HDAC Inhibition Attenuates Inflammatory, Hypertrophic, and Hypertensive Responses in Spontaneously Hypertensive Rats

Jeffrey P. Cardinale, Srinivas Sriramula, Romain Pariaut, Anuradha Guggilam, Nithya Mariappan, Carrie M. Elks, Joseph Francis

Abstract—Reactive oxygen species and proinflammatory cytokines contribute to cardiovascular diseases. Inhibition of downstream transcription factors and gene modifiers of these components are key mediators of hypertensive response. Histone acetylases/deacetylases can modulate the gene expression of these hypertrophic and hypertensive components. Therefore, we hypothesized that long-term inhibition of histone deacetylase with valproic acid might attenuate hypertrophic and hypertensive responses by modulating reactive oxygen species and proinflammatory cytokines in SHR rats. Seven-week-old SHR and WKY rats were used in this study. Following baseline blood pressure measurement, rats were administered valproic acid in drinking water (0.71% wt/vol) or vehicle, with pressure measured weekly thereafter. Another set of rats were treated with hydralazine (25 mg/kg per day orally) to determine the pressure-independent effects of HDAC inhibition on hypertension. Following 20 weeks of treatment, heart function was measured using echocardiography, rats were euthanized, and heart tissue was collected for measurement of total reactive oxygen species, as well as proinflammatory cytokine, cardiac hypertrophic, and oxidative stress gene and protein expressions. Blood pressure, proinflammatory cytokines, hypertrophic markers, and reactive oxygen species were increased in SHR versus WKY rats. These changes were decreased in valproic acid–treated SHR rats, whereas hydralazine treatment only reduced blood pressure. These data indicate that long-term histone deacetylase inhibition, independent of the blood pressure response, reduces hypertrophic, proinflammatory, and hypertensive responses by decreasing reactive oxygen species and angiotensin II type1 receptor expression in the heart, demonstrating the importance of uncontrolled histone deacetylase activity in hypertension. (Hypertension. 2010;56:437-444.)

Key Words: Ang II — cardiac hypertrophy — cytokines — hypertension — oxidative stress — HDAC

Essential hypertension is a condition associated with increased expression of proinflammatory cytokines (PICs).1,2 Studies from our laboratory and others have shown that PICs lead to an increase in reactive oxygen species (ROS), which upregulates nuclear factor (NF)κB activity, thus further increasing PIC and ROS transcription and amplifying their subsequent actions.3–5 Along with renin–angiotensin system (RAS) components, PICs also activate hypertrophic mediators, which can result in cardiac hypertrophy and altered cardiac remodeling and function.6,7 There are many triggers of hypertensive-induced inflammation resulting in both hypertrophic and hypertensive responses, many of which are through transcription factor NFκB activation, ultimately resulting in alterations of gene transcription and perpetuation of the hypertensive state.5,8 For transcription factors such as NFκB to activate their target genes, DNA and chromatin remodeling must occur. Posttranslational modifications of histone cores through a tightly regulated addition/removal of an acetyl tag on their N-terminal tails plays a major role in gene expression modulation.9 These additions/removals are accomplished by several members of the histone acetyltransferase (HAT) and histone deacetylase (HDAC) families, which either open or close DNA strands to the actions of transcription factors.10,11 Normally, this balance is tightly controlled, but during conditions of stress and inflammation, activation of PICs can result in increased HDAC activation and histone acetylation, correlating with an increase in NFκB activity and further increases in PIC expression, including tumor necrosis factor-α (TNF) and the interleukins (ILs).12,13 Although HDACs would appear to repress inflammatory responses through reduced gene expression, this view is too simplistic in regards to their nonhistone protein acetylation/deacetylation abilities, oftentimes having quite opposite effects with regard to the inflammatory response.14–17

Recent evidence indicates that the various HDAC classes respond differently toward inducing cardiac hypertrophy in nonhypertensive animal models16,18,19 and that global HDAC inhibition (HDACi) can prevent these hypertrophic changes.20 However, it is not known whether HDACi protects against

Received April 7, 2010; first decision April 22, 2010; revision accepted July 8, 2010.
From Comparative Biomedical Sciences (J.P.C., S.S., A.G., N.M., C.E., J.F.) and Veterinary Clinical Sciences (R.P.), School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA.
Correspondence to Joseph Francis, Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, E-mail jfrancis@lsu.edu
© 2010 American Heart Association, Inc.
Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.110.154567
cardiac hypertrophy and hypertensive response by modulating PICs and oxidative stress in spontaneously hypertensive (SHR) rats. A previous study used valproate, a derivative of valproic acid (VPA), to study hypertension in SHR rats. Although they showed a reduction in systolic blood pressure, the treatment was only carried out to 9 weeks of age, which is still 3 weeks too young to display the full systemic changes associated with hypertension in this animal model, including cardiac hypertrophy and inflammation. This study was established to assess the role of HDAC blockade on the inflammatory response and its effect on the pathogenesis of hypertension. Therefore, we hypothesize that chronic HDACi will attenuate the inflammatory and hypertensive responses associated with the hypertensive state. To test this, we administered VPA, a fairly novel HDAC inhibitor, especially class I HDACs, as a long-term treatment in SHR rats, for assessment of inflammatory, hypertrophic, and hypertensive changes associated with essential hypertension.

Methods
All the procedures in this study were approved by the Louisiana State University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and Experimental Design
Male Wistar–Kyoto (WKY) (n=20) and SHR (n=20) rats were randomly assigned to vehicle (water) or VPA (0.71% wt/vol. dissolved in water, prepared and provided daily) treatment groups. Another set of rats were treated with hydralazine (HYD) (25 mg/kg per day in drinking water). Rats received drug or vehicle for 20 weeks starting from 7 weeks of age. Rats were euthanized at 27 weeks of age with left ventricular (LV) tissue collected for molecular analyses. We performed the following experimental procedures: blood pressure measurements, echocardiographic analysis, real time RT-PCR, Western blot analysis, electron paramagnetic spin resonance (EPR) studies, electrophoretic mobility-shift assays (EMSA), colorimetric assays, immunofluorescence, and immunohistochemical and statistical analysis. For an expanded Methods section, see the online Data Supplement at http://hyper.ahajournals.org.

Results
VPA Treatment Attenuates the Blood Pressure Changes in SHR Rats
Blood pressure recordings showed that SHR+VPA maintained a lower blood pressure level from the prehypertensive to the more advanced hypertensive phases as compared with SHR controls (Figure 1). Following 10 weeks of treatment, SHR control mean arterial pressure (MAP) continued to rise, whereas SHR+VPA rats plateaued at a lower pressure (163±2.32 versus 127.5±2.35 mm Hg, respectively, P<0.05). The MAP of SHR+VPA rose slightly throughout the course of the study, becoming significantly elevated above WKY controls toward the end of the treatment period (141.2±4.5 versus 114.8±3.41, respectively, P<0.05), but still significantly lower than the MAP of SHR controls (141.2±4.5 versus 173.2±3.47, respectively, P<0.05). Mean diastolic and systolic pressures followed the same pattern as the MAP (data not shown), indicating that VPA attenuated all phases of blood pressure in SHR rats. Furthermore, VPA did not have any adverse effects on the health of the animals used in this study.

Another set of animals (n=5 for each SHR and WKY) were treated with HYD, a direct smooth muscle relaxant and vasodilator, to compare their effects against treatment with VPA (Figure S1 in the online Data Supplement). SHR+HYD demonstrated a decrease similar to SHR+VPA in MAP as compared with SHR (142.57±4.906 versus 173.2±3.47). WKY+HYD had no change in MAP. Mean diastolic and systolic followed the same pattern as the MAP in HYD treated rats (data not shown), indicating that HYD similarly attenuated all phases of increased blood pressure seen in SHR rats.

VPA Attenuates Cardiac Hypertrophy in SHR Rats
Echocardiographic assessment showed that SHR controls had significantly more concentric hypertrophy of the left ventricular posterior wall (LVPWT) during diastole and systole (Figure 2A) (2.48±0.11 and 3.43±0.2 mm, respectively) when compared with WKY controls (1.73±0.02 and 2.32±0.06, respectively). However, SHR+VPA (1.85±0.03, 2.52±0.04 mm) rats displayed no change in ventricular thickness as compared with WKY and WKY+VPA (1.75±0.06, 2.28±0.07 mm) rats, indicating that VPA had a beneficial effect on preventing the increased LVPWT in diastole/systole that is typically observed in SHR rats.

Heart weight (HW)/body weight (BW) and lung weight (LW)/BW ratios are often used to show phenotypic changes attributable to hypertension such as increased heart mass resulting from hypertrophy (HW/BW) and increased edema of the lungs (LW/BW) resulting from cardiac dysfunction and increased systemic circulatory resistance. Treatment with VPA or HYD alone did not have any effects on BW (Figure S2). SHR+VPA normalized both HW/BW and LW/BW indices versus SHR control rats (0.0032 and 0.0048 versus 0.0039 and 0.0062, respectively, P<0.05) as compared with WKY (0.0029 and 0.0038), reinforcing that VPA reduces cardiac hypertrophy and dysfunction (Figure 2B). However, the HW/BW ratio assessed for SHR+HYD did not improve cardiac hypertrophy (Figure S3) as compared with SHR controls (0.0036 versus 0.0039), indicating that improvements in cardiac hypertrophy attributable to VPA treatment were not pressure dependent.

Figure 1. Effects of VPA treatment on MAP. VPA attenuated the increase in MAP seen in untreated SHR rats. *P<0.05 vs WKY; #P<0.05 vs SHR (n=7 to 8 in each group).
VPA Reduces Hypertrophic Response Elements in the LV Tissue of SHR Rats

To further demonstrate the effects of VPA on hypertrophy, RT-PCR analysis was undertaken on hypertrophic response genes in the LV. Compared to WKY, untreated SHR rats had elevated levels of collagen IV and atrial natriuretic peptide (ANP), 2 markers of LV remodeling associated with cardiac hypertrophy (Figure 2C), as well as angiotensin type 1 receptor (AT1R). SHR/VPA reduced these levels to that of normotensive WKY controls ($P<0.05$), indicating HDACi has a positive effect on reducing molecular markers of cardiomyocyte and interstitial growth normally attributed to systemic hypertension. Furthermore, SHR+VPA had reduced percentage of fibrosis staining vs SHR rats, as indicated by picrosirius red staining collagen vs total area (D). Stained slides are representative of typical results for each group ($n=3$ group). d indicates diastole; s, systole. *$P<0.05$ vs SHR; #$P<0.05$ vs WKY ($n=7$ to 8 in each group).

Figure 2. Effects of VPA treatment on cardiac hypertrophy. VPA attenuated ventricular wall thickness as assessed by echocardiography (A) during both diastole and systole. VPA also attenuated the HW/BW (indicating reduction in cardiac hypertrophy) and LW/BW (indicating reduction in lung edema attributable to reduced cardiac function) ratio in SHR rats (B). SHR rats had increased levels of ANP and collagen IV mRNA expression which were reduced with VPA treatment (C). Graphs expressed as fold change vs WKY rats. VPA reduced cardiac fibrosis vs SHR rats, as indicated by picrosirius red staining collagen vs total area (D). Stained slides are representative of typical results for each group ($n=3$ group). d indicates diastole; s, systole. *$P<0.05$ vs SHR; #$P<0.05$ vs WKY ($n=7$ to 8 in each group).
VPA Reduces HDAC Activity in the LV of Treated Groups

To assess the effectiveness of VPA on HDAC activity in LV tissue, a colorimetric assay kit was used to analyze differences between the VPA treated and untreated groups (Figure 4A). Global HDAC activity was reduced in untreated WKY versus untreated SHR rats (17.87±1.06 versus 22.19±0.57 optical density/mg protein sample, respectively, P<0.05). Furthermore, WKY+VPA and SHR+VPA groups both exhibited a lower HDAC activity level (not significant and P<0.05, respectively) as compared with their own strain controls. Conversely, SHR and WKY rats treated with HYD did not have a decrease in HDAC activity when compared with their respective controls (Figure S5).

VPA Normalizes Inflammatory Response in the LV Tissue of SHR Rats

Immunohistochemistry revealed that SHR controls had increased protein expression of TNF and IL-1β in the LV as compared with WKY controls. This protein expression was reduced in SHR+VPA rats (Figure 5A). RT-PCR similarly indicated that SHR controls had increased expression of TNF, IL-1β, IL-6 (Figure 5B), and NFκB (Figure 4C) mRNA in LV tissue when compared with WKY controls. This increase was attenuated in SHR+VPA rats, whereas WKY+VPA exhibited no change. Furthermore, HYD had no effect on the PIC mRNA expression of TNF or IL-1β (Figure S6A and S6B). These combined results indicate that VPA treatment reduces and normalizes the inflammatory response observed in SHR rats.

VPA Reduces ROS and gp91phox in the LV of SHR Rats

Since inflammation during hypertensive response is associated with an increase in oxidative stress, both total ROS as assessed by EPR (Figure 6A) and the expression of gp91phox (Figure 6B and 6C) were examined in the LV. Untreated SHR rats experienced a significant increase in ROS when compared with WKY rats (0.54±0.05 versus 0.22±0.03 mmol/L per milligram protein per minute). SHR+VPA normalized this ROS increase (0.54±0.05 versus 0.21±0.03 mmol/L per milligram protein per minute). Furthermore, gp91phox, the major catalytic subunit of NADPH oxidase and a ROS contributor, was increased in untreated SHR rats versus WKY controls. This was subsequently reduced in SHR+VPA (3.39 versus 1.56-fold change/WKY) but not SHR+HYD (Figure S6C). The reduction in protein expression was confirmed by immunofluorescence of the LV tissue. These results indicate that treatment of SHR rats with VPA had beneficial effects in reducing oxidative stress in the LV tissue.
HDACi reduced AT1R expression, thereby limiting the role of inflammatory markers, including TNF and NF-κB expression; (4) untreated SHR rats had an increase in inflammatory and oxidative stress actions and contributed to hypertrophic and hypertensive responses in SHR rats. These findings suggest that long-term treatment with VPA reduces hypertension-induced PICs, thereby attenuating hypertrophic and hypertensive responses in SHR rats.

HDACs play an important role in inducing the structural remodeling of chromatin that exposes DNA to transcription factors, ultimately yielding changes in gene expression. Along with HATs, HDACs maintain a relative balance in cellular systems during normal physiology that allows typical function. However, during diseased conditions, this balance is tilted in favor of HDACs, which can lead to an elevation in inflammatory and immune responses. In this study, we also observed an elevated HDAC activity, which was accompanied by increased PIC and oxidative stress gene expression in the hearts of SHR rats.

Cardiac hypertrophy is a well-described consequence of systemic hypertension. Studies have shown the role of HDACi on controlling cardiac growth and remodeling. However, little is known about the holistic role of HDACi on hypertrophy during hypertensive response. As evidence indicates, pathological cardiac hypertrophy and function rely on the balanced abundance of α- and β-myosin heavy chain (MHC) protein throughout the heart. During the progression of cardiac hypertrophy, the adult isoform of MHC (α-MHC) undergoes a stressed-trigger switch to the fetal isoform (β-MHC), contributing to cardiac hypertrophy. HDACi blunts MHC isoform switching, therefore preserving ventricular function. These results are contradictory to earlier experiments showing that class II HDACs block progrowth genes through interaction with transcription factor myocyte enhancer factor-2 (MEF2). More recently though, research has indicated that the prohypertrophic class I HDACs, when activated, are more potent and take priority over the antihypertrophic class II HDACs, thus explaining how global HDACi can attenuate cardiac hypertrophy in various animal models. From these changes, it has been suggested that within the hypertrophied heart, hypertrophic stress signals cause the phosphorylation of HDACs bound to MEF2, causing their disassociation into the cytoplasm. Since these HDACs are not bound to the chromatin structure, their inhibition would have no overt effect on MEF2 transcription, indicating that HDACi has no direct effect on the depression of MEF2 controlled prohypertrophic genes and signifying that another mechanism must be in place.

HDACi using trichostatin A, an inhibitor of class I and II HDACs, either induced or blunted agonist-induced expression of ANP, a hypertrophic growth factor, in cultured neonatal myocytes. The present study also demonstrated that...
Hypertension

September 2010

442

Figure 6. Effects of VPA treatment on total ROS and gp91phox in the LV. SHR rats had increased total ROS levels as assessed by EPR. These levels were normalized in SHR+VPA (A). SHR rats also showed increased mRNA (B) and protein (C) expression of gp91phox, the catalytic subunit of NADPH oxidase, in the LV vs WKY rats. This was attenuated in SHR+VPA. Immunofluorescence of LV cardiomyocytes shows increased presence of gp91phox in SHR rats vs SHR+VPA. *P<0.05 vs SHR (n=7 to 8 in each group).

HDACi in SHR rats showed a significant reduction in ANP, suggesting that in SHR rats, ANP possibly plays an important role in attenuating cardiac hypertrophy, and that HDACi silences/blunts this signaling mechanism. Moreover, collagen IV, an indicator of cardiac remodeling, especially within the failing heart,32 was increased in SHR rats but not SHR+VPA rats. Finally, AT1R, an important component of the RAS which, when acted on by Ang II in the LV, causes cardiac hypertrophy,33 has been shown to be attenuated with VPA treatment,29,34 but the mechanism involved has not been fully investigated. The present study showed significantly reduced AT1R expression in SHR+VPA rats versus SHR controls, indicating that a possible hypertrophic mechanism more intimately involves AT1R activation.

There are several well-known modulators of pressure-independent cardiac hypertrophy including Ang II and sympathetic neurohormones.35 Recently, it was also shown that Ang II-induced cardiac hypertrophy can be prevented using HDACi.29 To determine whether the present study’s effects on cardiac hypertrophy were pressure-independent or -dependent of HDACi, we looked at the effect of HYD on blood pressure and cardiac hypertrophy, as well as on ANP and AT1R expression. HYD reduced MAP in SHR rats similar to VPA; however, cardiac hypertrophy was unaffected, including hypertrophic mediators ANP and AT1R. This indicates a possible pressure-independent mechanism regarding the effect of VPA on cardiac hypertrophy. These results are in agreement with a recent study where NFκB inhibition reduces cardiac hypertrophy in a pressure-independent manner.8 This may offer another mechanism whereby HDACi reduces cardiac hypertrophy, for results herein show a decrease in NFκB activity and expression following VPA treatment. Therefore, from these results, not only was cardiac hypertrophy alleviated through long-term VPA treatment, but several possible mechanisms that induce cardiac remodeling were also attenuated.

We and others have recently demonstrated that hypertensive drive is partially controlled through the overexpression of PICs, especially TNF, along with downstream alterations in NFκB, ROS, and RAS components, as regulated through AT1R activation.3–5,36 This study demonstrates that chronic HDACi attenuates PIC response in SHR+VPA rats. Moreover, VPA reduces the presence of ROS and AT1R, two components implicated in the inflammatory response observed in hypertension. HDACi has been increasingly identified as a possible therapeutic approach toward many inflammatory conditions,11,14,22,37 including cardiovascular diseases.12 Although this mechanism has not yet been entirely delineated, it is suggested that the use of a HDACi, such as VPA, blocks HDAC actions on protein function outside of its normal action on altering transcription,17,38 as subsets of these families possess the ability to act on nonhistone proteins, further complicating their roles in gene modulation.39 A recent study showed that HDACi can deactivate Akt, a potential mediator of cardiac hypertrophy and oxidative stress, via dephosphorylation by HDAC–protein phosphatase 1 complexes.17 However, other studies have shown that specific sets of Toll-like receptor–inducible genes are targeted by HDACi in macrophages and dendritic cells and that one of these is through the NFκB pathway,24,25 which concurs with the present study. It underscores the role of HDACi on PIC activation of NFκB, preventing a further, cyclically driven upregulation of PICs, including TNF, IL-1β, and IL-6.

The effect of inflammation on ROS in hypertension has been previously demonstrated.3,40 A number of proinflammatory mediators of this increased ROS have been identified, including TNF and IL-6, which, as demonstrated here, are attenuated with HDACi, confirming the roles of HDACs on inflammatory and oxidative stress responses on hypertension in SHR rats. Although the signaling pathway is not entirely clear on how HDACi attenuates ROS in SHR rats, either directly or indirectly through its blockade of PICs and the NFκB pathway, we postulate that by blocking PIC activation, with its subsequent downregulation of NFκB activity and gp91phox expression, ROS is inhibited.
The interaction between the RAS, PICs, and ROS has also been demonstrated by work in our laboratory and others. Presently, we show that untreated SHR rats have increased AT,R expression, an important component of the prohypertensive portion of the RAS. HDACi through VPA treatment attenuated this increase concomitant with that of PICs. The pathophysiological mechanism of hypertension intimately involves the action of Ang II, including vasoconstriction, increased aldosterone secretion, increased sympathetic nerve activity, tissue remodeling, and increased sodium and water intake, all of which are mediated through AT,Rs that are distributed throughout most organ systems, including the liver, brain, kidney, heart, and blood vessels. Reports indicate that Ang II is controlled by, and controls, HDAC-induced changes in gene and protein response. Here, we show a possible new mechanism involving the regulation of Ang II responses as directed through the AT,R. HDACi reduces AT,R gene expression and receptor density, thereby ameliorating the actions of Ang II. This alteration in AT,R expression could be either through direct HDACi effects on the production and function of the receptor, or through the effects of HDACi on inflammatory gene response during hypertension. This mechanism must be further investigated to determine the full effect of HDACi in attenuating responsive gene.

In conclusion, the results of the present study show that long-term HDACi through VPA attenuated MAP and cardiac hypertrophy, possibly through modulation of ANP, collagen IV, and AT,R. HDAC inhibition also attenuated the increased inflammatory response, including TNF and NFκB, as well as the increase in ROS and gp91phox. These findings suggest that HDACi with VPA reduced inflammation, ROS, and AT,R, thereby attenuating hypertension and its secondary consequences in SHR rats.

Perspectives

We chose to use VPA because of its current use in clinical settings as an antiseizure and bipolar drug, demonstrating its availability to patients. In our study, VPA was administered long-term without any adverse effects toward the treated animal groups. This outlines the importance of the continuous drug administration necessary for the successful treatment of hypertension and its consequences, including cardiac hypertrophy, systemic inflammation and end organ damage attributable to ROS. Although we cannot rule out the effects of VPA on blood pressure from its GABAergic actions or nonhistone protein interactions, we feel confident that this study provides sufficient evidence that the use of HDACi can reduce not only blood pressure, but cardiac hypertrophy and the inflammatory state associated with hypertension. The specific mechanisms involved in HDACi must be studied more closely. However, as the quest to find new therapeutic strategies in hypertensive control is ever pressing, this could present a possible new approach in future treatment options.

Sources of Funding

This work was supported by National Heart, Lung, and Blood Institute grant HL-80544 (to J.F.).

Disclosures

None.

References


HDAC Inhibition Attenuates Inflammatory, Hypertrophic, and Hypertensive Responses in Spontaneously Hypertensive Rats
Jeffrey P. Cardinale, Srinivas Sriramula, Romain Pariaut, Anuradha Guggilam, Nithya Mariappan, Carrie M. Elks and Joseph Francis

Hypertension. 2010;56:437-444; originally published online August 2, 2010;
doi: 10.1161/HYPERTENSIONAHA.110.154567

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/56/3/437

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2010/07/30/HYPERTENSIONAHA.110.154567.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/
HDAC Inhibition Attenuates Inflammatory, Hypertrophic and Hypertensive Responses in Spontaneously Hypertensive Rats

Jeffrey P Cardinale¹, Srinivas Sriramula¹, Romain Pariaut², Anuradha Guggilam¹, Nithya Mariappan¹, Carrie Elks¹ and Joseph Francis¹

¹Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA

²Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA

Running Title: HDACi attenuates blood pressure in SHR rats

Word Count: 1769  Figures: 6

Corresponding author: Joseph Francis, B.V.Sc, M.V.Sc, Ph.D.

Comparative Biomedical Sciences

School of Veterinary Medicine

Louisiana State University

Baton Rouge, LA 70803, USA

Phone: +1 225 578 9752

Fax: +1 225 578 9895

jfrancis@lsu.edu
Data Supplement

Materials and Methods

Animals

Adult male SHR or WKY rats were used for this study. They were housed in temperature- (23 ± 2°C) and light-controlled (lights on between 7 AM and 7 PM) animal quarters and were provided with chow ad libitum. Vehicle (water) or valproic acid (VPA, 0.71% wt/vol, Sigma) dissolved in water were prepared and provided daily. Another subset of animals (WKY and SHR; n=5 each) were administered hydralazine (HYD, 25mg/kg/day in drinking water, Sigma), a direct smooth muscle relaxant and vasodilator, to determine the pressure-independent effect of VPA on cardiac hypertrophy. The experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental protocol

SHR (n=20) and WKY (n=20) rats underwent echocardiographic assessment at the start and end of the treatment period. Rats received drug or vehicle treatment for 20 weeks. Rats were euthanized at 27 weeks of age. Wet heart and lung weights were measured and analyzed against body weight. Blood and left ventricular tissue (LV) was collected for molecular analysis.

Blood pressure measurement

A tail-cuff plethysmograph (CODA 6 Blood Pressure System, Kent Scientific System, Torrington, CT) was used for blood pressure measurement at baseline, and weekly thereafter. Blood pressure was measured for four consecutive days for determination of weekly average measures. The daily measurements were blindly analyzed for the most closely associated five consecutive runs for use as that day’s average value.

Echocardiographic assessment of LV hypertrophy

Echocardiography was obtained at baseline, and repeated at the end of the treatment period. Echocardiogram was performed as described previously. Briefly, transthoracic echocardiography was performed under isoflurane anesthesia, using a Toshiba Apio SSH770 (Toshiba Medical, Tustin, California) fitted with a PST 65A sector scanner (8 MHz probe) which generates two-dimensional images at a frame rate ranging from 300-500 frames per second. Left ventricular posterior wall thickness at end-systole and end-diastole (LVPWTs and LVPWTd, respectively) was measured digitally on the M-mode recordings and averaged from at least three cardiac cycles.

Assessment of LV hypertrophy through fibrosis staining

Paraffin sections (10μm) were obtained from heart specimens as previously described and stained with picrosirius red for the detection of collagen. The percent area of fibrosis was calculated using ImageJ software (NIH).
Detection of Total ROS and Superoxide (O$_2^{•−}$) in LV heart tissue

One of the most sensitive and definitive methods of superoxide production is electron spin resonance (ESR). In this study, we utilized an established technique for total ROS detection in tissue using ESR and spin traps. Different spin probes were used for the ESR studies. 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrroldine (CMH) was used to measure the O$_2^{•−}$ levels. All ESR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super-high quality factor (Q) microwave cavity (Bruker Company, Germany).

Sample preparation for ESR studies: The dissected LV tissue from each animal was placed into a 24-well plate containing Kreb’s HEPES buffer (KHB) (20mM, pH 7.4). Tissue pieces were then washed twice with the same buffer to remove any trace contamination. Samples were then incubated at 37°C with specific spin probes for 30 minutes.

Total tissue ROS production: Total ROS was determined as previously described. Tissue pieces were incubated at 37°C with CMH (200 μM) for 30 minutes. Aliquots of the incubated probe media were then taken in 50 μl glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS production, under the following ESR settings: field sweep 50 G; microwave frequency 9.78 GHz; microwave power 20 mW; modulation amplitude 2 G; conversion time 327 ms; time constant 655 ms; receiver gain 1 x 10$^5$. For superoxide production, samples were pre-incubated at 37°C with PEG-SOD (50 U/ml) for 30 minutes, then CMH (200μM) for an additional 30 minutes. Aliquots of the incubated probe media were taken in 50 μl glass capillary tubes for determination of total superoxide production. Addition of PEG-SOD to CMH allowed competitive inhibition of the O$_2^{•−}$-CMH oxidation reaction by the quenching of O$_2^{•−}$ radicals. Since it is cell permeable, PEG-SOD can competitively inhibit the CMH-O$_2^{•−}$ interaction both intracellularly and extracellularly, thus allowing accurate measurement of total tissue O$_2^{•−}$ production. To determine actual total tissue superoxide production, the values obtained from incubation with PEG-SOD and CMH were subtracted from the values obtained from incubation with CMH only.

RNA isolation and real-time RT-PCR

Total RNA was extracted from the LV using TRI reagent (Invitrogen), and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) as previously described. The mRNA expression levels of ANP, Collagen IV, TNF, IL-1β, IL-6, the p50 subunit of NF-κB, gp91phox and AT1-R were determined using previously published specific custom made primers. GAPDH was used as the housekeeping gene. Real-time RT-PCR (qRT-PCR) was performed in 384 well PCR plates using Bio-Rad PCR Master Mix (The iQ SYBR™ Green Supermix with ROX) and the ABI Prism 7900 sequence detection system (Applied Biosystems). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles (15 s at 95°C, 1 min, at 60°C). A dissociation step (15 s at 95°C, 15 s, at 60°C and 15 s at 95°C) was added to check the melting temperature of the specific PCR product.

HDAC activity analysis by colorimetric assay

Nuclear extracts of LV tissue were obtained with a Nuclear Extraction Kit (BioVision). Nuclear extract was then analyzed for HDAC activity with a Colorimetric HDAC Activity Assay Kit.
(BioVision), both according to manufacturer’s instructions. The plate was read by a Multiskan Spectrum system and values were determined as O.D./μg protein in samples.

**AT1-R protein analysis by Western blot**

The protein expression of AT1-R in the heart was analyzed by Western blot as previously described with the use of anti-AT1-R antibody (Santa Cruz). Bands were normalized to GAPDH.

**Localization of TNF and IL-1β by Immunohistochemistry**

Heart tissues were prepared as previously described. The sections were treated with respective primary antibodies TNF (1:100 dilution, anti-goat) and IL-1β (1:50 dilution, anti-rabbit) (Santa Cruz). Negative control sections were incubated with secondary antibody alone.

**Localization of gp91phox by Immunofluorescence**

For detection of gp91phox in left ventricular heart tissue, slides were incubated overnight at 4ºC with a 1:100 dilution of goat polyclonal anti-gp91phox (Santa Cruz) as previously described.

**Electrophoretic Mobility Shift Assay (EMSA) for assessment of NF-kB activity**

EMSA was used to assess the activity of NF-kB in the left ventricle as previously described.

**Statistical analysis of data**

All results are expressed as mean ± SEM. For statistical analysis of the data, student’s t test, one-way ANOVA or repeated measures ANOVA followed by Bonferroni’s post hoc test was performed using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California, USA, to determine differences among groups. Cardiac fibrosis measurements were compared by using nonparametric Kruskal-Wallis ANOVA followed by Mann-Whitney U post hoc. A value of p< 0.05 was considered statistically significant.

**References**


S1. Effects of VPA and HYD treatment on MAP. VPA and HYD attenuated the increase in MAP seen in untreated SHR rats. VPA: valproic acid, HYD: hydralazine, MAP: mean arterial pressure. n=7-8 in each group, *P<0.05 vs WKY, #P<0.05 vs SHR.
S2. Effects on VPA and HYD treatment on BW. There were no BW differences across SHR and WKY rats or between treatment groups. VPA: valproic acid, HYD: hydralazine, BW: body weight. n=7-8 in each group.
S3. Effects of VPA and HYD treatment on cardiac hypertrophy. VPA attenuated the HW/BW (indicating reduction in cardiac hypertrophy) ratio, whereas HYD had minimal effect in reducing cardiac hypertrophy in SHR rats. VPA: valproic acid, HYD: hydralazine, HW: heart weight, BW: body weight. n=7-8 in each group, *P<0.05 vs SHR, #P<0.05 vs SHR+HYD.
S4. Effects of HYD treatment on hypertrophic markers. SHR rats had increased levels of ANP (S4A) and AT1-R (S4B) mRNA expression compared to WKY controls. These levels remained unchanged following HYD treatment. HYD: hydralazine, ANP: atrial natriuretic peptide, AT1-R: angiotensin type 1 receptor. n=7-8 in each group, *P<0.05 vs SHR, #P<0.05 vs SHR+HYD.
S5. Effects of VPA and HYD on HDAC activity as assessed through a colorimetric detection assay. HDAC activity was elevated in both untreated WKY and SHR as compared to VPA treated WKY and SHR rats. HYD had no effect on HDAC activity in LV tissue of either WKY or SHR rats. Untreated SHR HDAC activity was elevated compared to untreated WKY rats. VPA: valproic acid, HYD: hydralazine, HDAC: histone deacetylase, LV: left ventricle. n=7-8 in each group, *P<0.05 vs WKY+VPA, #P<0.05 vs SHR and $P<0.05 vs SHR+HYD.
S6. Effects of HYD treatment on PIC and ROS gene expression. SHR rats had increased levels of TNF (S6A) and IL-1β (S6B) mRNA expression compared to WKY controls. These levels remained unchanged following HYD treatment. SHR rats also had elevated expression levels of gp91phox (S6C) when compared to WKY controls. HYD had a small but not significant effect on reducing these levels. HYD: hydralazine, TNF: tumor necrosis factor-alpha, IL-1β: Interleukin-1 beta. n=7-8 in each group, *P<0.05 vs SHR, #P<0.05 vs SHR.