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Up-Regulation of Human CYP2J2 in HepG2 Cells by Butylated Hydroxyanisole Is Mediated by c-Jun and Nrf2

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ABSTRACT

Cytochrome P450 2J2 oxidizes arachidonic acid to a series of epoxyeicosatrienoic acid (EET) isomers in human tissues. EETs regulate numerous homeostatic processes, including cytoprotective and proliferative responses against injurious stresses. There is little information currently available on the factors that regulate CYP2J2, but strategies to activate expression could use the beneficial effects of EETs in cells. The basic leucine zipper (bZIP) transcription factor c-Jun has been shown previously to maintain CYP2J2 expression in human HepG2 cells; c-Jun forms transcriptionally active dimers with the antioxidant-inducible bZIP factor Nrf2. In the present study, we tested the hypothesis that CYP2J2 expression may be activated in cells by c-Jun/Nrf2 heterodimers. Treatment of HepG2 cells with butylated hydroxyanisole elicited concentration- and time-dependent activation of CYP2J2 expression, as well as the bZIP factors Nrf2 and c-Jun; chromatin immunoprecipitation assays revealed a pronounced increase in binding of these bZIP factors to the CYP2J2 5′-flank. Transient transfection analysis using deletion constructs and gel-shift assays were consistent with a role for the −105/−88 region of CYP2J2 in c-Jun/Nrf2 responsiveness. Using a series of mutant expression plasmids, we identified c-Jun as the critical partner in CYP2J2 transactivation. Coimmunoprecipitation experiments confirmed the importance of the leucine zipper region of Nrf2 in the enhancement of c-Jun-dependent transactivation of CYP2J2. Agents that activate CYP2J2 expression may offer a new approach to using the beneficial effects of EETs in cells.

The arachidonic acid epoxygenase cytochrome P450 (P450) 2J2 is widely expressed in many human tissues, including liver, heart, lung, and intestine, and catalyzes the formation of all four regioisomeric epoxyeicosatrienoic acids (EETs) (Wu et al., 1996; Scarborough et al., 1999) that modulate a range of cellular processes. EETs not only regulate ion channel activity in cardiomyocytes (Lee et al., 1999) and inhibit inflammation (Node et al., 1999) but also inhibit apoptosis (Chen et al., 2001), enhance the recovery of endothelial cells from hypoxia-reoxygenation injury (Yang et al., 2001), and stimulate cell growth and migration (Chen et al., 1998; Potente et al., 2002; Sun et al., 2002). Thus, factors that have the potential to alter EET production may also modulate cell viability and influence the development of disease after injury (Chen et al., 2001; Yang et al., 2001; Kroetz and Zeldin, 2002). Unlike other P450 epoxygenases, CYP2J2 mediates the formation of 8,9-EET, which has been extracted from human liver (Zeldin et al., 1996) and heart (Wu et al., 1996) and is an important regulator of cell proliferation (Pozzi et al., 2005). Thus, CYP2J2 seems to be functionally important in the range of tissues in which it is expressed.

A more detailed understanding of the regulation of the CYP2J2 EET synthase may lead to the development of new pharmacological strategies to alter the pathogenesis of disease (Yang et al., 2001; Kroetz and Zeldin, 2002). The interplay between members of the activator protein-1 (AP-1) complex of leucine zipper (bZIP) transcription factors has been found to regulate basal *CYP2J2* expression in human liver-derived cells (Marden et al., 2003; Marden and Murray, 2005). Thus, c-Jun homodimers supported CYP2J2 expression in HepG2 cells cultured in a normoxic environment and activated *CYP2J2*-luciferase reporter constructs via AP-1-like gene elements in the *CYP2J2* upstream region. Under hypoxic conditions (~1% O_2), another bZIP factor, c-Fos, was activated, resulting in impaired regulation of *CYP2J2*; in accord with this finding, c-Jun/c-Fos heterodimers abrogated the activity of CYP2J2-driven luciferase reporters (Marden et al., 2003). Thus, the intracellular composition of bZIP

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ABBREVIATIONS: EET, epoxyeicosatrienoic acid; AP-1, activator protein-1; BHA, butylated hydroxyanisole; bZIP, basic leucine zipper; ChIP, chromatin immunoprecipitation; CoIP, coimmunoprecipitation; P450, cytochrome P450; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; NP, nuclear protein; Nrf2, NF-E2-related factor-2; PCR, polymerase chain reaction; DN, dominant negative; nt, nucleotide.

transcription factors has emerged as an important determinant of constitutive CYP2J2 expression in cells.

Strategies to activate CYP2J2 expression could enable the therapeutic exploitation of some of the beneficial actions of EETs in cells. Although the induction of many P450 genes by drugs and other xenobiotics is mediated by activated nuclear and aryl hydrocarbon receptors (Waxman, 1999), characteristic response elements for these transcription factors have not been detected in the *CYP2J2* upstream sequence (Marden et al., 2003). Consistent with this observation, *CYP2J2* seems unresponsive to typical xenobiotic inducers (Wu et al., 1996; Scarborough et al., 1999). Instead, because c-Jun is important in the maintenance of constitutive CYP2J2 expression and forms dimers with the antioxidant-inducible bZIP factor NF-E2-related factor-2 (Nrf2) (Yu et al., 2000; Yuan et al., 2006), we tested whether this system may regulate CYP2J2 expression. The principal finding to emerge from the present study was that treatment of HepG2 cells with butylated hydroxyanisole (BHA), an antioxidant chemical that activates Nrf2 (Yu et al., 1997; Dinkova-Kostova et al., 2002), increased CYP2J2 expression in HepG2 cells. In accord with these findings, *CYP2J2*-luciferase reporter constructs were also responsive to BHA, which was dependent on the binding of c-Jun and Nrf2 to an atypical AP-1-like element in the *CYP2J2* upstream region near −105/−88 relative to the translational start site. Using mutagenized expression constructs, a critical role for the transactivation domain of c-Jun in CYP2J2 activation was found, whereas enhanced transactivation by the Nrf2/c-Jun combination was dependent on dimerization between leucine residues in the bZIP region, but not on the DNA-binding or transactivation domains of Nrf2.

Materials and Methods

Chemicals and Reagents. The wild-type c-Jun and Nrf2 expression plasmids were generously provided by Dr. Kazuhiko Imakawa (Faculty of Agriculture, University of Tokyo, Tokyo, Japan) and Dr. Volker Blank (Faculty of Medicine, McGill University, Montreal, QC, Canada), respectively. TRI RNA extraction reagent was from Molecular Research Centre (Astral Scientific, Caringbah, NSW, Australia), restriction enzymes were from New England Biolabs (Arundel, QLD, Australia) unless otherwise specified, and Effectene transfection reagent was from QIAGEN (Doncaster, VIC, Australia). Oligonucleotides were synthesized by Geneworks (Adelaide, SA, Australia).

Antibodies directed against c-Jun, Nrf2, and ubiquitin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas anti-phosphoserine^{63}-c-Jun and anti-phosphoserine^{40}-Nrf2 were from Cell Signaling Technology (Danvers, MA) and Epitomics Inc. (Burlingame, CA), respectively. Rabbit anti-(rat CYP2J4) IgG, which is cross-reactive with human CYP2J2, was generously provided by Dr. Qing-Yu Zhang (New York State Department of Health, Albany, NY) (Xie et al., 2000). α-Tubulin antibody, protease inhibitors, 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), and other chemicals were from Sigma-Aldrich (Castle Hill, NSW, Australia). Gas chromatography-mass spectrometry indicated that the purity of the BHA preparation was at least 99%; potential contaminants such as *tert*-butylhydroquinone were not detected. SDS and other reagents for electrophoresis were from Bio-Rad (Richmond, CA). High-performance liquid chromatography-grade solvents were from Rhone-Poulenc (Baulkham Hills, NSW, Australia), and analytical reagents were from Ajax (Sydney, NSW, Australia). Hyperfilm-MP, Hybond-N^+ filters, and reagents for enhanced chemiluminescence were from GE Healthcare (Rydalmere, NSW, Australia).

Cell Culture and Western Blotting. HepG2 cells (American Type Culture Collection, Manassas, VA) at passage 6 were used in all experiments. Cells were treated with BHA (10–100 μM) in 0.1% dimethyl sulfoxide (DMSO) in serum-free Dulbecco's modified Eagle's medium and replenished at 6, 12, 18, and 24 h, and again at 48 h, after which cells were harvested at 72 h. Cell lysates (100 μg of protein/lane) were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and then subjected to Western immunoblotting with polyclonal anti-(rat CYP2J4) (16 μg/ml), anti-Nrf2 (2 μg/ml), anti-c-Jun (2 μg/ml), anti-α-tubulin (2 μg/ml), anti-phosphoserine^{63}-c-Jun (1:1000 dilution), or anti-phosphoserine^{40}-Nrf2 (1:1000 dilution) as described previously (Marden et al., 2003). Preliminary experiments established the linearity of the signal response on immunoblots under these conditions.

RNA Extraction and Real-Time PCR. Cellular RNA was extracted with TRI reagent and was subjected to real-time PCR analysis (SYBR Green PCR master mix; QIAGEN) in a Rotor Gene 6000 instrument (Corbett Life Science, Mortlake, NSW, Australia). Thermal cycling was carried out as follows: 50°C for 30 min, 95°C for 15 min as initial denaturing, followed by 40 cycles of 94°C for 15 s, 55 to 57.5°C for 30 s (CYP2J2, c-Jun, and β-actin at 55°C, and Nrf2 at 57.5°C; primer sequences are shown in Table 1), and 72°C for 30 s. Threshold cycles (CT values) were determined and normalized using appropriate cDNA plasmid inputs (pTOPO vector; Invitrogen) without reverse transcriptase polymerase mix. Product DNA was quantified from standards run under the same conditions.

Cloning of Mutagenized Expression Plasmids. A dominant-negative c-Jun expression plasmid (DN-c-Jun) analogous to TAM67 (Brown et al., 1993) was constructed by introducing BglII sites in the native sequence (mutagenesis primers in Table 1), followed by digestion and religation. Thus, the sequence corresponding to the first 120 amino acids was deleted, and the nt encoding Ala121 to Glu122 were mutagenized to encode methionine-threonine, which are the first two residues in the native sequence of c-Jun. Nrf2 mutant constructs were also prepared by site-directed mutagenesis. In Nrf2mtbZIP, the leucine residues in the fifth and sixth heptads of the bZIP domain (Leu537 and Leu544) were replaced by alanine residues, whereas in the DNA binding domain mutant Nrf2ΔDBD, the basic region between Arg499 and Lys518 encoded by nt 1495 to 1548, was deleted (mutagenesis primers in Table 1). The DN-Nrf2 construct was described previously by Yu et al. (2000), in which nt 4–1268 were deleted and that resulted in deletion of Asp2-Thr408, was prepared using the mutagenesis primers in Table 1. The pcDNA3.1-c-Jun-FLAG (c-Jun-FLAG) construct was prepared by directional PCR cloning using restriction sites HindIII and XbaI. All constructs were confirmed by bidirectional sequencing (ABI Prism Big Dye; Applied Biosystems, Foster City, CA).

Transient Transfection Analysis of the Activation of the Human CYP2J2 Promoter. The *CYP2J2*-luciferase reporter constructs p2J2(−2341/+98), p2J2(−1228/+98), p2J2(−152/+98), p2J2(−122/+98), and p2J2(−82/+98) were described previously (Marden et al., 2003; Marden and Murray, 2005). The construct p2J2(−152/+98; mt−105/−88), containing the mutagenized region −105/−88 was generated from p2J2(−152/+98) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Willoughby, NSW, Australia); oligonucleotide sequences are shown in Table 1.

HepG2 cells (5×10^4 cells/well in 96-well plates) were cotransfected (Effectene reagent) with *CYP2J2* promoter constructs (300 ng/well) and a phRG-TK-*Renilla reniformis* expression plasmid (60 ng/well to standardize transfection efficiency). Wild-type and mutagenized Nrf2 and c-Jun expression plasmids were added at 150 ng/well, with salmon sperm DNA used to ensure equivalent loading in transfections. Seventy-two hours later, luciferase and *R. reniformis* activities were measured by Vector 3 luminometry (Dual-Glo; Promega, Alexandria, NSW, Australia). Transfections were performed in quadruplicate and in three independent experiments.

Electromobility Shift Assay. Nuclear protein (NP) extracts were prepared from untransfected HepG2 cells or cells that had been transfected for 24 h with Nrf2 and c-Jun expression plasmids (500 ng of each/6×10^5 cells) and used in electromobility shift assay (EMSA)

analysis as described previously (Marden et al., 2003). Oligonucleotides used as ^{32}P-labeled probes or competitors in EMSAs are shown in Table 1. In supershift experiments, NP extracts were incubated overnight at 4°C with anti-c-Jun and anti-Nrf2 antibodies alone or in combination (5 μg each) before the binding reaction; the nonspecific anti-ubiquitin antibody was the negative control.

Chromatin Immunoprecipitation Assay. In vivo binding of the bZIP transcription factors c-Jun and Nrf2 to the human CYP2J2 promoter region was investigated by chromatin immunoprecipitation (ChIP) assay essentially as described previously (Fiala-Beer et al., 2007) with minor modifications. In brief, HepG2 cell monolayers were treated with 1% formaldehyde in Dulbecco's modified Eagle's medium for 10 min at 37°C. After treatment with 125 mM glycine to terminate the cross-linking reaction, cells were washed twice in ice-cold phosphate-buffered saline containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 μg/ml pepstatin A), and then lysed and sonicated as described previously (Fiala-Beer et al., 2007). Chromatin samples were incubated overnight at 4°C with antibodies directed against the N terminus of c-Jun or the C terminus of Nrf2 (5 μg), and PCR (primer sequences in Table 1) was used to amplify the −143/+52 region of *CYP2J2* from the purified DNA-protein immune complexes; PCR products were run on 2% agarose gels and visualized after ethidium bromide staining. Controls used in ChIP assays were preimmune homologous IgG and input template DNA. Chromatin samples were diluted 1000-fold before use as template for quantitative PCR analysis. A standard curve was constructed using a serial dilution of CYP2J2 promoter plasmid DNA spanning the −143/+52 region. The relative fold difference was calculated by the comparative quantitation method and normalized to α-tubulin.

Coimmunoprecipitation Assay. HepG2 cells were transfected for 24 h with c-Jun-FLAG (500 ng/6 × 10^5 cells) and either Nrf2, Nrf2mtbZIP, or Nrf2ΔDBD (500 ng) and then lysed in 25 mM HEPES, pH 7.9, containing 10% glycerol, 0.1% Igepal, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin. Lysates were incubated overnight with anti-Nrf2 IgG (6 μg) at 4°C before pull-down with beads that had been treated with lysis buffer containing 0.5 M KCl. After washing, bound pro-

TABLE 1
Primer and oligonucleotide sequences (5′-3′)

Primer	Sequence
Cloning and Mutagenesis	
p2J2(−152/+98; mt−105/−88)	
2J2-mt(−105/−88) F	GCGAAGGGGGAAAAAAAAAAAAAAAAAACGGCTGGGAGCGAGGCGGG
2J2-mt(−105/−88) R	CCCGCCTCGCTCCCAGCCGTTTTTTTTTTTTTTTTTTCCCCCTTCGC
DN-c-Jun	
cJun.mt616-618 F	GAAGTGACGGACCGTTCTAGATCTGCAAAGATGGAAACGAC
cJun.mt616-618 R	GTCGTTTCCATCTTTGCAGATCTAGAACGGTCCGTCACTTC
cJun.mt978-980 F	GCTTCGTGCGCGCCCTGAGATCTATGACTAGCCAGAACACGCTTCCC
cJun.mt978-980 R	GGGAAGCGTGTTCTGGCTAGTCATAGATCTCAGGGCGCGCACGAAGC
DN-Nrf2	
DN-Nrf2 F	GGAATTCCATGAACACACCAGAGAAAGAATT
DN-Nrf2 R	GGAATTCCCTAGTTTTTCTTAACATCTGGCTT
Nrf2mtbZIP	
mtNrf2.L536A F	CAAAAGCCTTCACCTGGCAAAAAAACAACTCAGC
mtNrf2.L536A R	GCTGAGTTGTTTTTTTGCCAGGTGAAGGCTTTTG
mtNrf2.L543A F	CAACTCAGCACCGCTTATCTTGAAGTGTTC
mtNrf2.L543A R	GAACACTTCAAGATAAGCGGTGCTGAGTTG
Nrf2ΔDBD	
Nrf2.mt1484-1489 F	CTGGAAAATATAGTAGAAGGTACCCAAGATTTAGATCATTTG
Nrf2.mt1484-1489 R	CAAATGATCTAAATCTTGGGTACCTTCTACTATATTTTCCAG
Nrf2.mt1549-1554 F	CTGAAAAAACAACTCAGCGGTACCTATCTCGAAGTTTTCAGC
Nrf2.mt1549-1554 R	GCTGAAAACTTCGAGATAGGTACCGCTGAGTTGTTTTTTCAG
c-Jun-FLAG	
FLAG F	AAGCTTGGATGACTGCAAAGATGGAAACGACC
FLAG R	TCTAGAGCGAATGTTTGCAACTGCTGCGTTAG
Real-Time PCR Primers	
CYP2J2 F	AGCTTAGAGGAACGCATTCAGGA
CYP2J2 R	CGAAGGTGATGGAGCAAATGAT
Nrf2 F	GGTAGCCCCTGTTGATTTAG
Nrf2 R	CGAAGGTGATGGAGCAAATGAT
c-Jun F	CCCCAAGATCCTGAAACAGA
c-Jun R	CCGTTGCTGGACTGGATTAT
β-Actin F	ACGGGGTCACCCACACTGTGC
β-Actin R	CTAGAAGCATTTGCGGTGGAC
Oligonucleotide Sequences Used in EMSA	
2J2(−118/−60) S	GCTGCGAAGGGGCGGGCTGGGAGGCGGGGCACGGCTGGGAGCGAGGCGGGGCGGGGACCGGG
2J2(−118/−60) A	GGTCCCCGCCCCGCCTCGCTCCCAGCCGTGCCCCGCCTCCCAGCCCGCCCCTTCGCAGCGGG
2J2(−105/−88) S	GGGCTGGGAGGCGGGGCA GGG
2J2(−105/−88) A	CGTGCCCCGCCTCCCAGC GGG
2J2(−105/−95) S	GGGCTGGGAGG GGG
2J2(−105/−95) A	CCTCCCAGCCC GGG
2J2(−99/−88) S	GGAGGCGGGGCA GGG
2J2(−99/−88) A	TGCCCCGCCTCC GGG
2J2(−83/−73) S	TGGGAGCGAGGC GGG
2J2(−83/−73) A	GCCTCGCTCCCA GGG
STAT5 S	GGACTTCTTGGAATTAAGGGA
STAT5 A	TCCCTTAATTCCAAGAAGTCC
Primers Used in ChIP Assays for CYP2J2 (−143/+52)	
Forward	CATGGACCACTGCCCAGAG
Reverse	CCAGCGCCTGGCATCTTC

F, forward; R, reverse; S, sense; A, antisense; STAT5, signal transducer and activator of transcription 5.

teins were eluted by heating at 55°C for 30 min in 50 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.1% bromphenol blue before resolution on 10% polyacrylamide gels and immunoblotting; lysates precleared with beads only were used as inputs.

Statistical Analysis. Data are expressed as means ± S.E.M. Differences between experimental groups were detected by one-way analysis of variance and Fisher's protected least difference test.

Results

Activation of *CYP2J2* Expression in HepG2 Cells by the Antioxidant BHA. Treatment of HepG2 cells with BHA (20 μM) increased CYP2J2 mRNA expression to 182 ± 10, 211 ± 20, and 206 ± 15% of control after 24, 48, and 72 h, respectively ($P < 0.01$; Fig. 1A). The concentration-dependence of CYP2J2 mRNA induction by BHA (10–100 μM) was established at 24 h ($P < 0.01$ in each case; Fig. 1B). After BHA treatment, HepG2 cell lysates were also prepared at timed intervals for the estimation of CYP2J2 immunoreactive protein using an antiserum directed against rabbit CYP2J4, which is cross-reactive with human CYP2J2 (Xie et al., 2000); α-tubulin was used to indicate protein loading (Fig. 1C). From Fig. 1D, CYP2J2 protein expression was increased at each time point by BHA treatment (100 μM). Treatment of HepG2 cells with the related chemical *tert*-butylhydroquinone (100 μM) also increased CYP2J2 mRNA and protein expression similarly to BHA over the time frame of 6 to 72 h in culture (data not shown).

BHA increases Nrf2 expression in cells, and the resultant heterodimers with c-Jun activate a number of phase II genes that are protective against a range of xenobiotics and reactive intermediates (Yu et al., 1997, 2000). c-Jun has been shown previously to activate the human *CYP2J2* gene (Marden and Murray, 2005). Consistent with previous reports (Yu et al., 1997; Yuan et al., 2006), c-Jun and Nrf2 mRNAs were both increased in HepG2 cells by treatment with BHA (100 μM) for 6 and 24 h ($P < 0.05$; Fig. 2A). c-Jun immunoreactive protein was also increased to 8.3 ± 2.3- and 15.5 ± 4.6-fold of control after 6 and 24 h of treatment, respectively ($P < 0.05$; Fig. 2B), and Nrf2 immunoreactive protein was increased to 1.9 ± 0.2- and 2.3 ± 0.1-fold of control after 6 ($P < 0.01$) and 24 h ($P < 0.001$; Fig. 2A). ChIP assays confirmed the direct binding of c-Jun and Nrf2 to the *CYP2J2* proximal promoter region that was strongly enhanced by treatment of cells with BHA (20 and 100 μM; Fig. 2C). In agreement with these findings, the expression of activated forms of these bZIP factors, phosphoserine^{63}-c-Jun and phosphoserine^{40}-Nrf2, was increased in lysates from HepG2 cells that had been treated with BHA (100 μM) for 0.5 to 6 h (Fig. 2D).

Molecular Analysis of CYP2J2 Transactivation by c-Jun and Nrf2. *CYP2J2* 5′-flank-luciferase reporter deletion constructs were used to characterize the region of the *CYP2J2* upstream sequence that mediated transactivation by c-Jun and Nrf2, which were up-regulated by BHA treatment (Fig. 3A). The construct p2J2(−2341/+98) was strongly activated by these factors after individual transfection and to 6.8 ± 0.6-fold of control by the combination of c-Jun/Nrf2 (Fig. 3B). Deletion constructs were similarly responsive down to the construct p2J2(−122/+98), but p2J2(−82/+98) was much less responsive. Thus, the region between −122 and −82 seemed to be responsible for c-Jun/Nrf2-dependent transactivation. Consistent with this finding, mutagenesis of

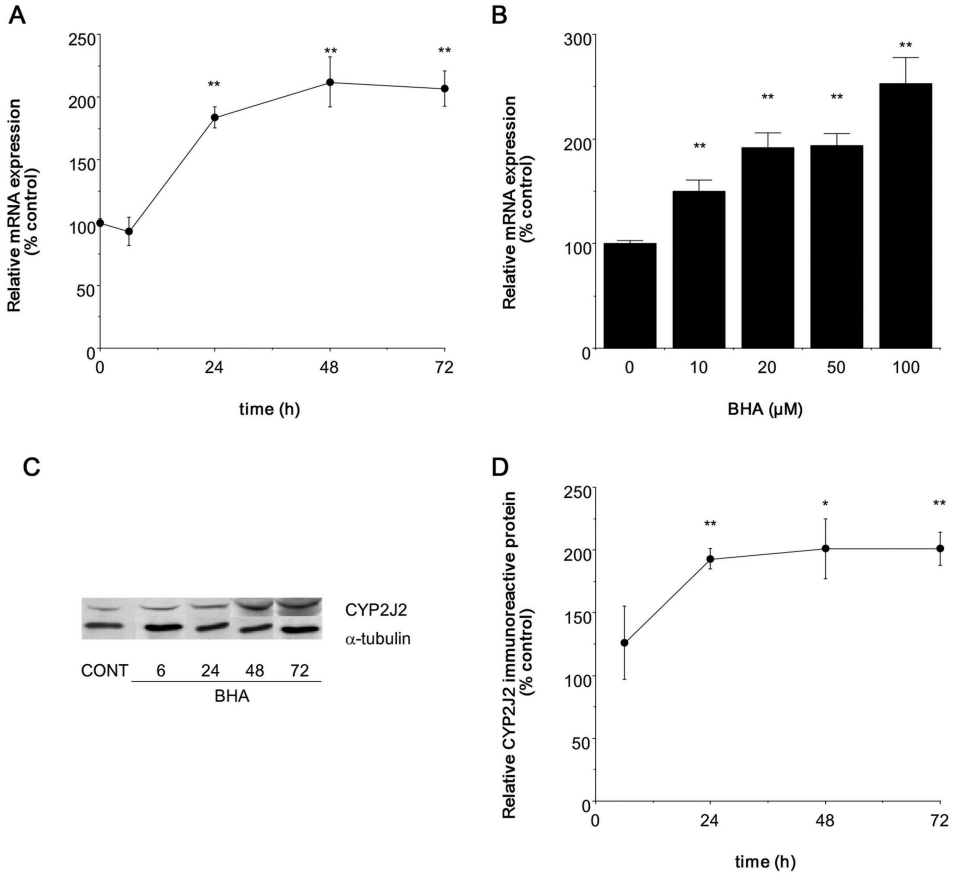

Fig. 1. Concentration- and time-dependent activation of CYP2J2 expression in HepG2 cells by BHA. A, time course of CYP2J2 mRNA activation by BHA (20 μM). B, effect of BHA concentration (0–100 μM; 24 h) on CYP2J2 mRNA expression. C and D, time-dependent activation of CYP2J2 immunoreactive protein expression after BHA (100 μM). Data are expressed relative to time-matched DMSO-control as means ± S.E.M. of three independent experiments performed at least in triplicate. Different from control, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Representative immunoblots in a series of lysates prepared at different time points are shown; the experiment was performed in triplicate.

the −105/−88 region of *CYP2J2* attenuated c-Jun/Nrf2 responsiveness [p2J2(−152/+98; mt−105/−88); Fig. 3B].

The −105/−88 Region in the *CYP2J2* 5′-Flank Is Involved in the Binding of c-Jun and Nrf2. BHA treatment activated Nrf2 and c-Jun expression in cells and, from ChIP analysis, increased the binding of these factors to the *CYP2J2* 5′-flank. Transient transfection studies strongly implicated the region between −105/−88 in the *CYP2J2* 5′-flank in c-Jun/Nrf2-mediated transactivation. This region has been shown previously to harbor an AP-1-like responsive region that binds c-Jun (Marden and Murray, 2005), but its involvement in Nrf2-mediated augmentation of transactivation by c-Jun has not been considered previously. In the present study, EMSA analysis with the −118/−60 probe that spans the putative responsive region (Fig. 4A) was undertaken to evaluate binding in NP from HepG2 cells, in which c-Jun and Nrf2 had been overexpressed. The resultant DNA-protein complex was competed by unlabeled self-probe but not by the unrelated STAT5 probe from the β-casein promoter (Fig. 4B). An antibody against c-Jun, but not anti-

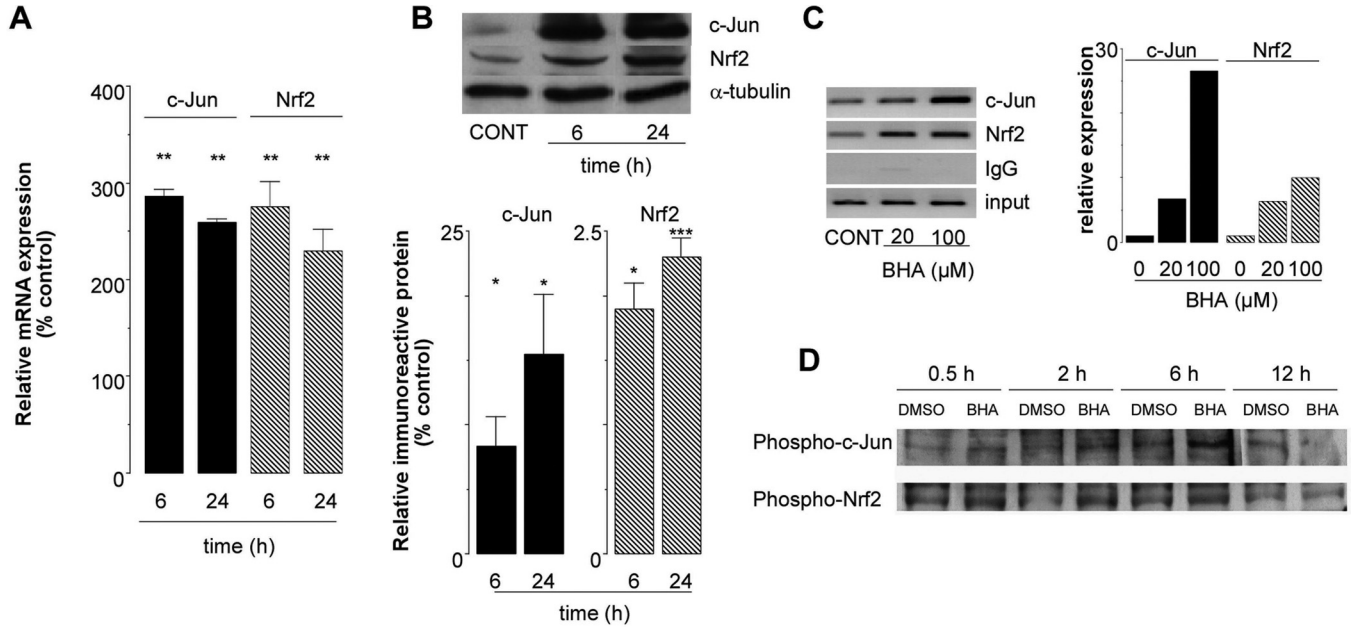

Fig. 2. Activation of c-Jun and Nrf2 expression in HepG2 cells after BHA treatment relative to time-matched DMSO-control. A, induction of c-Jun and Nrf2 mRNA in HepG2 cells after 6 and 24 h of treatment with BHA (100 μM). B, induction of c-Jun and Nrf2 immunoreactive protein expression in HepG2 cells after 6 and 24 h of BHA treatment (100 μM). Data are mean ± S.E.M. of three independent experiments performed in triplicate. Different from control, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Representative immunoblots from triplicate experiments are shown in B. C, ChIP assays performed in HepG2 cells that had been treated with BHA (20 or 100 μM) or DMSO control for 24 h. Signals on ethidium bromide stained agarose gels are shown with separate quantitation by real-time PCR, as described under *Materials and Methods*. Representative data are shown from a single experiment that was conducted in duplicate. D, activation of phosphoserine[63]-c-Jun and phosphoserine[40]-Nrf2 in lysates from untreated (DMSO) or BHA (100 μM)-treated HepG2 cells. Data from a representative experiment are shown; the experiment was conducted twice.

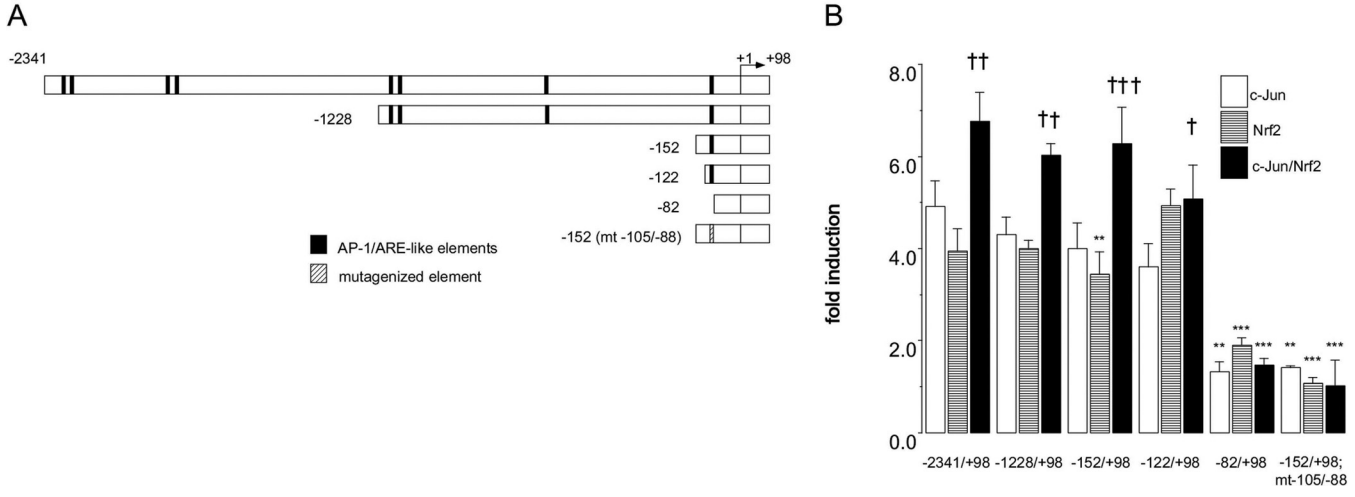

Fig. 3. A, CYP2J2 5′-flank-luciferase reporter constructs p2J2(−2341/+98) to p2J2(−82/+98) by c-Jun and Nrf2; the construct p2J2(−152/+98) in which the −105/−88 region is mutagenized is also shown. B, differential activation of the constructs by c-Jun and Nrf2, showing loss of responsiveness after mutagenesis of the −105/−88 region. Different from corresponding activation of the −2341/+98 construct by c-Jun, Nrf2, or c-Jun/Nrf2: **, $P < 0.01$; ***, $P < 0.001$; different from activity of constructs after cotransfection with c-Jun alone: †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$. Data are expressed as fold of reporter activity in the absence of cotransfected bZIP factors and are the means ± S.E.M. of three independent experiments performed at least in triplicate.

Nrf2, block-shifted the DNA-protein complex; the effect of the anti-c-Jun/anti-Nrf2 combination was similar to that of anti-c-Jun alone, thus suggesting a pivotal role for c-Jun binding (Fig. 4B). The nonspecific anti-ubiquitin antibody negative control did not shift the complex. Competitor oligonucleotides corresponding to the *CYP2J2* regions at −105/−95 and −99/−88, but not the adjacent sequence −83/−73, also blocked the binding interaction (Fig. 4C). Considered together, findings from EMSA, transient transfection, and ChIP assays implicated the −105/−88 region of the *CYP2J2* upstream region in binding and transactivation by c-Jun and Nrf2.

To corroborate the functional importance of this region of the *CYP2J2* 5′-flank HepG2 cells were transfected with the constructs p2J2(−152/+98), which contains the −105/−88 region, and p2J2(−49/+98), which does not. BHA treatment (100 μM) stimulated the reporter activity of the p2J2(−152/+98) construct but not that of p2J2(−49/+98) ($P < 0.01$; Fig. 5A). In further studies, NP fractions were extracted from BHA-treated HepG2 cells (20 and 100 μM) and used in EMSA studies with the −118/−60 probe. As shown in Fig. 5B, binding of the ^{32}P-labeled −118/−60 sequence to BHA-NP was enhanced (lanes 2 and 3) compared with control (DMSO-treated)-NP (lane 1). These data are consistent with a major role for the −105/−88 region in activation of the *CYP2J2* gene by BHA.

Interaction of c-Jun and Nrf2 in the Activation of the *CYP2J2* Promoter. ChIP assays established that BHA enhanced the binding of both c-Jun and Nrf2 to the *CYP2J2* regulatory region, and supershift EMSAs suggested that c-Jun may be the critical partner in the binding reaction. To further investigate the nature of the Nrf2/c-Jun interaction a series of mutant expression plasmids was constructed for use in transient transfection assays (Fig. 6A). As shown in Fig. 6B, the increase in c-Jun-mediated activation of the construct p2J2(−152/+98) by cotransfected Nrf2 (6.13 ± 0.36-fold of control relative to 3.32 ± 0.31-fold of control by cotransfected c-Jun alone; $P < 0.001$) was abolished by the DN-c-Jun plasmid (0.76 ± 0.12-fold of control). In contrast, cotransfection of Nrf2 mutants lacking either the Nrf2 DNA-binding domain (Nrf2ΔDBD), or the transactivation domain (DN-Nrf2), still augmented c-Jun-mediated transactivation of p2J2(−152/+98) ($p < 0.001$). However, replacement of wild-type Nrf2 with Nrf2mtbZIP, in which Leu537 and Leu544 in the bZIP domain were replaced with alanine residues, significantly attenuated the augmentation by Nrf2 of c-Jun-mediated transactivation (3.63 ± 0.11-fold of control; Fig. 6B). Thus, the bZIP regions of Nrf2 and c-Jun were implicated in the activation of *CYP2J2* reporter constructs, and this finding is consistent with an essential role for c-Jun in transactivation.

Coimmunoprecipitation (CoIP) assays were used to further evaluate the interaction between c-Jun and Nrf2. A FLAG-tagged c-Jun expression plasmid was coexpressed with plasmids encoding either wild-type Nrf2, Nrf2ΔDBD, or Nrf2mtbZIP. After immunoprecipitation with anti-Nrf2 IgG and immunoblotting for the FLAG tag, a weaker signal was observed in the case of the c-Jun-FLAG/Nrf2mtbZIP combination (Fig. 6C, lane 6) compared with that with wild-type Nrf2 (lane 4) and Nrf2ΔDBD (lane 5). Immune precipitates in Nrf2mtbZIP-transfected cells also contained less Nrf2, even though the signal from direct immunoblots was similar to that in cells transfected with Nrf2/c-Jun-FLAG. These findings from CoIP experiments support a role for bZIP leucine residues in the c-Jun/Nrf2 interaction, which enhances *CYP2J2* transactivation over that elicited by c-Jun alone.

Discussion

The present study has identified BHA as a novel inducer of *CYP2J2* expression in human cells. The time- and concentration-dependent increases in CYP2J2 mRNA and protein expression after BHA treatment were closely related to activation of the bZIP transcription factors c-Jun and Nrf2. Furthermore, ChIP analysis indicated that the binding of these factors to the *CYP2J2* proximal promoter region was strongly increased by BHA treatment of cells. This region of the *CYP2J2* gene has been shown previously to harbor elements responsive to c-Jun and to mediate CYP2J2 down-regulation in hypoxia (Marden et al., 2003; Marden and Murray, 2005), but the present findings now establish that heterodimers formed between Nrf2 and c-Jun up-regulate CYP2J2. Moreover, agents that activate the expression of

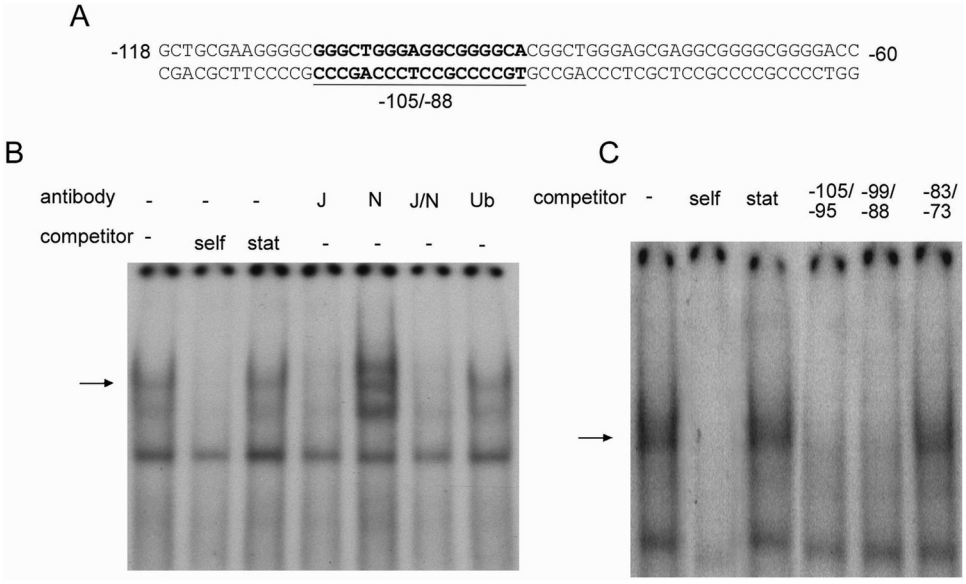

Fig. 4. A, sequence of the double-stranded oligonucleotide probe −118/−60 containing the −105/−88 region that is implicated in binding and transactivation by c-Jun/Nrf2 (shift indicated by arrow). B, EMSA analysis in nuclear protein fractions from c-Jun/Nrf2-transfected HepG2 cells, showing the block shift elicited by anti-c-Jun. C, the unlabeled competitor oligonucleotides −105/−95 and −99/−88 but not −83/−73 impaired binding of the −118/−60 probe to nuclear protein fractions. Representative EMSAs are shown from experiments performed at least in duplicate.

these bZIP factors may have the potential for development of strategies to up-regulate CYP2J2 and use the beneficial actions of EETs in cells.

bZIP transcription factors from the AP-1 family regulate a large number of genes, and response elements for AP-1 are common throughout the genome. However, a number of AP-1-responsive genes contain atypical AP-1 response elements such that heterodimerization between alternate bZIP factors may influence the fine-tuning of transcriptional responses (McBride and Nemer, 1998; Venugopal and Jaiswal, 1998; Vinson et al., 2006). In the case of the murine interleukin-4 gene, for example, the apparent AP-1-responsive region is unrelated to the AP-1 consensus sequence (Rooney et al., 1995). Atypical AP-1-responsive gene elements that may accommodate a number of alternate bZIP factors have also been found in the osteocalcin and secreted protein acidic and rich in cysteine genes, among others (Vial et al., 2000; Akhouayri and St-Arnaud, 2007). In the present study, transient transfection and EMSA analysis were used to identify the −105/−88-base pair region of the *CYP2J2* gene 5′-flank as an atypical bZIP binding sequence that was responsive to c-Jun/Nrf2 dimers.

c-Jun seemed to have the critical role in transactivation of *CYP2J2* by c-Jun/Nrf2 dimers. Thus, a dominant-negative mutant expression plasmid that lacked the transactivation domain abolished *CYP2J2* activation. In contrast, Nrf2 mutants lacking either the transactivation or DNA-binding domain behaved like wild-type Nrf2; thus, augmentation of c-Jun-dependent transactivation remained. Instead, an Nrf2 mutant in which alanine residues replaced critical fifth and sixth leucine residues within the bZIP domain at Leu537 and Leu544, which are essential for dimerization and nuclear retention (Li et al., 2008), significantly decreased *CYP2J2* transactivation. EMSA findings also supported a major role for c-Jun. Thus, anti-c-Jun elicited a block shift, consistent with disruption of the interaction between c-Jun and the labeled oligonucleotide probe, whereas an anti-Nrf2 antibody weakly supershifted the complex, consistent with a decrease in electrophoretic mobility. Taken together, these experiments strongly suggest that c-Jun and Nrf2-mediated activation of CYP2J2 at the −105/−88 element region is highly dependent on c-Jun.

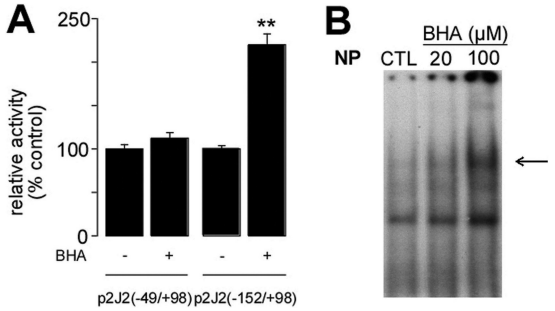

Fig. 5. A, activation of the reporter construct p2J2(−152/+98) but not p2J2(-49/+98) in transfected cells by BHA treatment (100 μM). B, EMSA analysis using the −118/−60 oligonucleotide probe in nuclear protein fractions from HepG2 cells that had been treated with BHA (20 or 100 μM), relative to DMSO-treated control (CTL; shift indicated by arrow). Reporter data are means ± S.E.M. of three independent experiments performed at least in triplicate. Different from corresponding control (reporter activity in the absence of BHA treatment): **, $P < 0.01$. Representative EMSAs are shown from experiments performed in duplicate.

The synthetic phenolic antioxidant BHA is an effective in vivo inducer of phase II genes in mammals and exerts protective effects against toxic xenobiotics (Chung et al., 1986; Williams and Iatropoulos, 1996). The underlying mechanism involves activation of the bZIP transcription factor Nrf2 (Yu et al., 1997; Venugopal and Jaiswal, 1998; Yuan et al., 2006). Thus, pro-oxidant and antioxidant agents modulate the cytoplasmic association of Nrf2 with the cysteine-rich scaffold protein kelch-like ECH-associated protein 1 that is attached to the cytoskeleton (Dinkova-Kostova et al., 2002; Wakabayashi et al., 2004). Modification of the cysteine sulfhydryls in kelch-like ECH-associated protein 1 enables Nrf2 dissociation and facilitates its entry to the nucleus, in which it undergoes phosphorylation and may dimerize with other bZIP factors and bind to antioxidant-response elements in the promoters of cytoprotective genes (Venugopal and Jaiswal, 1998; Huang et al., 2000). The present findings indicate that CYP2J2 is also an antioxidant-inducible Nrf2-regulated gene and that dimerization with c-Jun is important in transactivation. Other members of the antioxidant-inducible gene battery, including the γ-glutamylcysteine synthase-heavy subunit NADPH-quinone oxidoreductase-1 and glutathione transferase Ya genes, are also transactivated by Nrf2

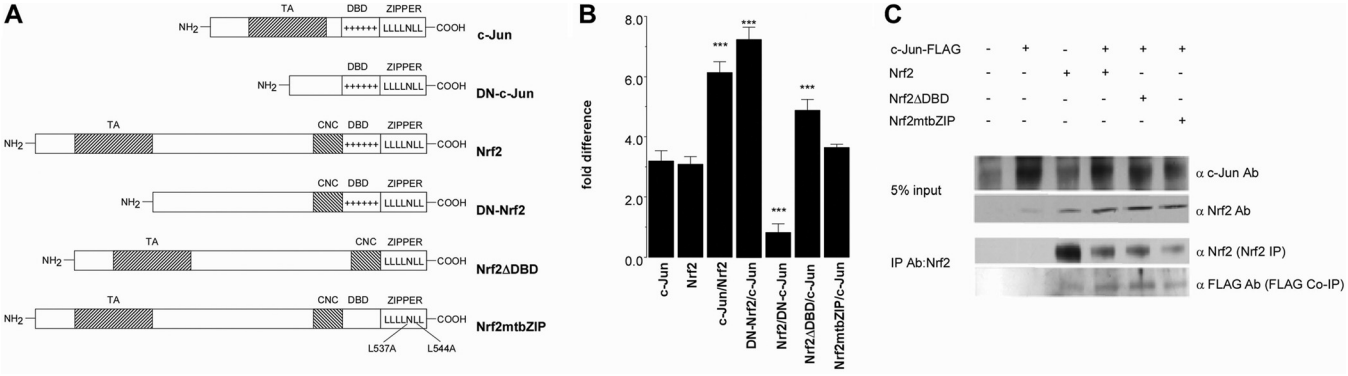

Fig. 6. A, domain structures of the bZIP transcription factors c-Jun and Nrf2 and the mutant expression plasmids prepared in this study, as described under *Materials and Methods*; TA, transactivation domain; DBD, DNA-binding domain; ZIPPER, bZIP region; CNC, cap'n'collar region. In the construct Nrf2mtbZIP, the leucines that are mutagenized are indicated. B, transactivation of p2J2(152/+98) by combinations of wild-type and mutagenized c-Jun and Nrf2 expression plasmids. Data are presented as fold of reporter activity in the absence of cotransfected bZIP plasmids and are means ± S.E.M. of three independent experiments performed at least in triplicate. Different from transactivation by c-Jun alone: ***, $P < 0.001$. C, CoIP experiments showing the interaction of c-Jun-FLAG and Nrf2 that is impaired in the Nrf2 bZIP double-leucine mutant (Nrf2mtbZIP). Representative CoIPs are shown from experiments performed in triplicate.

in processes that are highly dependent on cellular c-Jun expression (Jeyapaul and Jaiswal, 2000; Jaiswal, 2004). BHA is the first agent identified to date that activates *CYP2J2* gene expression in human cells. Alternate agents, such as sulforaphane, that also activate bZIP factor expression have been found to exert protective effects in cells and in in vivo models of experimental injury (Yoon et al., 2008; Higgins et al., 2009). In view of the proliferative and mitogenic actions of EETs, it is now of interest to evaluate the specific role of CYP2J2 in the overall cytoprotective actions of such agents.

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