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Acetylation regulates the stability of glutamate carboxypeptidase II protein in human astrocytes

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ABSTRACT

Glutamate carboxypeptidase II (GCPII) is known to be implicated in brain diseases such as schizophrenia and bipolar disorder, and dramatically increases in prostate cancer. Here, we investigated the regulation of GCPII expression in astrocytes and examined whether *GCPII* is epigenetically regulated through histone modification. In this study, valproic acid (VPA), a drug used for bipolar disorder and epilepsy and a known histone deacetylase (HDAC) inhibitor was used. We found that acute exposure of VPA for 4–6 h increased the GCPII protein level in human astrocyte U87MG cells but did not have a similar effect after 12–24 h exposure. Real-time polymerase chain reaction analysis revealed that VPA did not affect the *GCPII* mRNA expression. In contrast, decrease in GCPII protein level by cycloheximide treatment was blocked by VPA, indicating that VPA increases GCPII protein stability. Treatment with MG132, a proteasome inhibitor, suggested that the VPA-induced increase of GCPII protein level is dependent on the ubiquitin/proteasome pathway. In addition, immunoprecipitation analysis revealed that VPA increased the acetylation of GCPII protein at the lysine residues and facilitated a decrease of the poly-ubiquitinated GCPII level. Similarly, M344, a specific HDAC 1/6 inhibitor, also increased the GCPII protein level. In contrast, treatment with C646, a histone acetyltransferase inhibitor of p300/CBP, significantly reduced the level of GCPII protein. Taken together, this study demonstrated that the increase in GCPII induced by VPA is not due to the classical epigenetic mechanism, but via enhanced acetylation of lysine residues in GCPII.

1. Introduction

Glutamate carboxypeptidase II (GCPII) is a zinc-dependent metallopeptidase expressed in many tissues, including the kidney, brain, intestines, and prostate [1]. In the intestine, the function of GCPII is identical to that of folate hydrolase, which hydrolyzes pteroyl poly-γ-glutamate to facilitate the uptake of folate. GCPII is also known as prostate-specific membrane antigen (PMSA), a prostate cancer marker. In the brain, GCPII is known for its ability to hydrolyze a neurotransmitter, N-acetyl-L-aspartyl-L-glutamate (NAAG), to N-acetyl-L-aspartate (NAA) and L-glutamate [2–4]. While glutamate plays a key role in long-term potentiation and learning and memory, the role of NAAG is not still known. Nonetheless, NAAG has been shown to function as a partial N-methyl-D-aspartate (NMDA) receptor antagonist [5] and a metabotropic glutamate receptor 3 (mGluR3) agonist [6]. Thus, under normal conditions, inhibition of GCPII can cause an imbalance between NAAG and NAA, which could interrupt the function of glutamate receptors. It has been reported that aberrant NAAG/NAA levels in the brain are associated with psychiatric diseases such as schizophrenia and bipolar disorder [7,8]. Recent studies also implicated GCPII in other neurological disorders such as inflammatory pain [9], stroke [10], diabetic neuropathy [11], amyotrophic lateral sclerosis [12], and Alzheimer's disease [13]. However, the role of GCPII in the development of these diseases and the regulation of GCPII expression in the brain are not fully understood.

Valproic acid (VPA) is a therapeutic drug widely used in the treatment of epilepsy [14], bipolar disorder, and migraine [15]. Although the detailed molecular mechanism underlying the therapeutic effects of VPA is unclear, VPA is known as a class I histone deacetylase (HDAC) inhibitor that regulates the expression of neuronal genes via epigenetic regulation. Brain-derived neurotrophic factor (BDNF), which influences memory, is epigenetically regulated by VPA via increase in histone H4 acetylation at promoter

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IV of *BDNF* in the prefrontal cortex of fear-conditioned rats [16]. In addition, VPA is reported to regulate the expression of mood disorder-associated genes such as *reelin* and *GAD67* by histone hyperacetylation and DNA demethylation within the promoter regions [17]. In clinical studies on schizophrenia and bipolar patients, the level of acetylated H3 and H4 in lymphocyte nuclear protein was increased by VPA [18]. Therefore, VPA is likely to stimulate gene expression through epigenetic mechanisms.

In this study, we examined whether and how GCPII is regulated by VPA treatment in astrocytes where it is known to be highly expressed. We found that short-term treatment of VPA increases the GCPII protein level in human astrocyte U87MG cells. This increase in GCPII protein levels was probably not due to classical epigenetic regulation via histone modification but instead due to the enhanced stability of GCPII protein by acetylation at lysine residue(s).

2. Materials and methods

2.1. Cell culture and drugs

Human astrocyte U87MG cells were obtained from the American Type Culture Collection and maintained in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS; Hyclone; South Logan, UT), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Gibco; Carlsbad, CA) in a humidified atmosphere of 5% CO_2 at 37 °C. VPA was purchased as sodium valproate from Handok Inc. (Seoul, Korea). Cycloheximide, MG132, M344, C646, dithiothreitol (DTT), and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO).

2.2. Transfection

The wild type construct of *GCPII* was cloned using the human *GCPII* sequence into the pcDNA3.1 vector as described previously [13] and was tagged with hemagglutinin A (GCPII-HA). Transfection was performed according to the manufacturer's instruction (Lonza; Walkersville, MD). Briefly, U87MG cells were suspended in a 100 μL mixture of Nucleofector solution and supplement, and treated with 5 μg wild type GCPII-HA, to facilitate transfection using the Amaxa nucleofection technology (Amaxa; Koeln, Germany). Transfected U87MG cells were plated on 100-mm dishes containing MEM supplemented with 10% FBS and penicillin/streptomycin and allowed to grow for 48 h.

2.3. Reverse transcription and real-time polymerase chain reaction (PCR)

Total RNA was isolated from U87MG cells according to the manufacturer's instruction (NucleoSpin RNA; Macherey-Nagel; Düren, Germany). One microgram RNA was converted to cDNA using reverse transcriptase (RT; Promega; Madison, WI) and oligo dT. PCR was then conducted using 2 μL of RT sample in a 20-μL reaction mixture containing Fast SYBR®-Green Master Mix (Applied Biosystems; Foster City, CA) and primers. The following PCR primers pairs were designed to detect each gene: *GCPII*-F, 5′-AGC GTG GAA ATA TCC TAA ATC TGA A-3′; *GCPII*-R, 5′-GAC CAA CAG CCT CTG CAA TTC-3′; *GAPDH*-F, 5′-CCA TGG AGA AGG CTG GGG-3′; *GAPDH*-R, 5′-GGT CAT GAG TCC TTC CAC GA-3′. The reaction conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 15 s, and 72 °C for 20 s. At the end of each cycle the fluorescence was measured and analyzed on a 7500 Fast Real-time PCR system (Applied Biosystems).

2.4. Western blotting analysis

U87MG cells were treated with various doses (0.1, 1, 10, and 50 μM) of VPA for 6 h, washed with ice-cold Dulbecco's phosphate-buffered saline (DPBS; Hyclone), and then lysed for 30 min on ice in RIPA buffer containing 20 mM Tris–Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease inhibitor cocktail tablets (Roche; Penzberg, Germany), protein phosphatase inhibitor cocktail (Sigma), and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). The protein concentration was determined using a BCA protein assay kit (Pierce; Rockford, IL). Equal amounts of protein (20 μg) were dissolved in SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM DTT, 0.01% bromophenol blue, and 10% glycerol) and separated by 8% SDS–polyacrylamide gel electrophoresis under reducing conditions, after which they were electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore; Billerica, MA). The blots were then probed with appropriate antibodies: anti-GCPII (1:1000; Exbio; Praha, Czech Republic), anti-β-actin (1:1000; Abcam; Cambridge, UK), anti-ubiquitin, and anti-acetyl-lysine (1:1000; Cell signaling; Danvers, MA). Anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) was then used as a secondary antibody at 1:5000 dilution; the blots were subsequently developed using enhanced chemiluminescence reagents (Advansta; Menlo Park, CA).

2.5. Immunoprecipitation

U87MG cells were harvested with RIPA buffer containing protease inhibitor cocktail tablets, protein phosphatase inhibitor cocktail, and 1 mM PMSF. Lysate (500 μg) was collected and pre-cleared with 50% slurry Sepharose G beads (GE Healthcare; Uppsala, Sweden). The supernatant was incubated with 2 μg anti-GCPII or anti-mouse IgG antibody as a negative control in RIPA buffer containing protease inhibitor cocktail and 1 mM PMSF at 4 °C for 16 h. The immune-complex was purified by Sepharose G for 2 h at 4 °C, and subjected to 8% SDS–PAGE, followed by western blot analysis with acetyl-lysine and ubiquitin antibodies.

2.6. Statistical analysis

All results are expressed as mean ± SEM. One-way ANOVA followed by post hoc Student–Newman–Keuls test was used for analysis of data; different characters were considered significant at $p < 0.05$. The Student's *t*-test was applied for analysis of significant differences between the 2 groups. The procedures were performed using SPSS software (version 18.0, SPSS Inc, Chicago, IL).

3. Results

3.1. VPA increases GCPII protein stability

To examine the epigenetic effects of VPA on GCPII expression in astrocytes, U87MG cells were treated with VPA at various doses (0, 0.1, 1, 10, and 50 μM) and harvested at specified times (4, 6, 12, and 24 h). As shown in Fig. 1A exposure of VPA for 4 h significantly increased the GCPII protein levels in a dose-dependent manner. A significant increase in the GCPII protein level was detected when treated with 10 μM VPA for 4 h (1.9-fold relative to the control) and 1 μM VPA for 6 h (1.2-fold relative to control). However, after VPA treatment for 12–24 h, there was no change in the GCPII protein levels among the samples. Therefore, all subsequent experiments were performed using 10 μM VPA for 4–6 h. In contrast, real-time PCR analysis showed that treatment of 10 μM VPA for 6 h did not affect *GCPII* mRNA expression. These results indicate

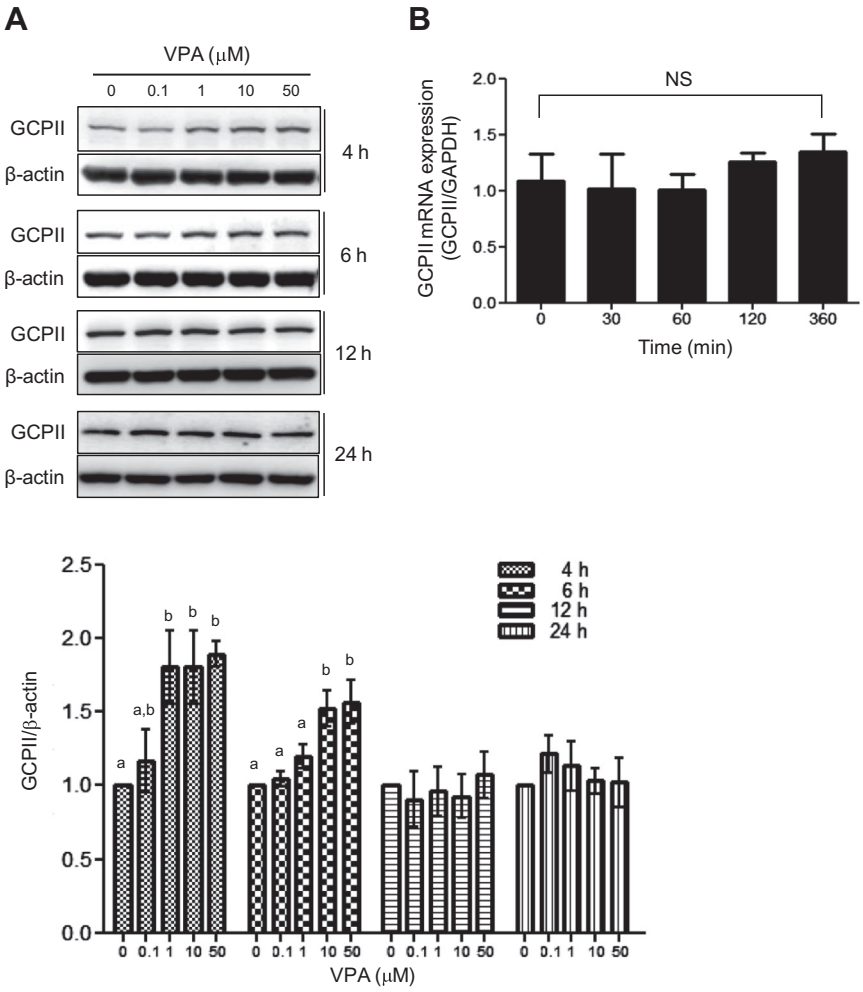

Fig. 1. VPA upregulates GCPII protein level, but does not affect the mRNA expression in human astrocyte U87MG cells. (A) Cells were treated with various doses (0, 0.1, 1, 10, or 50 μM) of VPA and harvested with RIPA buffer at 4, 6, 12, and 24 h as described in Section 2. The protein level of GCPII was detected by western blot analysis. The protein level of β-actin was measured for a control (top panel). Quantifications were performed using densitometry (Image J software) and the results were normalized to β-actin. The bar graph represents the mean ± SEM of 4–5 independent experiments. The different characters mean significant differences ($p < 0.05$) among the groups, which were determined by one-way ANOVA followed by post hoc Student–Newman–Keuls (bottom panel). (B) Total RNA was isolated from cells treated with 10 μM VPA for 0, 0.5, 1, 2, or 6 h. There were no significant (NS) changes in *GCPII* mRNA expression. The results from three independent experiments were normalized to *GAPDH* and expressed relative to the mRNA level of *GCPII* in no-treated control cells.

that acute exposure of VPA increases the GCPII protein levels, but this increase might be not due to the transcriptional regulation of *GCPII*. Based on this finding, we tested the effect of VPA on GCPII protein stability. Compared with control (cycloheximide 0 h) the GCPII protein level was significantly decreased by cycloheximide treatment for 6 h (0.71-fold of control), but this decrease was blocked by pretreatment with 10 μM VPA (1.2-fold of control) (Fig. 2A), suggesting that VPA increases the GCPII protein level via a posttranslational mechanism. In addition, MG132, an inhibitor of proteasome, increased the GCPII protein level and this increase was potentiated by co-treatment with VPA (Fig. 2B). Taken together, these results suggested that VPA regulates the stability of GCPII protein through the ubiquitin/proteasome-dependent pathway.

3.2. VPA enhances the acetylation of GCPII at lysine residues

It has been recently reported that HDACs such as SIRTs regulate FOXO3 protein degradation by directly increasing acetylation at lysine residues and consequently decreasing poly-ubiquitination [19]. Here, we tested whether VPA enhanced acetylation of GCPII protein at lysine residues. As shown in Fig. 3A, acetylated lysine residues on GCPII protein, which were immunoprecipitated with anti-GCPII antibody, were dramatically increased (1.8-fold compared to control) after 10 μM VPA treatment. We next measured the changes in the ubiquitination of GCPII. To this end, U87MG cells were transfected with HA-GCPII DNA were treated with 10 μM VPA for 6 h. As shown in Fig. 3B, GCPII immunoprecipitated with HA antibody was found to be highly acetylated and less ubiquitinated after VPA treatment. Our results indicate that VPA enhances acetylation at lysine residues of GCPII, which probably blocks ubiquitination of the protein.

3.3. Inhibition of both HDAC and histone acetyltransferase regulates the GCPII protein level

To clarify the role of acetylation in GCPII protein stability, we tested the effect of M344, a specific HDAC 1/6 inhibitor, on GCPII protein level in U87MG cells. As shown in Fig. 4A, acute exposure of M344 (0.1–10 μM) significantly increased the GCPII protein level in a dose-dependent manner. This result promptly led us to test the effect of deacetylation on GCPII protein level. Since histone acetyltransferases (HATs) are known to transfer acetyl groups to lysine residues of non-histones as well as histones, we used C646, an

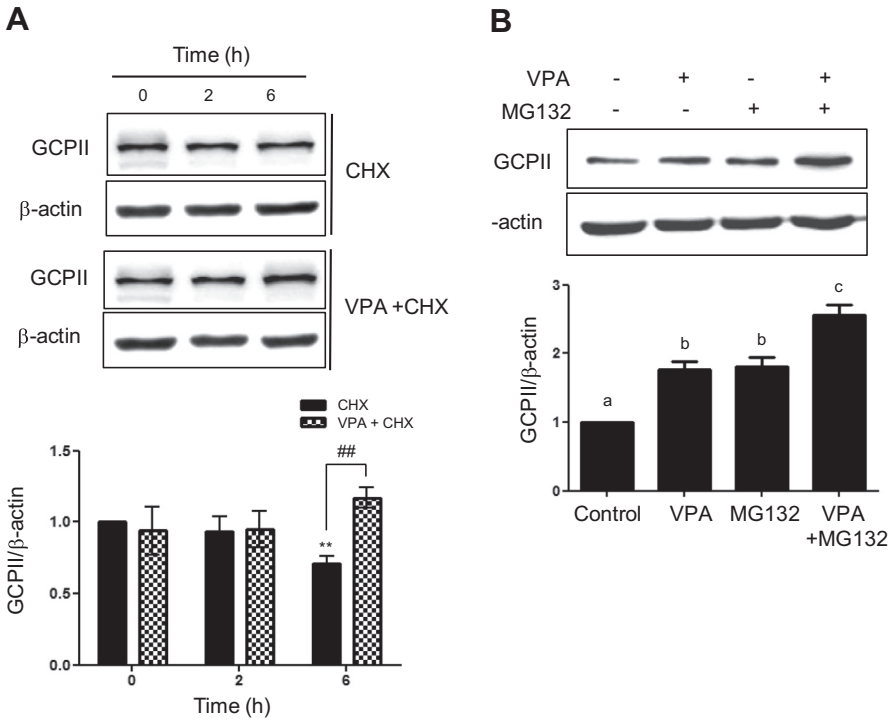

Fig. 2. VPA increases GCPII protein stability in U87MG cells. (A) To measure protein stability, cells were pretreated with 10 μM VPA for 1 h prior to cycloheximide (CHX) treatment. After CHX treatment, cells were harvested at specified times (0, 2, or 6 h). The results from three independent experiments were normalized to β-actin and expressed relative to GCPII protein level in no-treated control cells (CHX 0 h). Each bar represents the mean ± SEM of 3 independent experiments ($^{**}p < 0.05$ *vs* CHX 0 h; $^{\#\#}p < 0.01$ CHX 6 h *vs* VPA + CHX 6 h). (B) After pretreatment with VPA for 1 h, cells were exposed to 10 μM MG132 treatment for 4 h. The protein level of GCPII was detected by western blot analysis. Quantifications were performed using densitometry and the results were normalized to β-actin. The bar graph indicates the mean ± SEM of 3 independent experiments. The different characters mean significant differences ($p < 0.05$) among the groups, which were determined by one-way ANOVA followed by post hoc Student–Newman–Keuls test.

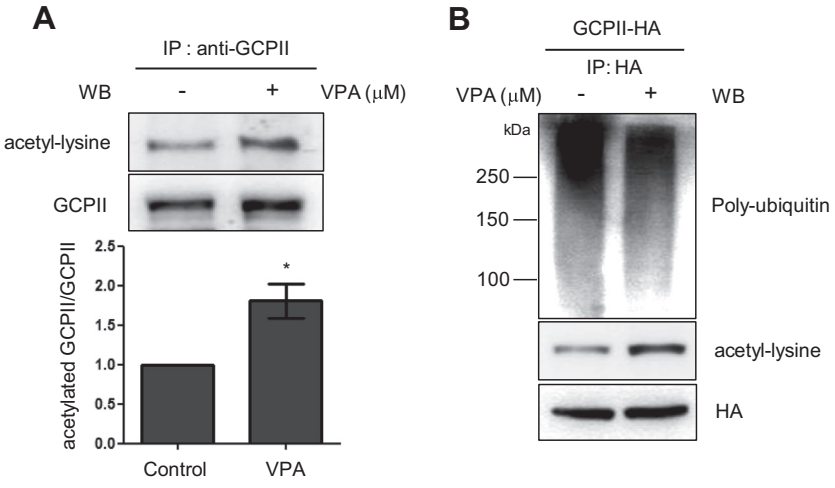

Fig. 3. VPA enhances the acetylation of GCPII at lysine residues, which may block ubiquitination. (A) After VPA treatment for 6 h, cells were harvested with RIPA buffer. Cell lysated were immunoprecipitated (IP) with anti-GCPII antibody as described in Section 2. Immunoprecipitates were dissolved with SDS sample buffer and separated by SDS-gel electrophoresis. The bar graph indicates the mean ± SEM of relative acetylated GCPII levels normalized to total GCPII and statistical significance is indicated by $^{*}p < 0.05$. (B) Cells were transfected with HA-GCPII DNA for 24 h. After transfection, cells were also treated with 10 μM VPA for 6 h. Cell lysated were immunoprecipitated with anti-HA antibody. Bound proteins in the immunoprecipitates were subjected to western blot analysis using the respective antibodies.

inhibitor of HAT p300/CBP, to block acetylation. As shown in Fig. 4B, acute treatment of C646 for 6 h significantly decreased the GCPII protein level in a dose-dependent manner. Taken together with previous results, our data strongly suggest that acetylation regulates the GCPII protein level in human astrocyte U87MG cells.

4. Discussion

In this study, we found that acute exposure of human astrocytes to VPA increases GCPII protein stability and that this increase is likely due to a direct acetylation of GCPII at its lysine residues rather than epigenetic regulation through histone modification.

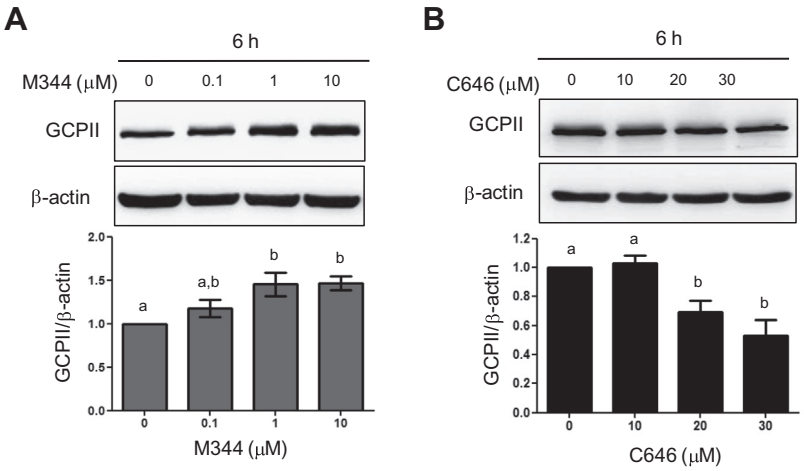

Fig. 4. Inhibition of HDAC or HAT regulates the GCPII protein level. U87MG cells were treated with M344, a specific 1/6 HDAC inhibitor, or C646, a HAT (p300/CBP) inhibitor, for 6 h. After treatment, cells were harvested with RIPA buffer as described in Section 2. The protein level of GCPII was detected by western blot analysis. The protein level of β-actin was measured for a control. Quantifications were performed using densitometry and the results were normalized to β-actin. The bar graph represents the mean ± SEM of 3 independent experiments. The different characters mean significant differences ($p < 0.05$) among the groups, which were determined by one-way ANOVA followed by post hoc Student–Newman–Keuls.

This conclusion is supported by the following evidences: (1) Increased level of GCPII induced by VPA was not correlated with increase in mRNA levels; (2) Precipitated GCPII from cell lysates isolated from VPA-treated cells showed more reactivity with anti-acetyl-lysine antibody; and (3) VPA increased acetylation of GCPII at lysine residues and reduced ubiquitination of GCPII. Therefore, our findings suggest a novel molecular mechanism by which enhanced acetylation of GCPII induced by VPA resists ubiquitination, which in turn, results in decreased degradation.

Our data show that GCPII protein was acetylated at lysine residues by VPA. HAT and HDAC are responsible for the transfer and removal of an acetyl group on the tail of histones, which is a well-known classical epigenetic mechanism regulating gene transcription. However, recent evidence shows that HAT and HDAC are also implicated in the acetylation of non-histone proteins. The first non-histone protein shown to be acetylated by HAT was p53 [20]. Sp1 is also known to be directly acetylated by an HDAC inhibitor, sodium butyrate, in a colon cell line [21]. Surprisingly, recent acetylomic studies have shown that thousands of proteins are acetylated in various cellular compartments [22,23]. These studies support our current results that GCPII is one such target protein and can be acetylated by an HDAC inhibitor, VPA.

Several studies investigating the role of protein acetylation in diverse biological processes have recently been reported. In particular, protein acetylation has been demonstrated to play an important role in protein stability. For example, the stability of hypoxia-inducing factor (HIF)-1α is increased through HDAC1/3 inhibitory activity in response to VPA [24], and via acetylation at lysine 709 (K709) of HIF-1α protein by HAT, p300 [25]. HDACs such as SIRTs regulate FOXO3 protein degradation by increasing the acetylation at lysine residues and consequently decreasing poly-ubiquitination. Under our experimental conditions, we also found that GCPII protein stability is increased by VPA, and is likely to be regulated by class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8). A search of acetylation and ubiquitination sites on GCPII using a posttranslational modification database (PHOSIDA, http://www.phosida.com) and ubiquitination prediction analysis (http://bdmpub.biocuckoo.org/prediction.php) showed four candidate lysine sites on human GCPII protein that can be acetylated and ubiquitinated (K207, K479, K491, and K699). Which specific lysine residue in GCPII is acetylated by VPA and thus less ubiquitinated warrants further investigation.

Regarding the regulation of GCPII expression, it has been reported in the prostate cancer cell line LNCaP that NFATc1 regulates PMSA expression by binding to a PMSA enhancer region localized within the third intron of the gene [26]. In addition, the LyF-1 transcription factor was reported to enhance transcription of the *GCPII* gene by binding to the core promoter region located in the proximal region (approximately 240 bp) of the transcription start site in LNCaP cells. However, this was not observed in astrocyte SVG [27], suggesting a cell-specific mechanism for regulating GCPII levels. Although many cells other than prostate cancer cells are known to express GCPII, the molecular mechanism regulating GCPII expression is poorly understood so far. Here, for the first time, we demonstrated that the GCPII protein level is regulated by acetylation at lysine residues in human astrocytes.

In summary, this study demonstrates a novel molecular mechanism by which VPA inhibits HDAC activity, which results in enhanced acetylation at lysine residues of the protein. Then, the acetylated protein is resistant to ubiquitination which leads to protein degradation.

Acknowledgments

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