hypo- (200 mOsmol/kg) to hypertonicity (450 mOsmol/kg). So were other human cells (primary human colon epithelia, a colon carcinoma-derived cell line (LS180), and a hepatoma-derived cell line (HepG2)) (Fig. 2), suggesting that this is probably a universal phenomenon among human cells. Unlike human CYP3A, however, mouse Cyp3a13 mRNA levels changed inversely to ambient tonicity, whereas Smit, an established NFAT5 target gene, showed similar responses in human (Fig. 2) and mouse cells (intestinal CMT93 and hepatic Hepa1c1c7 cells; data not shown). It is unclear whether the tonicity effect on mouse Cyp3a13 represents a system-wide phenomenon in mice or a response specific to Cyp3a13, because other mouse Cyp3a mRNAs were undetectable or were not examined in these cell lines.

Tonicity-Responsive Enhancer (TonE) Is Located in the CYP3A7 Intron 2 Region. We sought the CYP3A gene locus for existence of TonE, which is characterized by an 11-bp consensus sequence (tggaaNANNYNN; N, any nucleo-

### Fig. 1. Ambient hypertonicity induces CYP3A expression in C_bbe1 human intestinal cells. a, time course of gene expression induced by ambient hypertonicity. C_bbe1 cells were cultured under hypertonic conditions (400 mOsmol/kg), and mRNA levels of CYP3A4 (●), CYP3A7 (○), CYP3A5 (□), SMIT (■), and NFAT5 (▲) were measured with real-time PCR. Results are normalized to respective GAPDH levels and expressed as ratios to the value at time 0 of each gene (mean ± S.E.M., n = 3). b, time-dependent expression of CYP3A protein in hypertonic (400 mOsmol/kg) or isotonic (300 mOsmol/kg) conditions. Cell lysates were obtained from C_bbe1 cells incubated for the indicated periods, and immunoblotting was performed. c, dose-response of mRNA levels to increasing ambient osmolality. C_bbe1 cells were treated for 24 h in media of increasing osmolality using NaCl (□), sucrose (■), or glycerol (▲), and mRNA levels were measured with real-time PCR. GDPDH-standardized results are expressed as ratios to those of respective isotonic condition (mean ± S.E.M., n = 3). d, solute-dependent increase of NFAT5 and CYP3A protein expressions in hyperosmotic conditions. Western blotting was performed on cell lysates obtained from C_bbe1 cells after 24-h incubation under the above-mentioned isotonic or hyperosmolality conditions (400 mOsmol/kg) created with different solutes: NaCl, sucrose, or glycerol. e, immunohistochemical detection of NFAT5 intracellular distribution. f, mRNA expression of transcription factors and intestinal xenobiotics transporters upon hyperosmolarity changes. C_bbe1 cells were fixed, incubated with the NFAT5 antibody, and visualized with Cy3-conjugated anti-rabbit IgG. Arrows show NFAT5 staining, which is confined almost exclusively in the nuclear compartment under NaCl or sucrose treatment but is scattered in cytoplasm in isotonic or glycerol treatment. f, mRNA expression of transcription factors and intestinal xenobiotics transporters upon hyperosmolarity changes. C_bbe1 cells were fixed, incubated with the NFAT5 antibody, and visualized with Cy3-conjugated anti-rabbit IgG. Arrows show NFAT5 staining, which is confined almost exclusively in the nuclear compartment under NaCl or sucrose treatment but is scattered in cytoplasm in isotonic or glycerol treatment.
Analyses of the genome database revealed that there are 85 consensus TonE motifs in the CYP3A gene cluster. In our experiments (Figs. 1 and 2), tonicity-dependence was observed in CYP3A4, CYP3A7, and CYP3A5; therefore, we postulated that a functional TonE sequence is located in a relative vicinity of the transcription start site of each CYP3A gene cassette. To identify a responsible TonE(s) in the 230-kb-wide CYP3A gene locus, our first approach was to screen 10 kb of the transcription start site of CYP3A4, CYP3A7, and CYP3A5. As shown in Fig. 3a, 11 sense and 2 antisense TonE consensus sequences were located in these regions (CYP3A4: 3 sense and 1 antisense; CYP3A5: 4 sense; and CYP3A7: 4 sense and 1 antisense). Of those, 6 putative TonE consensus motifs were localized in the −10 kb of the 5′-flanking region of each gene; one motif for CYP3A4 (−7913/−7903 from the CYP3A4 transcription start site); two for CYP3A7 (−7900/−7890 and −551/−541); and three for CYP3A5 (−6341/−6331, −3051/−3041, and −1924/−1914). Some of their localizations were close to xenobiotic-responsive enhancer module and everted repeat 6, which contain known enhancers transactivated by PXR and CAR. However, luciferase reporter assays in C2bbe1 cells showed that constructs containing these motifs (Fig. 3a; CYP3A4, −7979/−7140, −7979/−7831, −7850/−7140, and −638/+53; CYP3A7, −7994/−7155, −7994/−7831, −630/+55, and −7856/−7155; and CYP3A5, −6543/−5985, −3245/−2940, and −2235/−1862), driven by SV40 promoter or each CYP3A promoter, were unresponsive to hypertonicity (data not shown). To further explore these negative findings, we examined tonicity-responsiveness of the two reporter constructs, CYP3A4[−10466/+53] and CYP3A7[−9302/+53] (Bertilsson et al., 2001), which span approximately 9 kb of the 5′-flanking regions of CYP3A4 or CYP3A7, respectively. As shown in Fig. 3b, these reporters were unresponsive to hypertonic stimuli.
in hepatic HepG2 cells. In contrast, rifampicin-induced PXR-dependent responses were clearly observed as expected, indicating that the PXR-mediated pathway is intact in these constructs. These reporters transfected in C2bbe1 cells were not responsive to either rifampicin or hypertonicity treatment (data not shown). Taken together, these findings suggest that a tonicity responsive element does not exist at least in the −10-kb 5′-flanking regions of the CYP3A genes.

Fig. 3. TonE exists in CYP3A7 intron 2. a, scheme of the CYP3A gene cluster and corresponding reporter constructs, which shows the ±10 kb of the CYP3A transcriptional start sites. Eleven sense and two antisense consensus TonE motifs in the regions are depicted as an open column on either the top (sense) or bottom (antisense) side of the genome (horizontal line) with the starting 5′ (sense TonE) and 3′ base pair positions (antisense TonE) relative to the transcription initiation site of each CYP3A gene. The reporter constructs (thick horizontal lines with or without TonE as a white dot) are shown with the 5′-/3′-ends of the sequence. The constructs with an asterisk (*) were responsive to hypertonicity and NFAT5. b, activity of the reporter constructs encompassing 9 to 10 kb of the 5′-flanking regions of CYP3A4 or CYP3A7. Luciferase reporter assays were conducted in hepatic HepG2 cells with cotransfection of human PXR or empty expression vector after 24-h incubation in the presence of 10 μM rifampicin (○) or 50 mM NaCl (□), hypertonic 400 mOsmol/kg). The normalized reporter responses are shown here as a ratio to the respective reporter activity value in control isotonic conditions (■). Results are expressed as mean ± S.E.M. (*, p < 0.05; **, p < 0.01; n = 6–9). c, reporter assays for constructs harboring regions within the transcription units of CYP3A4 and CYP3A7. Activity of the reporter constructs was measured in C2bbe1 cells after 24 h of control isotonic (□) or hypertonic conditions (■). Results are expressed as ratios to activity of the respective minimal promoters (mean ± S.E.M.: n = 3). d, activity of the reporter construct containing the CYP3A7 intron 2 region (+4910/+5590) with different CYP3A promoters in hypertonic or NFAT5 overexpression conditions. C2bbe1 cells were cotransfected with respective reporter constructs and either NFAT5 expression plasmids (□) or empty vector (○). After 24-h incubation under isotonic (□) or □ or hypertonic (■) condition, luciferase activity was measured. In this experiment, results are expressed as values relative to those of the promoterless construct with empty expression plasmid under isotonic condition (mean ± S.E.M., n = 3). e, loss-of-function assays on the CYP3A7 intron 2 region (+4910/+5590) reporter using dominant-negative (dnNFAT5, top) or siRNA (bottom) against NFAT5. C2bbe1 cells were cotransfected with the reporter plasmid and either dnNFAT5 or siRNA or an inverted 569R sequence (siRNA inv569R, ■) was used. Cells were treated with isotonic or hypertonic medium for 24 h. Results are normalized against the values of the CYP3A7 minimal promoter reporter under isotonic conditions (mean ± S.E.M.; *, p < 0.05; **, p < 0.01, compared with respective controls; n = 4).
We then examined the remaining 7 TonE consensus motifs within 10 kb downstream from the transcription start site of each of those CYP3A genes (Fig. 3a): two sense TonE sequences in the CYP3A4 exon 3/intron 3 (+6144/+6154 and +6169/+6179 from the CYP3A4 transcription start site); one antisense sequence in the CYP3A4 intron 2 (+5636/+5646); two sense sequences in the CYP3A7 intron 2 (+5076/+5086 and +5417/+5427 from the CYP3A7 transcription start site); one antisense sequence in the CYP3A7 intron 2 (+4688/+4788); and one sense sequence in CYP3A5 exon 3 (+5437/+5447 from the CYP3A5 transcription start site). Screening with SV40 promoter-driven luciferase reporters in C2bBe1 cells (Fig. 3a; CYP3A4, +5971/+6352; CYP3A7, +4910/+5590, and CYP3A5, +5313/+5689) showed that only CYP3A7 (+4910/+5590) with the CYP3A7 intron 2 sense TonE sequences resulted in robust responses under isotonic conditions and more so after hypertonicity exposures (data not shown). We then further examined comparable regions of CYP3A4 and CYP3A7 approximately 5 to 6 kb downstream of the transcription start sites (Fig. 3a). The CYP3A4 (+5971/+6352) reporter driven by the CYP3A4 promoter, which contains two TonEs at +6144 and +6169, was not responsive (Fig. 3c). Moreover, the CYP3A4 (+5043/+6429) construct containing the three TonEs (+5636, +6144, and +6169) did not show activity (Fig. 3c; CYP3A4, +5403/+6429). In contrast, the CYP3A7 (+5417/+5910) with the two sense intronic TonEs (CYP3A7, +5076 and +5417; see Fig. 3a), driven by the CYP3A7 promoter, was active under isotonic condition and responded to hypertonicity. However, inclusion of the antisense TonE (CYP3A7, +4688) did not modify tonicity-responsiveness (Fig. 3c; CYP3A7, +4656/+5597), and the reporter containing the antisense TonE (CYP3A7, +4688) alone was inactive (Fig. 3c; CYP3A7, +4656/+4909). These findings indicate that the CYP3A7 intron 2 region containing the two sense TonE motifs (+5076/+5086 and +5417/+5427) has a tonicity-responsive enhancer activity.

Transactivation of CYP3A7 Intron 2 TonE Is Dependent on NFAT5. The CYP3A7 intron 2 region (+4910/+5590) was placed in each of the CYP3A promoters in luciferase constructs and tested for its transactivation activity with and without NFAT5 expression vector cotransfection (Fig. 3d). Results showed that this region is capable of activating all CYP3A promoter constructs in response to hypertonicity or NFAT5 overexpression. Loss-of-function assays were also conducted using the CYP3A7 (+4910/5590) luciferase reporter cotransfected with dominant-negative NFAT5 (dnNFAT531–156; Tong et al., 2006) or empty pFLAG-CMV-2 expression vector (pFLAG). As shown in Fig. 3e, dnNFAT531–156 decreased reporter activity in both isotonic and hypertonic conditions (p < 0.01; n = 4). Likewise, reporter activity was examined with siRNA569si against NFAT5 (Na et al., 2003). siRNA569si significantly reduced reporter activity (p < 0.05, n = 4; Fig. 3e). In these experiments, siRNA569ca caused approximately 50% reduction of NFAT5 mRNA (data not shown). Together, these findings suggest that CYP3A7 intron 2 region may work in conjunction with other CYP3A promoters and that NFAT5 is required for enhancer activation.

The TonE Motif at +5417 in the CYP3A7 Intron 2 Region Is Required for Transactivation. Serial deletions of the CYP3A7 intron 2 fragment were conducted to determine the minimal sequence responsible for enhancer activity (Fig. 4a). Compared with the CYP3A7 (+4910/+5590) reporter plasmid with two TonE sense motifs, serial upstream deletions showed a gradual reduction in reporter signals. Constructs without the upstream TonE motif (constructs +5088/+5590 and +5361/+5590) still retained the reporter activity, suggesting that the upstream TonE motif and neighboring regions are dispensable but required for a full response. We found that the reporter +5361/+5590 was the shortest construct that responds to hypertonicity or NFAT5. Further deletion (construct +5428/+5590), which is devoid of both TonE motifs, abolished the response. This observation supports that the downstream motif (+5417/+5427) is required in the NFAT5-mediated transactivation. This notion is further supported by the +4910/+5204 construct, in which the upstream motif without the downstream segment was unresponsive. This indicates that the upstream TonE motif and immediate neighboring regions are insufficient for enhancer activity. Furthermore, mutations in the +5417/+5427 TonE motif (tggaagaattAaA; the 2 "A"s in the mutant represent adenine replacing thymine and cytosine, respectively) drastically reduced tonicity responsiveness (Fig. 4b), reaffirming the requirement of the downstream motif for robust enhancer activity. General enhancers have the ability to transactivate promoter constructs independent of their location. To test whether the CYP3A7 intron 2 region has such an enhancer characteristics, it was placed downstream of the reporter gene (+4910/+5590 3′-position). Note that the CYP3A7 (+4910/+5490) 3′ position reporter construct may mimic actual CYP3A7 promoter—intron 2 DNA configuration to some extent. Results show that this reporter plasmid still retained tonicity/NFAT5 responsiveness, although the magnitude of the response was smaller (Fig. 4b).

NFAT5 Specifically Binds to the CYP3A7 Intron 2 TonE at +5417. EMSAs showed in Fig. 5 that competition by unlabeled TonE DNA sequence (lanes 2 and 3), lack of effects of mutant TonE (lanes 4 and 5), and shift of NFAT5-TonE complex by NFAT5 antibody (lane 6) indicate specific binding of NFAT5 to the DNA sequence containing the +5417 TonE motif. The mutant TonE sequence shown to have low but detectable activity in the earlier reporter assay (Fig. 4b) was weakly competitive in inhibiting NFAT5-TonE binding in EMSA (data not shown). To further explore NFAT5 binding to the CYP3A7 intron 2 TonE site at +5417 in a native chromatin context, ChIP assay was performed (Fig. 6). As shown in Fig. 6a, Fragment B represents a DNA region surrounding the active TonE (+5417), whereas fragment A contains a region with an antisense TonE further upstream (+4688) that is inactive in luciferase reporters (Fig. 3C; CYP3A7, +4656/+4909). In our ChIP assay, fragment B containing the TonE at +5417 is clearly detectable and so is the TonE motif located upstream of the SMIT promoter (Rim et al., 1998), one of the NFAT5 target genes (Fig. 6b, lane 1). Neither the TonE-absent exon 14 region of NFAT5 gene (Fig. 6b, lane 4) nor antibody against CYP1A1 (Fig. 6b, lane 2 and 5) produced an amplicon, validating the specificity of NFAT5-TonE binding in our assay. Moreover, fragment A containing the neighboring inactive TonE motif is undetectable (Fig. 6c). Taken together, this is consistent with the notion that NFAT5 binds to the CYP3A7 intron 2 region at +5417 in a native DNA setting.
Discussion

We have discovered that the expressions of human CYP3A4, CYP3A5, and CYP3A7 are under the influence of ambient tonicity. Moreover, the phenomenon is not restricted to immortalized cell lines, because it is also seen in primary colonic cells (Fig. 2), suggesting that this unexpected link between ambient osmotic environment and human CYP3A expression may represent a process of physiological significance. Although highly speculative, CYP3A may mediate osmolyte production or catabolism of osmolytes to counterbalance increased intracellular concentrations of organic osmolytes upon ambient hypertonicity challenges. Further studies are required to elucidate biological and in vivo significance of this phenomenon. This is important particularly because basal CYP3A expressions in these cells in vitro, including primary cells, are lower than those in vivo.

Our data suggest that the increased CYP3A expression is the result of transcriptional activation mediated by NFAT5. Several transcriptional factors, such as PXR and CAR, have been well established in the CYP3A regulation network. It remains to be defined whether the tonicity-mediated CYP3A expression is modified by other transcription factors. Specifically, it awaits further studies to elucidate a hierarchy of...
these factors in regulation of CYP3A, which determines system characteristics such as additivity, synergism, or antagonism among them. As shown in Fig. 1e, ambient hypertonicity may cause mild alterations in mRNA expression for some of these transcription factors, which may contribute to toxicity-triggered CYP3A induction. However, relatively rapid induction of CYP3A mRNA within 4 h by ambient hypertonicity (Fig. 1a) suggests a direct toxicity-mediated transcriptional induction rather through a secondary mechanism, if any.

Compared with human CYP3A, responses of mouse Cyp3a13 were exactly opposite in the two cell lines tested (CMT93 rectal cells and hepe1c1c7 liver cells), although mouse Smit, a known NFAT5 target gene, responded in the same manner as human SMIT. Tonicity responses of other mouse Cyp3a remain elusive, because only Cyp3a13 was detectable in this study. No orthologous CYP3A pair exists between mouse and human, suggesting a species-specific independent expansion of the ancestral CYP3A gene cassette over the last 75 million years (Nelson et al., 2004). Rodent and human CYP3A share many regulatory factors such as the PXR and CAR, but distinct species differences still exist. Whether mouse Cyp3a in vivo similarly responds to toxicity remains to be examined.

Mammalian cellular responses to osmotic stresses are mediated by the osmoregulatory transcription factor called tonicity enhancer binding protein (NFAT5) (reviewed by Ho, 2006). Upon activation by increased osmotic pressure, NFAT5 is translocated to the nucleus and binds to the TonE of target genes. Our NFAT5 siRNA experiments (Fig. 1h) suggest that NFAT5 plays a central role in the tonicity-CYP3A pathway. There are 13 consensus TonE motifs within the ≥ 10 kb from the transcription start sites of CYP3A (Fig. 3a). The present study shows that among these DNA elements, only the 0.7-kb region within CYP3A7 intron 2 has robust enhancer activity, responsive to hypertonicity and NFAT5 overexpression or knockdown (Fig. 3, b–e). Deletion

Fig. 5. NFAT5 binds to the CYP3A7 intron 2 TonE motif (+5417/+5427) in in vitro binding assay. Nuclear extracts from C_bbe1 cells treated with hypertonic medium for 4 h were incubated with biotinylated DNA probes derived from the CYP3A7 intron 2 TonE motif (+5417) containing the surrounding region (+5409/+5435). EMSA was conducted with increasing concentrations of unlabeled competitor DNA or mutant DNA probes (see Materials and Methods). TonE probe signals in the absence of competing or mutant DNA sequences (lane 1) and those in the presence of increasing concentrations of the competing unlabelled TonE region (lanes 2 and 3) or mutant DNA sequence (lanes 4 and 5) are shown (Δ). A supershift band driven by NFAT5 antibody is demonstrated (a, lane 6). A representative figure is shown.
and mutation analyses showed that the CYP3A7 intron 2 TonE at +5417 is indispensable for the minimal enhancer activity, although a full response requires the neighboring region (Fig. 4, a and b). Using EMSA and ChIP assays, we further showed NFAT5-binding to this TonE motif (Fig. 5 and 6). Altogether, our findings indicate specific binding of NFAT5 to the region surrounding CYP3A7 intron 2 TonE at +5417 in a native cell context.

The CYP3A7 intron 2 region containing the responsive TonE site (+5417) placed immediate 3′-side of the luciferase reporter gene (CYP3A7+/−4910/+5590) 3′position, to mimic the natural genomic configuration is responsive to hypertonicity and NFAT5 overexpression (Fig. 4b). An enhancer located in the intron 3 of the human tumor necrosis factor-α gene, which interacts with the human tumor necrosis factor-α promoter, has been characterized in a similar experimental approach (Barthel and Goldfeld, 2003). The magnitude of response of the CYP3A7+/−4910/+5590 3′position, however, was lower than when placed at 5′ of the CYP3A7 promoter (Fig. 4b). It is presently unknown whether this quantitative difference is of any biological significance in native DNA context or is simply an experimental limitation in an artificial environment of gene reporter assays.

Our studies show that expressions of CYP3A4, CYP3A5, and CYP3A7 are all dependent on ambient tonicity and that the CYP3A7 intron 2 region houses the only TonE consensus motif within the ±10 kb from the transcription start sites of CYP3A. Based on these findings, we speculate that the CYP3A7 intronic TonE segment serves as an enhancer for CYP3A4 and CYP3A5 and possibly as a long-range enhancer for CYP3A4 and CYP3A5, as seen in some genes including β-globin. Alternatively, there may be unidentified TonEs, or similar enhancers, separately for each CYP3A gene. Involvement of other transcriptional factors in this phenomenon is largely speculative at this point. We think that at least PXR is not involved because overexpression of PXR does not increase toxicity responses of CYP3A mRNA (data not shown) and because xenobiotic-responsive enhancer module-containing 5′-flanking regions of CYP3A4 and CYP3A7 do not respond to toxicity (Fig. 3b). If PXR, CAR, or VDR is involved, lack of toxicity responses in genes such as MDR1 is also difficult to explain. Further studies are required to address these questions.

The implications of our findings are severalfold. First, because NFAT5 shows basal activity under physiological osmolarity (Miya kawa et al., 1999; Woo et al., 2000), normal osmotic environment may be one of the baseline stimuli for CYP3A expression. This implies that the tightly regulated constitutive osmotic environment provides consistency and stability to the basal transcriptional drive, thereby sustaining CYP3A basal expression. Second, intestinal CYP3A expression may be affected through this pathway by osmolality/tonicity changes within the intestinal lumen as a result of food or fluid intake. Intestinal lumen faces periodic surges of osmolality related to food intake (Ladas et al., 1983; Houpt, 1991; Kalantzi et al., 2006). Therefore, CYP3A mRNA expression in the intestine may increase after food intake, which then ejectives CYP3A protein levels. This may explain clinical observations in which human subjects receiving high-salt diets for 7 to 10 days showed significant increase in presystemic elimination and metabolite formation of orally administered CYP3A substrates quinidine and verapamil (Darbar et al., 1997, 1998). Third, the mechanism of urine volume control by NFAT5, which is exemplified in dehydration (Lam et al., 2004), may be supported by an increased CYP3A5 level because of its role in renal Na+ and water retention (Kuehl et al., 2001; Givens et al., 2003; Thompson et al., 2004). Given the potential role of kidney-predominant CYP3A5 in salt retention and hypertension by converting corticoids to β-hydroxysteroids with mineralocorticoid action, this pathway is likely to be part of a regulation loop for salt homeostasis. The renal NFAT5-CYP3A5 pathway may be crucial to the hypertension pathogenesis theory associated with genetic variation in renal CYP3A5 expression (Kuehl et al., 2001).

In summary, we discovered the ambient toxicity-driven, NFAT5-mediated expression of human CYP3A4, CYP3A5, and CYP3A7. Whether the CYP3A7 intron 2 TonEs are responsible for propagation of the toxicity-NFAT5 signal toward all three isoforms of CYP3A awaits further studies.

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References


