Histone deacetylase inhibition attenuates cardiac hypertrophy and fibrosis through acetylation of mineralocorticoid receptor in spontaneously hypertensive rats

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Running title: HDAC inhibition attenuates cardiac hypertrophy

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Abbreviations: PAI-1, plasminogen activator inhibitor-1; ChIP, chromatin immunoprecipitation; TNX, tenascin-X; ORM-1, orosomucoid-1; HDAC, histone deacetylase; MR, mineralocorticoid receptor; VPA, valproic acid
ABSTRACT

Inhibition of histone deacetylases (HDACs) by valproic acid (VPA) attenuates inflammatory, hypertrophic, and fibrotic responses in the hearts of spontaneously hypertensive rats (SHRs). However, the molecular mechanism is still unclear. We hypothesized that HDAC inhibition (HDACi) attenuates cardiac hypertrophy and fibrosis through acetylation of mineralocorticoid receptor (MR) in SHRs. Seven-week-old SHRs and Wistar-Kyoto rats (WKYs) were treated with an HDAC class I inhibitor (0.71% w/v in drinking water; VPA) for 11 weeks. Sections of heart were visualized after trichrome stain as well as hematoxylin and eosin stain. Histone modifications, such as acetylation (H3Ac) and fourth lysine trimethylation (H3K4me3) of histone 3, and recruitment of MR and RNA polymerase II (Pol II) into promoters of target genes were measured by quantitative real-time PCR (qRT-PCR) after chromatin immunoprecipitation (ChIP) assay. MR acetylation was determined by western blot with anti-acetyl-lysine antibody after immunoprecipitation (IP) with anti-MR antibody. Treatment with VPA attenuated cardiac hypertrophy and fibrosis. Although treatment with VPA increased H3Ac and H3K4me3 on promoter regions of MR target genes, expression of MR target genes as well as recruitment of MR and Pol II on promoters of target genes were decreased. Although HDACi did not affect MR expression, it increased MR acetylation. These results indicate that HDACi attenuates cardiac hypertrophy and fibrosis through acetylation of MR in spontaneously hypertensive rats.
INTRODUCTION

Cardiac hypertrophy and fibrosis are compensatory mechanisms to volume or pressure overload and frequently associated with chronic diseases such as hypertension (Gupta et al., 2005). Hypertension is a condition associated with increased expression of pro-inflammatory cytokines that activate hypertrophic mediators, which results in cardiac hypertrophy (Kudo et al., 2009; Tokuda et al., 2004). Cardiac fibrosis is also initiated by the actions of pro-inflammatory cytokines, and mediated by fibroblast activation (Kanzaki et al., 2001; Ratcliffe et al., 2000).

The mineralocorticoid receptor (MR) is a member of steroid hormone receptors known for its role in the development of cardiac diseases in response to ligand binding (Messaoudi et al., 2013; Pitt, 2004). MR regulates cardiac function through induction of inflammation and extracellular matrix, as well as expression of tenascin-X (TNX), plasminogen activator inhibitor-1 (PAI-1), orosomucoid-1 (ORM-1), and collagen IV (Fejes-Toth and Naray-Fejes-Toth, 2007). Expression of TNX, PAI-1, and extracellular matrix proteins including collagen IV is regulated by MR ligands, and is abundant in the heart (Chun and Pratt, 2005; Schellings et al., 2004). TNX is an essential regulator of collagen deposition by fibroblasts and is up-regulated during fibrosis in response to tissue injury. PAI-1 is an inhibitor of tissue-type plasminogen activator in plasma, which consequently suppresses the activity of plasmin. Increased PAI-1 expression is associated with greater risk of cardiovascular disease (Brown et al., 2002). MR ligand increased expression of ORM-1, which is induced by proinflammatory cytokines and glucocorticoid (Vannice et al., 1984). Patients with diabetes and metabolic syndrome have high plasma ORM-1 levels that may be
responsible for the increased incidence of cardiovascular problems in this syndrome (Engstrom et al., 2003). Therefore, MR is a potential target of hypertrophy and fibrosis.

The transcriptional activity of MR is mainly regulated by ligands such as aldosterone (Aldo). MR resides in the cytosol and translocates into the nucleus after ligand binding. The binding complex of MR with its ligand binds to hormone response elements in the promoter of target genes, recruits co-regulatory proteins results in gene transcription (Yang and Young, 2009). However, post-transcriptional modifications (PTMs) such as acetylation, phosphorylation, ubiquitylation, and sumoylation play critical roles in regulating its transcriptional activity (Faus and Haendler, 2006). Phosphorylation of MR enhances its binding affinity for DNA response elements (Massaad et al., 1999) whereas acetylation of MR inhibits recruitment of MR and Pol II on promoter of MR target genes and prevents development of hypertension (Lee et al., 2013).

Histone deacetylases (HDACs) regulate pathological cardiac conditions such as fibrosis (Kee et al., 2006) and hypertrophy (Antos et al., 2003; Kook et al., 2003). Several groups have showed that HDAC inhibitors can prevent cardiac hypertrophy in various animal models. For example, HDAC inhibitors increase expression of anti-hypertrophic transcription factors such as Kruppel-like factor 2 (KLF2) in cultured cardiomyocytes, which prevents cardiac hypertrophy in culture (Kee and Kook, 2009; Liao et al., 2010). When transgenic mice that overexpress Hop are treated with HDAC inhibitor, increased cardiac mass is significantly reduced (Kook et al., 2003). HDAC inhibitors block cardiac fibrosis through multiple mechanisms. Induction of collagen synthesis mediated by transforming growth factor-β (TGF-β) is prevented by HDAC inhibitor in cultured rat ventricular fibroblasts
(Kong et al., 2006). HDAC inhibitor is capable of suppressing differentiation of fibroblasts, which involves cardiac fibrosis (Guo et al., 2009). Recently, other groups also have reported that HDAC inhibitor can prevent cardiac hypertrophy and fibrosis in animal models of hypertension such as spontaneously hypertensive rats (SHRs) (Cardinale et al., 2010) and deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Iyer et al., 2010). However, the mechanism for prevent cardiac hypertrophy and fibrosis in SHRs by HDAC inhibitor is still unclear. We hypothesized that HDACi attenuates cardiac hypertrophy and fibrosis through MR acetylation in SHRs.
MATERIALS AND METHODS

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, and every effort was made to minimize both the number of animals used and their suffering. Seven-week–old male WKYs and SHRs were purchased from SLC Co. (Shizuoka, Japan). Valproic acid (VPA) was purchased from Sigma-Aldrich (St. Louis, MO). Rats were administered VPA (0.71% w/v) via their drinking water for 11 weeks. Rats were intraperitoneally anesthetized with sodium pentobarbital (50 mg/kg). Wet heart and lung weight were measured and normalized against tibia length. Tissues were frozen in liquid nitrogen and stored at -80°C until further study.

Histology
For hematoxylin and eosin (H&E) and trichrome stains, heart tissues were fixed in 4% formalin for overnight, dehydrated, and embedded in paraffin. The paraffin-embedded samples were sectioned at 3 μm thickness. The slides were examined using light microscopy.

Quantitative real-time PCR and Microarray
Tissues (about 100 mg) were homogenized in liquid nitrogen with a glass homogenizer. RNA was extracted by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s recommendations. Total RNA (2 μg) was reverse-transcribed into cDNA by using RevertAidTM first strand cDNA synthesis (Fermentas, EU) in 20 μl reaction volume according to manufacturer’s instructions. Quantitative real-time PCR (qRT-
PCR) was performed using ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). 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. The primer sets used in the RT-TCR were shown in table 3.

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-ΔΔCt values. For microarray, RNA was extracted from left heart. Microarray analysis was performed using Affymetrix Rat ST1.0. All primer sets used in qRT-PCR are shown in table 3.

**Chromatin immunoprecipitation (ChIP) assay**

Chromatin immunoprecipitation (ChIP) analysis was performed according to the manufacturer’s instructions with minor modification using EZ ChIP kit (Upstate Biotechnology, Lake Placid, NY). In brief, tissues were fixed with 1% formaldehyde and washed with ice-cold phosphate-buffered saline (PBS). After homogenization, tissues were incubated in sodium dodecyl sulfate (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 antibodies were added and incubated at 4°C overnight. Antibodies to MR and trimethyl-H3-K4 were obtained from Abcam (Cambridge, UK). Antibody to acetyl-histone H3 was obtained from Upstate Biotechnology. Antibody to Pol II was obtained from Millipore. 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. All primer sets used in ChIP assay are shown in Table 3.

**Immunoprecipitation and Western blot**

The frozen tissues were homogenized in RIPA buffer containing protease inhibitors. 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 (Abcam, Cambridge, UK) or ac-K antibody (Abcam, Cambridge, UK) at 4°C for overnight. The immunocomplexes were washed three times with lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1% NP-40, 0.5% SDS, 0.1% sodium dodecyl sulfate and 1x proteinase inhibitor cocktail) and subjected to western blot analysis. For western blot 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 1 mg/ml of MR or ac-K antibody at 4°C for overnight. Secondary antibody (1:5000 diluted) was incubated at room temperature for 1 h and then washed three times for 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.

Statistics

Results are expressed as means ± S.E. Kruskal-Wallis test or one-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 two groups. The procedures were performed using SPSS software (release 19.0, SPSS Inc., Chicago, IL).
RESULTS

HDACi attenuated left heart weight.

Ratios of heart weight (HW)/tibia length (TL) were used to show phenotypic changes attributable to hypertrophy-induced increased heart mass since it is not affected by change of body weight. To determine whether SHRs showed cardiac hypertrophy, we analyzed the HW/TL and left heart weight (LHW)/TL. SHRs showed increased HW/TL and LHW/TL ratio when compared with that of WKYs, which was attenuated by VPA treatment (Fig. 1A and 1B). Weights of lung and right hearts were similar between WKY and SHRs. VPA treatment did not affect lung weight (LW)/TL and right heart weight (RHW)/TL in SHRs (Fig. 1C and 1D). Thus, SHRs showed cardiac hypertrophy when compared with WKYs, which could be attenuated by VPA treatment.

HDACi attenuated cardiac hypertrophy and fibrosis.

To confirm cardiac hypertrophy microscopically in SHRs, we performed H&E stain. SHRs had hypertrophy when compared with WKYs, which was restored by VPA treatment (Fig. 2A). Collagen deposition is shown in blue color in trichrome stain. Trichrome stain revealed that SHRs had increased cardiac fibrosis when compared with WKYs. Cardiac fibrosis of SHRs was attenuated by VPA treatment (Fig. 2B). Expression of atrial natriuretic peptide A (Nppa) and B (Nppb), markers of cardiac hypertrophy, were detected by real-time PCR. Nppa and Nppb mRNA expression were increased in SHRs, consistent with the hypertrophy seen by histology. Nppa and Nppb mRNA expression were decreased with VPA treatment in SHRs (Fig. 2C and 2D).
**HDACi attenuated MR target gene expression.**

We analyzed expression of four major MR target genes, TNX, Collagen IV, ORM-1 and PAI-1 in the left hearts. Expression of MR target gene was investigated by qRT-PCR. MR target gene expression levels were higher in hearts of SHRs than those of WKY rats. Treatment of VPA resulted in attenuated expression of TNX (Fig. 3A), Collagen IV (Fig. 3B), ORM-1 (Fig. 3C) and PAI-1 (Fig. 3D).

**HDACi changed histone code modifications in ORM-1 and PAI-1 promoters.**

We investigated enrichment of H3Ac and H3K4me3 in ORM-1 and PAI-1 promoters by using ChIP assay. Result of conventional PCR and qRT-PCR show that enrichments of H3Ac and H3K4me3 in ORM-1 and PAI-1 promoters were comparable between WKYs and SHRs. Upon VPA treatment, enrichments of H3Ac and H3K4me3 in the ORM-1 (Fig. 4A and 4B, respectively) and PAI-1 (Fig. 4C and 4D, respectively) promoters significantly increased in the hearts of WKYs and SHRs.

**HDACi attenuated recruitment of MR and Pol II on promoters of target genes.**

Enrichment of MR and Pol II on promoters of ORM-1 and PAI-1 was analyzed by ChIP assay. Results of conventional PCR showed that enrichment of MR and Pol II on the ORM-1 promoter was higher in SHRs than those of WKYs. Treatment of VPA resulted in decreased enrichment of MR and Pol II on the ORM-1 promoter (Fig. 5A). Recruitment of
MR and Pol II on the ORM-1 promoter was confirmed by qRT-PCR (Fig. 5B). Results of conventional PCR (Fig. 5C) and qRT-PCR (Fig. 5D) showed that enrichment of MR and Pol II on the PAI-1 promoter was increased in SHRs when compared with WKYs, which was attenuated by VPA treatment.

**HDACi increased MR acetylation.**

MR acetylation was investigated by western blot with anti-acetyl-lysine antibody after immunoprecipitation with anti-MR antibody. Expression of MR was not different between WKYs and SHRs. Treatment of VPA did not affect protein levels of MR; however, MR acetylation was significantly increased by VPA treatment (Fig. 6A and 6B). Also, we performed western blot with anti-MR antibody after immunoprecipitation using anti-ac-K antibody. Results of reverse immunoprecipitation showed that MR acetylation was increased by VPA treatment (Fig 6C and 6D).
DISCUSSION

In the present study, we demonstrated that HDACi attenuates cardiac hypertrophy and fibrosis through MR acetylation in SHRs. Our results show that HDACi increases MR acetylation which decreases the recruitment of MR and Pol II on target gene promoters, reduces the expression of MR target genes such as TNX, collagen IV, ORM-1, and PAI-1, and attenuates cardiac hypertrophy and fibrosis.

HDACs and histone acetylases (HATs) play critical roles in remodeling chromatin structures (Delcuve et al., 2012). The status of acetylated histone and non-histone proteins such as transcription factors is controlled by the opposing actions of HATs and HDACs (Patel et al., 2011; Yang and Seto, 2007). HDACs are emerging as targets for the treatment of several diseases including cardiac hypertrophy and heart failure (Cao et al., 2011; Cho et al., 2010; Kee et al., 2006). In this study, we showed that VPA, an HDAC inhibitor, attenuated cardiac hypertrophy and fibrosis in SHRs. HDACi not only decreases heart weight, but also reduces expression of Nppa and Nppb (Fig. 1 and 2). Although HDACs are known to induce chromatin condensation and transcriptional repression (Kuo and Allis, 1998), HDAC increases the transcriptional activity of MR in the kidneys of DOCA-salt-induced hypertensive rat. In accordance with the result, treatment of HDACi resulted in increased acetylation of MR in the hearts of SHRs (Fig. 6) and adult human cardiomyocyte cells (Supplemental Figure. 3). Acetylation of lysine in the hinge region of MR resulted in transcriptional repression of MR (Lee et al., 2013).

Several studies have reported that HDAC inhibition reduces expression of collagen, tumor necrosis factor (TNF), and nuclear factor κB (NFκB) in spontaneously hypertensive rats (Cardinale et al., 2010) and DOCA-salt induced hypertensive rats (Iyer et al., 2010). Our
results also show that the rate of down-regulated genes was the highest in extracellular matrix and inflammation-related genes (Supplemental Figure. 1). In particular, expression of MR target genes such as TNX, collagen IV, ORM-1, and PAI-1 were decreased by VPA treatment (Fig. 3). VPA would likely up-regulate MR corepressors which down-regulate expression of MR target genes. However, VPA treatment did not affect the expressions of MR corepressor genes (Supplemental Figure. 2).

Acetylation of steroid hormone receptors is a PTM that plays an important role in regulating their activity (Wang et al., 2011). The androgen receptor (AR) is acetylated by Tip60, PCAF, and P300 in its hinge region (Fu et al., 2000). Mutations in AR acetylation sites dramatically impair AR function, stimulating the expression of AR target genes that regulate prostate cancer cell growth (Gaughan et al., 2002). When estrogen receptor alpha (ERα) is acetylated by P300 in lysine residues 266 and 268, it stimulates DNA binding activity (Kim et al., 2006). On the other hand, acetylation of lysine residues 302 and 303 prevents its binding to ligand (Fuqua et al., 2000; Wang et al., 2001). Glucocorticoid receptor (GR) is acetylated by CLOCK/BMAL1, which reduces its transcriptional activity (Nader et al., 2009), whereas it is deacetylated by HDAC2 (Ito et al., 2006). In the present study, MR interacts with HDAC1 and HDAC2 regardless of Aldo exposure (Supplemental Figure. 4).

Treatment of HDACi resulted in decreased recruitment of MR and Pol II on the promoters of target genes in vivo (Fig. 5). Ligand-bound MR is transported into the nucleus where it binds to specific hormone response elements (HRE) (Drouin et al., 1992; Walther et al., 2005), which are located up to 10 kb upstream, or downstream from the transcriptional start site of target genes and regulates transcriptional activity (van der Laan et al., 2008; Wang et al., 2004). Taken together, these results suggest that HDAC increases the
transcriptional activity by deacetylation of MR in SHRs whereas HDACi prevents action of MR by acetylation of MR in SHRs.

Histone code modifications are important mechanisms for epigenetic regulation. Expression of genes is epigenetically regulated by DNA methylation as well as histone modifications including methylation, acetylation, phosphorylation, and ubiquitylation (Kouzarides, 2007). In particular, hyperacetylated histones by treatment with HDAC inhibitor increased the degree of H3Ac, which is linked with H3K4 methylation. Thus, H3K4 methylation (mono methylation, dimethylation, and trimethylation) is also increased (Nightingale et al., 2007). We previously reported that expression of angiotensin converting enzyme (ACE), a component of the renin-angiotensin system (RAS), is regulated by histone acetylation (Lee et al., 2012). Expression of ACE1 was up-regulated by enrichment of activating chromatin marks such as H3Ac and H3K4me3 on ACE1 promoter. Thus, HDAC inhibition had been expected to mostly increase expression of gene through increased histone acetylation. However, gene expression profiles elucidated by microarray analyses show that, among the 27,997 genes identified, 73 were up-regulated and 106 were down-regulated in the hearts of SHRs upon VPA treatment (Table 1 and 2). The number of down-regulated genes was much more than that of up-regulated genes in SHRs after VPA treatment. Although VPA treatment increased enrichment of H3Ac and H3K4me3 on promoters of MR target genes in WKYs and SHRs (Fig. 4), actual expression of MR target genes was decreased by VPA treatment (Fig. 3). These findings suggest that enrichment of the promoters with H3Ac and H3K4me3 is not a sufficient condition for gene expression in the hearts of SHRs.

MR expression is detected in epithelial and non-epithelial tissues which imply for roles in physiology and pathophysiology (Viengchareun et al., 2007). MR activation by mineralocorticoid or glucocorticoid produces oxidative stress and vascular inflammation,
which eventually contributing to development of heart failure (Young et al., 2007). MR expression levels in epithelial cells plays a central role in controlling sodium transport. For example, Aldo and deoxycorticosterone acetate significantly induced Na\(^+\)-K\(^+\)-ATPase subunit \(\alpha_1\) (ATP1a1), glucocorticoid-induced leucine zipper (GILZ) and serum and glucocorticoid-regulated kinase 1 (SGK-1) in vitro and in vivo (Cardinale et al., 2010). In hearts, Aldo not only induces genes encoding extracellular matrix proteins such as TNX, and collagen IV, but also those related to inflammation such as ORM-1 and PAI-1 (Fejes-Toth and Naray-Fejes-Toth, 2007). MR blockers spironolactone and eplerenone significantly reduce morbidity and mortality in patients with heart failure. Therefore, HDACi and MR antagonists may be novel strategies for treating cardiac hypertrophy and fibrosis.

In summary, this study presents a mechanism by which HDACi attenuates cardiac hypertrophy and fibrosis through MR acetylation in SHRs. Acetylation of MR not only decreases the recruitment of MR and Pol II, but also expression of MR target genes which regulate hypertrophy and fibrosis. Therefore, HDACs may be a potential therapeutic target for cardiac hypertrophy and fibrosis treatments.
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Authorship contributions

Participated in research design: Kang, Lee, Kim

Conducted experiments: Kang, Song

Contributed new reagents or analytic tools: Lee, Seok

Performed data analysis: Kang, Song, Kim

Wrote or contributed to the writing of the manuscript: Kang, Kim, Kurz.
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Gaughan L, Logan IR, Cook S, Neal DE and Robson CN (2002) Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor.


Footnotes

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Seol-Hee Kang and Young Mi Seok contributed equally to this work.
FIGURE LEGENDS

**Figure 1.** Effect of valproic acid (VPA) treatment on heart weight. Cardiac hypertrophy was analyzed based on heart weight (HW)/tibia length (TL) ratios in Wistar-Kyoto rats (WKYs) (n=6) and spontaneously hypertensive rats (SHRs) (n=6). VPA was administered with 0.71% drinking water to seven-week-old WKYs and SHRs for 11 weeks. (A and B) HW/TL and LHW/TL ratios were increased in SHRs as compared with WKYs. Administration of VPA results in restoration of HW/TL and left heart weight (LHW)/TL ratios. (C and D) Lung weight (LW) and right heart weight (RHW) were similar in the WKY and SHR groups. VPA administration did not affect RHW/TL and LW/TL ratios. Data show the mean± standard error (SE) of six independent experiments (*p < 0.05 and **p < 0.01 vs. WKY, and #p < 0.05 and ##p < 0.01 vs. SHR).

**Figure 2.** Effect of VPA treatment on cardiac hypertrophy and fibrosis. Analyses of histology of WKY (n = 6) and SHR (n = 6) hearts were performed using hematoxylin and eosin (H&E) and trichrome stains. (A) SHRs had increased levels of hypertrophy when compared with WKY rats as shown by H&E staining, which were attenuated by VPA treatment. (B) SHRs had increased cardiac fibrosis seen in blue when compared with WKYs. VPA treatment results in attenuated cardiac fibrosis in SHR. Scale bar 50 μm. (C and D) Expression of ANP and BNP, cardiac hypertrophy markers, were detected by Quantitative real-time PCR (qRT-PCR). SHRs had increased expression of ANP and BNP mRNA which were decreased with VPA treatment. Data show the mean± standard error (SE) of six independent experiments (*p < 0.05 and **p < 0.01 vs. WKY, and #p < 0.05 and ##p < 0.01 vs. SHR).
**Figure 3.** Effect of VPA treatment on expression of mineralocorticoid receptor target genes. qRT-PCR analysis for mineralocorticoid receptor (MR) target genes in WKYs (n = 6) and SHRs (n = 6) was performed. SHRs showed increased gene expression of TNX (A), collagen IV (B), ORM-1 (C), and PAI-1 (D) in hearts, which was attenuated by VPA treatment. Data show the mean± standard error (SE) of six independent experiments (*p < 0.05 and **p < 0.01 vs. WKY, and #p < 0.05 and ##p < 0.01 vs. SHR).

**Figure 4.** Effect of VPA on histone code modifications in ORM-1 and PAI-1 promoters. Histone code modification was analyzed by chromatin immunoprecipitation (ChIP) assay. Homogenized tissues were sonicated and precipitated with specific antibodies with which DNA was extracted. Representative gels of conventional PCR (input: 27 cycles; others: 33 cycles) and real-time PCR showed that treatment of VPA resulted in increased enrichment of H3Ac and H3K4me3 on ORM-1 (A and B) and PAI-1 (C and D) promoter. Data show the mean± standard error (SE) of six independent experiments (*p < 0.05 and **p < 0.01 vs. WKY, and #p < 0.05 and ##p < 0.01 vs. SHR).

**Figure 5.** Treatment of VPA resulted in attenuated recruitment of MR and Pol II on target gene promoters in the hearts of SHRs. ChIP assay was performed to investigate the enrichments of MR and Pol II on promoters of target genes. (A and C), Representative gels of conventional PCR (input: 27 cycles; others: 33 cycles) show that enrichment of MR and Pol II was increased on the ORM-1 and PAI-1 promoters in SHRs, which were decreased by VPA. (B and D) The chromatin immunoprecipitation (ChIP) assays were quantified by real-time PCR. Data show the mean± standard error (SE) of six independent experiments (*p < 0.05 and **p < 0.01 vs. WKY, and #p < 0.05 and ##p < 0.01 vs. SHR).
**Figure 6. VPA increased acetylation of MR.** A representative immunoblot (A and C) and densitometry (B and D) show that MR acetylation was increased by VPA treatment. Data show the mean± standard error (SE) of six independent experiments (*p < 0.05 and **p < 0.01 vs. WKY, and *p < 0.05 and **p < 0.01 vs. SHR).
Table 1. Genes down-regulated by VPA treatment

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<td>myosin binding protein H-like</td>
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<tr>
<td>3.287</td>
<td>Scd1</td>
<td>stearoyl-Coenzyme A desaturase 1</td>
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<tr>
<td>3.558</td>
<td>Thrsp</td>
<td>thyroid hormone responsive</td>
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Table 3. Primers for qRT-PCR and ChIP assay

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Primer sequence (5′ to 3′)</th>
<th>Tm</th>
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<tr>
<td>qRT-PCR (for rat)</td>
<td></td>
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<tr>
<td>Nppa (NM_012612)</td>
<td>F: ATCTGATGGATTTCAGAAAACC 60</td>
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<tr>
<td></td>
<td>R: CTCTGAGACGGTTGACTTC</td>
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<tr>
<td>Nppb (NM_031545)</td>
<td>F: ACAATCCACGATGCAAGCT 60</td>
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<tr>
<td></td>
<td>R: GGGCCTTGTCCTTTGAGA</td>
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<tr>
<td>TNX (NC_005119)</td>
<td>F: TATGGGAGCAGCGTGGATCA 60</td>
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<td></td>
<td>R: TCAGTGCTCGGCAGTCATAC</td>
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<tr>
<td>Collagen-IV (NM_001135009)</td>
<td>F: GCCCTACGTTAGCAGATGTACC 60</td>
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<td></td>
<td>R: TATAATGGACTGGCTCGGAAT</td>
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<tr>
<td>ORM-1 (NM_053288)</td>
<td>F: GTGTGCAGGAGCAGTGAAAA 60</td>
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<tr>
<td></td>
<td>R: CATGCCCACATCTTTGACAG</td>
<td></td>
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<tr>
<td>PAI-1 (NM_012620)</td>
<td>F: AGGGCGACGAGATAGACAGA 60</td>
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<td></td>
<td>R: CACAGGGAGACCCAGGATAA</td>
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<tr>
<td>GAPDH (NM_017008)</td>
<td>F: GTGGACCTCATGGCCTACAT 60</td>
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<td></td>
<td>R: TGTTGGAGGAGATGCTCGTG</td>
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<tr>
<td>ChIP assay (for rat)</td>
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<tr>
<td>ORM-1</td>
<td>F: GGGAGGTGGCTCAACTCAG 60</td>
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<tr>
<td></td>
<td>R: TCCCAGTCAAGGTTGTTAG</td>
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<tr>
<td>PAI-1</td>
<td>F: CTCTGTAGTTGCTGGTCTCA 60</td>
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<tr>
<td></td>
<td>R: CTTCCCTCCTCCAGTAAC</td>
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Figure 1.

(A) Heart Weight/Tibia Length (g/mm) for WKY, WKY+VPA, SHR, and SHR+VPA.

(B) Left Heart Weight/Tibia Length (g/mm) for WKY, WKY+VPA, SHR, and SHR+VPA.

(C) Right Heart Weight/Tibia Length (g/mm) for WKY, WKY+VPA, SHR, and SHR+VPA.

(D) Lung Weight/Tibia Length (g/mm) for WKY, WKY+VPA, SHR, and SHR+VPA.
Figure 5.

(A) Western blots showing the expression of MR and Pol II in WKY, WKY+VPA, SHR, and SHR+VPA groups.

(B) Quantification of MR and Pol II enrichment on ORM-1 gene promoter normalized by input. ** indicates p < 0.01 compared to WKY group, # indicates p < 0.05 compared to WKY+VPA group.

(C) Western blots showing the expression of MR and Pol II in WKY, WKY+VPA, SHR, and SHR+VPA groups.

(D) Quantification of MR and Pol II enrichment on PAI-1 gene promoter normalized by input. ** indicates p < 0.01 compared to WKY group, # indicates p < 0.05 compared to WKY+VPA group.
Molecular pharmacology

Histone deacetylase inhibition attenuates cardiac hypertrophy and fibrosis through acetylation of mineralocorticoid receptor in spontaneously hypertensive rats

Seol-Hee Kang, Young-Mi Seok, Min-ji Song, Hae-Ahm Lee, Thomas Kurz, and Inkyeom Kim

Supplemental Results

Supplemental Figure 1. VPA down-regulated extracellular matrix and inflammation-related genes

Microarray analyses were performed to investigate the expression of genes in SHRs after VPA treatment. Of the 27,997 genes analyzed, 73 were upregulated and 106 were downregulated in SHR hearts in response to VPA treatment. Some of these genes are shown in Table I. VPA is supposed to induce transcriptional activation through increased histone acetylation. However, the number of down-regulated genes is more than that of up-regulated genes in SHRs after VPA treatment. In particular, the rate of down-regulated genes was the highest in extracellular matrix and inflammation-related genes.

Supplemental Figure 2. VPA does not affect the expression of MR corepressors

The expression of MR corepressor genes such as death-domain-associated, protein (DAXX), nuclear transcription factor Y subunit γ (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. The expression of DAXX (A), PIAS1 (B), NFYC (C) and SMRT (D) were not different between WKYs and SHRs. VPA treatment did not affect the expressions of MR corepressor genes. Data show the mean± standard error (SE) of six independent experiments (*p < 0.05 and **p < 0.01 vs. WKY, and #p < 0.05 and ##p < 0.01 vs. SHR).
Supplemental Figure 3. *MR acetylation increased* in response to HDAC inhibitor treatment *in adult human ventricular cardiomyocyte cells.*

Adult human ventricular cardiomyocyte cells were overexpressed with plasmids carrying MR. Although treatment with Aldo or HDAC inhibitor (VPA and MS-275) alone had no effect on MR acetylation, pretreatment with VPA or MS-275, followed by treatment with Aldo resulted in significantly increased MR acetylation.

Supplemental Figure 4. *MR interacts with HDAC1 and HDAC2 regardless of Aldo treatment in adult human ventricular cardiomyocyte cells.*

We investigated interaction between MR and class I HDACs (HDACs 1, 2, 3, and 8) using co-immunoprecipitation (Co-IP). Adult human ventricular cardiomyocyte cells were overexpressed with plasmids carrying MR and class I HDACs. Co-IP data showed that overexpression of HDAC1 (A) and HDAC2 (B), but not HDAC3 (C) or HDAC8 (D), interacts with MR regardless of Aldo treatment. MR was enriched in the nucleus after Aldo stimulation, whereas HDACs protein in the nucleus was not changed by Aldo treatment.
Supplemental Figure 1. VPA induces up- or down-regulation of genes expression. Microarray analyses were performed to investigate the expression of genes in SHRs after VPA treatment. Up-regulated genes are indicated in black whereas down-regulated genes are shown in white. Analyses of gene ontology (A) and gene cards (B) show that the number of down-regulated genes was more than that of up-regulated genes in SHRs after VPA treatment. Data show the mean±SE of six independent experiments (*p < 0.05 and **p < 0.01 vs. WKY, and #p < 0.05 and ##p < 0.01 vs. SHR).
Supplemental Figure 2. VPA does not affect the expression of MR corepressors.

The expression of MR corepressors such as DAXX (A), PIAS1 (B), NFYC (C) and SMRT (D) were analyzed by qRT-PCR. VPA treatment did not affect the expressions of MR corepressor genes.
Supplemental Figure 3. MR acetylation increased in response to treatment with HDAC inhibitors in adult human ventricular cardiomyocytes cells. Adult human ventricular cardiomyocyte cells transfected with HA-tagged MR were treated with aldosterone (Aldo) (10 nmol/L) for 30 min after pretreatment with VPA or MS-275 (Cayman, USA) for 6 h. MR acetylation was evaluated by western blot with anti-acetyl-lysine antibody after immunoprecipitation with anti-MR antibody. A representative immunoblot shows that treatment with VPA or MS-275 increased MR acetylation upon stimulation with Aldo.
Supplemental Figure 4. MR interacts with HDAC1 and HDAC2 in adult human ventricular cardiomyocytes cells. Adult human ventricular cardiomyocyte cells were co-transfected with HA-tagged MR and one of Flag-tagged class I HDACs. The cell nuclear fraction was isolated after stimulation with Aldo for 30 min. The interaction between MR and Class I HDACs was determined by western blot with anti-HDACs antibodies after immunoprecipitation with anti-MR antibody. Co-immunoprecipitation (Co-IP) shows that HDAC1 (A) and HDAC2 (B), but not HDAC3 (C) and HDAC8 (D), interacts with MR regardless of Aldo treatment.