Histone Deacetylase Inhibition Attenuates Transcriptional Activity of Mineralocorticoid Receptor Through Its Acetylation and Prevents Development of Hypertension

Hae-Ahm Lee, Dong-Youb Lee, Hyun-Min Cho, Sang-Yeob Kim, Yasumasa Iwasaki, In Kyeom Kim

Rationale: Inhibition of histone deacetylases (HDACs) results in attenuated development of hypertension in deoxycorticosterone acetate–induced hypertensive rats and spontaneously hypertensive rats. However, the molecular mechanism remains elusive.

Objective: We hypothesized that HDAC inhibition attenuates transcriptional activity of mineralocorticoid receptor (MR) through its acetylation and prevents development of hypertension in deoxycorticosterone acetate–induced hypertensive rats.

Methods and Results: Expression of MR target genes was measured by quantitative real-time polymerase chain reaction. Recruitment of MR and RNA polymerase II on promoters of target genes was analyzed by chromatin immunoprecipitation assay. Live cell imaging was performed for visualization of nuclear translocation of MR. MR acetylation was determined by Western blot with anti-acetyl-lysine antibody after immunoprecipitation with anti-MR antibody. Transcriptional activity of MR was determined by luciferase assay. For establishment of a hyperaldosteronism animal, Sprague-Dawley rats underwent uninephrectomy and received subcutaneous injection of 40 mg/kg per week of deoxycorticosterone acetate and drinking water containing 1% NaCl. Treatment with a HDAC class I inhibitor resulted in reduced expression of MR target genes in accordance with reduced recruitment of MR and RNA polymerase II on promoters of target genes. HDAC inhibition promoted MR acetylation, leading to decreased transcriptional activity of MR. Knockdown or inhibition of HDAC3 resulted in reduced expression of MR target genes induced by mineralocorticoids.

Conclusions: These results indicate that HDAC inhibition attenuates transcriptional activity of MR through its acetylation and prevents development of hypertension in deoxycorticosterone acetate–induced hypertensive rats. (Circ Res. 2013;112:1004-1012.)

Key Words: animal model cardiovascular disease ■ gene transcription ■ HDAC inhibitor ■ hypertension ■ mineralocorticoid receptor

Histone deacetylase inhibitors (HDACi) have exhibited antifibrotic, anti-inflammatory, antihypertrophic, and antihypertensive effects.1,2 HDACs are widely implicated in production of inactive heterochromatin through deacetylation of localized chromatin.3 Most genes localized in heterochromatin are inactivated in terms of expression.3,4 However, recent studies have reported that many genes are repressed by HDACi,5,6 suggesting a more complex function of HDACs in regulation of gene expression. HDACi, which causes hyperacetylation on chromatin, represses gene expression in spite of activated promoters,7 indicating that HDACi affects acetylation of nonhistone proteins such as transcription factors. Direct acetylation of transcription factors results in either inhibition8 or activation9 of gene expression. Several studies have reported that some HDACs act as a coactivator of transcription factors, including nuclear receptors.10,11

Human nuclear receptor superfamily has 48 members, among which the androgen receptor (AR), the estrogen receptor (ER), the progesterone receptor, the glucocorticoid receptor (GR), and the mineralocorticoid receptor (MR) belong to the steroid receptor family.12 The transcriptional activity of steroid receptors is mainly regulated by ligand; however, post-translational modifications, such as phosphorylation, acetylation, ubiquitylation, and sumoylation, also

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play an important role in their stabilization, nuclear translocation, or transcriptional activity.\textsuperscript{13} The post-translational modifications of estrogen receptor and AR, which regulate transcriptional activity, have been well investigated in breast cancer and prostate cancer, respectively, whereas that of MR is not known.\textsuperscript{14}

Aldosterone (Aldo), a mineralocorticoid, is one of the final effectors of the renin–angiotensin–aldosterone system and mediates genomic and nongenomic effects of MR. Aldo is a regulator of blood volume and pressure by handling sodium and potassium homeostasis. Up to 15\% of patients with essential hypertension have inappropriate regulation of Aldo.\textsuperscript{15} MR modulates kidney function through induction of oxidative stress, as well as expression of epithelial Na\textsuperscript{+} channel, Na\textsuperscript{+}-K\textsuperscript{+}-ATPase subunit α1 (ATP1a1), glucocorticoid-induced leucine zipper (GILZ), and serum and glucocorticoid-regulated kinase 1 (SGK-1). Epithelial Na\textsuperscript{+} channel and ATP1a1 are major Na\textsuperscript{+}-transporting proteins whose expression and activities are regulated in the kidneys by mediators such as GILZ and SGK-1.\textsuperscript{16} MR can also induce extracellular matrix proteins, such as collagen I, III, and IV and proinflammatory factors, including intercellular adhesion molecule, monocyte chemoattractant protein, cytokines, and cyclooxygenase-2 in cardiac and renal tissues.\textsuperscript{17,18} Therefore, MR is a potential target of hypertension, hypertrophy, inflammation, and fibrosis. All of the above pathological conditions are effectively attenuated by HDACi\textsuperscript{1,2,19} however, the molecular mechanisms are still unclear.

We hypothesized that HDACi attenuates transcriptional activity of MR through its acetylation and prevents development of hypertension in deoxycorticosterone acetate (DOCA)–induced hypertensive rats. In addition, certain HDAC is responsible for MR deacetylation, which is critical for transcriptional activity of MR.

### Methods

An expanded Methods section is available in the online Data Supplement.

### Animals

The investigation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the institutional review board of Kyungpook National University School of Medicine, and every effort was made to minimize both the number of animals used and their suffering. Sixteen-week-old male Sprague-Dawley rats were unilaterally nephrectomized under ketamine (150 mg/kg, Yuhan, Seoul, Korea) and xylazine (18 mg/kg; Bayer, Seoul, Korea) anesthesia. After unilateral nephrectomy, rats were allowed to recover overnight before being randomly assigned to one of 3 groups: control (n=4), DOCA-salt (n=4), and DOCA-salt plus valproic acid (VPA; n=4). DOCA-salt was injected subcutaneously at 40 mg/kg per week for a period of 4 weeks. The animals had free access to 1\% NaCl with or without 0.71\% VPA.

### Statistics

Results are expressed as mean±SE. Kruskal–Wallis test and 1-way ANOVA followed by post hoc Tukey’s comparison test were used for analysis of data; differences were considered significant at \(P<0.05\). The Student \(t\) test was applied for analysis of significant differences.
between the 2 groups. The procedures were performed using SPSS software (release 19.0, SPSS Inc, Chicago, IL).

**Results**

**HDACi Attenuated Expression of ATP1a1, GILZ, and SGK-1 Induced by Aldosterone**

Expression of MR target genes was investigated by quantitative real-time polymerase chain reaction (PCR). Treatment of HEK293 cells with Aldo (10 nmol/L) resulted in significantly increased expression of *ATP1a1* and *GILZ* after 16 hours of Aldo stimulation. Expression of *SGK-1* showed a significant increase after 24 hours (Figure 1A). MR target genes were induced by Aldo in a dose-dependent manner. Pretreatment with VPA (1 mmol/L, 6 hours) resulted in significantly attenuated Aldo-induced expression of *ATP1a1*, *GILZ*, and *SGK-1* (Figure 1B–1D). MS-275, a specific inhibitor of class I HDAC, also attenuated Aldo-induced expression of these genes (Online Figure I).

**HDACi Attenuated Aldo-Induced Recruitment of Wild-Type MR and Polymerase II on Target Gene Promoters in HEK293 Cells**

Enrichment of MR and RNA polymerase II (Pol II) on promoters of *GILZ* and *SGK-1* was analyzed by chromatin immunoprecipitation assay. A consensus glucocorticoid response element (GRE) sequence (CATACACACTGTTCT) was found in the human *GILZ* promoter at position -3275. Valproic acid (VPA) attenuates recruitment of mineralocorticoid receptor (MR) and polymerase II (Pol II) on target gene promoters induced by aldosterone (Aldo) in HEK293 cells. Schematic diagrams show the locations of glucocorticoid response element (GRE) and polymerase chain reaction (PCR) amplification after chromatin immunoprecipitation (ChIP) in *GILZ* (A) and *SGK-1* (C) promoters (upper). Representative gels of conventional PCR (input, 27 cycles; others, 32 cycles) show that Aldo (10 nmol/L) increased the enrichment of MR and Pol II on GILZ and SGK-1 promoters, which were decreased by VPA (1 mmol/L, 6 h) pretreatment (lower). B and D, The ChIP assays were quantified by real-time PCR. Graph, Mean±SE of 4 independent experiments (*P*<0.05, **P*<0.01 vs vehicle, and #P*<0.05 Aldo vs VPA pretreatment+Aldo [10 nmol/L]). TSS indicates transcription start site.

**HDACi Does Not Affect Nuclear Translocation but Increases Acetylation of Mineralocorticoid Receptor (MR).**

HEK293 cells were transfected with green fluorescent protein (GFP)-tagged MR expression vector and stimulated with aldosterone (Aldo; 10 nmol/L) after pretreatment with vehicle (A) or VPA (1 mmol/L, 6 h) (B). Most MR moved into the nucleus within 30 min after Aldo stimulation. HEK293 cells transfected with hemagglutinin (HA)-tagged MR were treated with Aldo for 30 min after pretreatment with VPA or vehicle for 6 h and then lysed for Western blotting (WB). MR acetylation was probed with antiacetyl-lysine antibody after immunoprecipitation with anti-MR or anti-HA antibodies. A representative immunoblot (C) and densitometry (D) show that treatment with VPA resulted in increased MR acetylation only after stimulation with Aldo. Graph, Mean±SE of 4 independent experiments (**P*<0.01 vs con).
Results of conventional PCR showed that treatment with Aldo resulted in increased enrichment of wild-type MR and Pol II on the GRE sequence, which was decreased by pretreatment with VPA (Figure 2A, lower). However, VPA pretreatment did not attenuate the enrichment of mutant MR induced by Aldo on the GILZ promoter (Online Figure IIB).

Recruitment of wild-type MR, mutant MR, and Pol II on the GILZ promoter was confirmed by quantitative real-time PCR (Figure 2B; Online Figure IIC). A consensus GRE sequence (TGTTCATTGTGTTCT) was found in the human SGK-1 promoter at position −429/−414 from transcription start site (Figure 2C, upper). Results of conventional PCR (Figure 2C, lower) and quantitative real-time PCR (Figure 2D) showed that recruitment of wild-type MR and Pol II enriched by Aldo was significantly attenuated by pretreatment with VPA. Pretreatment of VPA showed little effect on enrichment of mutant MR induced by Aldo on the SGK-1 promoter (Online Figure IIE and IIF).

VPA Had Little Effect on MR Translocation but Increased MR Acetylation

Live cell imaging was performed for visualization of nuclear translocation of MR. The green fluorescent protein (GFP)-tagged MR was traced by fluorescence microscopy every 10 minutes (Figure 4).

Figure 4. Valproic acid (VPA) increased acetylation at the hinge region of mineralocorticoid receptor (MR) and reduced its transcriptional activity. A, HEK293 cells were cotransfected with expression vectors of wild-type MR, AA-mutant MR, or RR-mutant MR, as well as luciferase vector conjugated with ATP1a promoter. Pretreatment with VPA (1 mmol/L, 6 h) resulted in significant decrease of Aldo-induced promoter activity in HEK293 cells transfected with wild-type MR, but not mutant MR. Graph, Mean±SE from 3 independent experiments. The different character mean significant differences (P<0.05) among the groups, which were determined by 1-way ANOVA followed by post hoc Tukey's comparison test. B, Comparison of amino acid sequences between human and rat MR hinge regions. C, Pretreatment with VPA followed by treatment with Aldo resulted in increased MR acetylation in HEK293 cells transfected with wild-type MR, but not AA-mutant MR. Graph, Mean±SE of 4 independent experiments. (*P<0.05 and **P<0.01).

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Figure 5. HDAC3 is responsible for mineralocorticoid receptor (MR) deacetylation. A, HEK293 cells were cotransfected with HA-tagged MR and a Flag-tagged class I histone deacetylase (HDAC). The cell nuclear fraction was isolated after stimulation with Aldo (10 mmol/L) for 30 min. MR was precipitated by anti-HA antibody to which Flag antibodies were immunoblotted for detection of HDAC. HDAC3 interacted with MR. B, The interaction among MR, NcoR, and HDAC3 was analyzed by Co-immunoprecipitation and Western blot (WB). Aldosterone (Aldo) stimulation (10 mmol/L) reduced the interaction between MR and NCoR. Pretreatment with valproic acid (VPA; 1 mmol/L) resulted in near inhibition of the interaction between MR and HDAC3. C, HEK293 cells were transfected with HDAC3 siRNA (10 nmol/L) for 48 h, which resulted in decreased expression of HDAC3 protein (upper) and increased MR acetylation (lower). Knockdown of HDAC3 resulted in significantly attenuated expression of GILZ (D) and SGK-1 (E) induced by Aldo (*P<0.05 vs con and #P<0.05, NT vs HDAC3 siRNA). N.T indicates non targeting.
seconds. The cells were stimulated with Aldo (10 nmol/L) after pretreatment with VPA (1 mmol/L, 6 hours) or vehicle. The GFP-tagged MR was diffusely distributed in the cells before stimulation with Aldo. Aldo stimulation immediately triggered nuclear translocation of MR, which was almost completed within 30 minutes of Aldo stimulation (Figure 3A). Pretreatment with VPA had little effect on MR nuclear translocation (Figure 3B). Even though treatment with Aldo or HDACi alone had little effect on MR acetylation, pretreatment with HDACi, followed by treatment with Aldo, resulted in significantly increased MR acetylation, compared with other cells (Figure 3C and 3D; Online Figure IIIA and IIIB).

Transcriptional Activity of MR Was Decreased by Acetylation

The luciferase assay was used for evaluation of promoter activity in response to Aldo. The ATP1a1 promoter was fused with the pA3luc vector and induced by Aldo. Treatment with Aldo resulted in profound induction of promoter activity, which was significantly decreased by pretreatment with VPA (P<0.05) only when HEK293 cells were transfected with wild-type MR (Figure 4A). Using a site-directed mutagenesis kit, 2 lysines in the hinge region of MR were substituted with alanine or arginine (Figure 4B). Aldo also induced ATP1a1 promoter-fused pA3luc vector in HEK293 cells transfected with mutant MR in which VPA did not inhibit transcriptional activity induced by Aldo (Figure 4A). Mutation of MR resulted in significantly decreased MR acetylation even after pretreatment with VPA, followed by stimulation with Aldo, compared with wild-type MR (Figure 4C). MS-275 showed similar effect on transcriptional activities of wild-type and mutant MR (Online Figure IIIC).

HDAC3 Enhanced Transcriptional Activity of MR

VPA primarily inhibits HDAC class I (1, 2, 3, and 8) and class IIa (4, 5, and 7). We investigated interaction between MR and HDAC class I because class I HDACs are mainly located in the nucleus. Co-immunoprecipitation data showed that MR interacts with HDAC3 regardless of Aldo treatment (Figure 5A). MR was enriched in the nucleus after Aldo stimulation (Figure 5A, input, upper), whereas HDAC protein in the nucleus was not changed by Aldo treatment (Figure 5A, input, lower). Treatment with Aldo resulted in reduced interaction among HDAC3, MR, and nuclear receptor co-repressor. Pretreatment with VPA resulted in almost complete blockade of the interactions of these proteins (Figure 5B). Knockdown of HDAC3 by siRNA resulted in increased acetylation of MR (Figure 5C), leading to decreased expression of MR target genes, including GILZ (Figure 5D) and SGK-1 (Figure 5E) induced by Aldo.

VPA Attenuated DOCA-Salt–Induced Hypertension and MR Target Gene Expression

Using the tail-cuff method, systolic blood pressure was measured and recorded for 4 weeks. Unilateral nephrectomy followed by administration of 1% NaCl had little effect on systolic blood pressure. Addition of DOCA-salt infusion resulted in significantly increased systolic blood pressure after 3 weeks (P<0.05 versus vehicle), which was abrogated by

![Figure 6. Effect of valproic acid (VPA) administration on blood pressure, body weight, and mineralocorticoid receptor (MR) target gene expression in deoxycorticosterone acetate (DOCA)-salt–induced hypertensive rats. A, Blood pressures were measured using the tail-cuff method in the vehicle group (n=4), DOCA-salt infusion group (n=4), and DOCA-salt infusion and VPA administration group (n=4) for a period of 4 weeks. Administration of VPA resulted in restoration of DOCA-salt–induced hypertension (P<0.05 vs vehicle). B, Body weights were monitored for 4 weeks. DOCA-salt infusion with or without VPA administration did not have an effect on body weight gain. DOCA-salt infusion resulted in increased gene expression of ATP1a1 (C), GILZ (D), and SGK-1 (E) in rat kidneys, which was attenuated by VPA administration (P<0.05, **P<0.01 vs vehicle, and #P<0.05 vs DOCA group).](http://circres.ahajournals.org/doi/fig/10.1161/01.RES.0000471135.55927.d2)
administration of VPA (Figure 6A). Neither DOCA-salt infusion nor VPA administration affected gain of body weight (Figure 6B). We analyzed expression of 3 major MR target genes, *ATP1a1*, *GILZ*, and *SGK-1*, in the kidney. DOCA-salt infusion resulted in profoundly induced expression of *ATP1a1* (Figure 6C; *P*<0.05 versus vehicle), *GILZ* (Figure 6D; *P*<0.05 versus vehicle), and *SGK-1* (Figure 6E; *P*<0.01 versus vehicle), which was attenuated by administration of VPA (Figure 1C–1E; *P*<0.05 versus DOCA-salt infusion group).

### VPA Attenuated Enrichment of MR and Pol II on Target Gene Promoters Induced by DOCA In Vivo

The GREs in the promoter of rat *GILZ* were searched by chromatin immunoprecipitation scanning in rats.22 Two GRE sequences (-2370/-2355 and -1873/-1853) were found in the *GILZ* proximal promoter from transcription start site to -5 kb upstream (Figure 7A, upper). Infusion of DOCA salt resulted in enrichment of MR and Pol II on a GRE in the *GILZ* proximal promoter, which was attenuated by administration of VPA (*P*<0.01) (Figure 7A, lower). Infusion of DOCA salt also resulted in enrichment of MR (*P*<0.05 versus vehicle) and Pol II (*P*<0.01 versus vehicle) on a GRE in the *SGK-1* promoter, which was attenuated by administration of VPA (*P*<0.05; Figure 7B).

### Discussion

Results of the current study demonstrate that HDACi attenuates transcriptional activities of MR in vitro and in vivo. HDACi increases MR acetylation, which reduces transcriptional activity of MR and prevents development of hypertension. Among the HDAC class I, HDAC3 catalyzes MR deacetylation, which restores transcriptional activity of MR.

HDACs are known to play an important role in remodeling of chromatin structures. HDAC induces hypoacetylation of localized chromatin domain, which widely participates in gene repression/silencing. Intriguingly, the ability of several HDACs and histone acetyltransferases (HATs) to deacetylate and acetylate nonhistone protein substrates (eg, transcription factors), respectively, has been demonstrated, suggesting the possibility that protein acetylation provides a rapid and reversible regulatory mechanism like protein phosphorylation.23 HDACs and HATs maintain a relative balance in a normal physiological condition. However, HDAC activity is elevated in pathological conditions, including hypertension.2 Although HDACs have long been known as corepressors, recent study has shown that HDAC serves as a coactivator of GR.10 Acetylation of lysine at a specific motif (KTKK) in the hinge region of GR by circadian locomotor output cycles kaput/brain and muscle aryl hydrocarbon receptor nuclear translocator–like protein 1 (BMAL1) complex resulted in repression of transcriptional activity of GR.24 Similar to this result, treatment with HDACi resulted in increased acetylation of MR in HEK293 cells (Figure 3C and 3D; Online Figure IIIA and IIIB) and kidneys of DOCA-salt–induced hypertensive rats (Online Figure VIIB). Point mutations of 2 lysines in the hinge region of MR attenuated the HDACi effect, which repressed transcriptional activity of MR (Figure 4A; Online Figure IIIIC). HDACi reduces expression of extracellular matrix protein, such as collagen and inflammatory markers, including several interleukins, tumor necrosis factor, and nuclear factor κB in spontaneously hypertensive rats2 and DOCA-salt–induced hypertensive rats.1 Our results also showed that HDACi resulted in significantly decreased expression of some MR target genes, including *ATP1a1*, *GILZ*, and *SGK-1* (Figure 1C–1E; Online Figure III).
The treatment of HDACi resulted in decreased recruitment of wild-type MR and Pol II on the promoters of target genes in vitro (Figure 2) and in vivo (Figure 7). Although the recruitment of mutant MR on the promoters of target genes was increased by Aldo, HDACi showed little effect on the recruitment (Online Figure II). Ligand-bound MR translocates into the nucleus to act as a transcription factor by binding to specific hormone-responsive elements in target genes, which are potentially located up to 10 kb upstream or downstream from transcription start site.22,27 Hormone-bound receptor recruits the coactivator complex, which facilitates target gene transcription by recruitment of RNA polymerase.28 Several studies have reported that, among the components of MR coactivator complex, steroid receptor coactivator-1 and p300/ CBP, which belong to the HAT families, play a crucial role in ligand-bound MR activity.29 Up to date, little is known about how MR acetylation regulates its transcriptional activity. We suggest that HDAC enforces transcriptional activity of MR in hyperaldosteronism-induced hypertension because HDACi ameliorates the actions of mineralocorticoid. Among the class I HDAC, HDAC3 is responsible for regulation of MR transcriptional activity because MR interacted with only HDAC3, of which knockdown resulted in increased MR acetylation and suppressed expression of MR target genes (Figure 5).

Some steroid hormone receptors are known to regulate their activity by acetylation. Elimation of acetylation showed strong AR activity on the promoter of prostate-specific antigen, but not of the Pem homeodomain gene; therefore, the effect of acetylation on AR is promoter-specific.31 Acetylation of estrogen receptor-α normally results in suppression of ligand sensitivity, which is revealed by mutation of lysine residues 302 or 303 to arginine.32,33 Progesterone receptor hinge region regulates the kinetics of nuclear translocation and transcriptional responses of progesterone receptor through acetylation and phosphorylation.34 Acetylation by circadian locomotor output cycles kaput/BMAL1 in the hinge region of GR results in reduced transcriptional activity.24 However, acetylation of MR and its effect on physiology are unknown. Potential consensus sequences of acetylation in the MR hinge region are found in several species, including human and rat (Figure 4B). Treatment of HDACi resulted in increased acetylation of MR in HEK293 cells (Figure 3C and 3D; Online Figure IIIA and IIIB) and the kidneys of DOCA-salt–induced hypertensive rat (Online Figure VI). Inhibition or knockdown of HDAC3 resulted in decreased transcriptional activity of MR. Point mutations of lysines in the hinge region of MR attenuated the effect of HDACi in decreasing transcriptional activity of MR (Figure 4). Together with the above results, we suggest that HDAC3 increases the transcriptional activity of MR by deacetylation in the hinge region of MR, as summarized in Figure 8.

MR expression has been reported in many tissues, both epithelial and nonepithelial tissues. In epithelial tissues, such as kidney and colon, MR regulates salt balance and water homeostasis through induction of specific ion channels and transporters, for example, epithelial Na+ channel and ATP1a1, as well as specific mediators, including GLIZ and SGK-1.35 Aldo and DOCA significantly induced ATP1a1, GLIZ, and SGK-1 in vitro (Figure 1) and in vivo (Figure 6C–6E), respectively. Owing to abundant expression of 11βHSD2, which catalyzes active cortisol to inactive cortisone, kidney and colon tissues are highly sensitive to mineralocorticoid. The discovery of new sites of MR expression in nonepithelial tissues, such as the heart, vasculature, brain, and adipocytes, has led to identification of novel MR target genes with unexpected biological functions in the tissues.16 For example, in aortic endothelial cells, treatment with Aldo has been shown to result in increased ACE expression, which may be involved in the development of endothelial dysfunction, elevated blood pressure, and vascular injury induced by this steroid.36 In addition, the adverse effects of Aldo are increments of proinflammatory molecules, extracellular matrix protein, and reactive oxygen species in nonepithelial tissues. The Randomized Aldactone Evaluation Study showed 30% reduction in mortality and 35% reduction in morbidity with low-dose spironolactone in patients with severe heart failure.37 The Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study showed substantial improvements in patient outcomes with eplerenone after myocardial infarction with systolic dysfunction.38 Moreover, a blockade of MR receptor improves the clinical outcomes of proteinuric kidney diseases.39 It is speculated that HDACi or combination with MR antagonist would be one of new strategies for treating cardiovascular disease.

In summary, the present study reveals one of the mechanisms that HDACi attenuates transcriptional activity of MR through increasing its acetylation. MR acetylation reduced hormone-responsive element binding affinity, RNA polymerase recruitment, and expression of target genes, which regulate intracellular salt balance. HDAC3 enhances MR activity via deacetylation in the hinge region of MR. Although exact mechanism of HDACi for treating cardiovascular disease remains elusive, HDAC is a potential target of metabolic disorder–associated diseases, including hypertension.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Mineralocorticoid receptor (MR) plays a central role in the control of blood pressure.
- MR acts as a transcription factor, which on aldosterone binding, translocates from the cytosol into the nucleus.
- Histone deacetylases (HDACs) catalyze deacetylation of histones and other nonhistone proteins.

**What New Information Does This Article Contribute?**

- Acetylation of MR negatively regulates its transcriptional activity.
- HDAC3 catalyzes deacetylation of MR.
- Inhibition of HDAC attenuates mineralocorticoid-induced hypertension by inducing MR acetylation.

HDAC inhibitors attenuate fibrosis, hypertrophy, inflammation, and hypertension in several animal models. However, the molecular mechanism is unclear. The present study was conducted to elucidate the molecular mechanism by which class I HDAC inhibitors attenuate mineralocorticoid-induced hypertension. Though inhibitors of class I HDAC had little effect on nuclear translocation of MR, these inhibitors attenuated transcriptional activity of MR by increasing acetylation of its hinge region. Activated MR elevated blood pressure by increasing sodium reabsorption in the kidney. The results of our study show that inhibitors of class I HDAC ameliorate mineralocorticoid-induced hypertension in part by reducing transcriptional activity of MR in rat kidney. Traditionally, HDAC3 complex has been considered as a corepressor of nuclear receptors. The present study, however, reveals that HDAC3 can be one of coactivators of MR as well. HDAC3 eliminates acetylation in the hinge region of MR, which increases binding affinity of MR on promoter of target genes and recruitment of RNA polymerase. Although the beneficial and adverse effects of these HDAC inhibitors need to be further evaluated, our findings suggest that class I HDAC inhibitors are potential antihypertensive agents that may be useful in treating patients with hyperaldosteronism.
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SUPPLEMENTAL MATERIAL

Histone Deacetylase Inhibition Attenuates Transcriptional Activity of Mineralocorticoid Receptor through its Acetylation and Prevents Development of Hypertension

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Short title: HDAC inhibition attenuates hypertension

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Supplemental Materials and Methods

Cell culture
Human embryonic kidney cells (HEK293) were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS), 50 μg/ml penicillin and streptomycin at 37 °C in a 5% CO₂ humidified incubator. The growth media was changed with 2.5% charcoal-stripped FBS before aldosterone (10 nmol/L) treatment with or without pretreatment of VPA (1 mmol/L) and MS-275 (10 μmol/L) for 6 h.

Blood pressure measurement
The blood pressure of the rats was measured by tail cuff method. Rats were preheated on a hotplate at 35 °C for 10 min and then placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail. Blood pressure values were recorded on a NIBP controller system (ADInstruments Pty Ltd, Castle Hill, NSW, Australia) with heating and were averaged from at least five consecutive readings obtained from each rat.

Quantitative real-time PCR (qRT-PCR)
Tissues (about 100 mg) were homogenized in liquid nitrogen with a glass homogenizer. RNA was extracted by using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s recommendations from HEK cells or homogenized tissues. Total RNA (2 μg) was reverse-transcribed into cDNA by using RevertAid™ first strand cDNA synthesis kit (Fermentas, EU) in 20 μl reaction volume according to manufacturer’s instructions. Quantitative real-time-PCR (qRT-PCR) was performed using ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). Ten micro liter of SYBR Green PCR master mix (TaKaRa, Japan), 4 μl of cDNA, and 200 nmol/L primer set were used for amplification in 20 μl reaction volume. All samples were amplified in triplicates in a 96-well plate and the cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s followed by 1 min at 60°C. The relative mRNA expression level was determined by calculating the values of Δcycle threshold (ΔCt) by normalizing the average Ct value compared with its endogenous control (Gapdh) and then calculating $2^{-ΔCt}$ values. All primer sets used in qRT-PCR are shown in Table I.

Chromatin immunoprecipitation (ChIP) assay
ChIP analysis was performed according to the manufacturer’s instructions with minor modification using EZ ChIP kit (Upstate Biotechnology, Lake Placid, NY). In brief, HEK cells or tissues were fixed with 1% formaldehyde, and washed with ice-cold PBS. After homogenization, tissues were incubated with SDS lysis solution for 10 min on ice. The lysate were sonicated with 15 cycles of 100 amplitude of sonication for 10 s followed by cooling on ice for 50 s. The lysate were pre-cleared with protein G agarose beads for 2 h. Then 1 μg of MR antibodies (Abcam, Cambridge, UK) were added and incubated at 4°C overnight. Soluble chromatin captured by specific antibodies was harvested by protein G agarose bead. The beads were washed serially with a low-salt solution, high-salt solution, LiCl solution, and TE solution twice. The antibody-chromatin complexes were eluted from the beads with a solution containing 1% of SDS and 0.1 mol/L of NaHCO₃. To reverse the crosslinking between DNA and chromatin, elutes were incubated at 65°C for 5 h after addition of NaCl to a final concentration of 0.2 mol/L. The proteins were eliminated by digestion with proteinase K at 45°C for 2 h, and the DNA was purified with a spin column. A specific promoter DNA was quantified by real-time PCR. All samples were amplified in triplicates in a 96-well plate and the cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s followed by 1 min at 60°C. The primer set used in ChIP assay is shown in Table I.
Immunoprecipitation and Western blot

The frozen tissues were homogenized in lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1x proteinase inhibitor cocktail). The cell lysates were precleared with protein G agarose at 4°C for 2 h. The supernatant were incubated with 1 μg of MR antibody (Cell Signaling Technology, Beverly, MA) at 4°C for overnight. The immunocomplexes were washed three times with lysis buffer, and subjected to western blotting analysis. For western blotting analysis, protein-matched samples (Bradford assay) were electrophoresed (SDS-PAGE), and then transferred to nitrocellulose (NC) membranes. The NC membranes were blocked with 5% skim milk in TBS (25 mmol/L Tris base and 150 mmol/L NaCl) for 2 h at room temperature, and then incubated with 0.2 μg/ml of MR antibody at 4°C for overnight. Secondary antibody (1:2000 diluted) was incubated at room temperature for 1 h, and then washed three times, 10 min each in TBST. The target proteins were detected with ECL plus detection reagents (Amersham, Pittsburgh, PA). The expression levels were quantified by an optical densitometry, ImageJ software.

Vector constructions, site directed mutagenesis and luciferase assay

The pEGFP-C1 vectors containing MR were kindly donated by Pearce D. (University of California San Francisco), pCMV-HA vectors containing human MR were kindly donated by Tirard M. (Max-Planck-Institute), and pA3Luc vectors containing Na⁺-K⁺-ATPase alpha and beta promoters were kindly donated by Iwasaki Y. (Kochi Medical School). The lysines at hinge region of MR (K677 and K678) were substituted to alanine or arginine by using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). For luciferase assay, 2 × 10⁵ HEK cells were seeded on collagen I coated 12-well plates, cultured overnight and transfected with vector constructs by using Superfect agent (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. The next day, cells were exposed 10 nmol/L aldosterone for 24 h with or without pretreatment of 1 mmol/L VPA or 10 μmol of MS-275 for 6 h. The cells were lysed with a lysis buffer and the luciferase activity was analyzed by Dual-glo luciferase reporter assay system kit (Promega, Madison, WI) according to the manufacturer's instructions. Luminescence was measured by Veritas™ Microplate Luminometer (Turner Biosystem, Sunnyvale, CA).

Live cell imaging

HEK293T cells were cultured on a collagen-coated 35-mm glass-base dish (Asahi techno glass, Tokyo, Japan) and grown overnight at 37°C in DMEM supplemented with 10% fetal bovine serum. For time-lapse analysis, HEK293T cells transfected with MR-GFP were imaged with a Nikon Ti-E inverted microscope (Nikon, Tokyo, Japan) equipped with CoolSNAP HQ camera (Roper Scientific, Trenton, NJ), excitation and emission filter wheels. All systems were controlled by MetaMorph software (Universal Imaging, Downingtown, PA). Filter sets and ND filters used were purchased from Semrock (Rochester, NY). Images were acquired by using the 4X4 binning mode and 200 ms exposure time. All images were processed using MetaMorph software.
Supplemental results

MS-275 attenuated expression of ATP1a1, GILZ, and SGK-1 induced by aldosterone

Expression of MR target genes was investigated by quantitative real-time PCR (qRT-PCR). Treatment of HEK293 cells with Aldo (10 nmol/L) increased expression of ATP1a1, GILZ, and SGK-1 after Aldo stimulation for 24 h. Pretreatment with MS-275 (1 umol/L, 6 h) resulted in significantly attenuated Aldo-induced expression of ATP1a1 (Fig. IA), GILZ (Fig. IB), and SGK-1 (Fig. IC).

VPA had little effect on Aldo-induced recruitment of mutant MR and Pol II on target gene promoters.

Enrichment of mutant MR and RNA polymerase II (Pol II) on promoters of GILZ and SGK-1 was analyzed by chromatin immunoprecipitation (ChIP) assay. Results of conventional PCR showed that treatment with Aldo resulted in increased enrichment of MR and Pol II on the GRE sequence in the promoter of GILZ and SGK-1, which were little affected by pretreatment with VPA (Fig. IIB and IIE). Recruitment of MR and Pol II on the promoters was confirmed by qRT-PCR (Fig. IIC and IIF).

MS-275 increased acetylation and reduced transcriptional activity of wild type MR

MR acetylation was investigated by Western blotting with anti-acetyl-lysine antibody after immunoprecipitation with anti-MR antibody. Treatment with Aldo or MS-275 alone had little effect on MR acetylation, whereas pretreatment with MS-275, followed by treatment with Aldo resulted in significantly increased MR acetylation (Fig. IIIA and IIIB). Transcriptional activities of wild type MR and mutant MR were analyzed by luciferase assay. Aldo induced ATP1a1 promoter-fused pA3luc vector in HEK293 cells transfected with wild type or mutant MR. MS-275 reduced transcriptional activity of wild type MR induced by Aldo, however transcriptional activity of mutant MR induced by Aldo was not affected by MS-275 (Fig. IIIC).

VPA had little effect on the expressions of MR corepressors

The expressions of MR corepressor genes such as death-domain-associated protein (DAXX), nuclear transcription factor Y subunit gamma (NF-YC), protein inhibitor of activated STAT-1 [signal transducer and activator of transcription-1] (PIAS1), and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) were analyzed by qRT-PCR. DOCA-salt infusion decreased the expression of DAXX (Fig. IVA, p<0.05 vs. vehicle), NF-YC (Fig. IVB, p<0.05 vs. vehicle), and PIAS1 (Fig. IVC p<0.01 vs. vehicle). The expression of SMRT was significantly decreased only in the DOCA+VPA group (Fig. IVD, p<0.05 vs. vehicle). However, VPA administration had little effect on the expressions of these MR corepressor genes compared with DOCA-salt-infused group.

VPA reversed the expressions of renin and angiotensin converting enzyme (ACE)

The expressions of rennin and ACE were analyzed by qRT-PCR. DOCA-salt-infusion decreased the expressions of rennin (p<0.01 vs. vehicle) and ACE (p<0.05 vs. vehicle). VPA administration reversed the expressions of rennin (p<0.01 vs. DOCA) and ACE (p<0.01 vs. DOCA) in those of the kidneys (Fig. V).

VPA administration increased MR acetylation in the kidneys of DOCA-salt-infused rats

MR acetylation was investigated by immunoprecipitation with anti-MR antibody followed by Western blot with anti-acetyl lysine antibody. DOCA-salt infusion with or without VPA administration did not affect protein expression of MR (Fig. IVA). MR acetylation was significantly increased by VPA administration (Fig. IVB).
## Online Table I. Primers for qRT-PCR and ChIP assay

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<th>Gene (Accession No.)</th>
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Supplemental Figures

Online Figure I. Effect of MS-275 on expression of ATP1a1, GILZ, and SGK-1 induced by aldosterone in HEK293 cells. HEK293 cells were incubated with 10 nmol/L of Aldo after pretreatment with MS-275 or vehicle for 24 h. Treatment with Aldo resulted in increased expressions of ATP1a1 (A), GILZ (B), and SGK-1 (C) which were decreased by pretreatment with MS-275. Data show mean±SE of four independent experiments [*p<0.05 vs. con, #p<0.05 and ##p<0.01 Aldo (10 nmol/L) vs. MS-275 (10 umol/L) pretreatment+Aldo (10 nmol/L)]
Online Figure II. VPA has little effect on enrichments of mutant MR and Pol II on target gene promoters induced by aldosterone. Schematic diagrams show the locations of GRE and PCR amplification after chromatin immunoprecipitation (ChIP) in \textit{GILZ} (A) and \textit{SGK-1} (D) promoters (upper). TSS; transcription start site. Representative gels of conventional PCR (Input: 27 cycles, others: 32 cycles) show that Aldo (10 nmol/L) increased the enrichments of mutant MR and Pol II on \textit{GILZ} (B) and \textit{SGK-1} (E) promoters, which were not affected by MS-275 (10 umol/L, 6 h) pretreatment (middle). C and F, The ChIP assays were quantified by real-time PCR. Graph shows mean±SE of four independent experiments (*p<0.05 and **p<0.01 vs. vehicle).
Online Figure III. MS-275 increased acetylation and reduced transcriptional activity of wild type MR. HEK 293 cells transfected with HA-tagged MR were treated with Aldo for 30 min after pretreatment with MS-275 or vehicle for 6 h. MR acetylation was probed by western blot with anti-acetyl lysine antibody after immunoprecipitation with anti-MR. A representative immunoblot (A) and densitometry (B) show that treatment with MS-275 (10 umol/L) resulted in increased MR acetylation only after stimulation with Aldo. Graph shows mean±SE of four independent experiments (*p<0.05 vs. con). C, HEK293 cells were transfected with expression vectors of wild-type MR, AA-mutant MR, or RR-mutant MR, as well as luciferase vector conjugated with ATP1a1 promoter. Pretreatment with MS-275 resulted in significant decrease of Aldo-induced promoter activity in HEK293 cells transfected with wild-type MR, but not mutant MR. The graphs show mean±SE from three 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 Tukey's comparison test.
Online Figure IV. VPA administration had little effects on the expressions of MR corepressors. The expressions of MR corepressors such as DAXX (A), NF-YC (B), PIAS1 (C), and SMRT (D) were analyzed by qRT-PCR. DAXX, NF-YC, and PIAS1 were decreased by DOCA-salt infusion in the kidneys (*p<0.05 and **p<0.01 vs. vehicle). SMRT was decreased only in rats of DOCA-salt-infusion with VPA administration (*p<0.05 vs. vehicle).
Online Figure V. VPA administration reversed renin and ACE expressions in DOCA-salt-induced hypertensive rats. Uninephrectomized-rats were infused DOCA-salt with or without VPA administration of VPA for 4 weeks. Renin and ACE expressions were analyzed by qRT-PCR. Data show mean±SE of 4 rats. DOCA-salt-infusion decreased the expressions of renin (**p<0.01 vs. vehicle) and ACE (*p<0.05 vs. vehicle). VPA administration reversed the expressions of renin and ACE (##p<0.01 vs. DOCA).
Online Figure VI. VPA administration increases acetylation of MR in the kidneys of DOCA-salt-infused hypertensive rats. A, MR protein expression was not affected by DOCA or VPA administration. B, MR acetylation was significantly increased in the kidneys of VPA-administered DOCA group. **p<0.01 vehicle or DOCA vs. VPA+DOCA