Valproic Acid–Induced Gene Expression through Production of Reactive Oxygen Species

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Abstract

Valproic acid (VPA) is a widely used anticonvulsant agent that has profound antiproliferative effects in many cell types, as well as inductive effects on a number of genes. The mechanism of its gene-inducing effect has been reported to involve transcription factors, Sp1 and activator protein-1. Using two well-characterized antioxidant response element (ARE)–driven gene promoters, i.e., mouse heme oxygenase-1 and human NAD(P)H quinone oxidoreductase 1 genes as tools to monitor the transcriptional response to VPA, we show here that VPA-induced gene transcription was abrogated by antioxidants. With the human G0s2 gene promoter, which was previously used to establish the involvement of Sp1 in the transcriptional action of VPA, we found that VPA-induced gene transcription was also blocked by antioxidants. Mutation of the ARE (5′-TGACTggGC-3′) in this promoter abrogated the transcriptional response to VPA. With such mutants, the NADPH oxidase inhibitor, diphenyleneiodonium, had no effect on VPA-induced transcription. In gel mobility shift assays, VPA-induced binding of nuclear proteins to a DNA probe containing the relevant ARE sequence in the G0s2 gene promoter was decreased in nuclear extracts from cells pretreated with antioxidants. Chromatin immunoprecipitation assays showed that the prototype redox-sensitive transcription factors, Nrf2, small Maf protein(s), and c-Fos, were recruited to this promoter in VPA-treated cells. Overall, this study reveals that the mechanism of the transcriptional action of VPA, we found that VPA-induced gene transcription was abrogated by antioxidants. With the human G0s2 gene promoter, which was previously used to establish the involvement of Sp1 in the transcriptional action of VPA, we found that VPA-induced gene transcription was also blocked by antioxidants. Mutation of the ARE (5′-TGACTggGC-3′) in this promoter abrogated the transcriptional response to VPA. With such mutants, the NADPH oxidase inhibitor, diphenyleneiodonium, had no effect on VPA-induced transcription. In gel mobility shift assays, VPA-induced binding of nuclear proteins to a DNA probe containing the relevant ARE sequence in the G0s2 gene promoter was decreased in nuclear extracts from cells pretreated with antioxidants. Chromatin immunoprecipitation assays showed that the prototype redox-sensitive transcription factors, Nrf2, small Maf protein(s), and c-Fos, were recruited to this promoter in VPA-treated cells. Overall, this study reveals that the mechanism of the transcriptional response to VPA includes VPA-induced production of reactive oxygen species which induce the activation of redox-sensitive transcription factors that interact with the ARE. (Cancer Res 2006; 66(13): 6563-9)

Introduction

Valproic acid (2-propylpentanoic acid; VPA) is widely used clinically as an anticonvulsant agent for suppressing epileptic seizures as well as a mood stabilizer in the treatment of manic depression (bipolar affective disorder; refs. 1–3). VPA induces histone acetylation (4–7), DNA demethylation (8), chromatin decondensation (9), and the expression of a variety of genes (10–12). It has antiproliferative effect on cells, and is known to induce differentiation of cell lines derived from myeloid leukemia (4, 7), teratocarcinoma, glioma, and neuroblastoma (13). Other inhibitors of histone deacetylases (HDAC), such as sodium butyrate, trichostatin A, and Helminthosporium carbonum toxin (HC toxin), also exhibit antiproliferative properties, an effect that has garnered increasing attention for HDAC inhibitors in cancer research (4, 7, 14–16).

Although not much has been published on the molecular mechanism of the various actions of VPA, the few reports that VPA increases the expression of genes regulated by the transcription factor, activator protein-1 (AP-1; refs. 17, 18), seem to be the basis for the conclusion that the molecular mechanism of VPA-induced gene expression is via DNA-binding activity of AP-1 transcription factors to the AP-1 response element (5, 19). We have shown that the G0s2 gene promoter, which does not contain the canonical AP-1-binding motif, could be transactivated by VPA through Sp1-binding sites in the promoter (11). Mutations at the relevant Sp1-binding sites did not completely abolish VPA-induced promoter activity, suggesting that there might be other mechanisms contributing to VPA-induced gene transcription.

VPA (20–22) and other class I/II HDAC inhibitors, i.e., suberoylanilide hydroxamic acid (SAHA; ref. 23), MS-275 (24, 25), and sodium butyrate (26), have been reported to elevate cellular levels of reactive oxygen species (ROS). Neural tube defects induced by VPA are prevented by vitamin E (27), a potent antioxidant; this effect is consistent with the idea that ROS production is part of the biological action of VPA. Although at high levels, ROS induce toxicity to cells, at physiologic levels, they function as part of normal cell signaling. There has been recent interest in unraveling molecular mechanisms of ROS-mediated gene expression. For example, Das et al. (28) showed that ROS generated by the exposure of HCT116 human colon carcinoma cells to glucose oxidase resulted in the induction of a mammalian DNA endonuclease through CRE/AP-1 binding sites in the promoter that binds the transcription factors c-Jun and CREB/ATF2. Others have shown that the electrophile tert-butylhydroquinone (tBHQ), which also elicits the generation of ROS, activates transcription through the interaction of the transcription factor Nrf2 (nuclear factor erythroid 2–related factor 2) at antioxidant response elements (ARE) on target gene promoters (for review, see refs. 29, 30). We showed recently that tBHQ-induced transcription from the human gene promoter for the signal transduction protein G0s2 occurred through redox-sensitive transcription factor interaction with the core ARE in the promoter (31). In the present study, we have used prototypical ARE-containing gene promoters, i.e., mouse heme oxygenase-1 gene, human NAD(P)H quinone oxidoreductase 1 gene, as well as the human G0s2 gene promoter, as tools to show that VPA can induce gene transcription through the ARE. This effect was independent of VPA-induced promoter activity that results from Sp1 interaction at Sp1-binding sites, as evident from studies with the human G0s2 gene promoter. Overall, this work reveals that the gene expression–inducing effect of VPA can be accounted for, in part, by the interaction of redox-sensitive transcription factors with the ARE.
Materials and Methods

Cell culture and transfection studies. K562 cells, obtained from the American Type Culture Collection (Manassas, VA), were maintained in culture as previously described (11, 32). For transfection experiments, the cells (1 × 10^5 cells per well in 1 mL of medium) were seeded in 24-well plates; wells on the periphery of the plates were not used. After 24 hours, the cells were transfected with 0.5 μg of plasmid DNA (containing the gene promoter or mutant) and 1.5 μL of FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN) 1 hour before the addition of sodium valproate (Sigma, St. Louis, MO) or other HDAC inhibitors. When chemical inhibitors such as N-acetyl-cysteine (NAC), diphenyleneiodonium (DPI), and pyrrolidine dithiocarbamate (PDTC) were used, they were added 30 minutes or 1 hour prior to the addition of valproate or other HDAC inhibitors. The cells were harvested 20 to 24 hours later, by centrifugation at 12,000 × g (45 seconds) in 1.5 mL microcentrifuge tubes, and washed once with 1 × PBS. The cell pellets were then lysed with 150 μL of 1 × Cell Lysis Beagent (Promega, Madison, WI) and kept at room temperature for 10 minutes. The lysed material was then centrifuged for 2 minutes at 12,000 × g, and the luciferase activity and protein content of each lysate were measured as previously described (11, 31).

Immunoblotting. Using 10 μg of protein, immunoblotting of G\textsubscript{i2} in whole-cell extracts was carried out as previously described (32, 33). The blots were reprobed for β-tubulin, as a loading control.

Measurement of ROS. The total content of ROS was measured by using the nonfluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), which upon oxidation, becomes a highly fluorescent species, 2′,7′-dichlorofluorescein (DCF). The cells (5,000 cells in 0.5 mL/well in a 24-well plate) were loaded with 10 μmol/L of DCFDA for 30 minutes prior to the addition of VPA (5 mmol/L). DCF fluorescence was measured at various time points thereafter, using CytoFluor Series 4000 Fluorescence Multi-Well Plate Reader (Applied Biosystems, Inc., Foster City, CA), at excitation and emission settings of 485 and 530 nm, respectively.

Site-directed mutagenesis. Specific nucleotides in the G\textsubscript{i2} gene promoter were mutated or deleted by using QuikChange Mutagenesis kit (Stratagene, Inc., La Jolla, CA). Mutations were confirmed by DNA sequencing at the Molecular Biology Core Facility, Meharry Medical College.

Preparation of nuclear extracts and electrophoretic mobility shift assays. Nuclear extracts were prepared as described previously (11, 31). For electrophoretic mobility shift assays (EMSA), annealed 5'-overhang oligonucleotide sequence, containing ARE-binding motif, was labeled with \([\alpha\textsuperscript{32P}]dCTP\), using the Klenow fill-in reaction, and purified (33). The labeled probe was used for the EMSA reaction as described in the legend to Fig. 5. After electrophoresis, the gel was dried, and then exposed to Kodak XAR5 film at −80°C. The radiolabeled bands were detected by autoradiography.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) analysis to monitor association of transcription factor(s) with the G\textsubscript{i2} gene promoter in vivo was done (31), in principle, according to the protocols recommended by Upstate Biotechnology (Lake Placid, NY) and by the Farnham Laboratory (http://www.genomecenter.ucdavis.edu/farnham/).

Results

Antioxidants inhibit VPA-induced expression of G\textsubscript{i2}. We have previously shown that VPA increases mRNA and protein levels of the signal transduction protein G\textsubscript{i2} in K562 cells; the induction was confirmed by reporter gene assay, using human G\textsubscript{i2} gene promoter linked to luciferase reporter gene (11). In that study, we showed that, like other HDAC inhibitors tested (i.e., sodium butyrate and trichostatin A), the mechanism of this induction involved the binding of the Sp family of transcription factors to three Sp1 consensus sequence-binding sites in the promoter. However, the possible involvement of other transcription factors binding at other response elements could not be ruled out because deletion or point mutation of the relevant Sp1-binding motifs in the promoter did not result in total loss of promoter activity (11). In further exploration, we have observed that antioxidants block the gene expression–inducing action of VPA. For example, the classic antioxidants NAC and PDTC, which are potent scavengers of ROS (34), inhibited VPA-induced expression of G\textsubscript{i2}, as evidenced from the decreased level of G\textsubscript{i2} protein, measured by Western blotting (Fig. 1).

VPA induces the generation of ROS. The inhibitory effect of antioxidants on G\textsubscript{i2} levels suggests that the treatment with VPA most likely resulted in the generation of ROS. We confirmed this by using the nonfluorescent compound, DCFDA, a cell-permeable dye that turns into a highly fluorescent species (DCF) upon oxidation, to monitor VPA-induced changes in the cellular content of ROS. As seen in Fig. 24, VPA treatment increased DCF fluorescence in a time-dependent manner, indicating increased ROS production in these cells. The data are consistent with reports in other cell systems that VPA induces the production of ROS (20–22). Other HDAC inhibitors that are of interest as cancer chemotherapeutic agents, i.e., SAHA (23), MS-275 (24, 25), and sodium butyrate (26) have also been reported to increase the production of ROS, although in some cases, ROS production occurred only in transformed cells (25). However, none of these studies explored how the ROS production results in HDAC inhibitor–induced regulation of any gene. We addressed this question in this study.

Inhibition of class I/II HDACs induces G\textsubscript{i2} gene promoter activity in an antioxidant-sensitive manner. Based on the premise that two major sources of ROS generation in cells are mitochondria and the activity of NADPH oxidase (35), we measured VPA-induced promoter activity in cells pretreated with DPI, which is often used to implicate the activity of NADPH oxidase in various processes (36, 37), but which also inhibits the production of oxygen...
production by these compounds could trigger the activation of redox-sensitive transcription factors that mediate response to oxidative stress in cells.

**VPA stimulates ARE-driven gene transcription.** The transcription factor Nrf2, in conjunction with Keap 1 (Kelch-like ECH-associated protein 1), has been characterized to be intimately involved in redox sensing in oxidatively stressed cells (29, 30). Electrophiles, such as tBHQ, or ROS are known to promote the inactivation of the Nrf2-Keap 1 complex, leading to Nrf2 binding to ARE on target gene promoters. The ARE originally was defined as TGACnmmGC (39). This definition has been extended (TMANNRTGAYnnmGCRwww) to include sequences flanking the core sequence (RTGACnmmGC; refs. 40, 41). Variants in flanking sequences have been reported (42). Mutations in the core sequence (39, 41–43) as well as in the flanking sequences (42, 43) impair the ability of the ARE to function optimally.

To test the idea that the ARE is involved in VPA-induced transcription, we used two reporter gene constructs (see schematics in Fig. 3A) that are driven by well-characterized prototypical ARE, and have been previously shown to contain sufficient information to modulate Nrf2-dependent gene transcription (37, 44, 45): (a) a luciferase reporter construct of the heme oxygenase-1 gene (designated HO-1-ARE-Luc) in which three copies of the ARE sequence were cloned upstream of the mouse heme oxygenase-1 minimal promoter (37); and (b) hNQO1-ARE-Luc [human NAD(P)H:quinone oxidoreductase 1-ARE-Luc] reporter construct that contains a single copy of ARE, derived from the human NQO1 promoter, placed upstream of a minimal promoter containing a TATA box fused to the luciferase gene (45). We found that VPA increased the ARE-driven promoter activity of HO-1-ARE-Luc (Fig. 3B) as well as that of hNQO1-ARE-Luc (Fig. 3C). In both cases, the VPA effect was abrogated by DPI as well as by PDTC. In addition, other HDAC inhibitors tested (sodium butyrate, HC toxin, and SAHA) also induced transcription from these reporter gene constructs in an antioxidant-sensitive manner. These results (data not shown) resembled the data obtained with the G\textsubscript{i2} gene promoter, which is shown in Fig. 2B. Therefore, we confirmed the involvement of the ARE by extending the transcriptional analysis to the G\textsubscript{i2} gene promoter which we have previously shown (31) to be induced by Nrf2, and to be unresponsive to this transcription factor in the absence of the ARE sequence (5′-TGA-CtgGC-3′) that maps at −84/−76 in that promoter. This sequence is exactly identical to that described as short ARE by Katsuoka et al. (43), or as core sequence by Nguyen et al. (29) and was shown to be essential for both basal and/or inducible activity (39).

Using site-directed mutagenesis, we deleted the ARE in the G\textsubscript{i2} gene promoter or mutated it to 5′-GAC4tggGC-3′, and then compared promoter activity of the mutants to that of the wild-type promoter, not only in cells treated with VPA but also in cells treated with tBHQ, which we used as a positive control because it is known to activate Nrf2 (29), and also because it enhances the Nrf2-induced transcription from this promoter (31). Additionally, we monitored VPA-induced G\textsubscript{i2} promoter activity of the mutant in cells pretreated with DPI, the rationale being that DPI, which inhibited VPA-induced promoter activity (Figs. 2B and 3B) should have no effect in the absence of the ARE. As seen in Fig. 4A, substitution mutation of this short ARE to 5′-GAC4tggGC-3′ resulted in 55% loss of promoter activity. As expected, loss (65%) of promoter activity was also evident when Sp1-binding sites were mutated (see also ref. 11). Double mutants in which both the Sp1-binding sites and the ARE site were mutated, resulted in loss of

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Figure 2. VPA-induced generation of ROS, and effects of DPI and PDTC on transcription from the G\textsubscript{i2} gene promoter induced by various HDAC inhibitors. A, detection of VPA-induced generation of ROS by DCF fluorescence. K562 cells (5,000 cells in 0.5 mL/well in 24-well plates) were loaded with 10 μmol/L of DCFDA for 30 minutes prior to the addition of VPA (5 mmol/L). DCF fluorescence was measured at various time points thereafter, up to 60 minutes, using CytoFluor Series 4000 Fluorescence Multi-Well Plate Reader (Applied Biosystems), at excitation and emission settings of 485 and 530 nm, respectively. VPA-induced DCF fluorescence at each time point is expressed as the fold increase, relative to cells that were not treated with valproate (defined as 1.0). Basal DCF fluorescence was relatively constant over the 60-minute measurement period. Points, mean for duplicate assays from four different experiments; bars, ±SE. B, PDTC and the NADPH oxidase inhibitor, DPI, inhibit transcriptional response to various HDAC inhibitors (valproate, sodium butyrate, HC toxin, and SAHA). PDTC (10 μmol/L) or DPI (10 μmol/L) was added to the cell cultures 1 hour after transfection, followed by the addition of either VPA (2 mmol/L), sodium butyrate (2 mmol/L), HC toxin (40 mmol/L), or SAHA (10 μmol/L) 1 hour later. The cells were harvested 20 hours after the addition of the HDAC inhibitors, and processed for luciferase assay as described under "Materials and Methods." Promoter activity is expressed as fold stimulation, relative to cells that were not treated with HDAC inhibitors. Columns, mean for duplicate assays from four to six different experiments; bars, ±SE. Con, appropriate controls not treated with any HDAC inhibitor.

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radical by complex I (NADH dehydrogenase) of mitochondria (38). The results show that DPI (used at 10 μmol/L) inhibited VPA-induced promoter activity by ~80% (Fig. 2B); PDTC (10 μmol/L) also inhibited VPA-induced G\textsubscript{i2} gene promoter activity (Fig. 2B). Sensitivity of the VPA-induced gene expression to antioxidants seen in K562 cells is not unique to transformed cells, as similar results were also obtained in endothelial cells isolated from mouse aorta (data not shown). We found that the G\textsubscript{i2} gene promoter-inducing activities of three other HDAC inhibitors (sodium butyrate, SAHA, and HC toxin) were also abrogated by DPI or PDTC (Fig. 2B). We speculated that the induction of ROS
promoter activity that was somewhat higher (~70%) than the single mutants. As expected (31), tBHQ-induced transcription was substantially decreased (88%) by deletion of the ARE site (ARE mtΔ) or by substitution mutation (ARE mtΔ) at that site (75%; Fig. 4B). The latter compares favorably with results obtained with VPA (Fig. 4A), using ARE mtΔ. Mutations at Sp1 sites had no effect on tBHQ-induced transcription (Fig. 4B), indicating that Sp1 does not mediate the effect of tBHQ. Consistent with our prediction, DPI had no effect on VPA-induced promoter activity if the promoter was mutated at the ARE (data not shown). Overall, these results reveal that the ARE is involved in the ROS-dependent, VPA-induced transcription from this gene promoter, and that the ARE response is independent of the Sp1 response. Together with the data in Fig. 2, these studies establish an interesting mechanistic link between ROS production induced by VPA or by other HDAC inhibitors, and transcription from ARE-containing target gene promoter(s). To our knowledge, this is the first evidence that the ARE is involved in the transcriptional effect of VPA on any gene promoter. Taken together with our previous study implicating Sp1 in the transcriptional action of VPA (11), and the results by others implicating canonical AP-1 motif in AP-1 consensus sequence-containing promoters (17, 18), it seems clear that VPA-induced gene transcription involves multiple mechanisms.

**Figure 4.** Mutations at ARE or Sp1-binding elements inhibit VPA-induced transcription from the Gαi2 gene promoter. The ARE-binding site (5’-TGACTGGGC-3’) at −84/−76 in the promoter was mutated by either deletion (ARE mtΔ) or by substitution (ARE mtΔ) to 5’-GACATGGGC-3’ (31). Mutated bases are indicated in boldface, and are italicized. The Sp1 mutant is a triple mutant in which all three relevant Sp1-binding sites (−92/−85, −50/−36, and +68/+75) were mutated (11, 33). The promoter construct (0.5 µg) was transfected 1 hour before the addition of VPA (2 mmol/L) or tBHQ (20 µmol/L), and the cells were harvested 24 hours later, and processed for luciferase assay as described under “Materials and Methods.” A, substitution mutations at Sp1 sites (Sp1 mtΔ) or the ARE (ARE mtΔ) inhibit VPA-induced transcription from the Gαi2 gene promoter. DMt, promoter mutated at both Sp1-binding sites and ARE-binding site. Columns, mean for duplicate assays from six different experiments; bars, ± SE. B, tBHQ-induced Gαi2 gene promoter activity is inhibited by the deletion of, or substitution mutations at the ARE, but not by mutation at Sp1 sites (Sp1 mtΔ). Columns, mean for duplicate assays from four different experiments; bars, ± SE. Con, appropriate control in the absence of inducer (tBHQ or VPA).
proteins or other bZIP proteins, to the ARE (29, 30, 46–49), we did EMSA as well as ChIP analysis, to show that VPA induces binding of these transcription factors to the ARE region in the Gcl2 gene promoter. In the in vitro binding assay (i.e., EMSA), using nuclear extracts from VPA-treated cells and a double-stranded DNA probe containing the corresponding ARE sequence in the Gcl2 gene promoter, the protein-DNA complex could be clearly detected. The binding activity increased with time after treating cells with VPA, exhibited dose dependence, and was greater in VPA-treated cells than in control cells. Binding was competitively abolished by an unlabeled oligonucleotide (Fig. 5A, compare lanes 3 with lane 2, or lane 10 with lane 9), but was not affected by an unrelated oligonucleotide (consensus Sp1 oligonucleotide; Fig. 5A, compare lane 5 with lane 2, or lane 12 with lane 9) or a mutated oligonucleotide probe (Fig. 5A, compare lane 4 with lane 2, or lane 11 with lane 9). Furthermore, pretreatment of the cells with PDTC or NAC decreased the binding to basal levels or less (Fig. 5B). The sensitivity to antioxidants supports the idea that VPA-induced binding to this element required the activation of redox-sensitive transcription factors.

To verify that Nrf2 was contained in the protein-DNA complex, antibody to Nrf2 was added in the assay. The antibody depressed the band intensity of the complex, indicating that it prevented the interaction of Nrf2 with the ARE probe (Fig. 5C, compare lane 7 with lane 3). Interestingly, antibodies to small Maf proteins dramatically depressed the band density of the protein-DNA complex, not only in the basal condition (data not shown), but also in VPA-treated cells (Fig. 5C, compare lanes 4–6 with lane 3). Components of the AP-1 transcription factor complex (Fos and Jun) effectively form heterodimers with Maf through their leucine zipper structures (46). Therefore, it was not surprising that antibody to c-Fos also caused reduction in band intensity (Fig. 5C, compare lane 8 with lane 3). These results indicate the presence of Nrf2, small Maf(s), and c-Fos in the protein-DNA complex detected with the ARE probe.

Next, we used ChIP analysis to confirm the association of these transcription factors with the Gcl2 gene promoter, in the context of the intact cell. For the PCR analysis of the target region, we used a primer set that bracketed a 170 bp fragment (−234/−65) containing the ARE site (see Fig. 6A). As shown in Fig. 6B, PCR analysis of the target region showed that, within 1 to 2 hours of VPA treatment, there was increased association of a variety of redox-sensitive transcription factors with the promoter; these included c-Fos, Nrf2, and nuclear factor-κB (NF-κB), as well as small Maf proteins, which are known to be heterodimer partners of Nrf2 on the ARE on target gene promoters (29, 49). The PCR analysis also indicated the association of Sp1 with the promoter; this is expected because the area amplified in the ChIP analysis (see Fig. 6A) contained one (−92/−85) of the Sp1-binding sites that we previously identified to be involved in VPA-induced transcription from this promoter (ref. 11; see also Fig. 4A). No association was detected in the nontarget region (+1099/+1347) used as negative control (Fig. 6B, right) or in samples treated with normal IgG (Fig. 6B, left and right). These data complement the gel shift assays, and show that redox-sensitive transcription factors as well as Sp1 are recruited to the Gcl2 gene promoter in vivo during VPA-induced transcription from this promoter.

Figure 5. Binding of nuclear extract proteins to DNA probe containing ARE sequence. EMSA was done with nuclear extracts from control (cells that were not treated with VPA) and VPA-treated K562 cells, using the protocol described in detail elsewhere (31). The EMSA reactions were carried out with 2 μg of nuclear extract protein for each lane. When antibodies were used, the nuclear extract was incubated with the labeled probe for 20 minutes at 25°C prior to addition of each antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then incubated for an additional 30 minutes, followed by electrophoresis. The EMSA was done with a labeled double-stranded DNA probe 5′−GCCCGCCCCGAGCCAGTCACACAGGCTTGGTTC−3′, which contains the canonical ARE (underlined) that maps at −84/−76 in the Gcl2 gene promoter. The sequence of the mutated oligonucleotide was 5′−GCCCGCCCCGAGCCAGTCACACAGGCTTGGTTC−3′ (mutated bases are in boldface and italic). EMSA results shown are representative of three different nuclear extract preparations. A, DNA binding competition experiments with 20-fold excess of the following oligonucleotides: unlabeled oligonucleotide identical (in sequence) to the labeled oligonucleotide probe (lanes 3 and 10), mutated oligonucleotide (lanes 4 and 11), and Sp1 consensus oligonucleotide (lanes 5 and 12). Consensus Sp1 oligonucleotide was from Santa Cruz Biotechnology. Antibodies to Nrf2 and c-Fos were used at 2 μg (lanes 6, 7, 13, and 14). In VPA-treated cells, nuclear extracts were prepared 1 hour after the VPA (5 mmol/L) treatment. B, pretreatment of cells with antioxidants results in decreased binding to the ARE. K562 cells were incubated with PDTC (50 μmol/L) or NAC (5 mmol/L) for 1 hour before the addition of VPA (5 mmol/L). Lane 5, VPA; Lane 6, VPA + PDTC; Lane 7, VPA + NAC. C, effect of increasing amounts of antibodies to small Maf proteins. Use of antibodies to Nrf2 and c-Fos was repeated in this assay, for reference purposes. The amounts of anti–small Maf antibodies used in lanes 4, 5, and 6 were 0.5, 1, and 2 μg, respectively. Antibodies to Nrf2 and c-Fos were used at 2 μg (lanes 7 and 8).

Ab, antibody; NE, nuclear extract.
The present study provides an additional understanding of the transcriptional mode of action of VPA, with regard to elevation of other HDAC inhibitor–induced ROS production vis-a-vis gene expression; it provides a link between ROS-induced activation of redox-sensitive transcription factors (typified by Nrf2) and their subsequent interaction with the ARE on specific gene promoters. In the absence of the ARE sequence, VPA-induced transcription was not sensitive to antioxidants.

Although much remains to be learned about the ROS targets that are involved in signaling response(s) to HDAC inhibitor(s), it seems clear from the studies reported here that targeting through the ARE underlies the VPA-induced, ROS-mediated gene transcription. There may be additional means by which ROS production may indirectly influence HDAC inhibitor–induced gene transcription. For example, upon ROS-mediated activation of a redox-sensitive transcription factor, the transcription factor could become acetylated by acetylases such as PCAF/p300 to yield a transcriptionally active species. The transcriptionally active and acetylated forms of such factors would then be maintained in the nucleus by inhibition of HDACs if the cells were treated with HDAC inhibitor(s). This seems to be the case with SAHA-induced NF-κB-mediated gene transcription in lung cell carcinoma (23). Whether this applies to VPA-induced activation of Nrf2 or other redox-sensitive transcription factors is yet to be investigated. Taken together with the fact that hyperacetylation (5), Sp1–(11), and AP-1-binding motifs (5, 17–19) have already been implicated in VPA induction of gene expression, our present study highlighting the role of Nrf2 and the ARE clearly indicates that multiple mechanisms underlie the transcriptional response to VPA, and perhaps other HDAC inhibitors.

At the present time, the mechanism(s) by which VPA or any other HDAC inhibitor induces generation of ROS are unknown. Nonetheless, ROS generation by these compounds should be an
important consideration in their use as chemotherapeutic agents, especially because ROS are increasingly recognized as potent activators of signaling cascades culminating in the up-regulation of stress-responsive genes.

Acknowledgments


References

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