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Inhibition of histone deacetylase on ventricular remodeling in infarcted rats

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Lee T-M, Lin M-S, Chang N-C. Inhibition of histone deacetylase on ventricular remodeling in infarcted rats. *Am J Physiol Heart Circ Physiol* 293: H968–H977, 2007. First published March 30, 2007; doi:10.1152/ajpheart.00891.2006.—Histone deacetylase (HDAC) determines the acetylation status of histones and, thereby, controls the regulation of gene expression. HDAC inhibitors have been shown to inhibit cardiomyocyte growth in vitro and in vivo. We assessed whether HDAC inhibitors exert a beneficial effect on the remodeling heart in infarcted rats. At 24 h after ligation of the left anterior descending artery, male Wistar rats were randomized to vehicle, HDAC inhibitors [valproic acid (VPA) and tributyrin], an agonist of HDAC (theophylline), VPA + theophylline, or tributyrin + theophylline for 4 wk. Significant ventricular hypertrophy was detected as increased myocyte size at the border zone isolated by enzymatic dissociation after infarction. Cardiomyocyte hypertrophy and collagen formation at the remote region and border zone were significantly attenuated by VPA and tributyrin with a similar potency compared with that induced by the vehicle. Left ventricular shortening fraction was significantly higher in the VPA- and tributyrin-treated groups than in the vehicle-treated group. Increased synthesis of atrial natriuretic peptide mRNA after infarction was confirmed by RT-PCR, consistent with the results of immunohistochemistry and Western blot for acetyl histone H4. The beneficial effects of VPA and tributyrin were abolished by theophylline, implicating HDAC as the relevant target. Inhibition of HDAC by VPA or tributyrin can attenuate ventricular remodeling after infarction. This might provide a worthwhile therapeutic target.

hypertrophy; infarction

HISTONE ACETYLATION PLAYS a critical role in cardiac development and disease. Acetylation of histone residues results in unwinding of the DNA, which allows transcription factors and RNA polymerase II to bind more readily to DNA and, thereby, increase gene transcription (40). Histone deacetylase (HDAC) inhibitors have been shown to cause cell cycle arrest. Class I and II HDACs are involved in the control of cardiac hypertrophy (4, 16, 40). Class I HDACs can be recruited by homeodomain-only protein or other hypertrophic stimuli, resulting in inhibition of serum response factor and the antihypertrophic gene program (16). Class II HDACs can act as signal-responsive repressors of cardiac hypertrophy by inhibiting expression of the gene that is dependent on myocyte enhancer factor 2C (4, 40). Consistent with this repressive role, mutant mice lacking the class II HDAC9 are hypersensitive to hypertrophic signals (26, 40). Paradoxically, HDAC inhibitors such as valproic acid (VPA) (33) and tributyrin induce growth arrest, differentiation, and/or apoptosis of cardiomyocytes in vitro (3). These inhibitory effects are believed to be caused, in part, by

accumulation of acetylated proteins, such as nucleosomal histones, which appear to play a major role in regulation of transcription of genes such as atrial natriuretic peptide (ANP) (1, 14, 18).

Cardiac remodeling has been associated with myocardial hypertrophy and left ventricular (LV) dilation following myocardial infarction (MI) (39). Although traditionally considered an adaptive response to pathological signaling, cardiac hypertrophy has generally been thought to induce reactivation of fetal genes such as ANP, resulting in maladaptive changes in cardiac contractility and sudden death (23). For more effective prevention of cardiac hypertrophy and more successful application of therapeutic interventions, better attenuation of ventricular growth at the early stage of cardiac hypertrophy, rather than at the established stage, is important. Although cardiac hypertrophy induced by isoproterenol infusion, angiotensin II infusion, and aortic banding can be significantly attenuated by the HDAC inhibitor trichostatin A (15, 16), whether HDAC inhibition would also prevent ventricular remodeling, a process of hypertrophy induced by a different mechanism, remained unknown. Thus we investigated the effects of VPA-induced HDAC inhibition on myocardial alterations of post-MI remodeling. Although VPA is an antagonist of HDAC, VPA has multiple cellular and molecular mechanisms, including the increase of reactive oxygen species (38). These alternative effects could confound the interpretation of the present study. To further confirm whether HDAC inhibition is mandatory for LV remodeling, we treated infarcted rats with HDAC inhibitors that differed structurally from VPA, e.g., tributyrin, and measured the hemodynamic, biochemical, molecular, functional, and morphological changes. Also, to assess the direct cause-effect relationship between HDAC and ventricular remodeling, we studied an agonist of HDAC, theophylline.

MATERIALS AND METHODS

Animals. Male Wistar rats (300–350 g body wt) were subjected to infarction of the LV free wall via ligation of the anterior descending artery, as previously described (19). At 24 h after MI, the rats were randomly separated into 6 groups of 10 rats each: 1) vehicle, 2) VPA (100 mg·kg⁻¹·day⁻¹), 3) tributyrin (0.4 g·kg⁻¹·day⁻¹), 4) 8-(p-sulfophenyl)-theophylline (20 mg·kg⁻¹·day⁻¹; Sigma, St. Louis, MO), 5) VPA + 8-(p-sulfophenyl)-theophylline, and 6) tributyrin + 8-(p-sulfophenyl)-theophylline. The dose of HDAC inhibitors has been reported to inhibit endogenous HDAC activity (8, 24). Tributyrin, a triglyceride with butyrate molecules esterified at the 1, 2, and 3 positions, induces differentiation and/or growth inhibition of a number of cell lines in vitro. When given orally to rodents, tributyrin produces substantial plasma butyrate concentrations (10). The dose of

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theophylline used in this study was chosen to achieve a plasma theophylline concentration of 6–10 μM (29), a therapeutic concentration for activation of HDAC. The drugs were administered by daily oral gavage for 4 wk starting on the day of randomization. Sham-operated rats served as controls. With this method, the 24-h mortality rate was 50% in the infarcted rats according to our experience (19). The study duration was designed to be 4 wk, because the majority of the myocardial remodeling process in the rat (70–80%) is complete within 3 wk (32). The animal experiment was approved and conducted in accordance with local institutional guidelines for the care and use of laboratory animals in the Chi-Mei Medical Center.

Echocardiogram. At 28 days after operation, the rats were lightly anesthetized with ketamine HCl (25 mg/kg ip). Echocardiographic measurements were done with an HP Sonos 5500 system with a 15-6L probe (6–15 MHz; Agilent Technologies, Palo Alto, CA). An M-mode trace of the LV was obtained from the parasternal long-axis view for measurement of LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD), and fractional shortening (FS, %) was calculated. After systemic heparinization, hemodynamic measurements were obtained.

Hemodynamics and infarct size measurements. Hemodynamic parameters were measured in ketamine (90 mg/kg ip)-anesthetized rats at the end of the study, as previously described (19). A Millar polyethylene catheter was inserted into the right carotid artery and connected to a transducer (model SPR-407, Millar Instruments, Houston, TX) for measurement of LV systolic and diastolic pressure as the mean of measurements of five consecutive pressure cycles. The maximal rate of LV pressure increase ($+dP/dt$) and decrease ($-dP/dt$) was measured. After the arterial pressure measurement, the heart was rapidly excised. The atria, right ventricle, and LV were rinsed in cold physiological saline, weighed, and immediately frozen in liquid nitrogen after coronal sections of the LV were obtained for estimation of infarct size. Sections (5 μm) from the equator of the LV were fixed in 10% formalin and stained with Masson's trichrome for determination of infarct size. The boundary lengths of the infarcted and noninfarcted endocardial and epicardial surfaces were traced with a planimeter digital image analyzer. Infarct size was calculated as the ratio average of external and internal perimeters of the scar to all fields of the LV sections, as previously described (19). Only rats with large infarction (>30%) were selected for analysis.

Real-time RT-PCR. To further confirm the degree of ventricular hypertrophy, ANP mRNA was measured by real-time quantitative RT-PCR from samples obtained from the border and remote zones with the TaqMan system (Prism 7700 Sequence Detection System, PE Biosystems), as previously described (19). The primers were 5'-GCCCTTGCGGTGTGTCA and 5-TGCAGCTCCAGGAGGGTATT for ANP and 5'-CTTCACCACCATGGAGAAGGC and 5'-GGCATGGACTGTGGTCATGAG for GAPDH. For quantification, ANP expression was normalized to the expressed housekeeping gene GAPDH. Reaction conditions were programmed on a computer linked to the detector for 40 cycles of the amplification step.

Western blotting. Accumulation of acetylated histones can be used as a marker of biological activity. All procedures used for Western blotting analysis of protein are described elsewhere (20). Briefly, samples from the border zone were homogenized with a kinematic Polytron blender in 100 mM Tris-HCl, pH 7.4, supplemented with 20 mM EDTA, 1 mg/ml pepstatin A, 1 mg/ml antipain, and 1 mM benzamide. Homogenates were centrifuged at 10,000 g for 30 min to pellet the particulate fractions. The supernatant protein concentration was determined using the bicinchoninic acid protein assay reagent kit (Pierce). Fifty micrograms of protein were separated by 8% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. After incubation with a primary polyclonal antibody against acetyl histone H4 (Upstate Biotechnology, Lake Placid, NY), the nitrocellulose membrane was rinsed with a blocking solution and incubated for 2 h at room temperature. A scanning densitometer was used for volume integration of the films within the linear range of the exposure.

Experiments were replicated three times, and results are expressed as mean values.

Immunohistochemical analysis of acetyl histone H4. For investigation of the spatial distribution of acetyl histone H4, immunohistochemical staining was performed on LV muscle from the border zone (0–2 mm outside the infarct). Hearts were snap frozen in liquid nitrogen and embedded in OCT compound (Tissue-Tek), and 5- μm -thick cryosections were obtained. The slides containing the sectioned tissues were fixed in 10% formalin and rinsed in PBS. Endogenous peroxidase activity was blocked by immersion of the sections in 3% H_2O_2 for 12 min at room temperature. The sections were blocked with 10% normal goat serum in PBS for 15 min. Tissues were incubated with a rabbit polyclonal anti-acetyl histone H4 antibody (1:10 dilution; Upstate Biotechnology) in 1% normal goat serum in PBS overnight at 4°C. Immunostaining with acetyl histone H4 antibodies was performed using a standard immunoperoxidase technique (N-Histofine Simple Stain Rat MAX PO kit, Nichirei, Tokyo, Japan). The antibody had been tested for specificity in the rat. Isotype-identical directly conjugated antibodies served as a negative control. Because of the wide variability of structural composition of border zone regions, which resulted in intercellular connection ranging from total disruption in fully scarred regions to negligible alterations with normal-appearing myocytes, we selected samples for analysis that were composed of cardiomyocytes separated by diffuse interstitial fibrosis.

The slides were coded so that the investigator was blinded to the identification of the rats. The area of immunoreactive acetyl histone H4 was qualitatively estimated from 10 randomly selected fields at $\times 400$ magnification and quantified by computer-assisted image analysis (Image Pro Plus), as previously described (20). The value was expressed as the ratio of H4-stained area to total area.

Morphometric histological determination of myocyte size and fibrosis. The effect of HDAC inhibitors on ventricular remodeling after infarction was further confirmed by pathological examination. To exclude differences in cardiomyocyte size in different regions of the LV (36), we obtained samples from the middle part of the LV and used hematoxylin-and-eosin stain. For consistency of results, myocytes positioned perpendicularly to the plane of the section with a visible nucleus and a clearly outlined and unbroken cell membrane were selected for the cross-sectional area measurement, as previously described (21). This area was determined by manual tracing of the cell contour on a digitized image acquired on the image-analysis system at $\times 400$ magnification using computerized planimetry (Image Pro Plus), as described previously (20). A total of 100 myocytes were selected in the LV of each heart and analyzed by an observer blinded to the experimental treatment.

Additionally, heart sections were stained with Masson's trichrome for assessment of the degree of fibrosis at the remote zone and border zone. The percentage of blue staining, indicative of fibrosis, was measured (10 fields randomly selected on each section). The value was expressed as the ratio of trichrome-stained fibrosis area to total infarct area. Furthermore, to evaluate the scar dynamics, we defined the thinning index as minimal wall thickness in the infarct normalized to maximal septal wall thickness. All sections were evaluated without prior knowledge of which section belonged to which rat.

Cell isolation. Because cardiac hypertrophy is a combination of reactive fibrosis and myocyte hypertrophy, we used cardiomyocyte size at the border zone, in addition to myocardial weight, to avoid the interference of nonmyocytes on postinfarction hypertrophy. Since the infarct size measurement procedure does not permit quantitation of cardiomyocyte sizes, additional groups of rats were infarcted using the same procedures and used for measurement of cell sizes at the end of the study. Myocytes were enzymatically isolated with collagenase (type II; Sigma) and protease (type XIV; Sigma), as previously described (19). Random high-power fields of the rodlike relaxed myocytes with clear striations were chosen to eliminate selection bias. At least 100 cells from each section were selected for measurement of

cell length, width, and area, and the mean value was used as the individual value for each section. In the sham-operated group, cell width and length were measured from the LV free wall for comparisons.

Plasma levels of theophylline and VPA. Blood samples from the inferior vena cava were obtained for measurements of theophylline and VPA at the end of the study. The blood samples were immediately centrifuged at 3,000 g for 10 min, and the plasma samples were stored at -70°C . Plasma VPA concentrations were analyzed by a fluorescence polarization immunoassay system using an AxSym analyzer (Abbott Diagnostic Division, Irving, TX). The sensitivity of the assay was $5\text{ }\mu\text{mol/l}$, and the intra- and interassay coefficients of variation were 1.8 and 2.7%, respectively.

Statistical analysis. Values are means $\pm$ SD. Statistical analysis was performed using the SPSS statistical package (version 10.0, SPSS, Chicago, IL). A two-way ANOVA was used to search for possible effects of VPA, tributyrin, and theophylline on the measurements of hemodynamics and myocyte sizes, and if an F value was found to be significant, a two-tailed Student's t -test for paired observation with Bonferroni's correction was used to test differences. Interaction term of VPA, tributyrin, and theophylline was incorporated into the model. $P < 0.05$ was considered to be statistically significant.

RESULTS

Daily administration of VPA or tributyrin for a given period was well tolerated, because it did not affect overall body weight compared with vehicle (Table 1). No differences in mortality between vehicle and treated groups were found throughout the study. No sham-operated rats showed evidence of cardiac damage. Blood pressure, heart rate, and infarct size did not differ among the infarcted groups.

Morphometric studies. VPA, tributyrin, and theophylline had little effect on cardiac gross morphology in the sham-operated rats, whereas their effects on the cardiac morphology after MI were significant (Table 1). At 4 wk after MI, the infarcted area of the LV was very thin and was totally replaced by fully differentiated scar tissue. Right ventricular weight-to-body weight ratio and lung weight-to-body weight ratio were increased in rats treated with vehicle, theophylline, VPA + theophylline, and tributyrin + theophylline compared with sham-operated rats. The weight of the LV, including the septum, remained essentially constant among the infarcted groups 4 wk after coronary artery occlusion.

To characterize the cardiac hypertrophy on a cellular level, we isolated cardiomyocytes from rats subjected to the different treatments (Table 2). The cells isolated from the border zone in the vehicle-treated group significantly increased by 41% compared with those from the same area of sham-operated hearts ($5,127 \pm 273$ vs. $3,002 \pm 201\text{ }\mu\text{m}^2$, $P < 0.0001$). VPA and tributyrin reduced cell areas 30% and 36% compared with the vehicle group (both $P < 0.0001$, respectively). In contrast to the VPA- and tributyrin-treated rats, theophylline-treated rats demonstrated cardiomyocyte hypertrophy. The characteristics of cardiomyocytes from remote sites were distinct from those from the border zones: myocyte length, width, and area were less in cardiomyocytes of infarcted groups treated with vehicle, theophylline, VPA + theophylline, and tributyrin + theophylline from remote sites. To further confirm cardiac hypertrophy after infarction, our histological measurement of cardiomyocyte cross-sectional area (Fig. 1) showed results consistent with those from cardiomyocytes isolated by the enzymatic method.

Table 1. Cardiac morphometry, hemodynamics, and concentrations of VPA and theophylline at the end of study

Parameters	Sham					Infarction						
	Vehicle (n= 11)	VPA (n = 11)	Tri (n = 11)	Theo (n = 11)	VPA + Theo (n = 11)	Tri + Theo (n = 11)	Vehicle (n = 9)	VPA (n = 10)	Tri (n = 12)	Theo (n = 11)	VPA + Theo (n = 11)	Tri + Theo (n = 10)
Body wt, g	405±28	421±30	425±21	408±22	410±21	412±22	399±40	414±25	416±25	422±32	423±23	419±27
HR, beats/min	413±23	429±25	421±31	427±25	429±25	437±25	429±25	432±23	414±24	429±28	428±25	427±21
LVESP, mmHg	111±11	105±8	112±10	109±12	107±14	108±12	104±10	112±11	107±10	107±11	104±12	112±11
LVDP, mmHg	5±3	5±3	6±2	6±3	5±4	5±4	17±3*	17±5*	15±5*	19±5*	19±4*	17±5*
LV wt mg	871±85	935±105	948±94	926±98	984±82	922±144	1,189±84*	1,217±196*	1,202±144*	1,317±135*	1,298±118*	1,265±105*
RV wt, mg	207±23	219±25	225±34	232±20	213±21	218±20	279±64*	232±50†	241±37‡	304±63*	321±55*	306±67*
Lung wt, g	1.63±0.21	1.78±0.25	1.85±0.31	1.76±0.21	1.78±0.21	1.80±0.30	2.19±0.33*	1.89±0.26†	1.78±0.24‡	2.35±0.25*	2.38±0.27*	2.29±0.23*
+dP/dt, mmHg/s	7,852±263	8,726±352	7,692±302	7,392±483	7,372±439	7,820±428	3,726±382*	5,428±372*†	4,982±273*‡	3,172±321*	3,271±302*	3,682±362*
-dP/dt, mmHg/s	6,326±341	5,084±285	6,527±487	5,982±392	6,720±259	6,582±528	3,172±452*	4,084±272*†	5,092±422*‡	2,672±268*	2,482±256*	2,157±354*
Infarct size, %							40±5	41±5	39±3	41±6	41±4	40±3
Theo, µM				7.4±1.1	8.1±1.4	8.9±1.2				8.8±1.2	7.6±2.1	7.6±1.8
VPA, µg/ml		78±19			74±25			93±27			88±22	

Values are means $\pm$ SD. HR, heart rate; LVESP, left ventricular (LV) end-diastolic pressure; LVDP, LV end-systolic pressure; RV, right ventricle; Theo, theophylline; Tri, tributyrin; VPA, valproic acid. * $P < 0.05$ vs. respective groups without infarction. † $P < 0.05$ vs. infarction with vehicle and VPA + Theo. ‡ $P < 0.05$ vs. infarction with vehicle and Tri + Theo.

Table 2. Characteristics of isolated cardiomyocytes

Parameters	Sham	Infarction					
		Vehicle	VPA	Tri	Theo	VPA + Theo	Tri + Theo
Border zone							
Myocyte length, μm	135±10	182±25*	152±15*†	151±21*†	175±27*	170±21*	175±19*
Myocyte width, μm	21±3	24±5*	22±2†	21±3†	26±5*	25±5*	26±5*
Measured myocyte areas, μm^2	3,002±201	5,127±273*	3,592±252*†	3,281±243*†	4,983±262*	4,692±234*	5,201±218*
Remote zone							
Myocyte length, μm	132±12	143±15*‡	135±19‡	137±21‡	146±20*‡	143±19*‡	145±20*‡
Myocyte width, μm	20±3	21±3‡	20±4	20±3	21±4‡	20±2‡	21±2‡
Measured myocyte areas, μm^2	2,763±218	3,128±214‡	2,873±168	2,769±189	3,367±275*‡	3,111±237‡	3,271±246‡

Values are means $\pm$ SD ($n = 5$). * $P < 0.05$ vs. sham. † $P < 0.05$ vs. vehicle, Theo, VPA + Theo, and Tri + Theo. ‡ $P < 0.05$ vs. respective data from border zone within the same group.

At 4 wk after MI, collagen volume fraction increased by 2.90-fold ($P < 0.0001$) at the remote zone in the vehicle-treated group compared with the sham group (Fig. 2). In the remote zone, VPA and tributyrin significantly reduced collagen area fraction (51% and 48%, respectively, both $P < 0.05$) compared with vehicle. Quantitative analysis showed a significant increase of collagen formation in the LV at the remote zone in rats treated with VPA + theophylline compared with those treated with VPA alone. Similarly, collagen formation at the border zone showed a significantly higher fibrosis fraction in infarcted rats treated with vehicle, theophylline, VPA + theophylline, and tributyrin + theophylline than in rats treated with VPA and tributyrin. As expected, the thinning index decreased from 0.95 ± 0.06 in the sham-operated group to 0.28 ± 0.08 in the vehicle-treated infarcted group ($P < 0.0001$; Fig. 3). The thinning index was significantly higher in infarcted rats treated with VPA and tributyrin than in rats treated with vehicle, theophylline, VPA + theophylline, and tributyrin + theophylline.

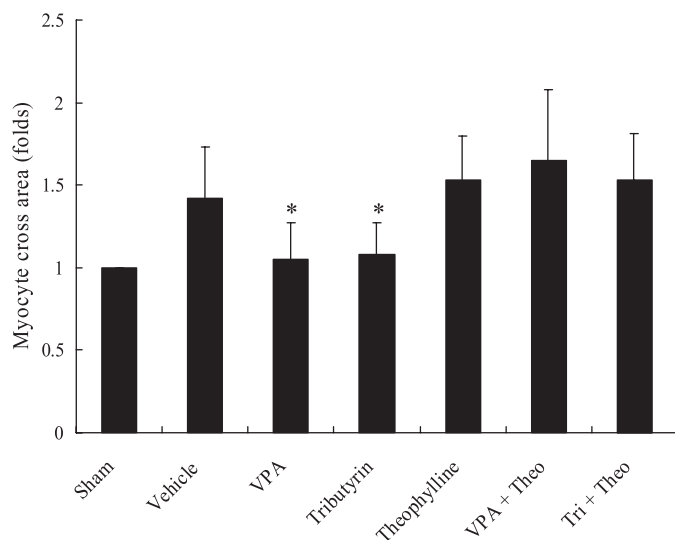


Fig. 1. Morphometric analysis of left ventricular (LV) cardiomyocyte cross-sectional areas (CSA) in rats treated with vehicle, valproic acid (VPA), tributyrin, theophylline, VPA + theophylline (Theo), and tributyrin (Tri) + theophylline in hematoxylin-and-eosin-stained cardiac sections from the border zone at 4 wk after infarction. Relative myocyte cross-sectional area was normalized to the mean value of sham-operated rats at the end of the study. * $P < 0.05$ vs. vehicle, theophylline, VPA + Theo, and Tri + Theo.

Echocardiographic data. After 4 wk of drug intervention, LV structure and function were evaluated in vivo by echocardiographic analysis (Table 3). Compared with sham-operated hearts, MI hearts showed structural changes such as increased LV diastolic and systolic diameters, consistent with LV remodeling (Fig. 4). LVEDD and LVESD in rats with MI were significantly reduced by VPA or tributyrin ($P < 0.05$). LV FS was significantly higher in the VPA- and tributyrin-treated groups than in the vehicle-treated group. Conversely, rats treated with theophylline developed impaired LV systolic function and progressive LV dilation compared with those treated with VPA and tributyrin. These data were corroborated by significant improvement of $+dP/dt$ and $-dP/dt$ in the VPA- and tributyrin-treated groups compared with the groups treated with VPA + theophylline, and tributyrin + theophylline. The interventricular septum was totally replaced by scar tissue, the thickness of which was not significantly different among the infarcted groups. LV posterior wall thickness in infarcted rats was significantly reduced by VPA or tributyrin ($P < 0.05$), consistent with the attenuated cardiac hypertrophy, as shown in isolated cardiomyocytes.

Immunohistochemical analyses and Western blot of histone H4. Immunohistochemical analysis of the infarcted myocardium revealed acetyl histone H4 immunoreactivity in the myocardial tissue (Fig. 5). Acetyl histone H4 signals at the border zone of vehicle-treated rats were stronger than those in the same region of sham rats ($4.5 \pm 1.2\%$ vs. $1.4 \pm 0.2\%$, $P < 0.0001$). The intensity of the immunoreaction was reduced in

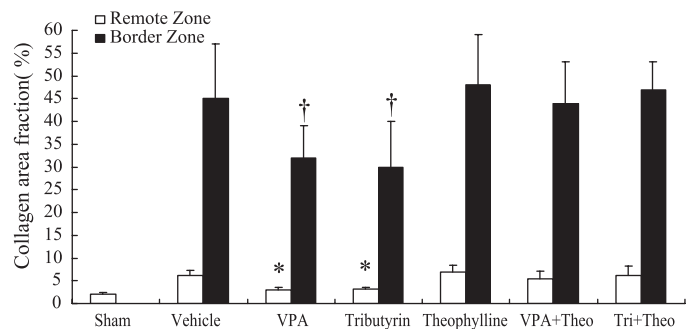


Fig. 2. LV collagen area fraction (%) at remote and border zones. Values are means $\pm$ SD. Collagen deposition within the LV is reduced after administration of VPA or tributyrin. * $P < 0.05$ vs. vehicle, theophylline, VPA + Theo, and Tri + Theo at the remote zone. † $P < 0.05$ vs. vehicle, theophylline, VPA + Theo, and Tri + Theo at the border zone.

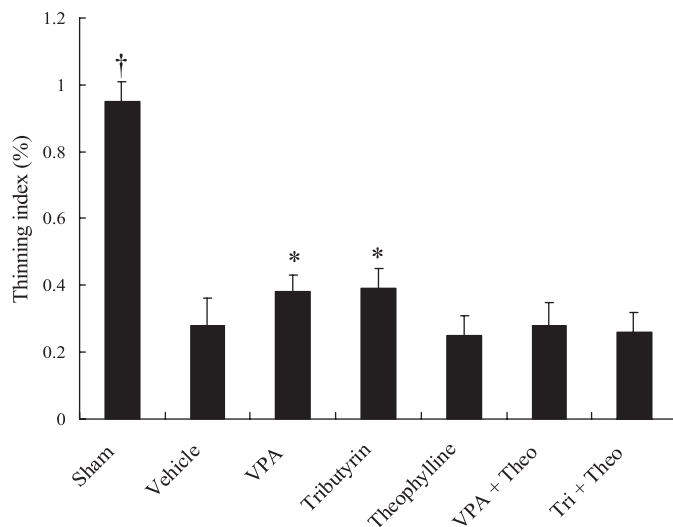


Fig. 3. VPA or tributyrin significantly decreased ventricular wall thinning, i.e., thinning index (ratio of minimal infarct wall thickness to maximal septal wall thickness). * $P < 0.05$ vs. vehicle, theophylline, VPA + Theo, and Tri + Theo. † $P < 0.05$ vs. all infarcted groups.

the VPA- and tributyrin-treated groups compared with the vehicle-treated group. The beneficial effects of VPA were reversed by the addition of theophylline, implicating HDAC as the relevant target.

To determine whether regression of cardiac hypertrophy by HDAC inhibitors correlated with increases in histone acetylation in the cardiomyocytes, the effect of VPA and tributyrin on histone H4 acetylation was determined by immunoblot analysis. Western blot shows that acetyl histone H4 levels were significantly upregulated 2.1-fold at the border zone in vehicle-treated rats compared with sham-operated rats ($P < 0.0001$; Fig. 6). LV acetyl histone H4 levels were significantly lower at the border zone in VPA- and tributyrin-treated rats than in vehicle-treated rats.

Real-time PCR of ANP. Regional myocardial expression of ANP was measured by competitive RT-PCR (Fig. 7). Ventricular remodeling reactivates the fetal gene program, characterized by increased ANP transcripts after infarction. These increases were blunted by administration of VPA or tributyrin at the remote and border zones. The changes in ANP mRNA levels were confirmed by quantification, and both VPA and tributyrin significantly blunted the increase, consistent with the changes in cellular dimensions.

DISCUSSION

Our present study is the first to investigate the effect of HDAC inhibitors on ventricular remodeling, including cardiac hypertrophy and fibrosis, at the remote and border zones after infarction. These new observations strengthen the concept that HDAC plays a central role in the remodeling process and may improve our understanding of the beneficial effect of early administration of HDAC antagonists in postinfarction remodeling.

The beneficial effects of HDAC inhibitors on LV remodeling were concordant, as documented structurally by reduction of myocyte size, molecularly by myocardial acetyl histone H4 protein and ANP mRNA levels, and functionally by improvement of echocardiography-derived systolic function. The effects of HDAC inhibitors appear to be specific, because no effect of drug on ventricular remodeling can be demonstrated in groups without intervening infarction. The importance of HDACs in this process was confirmed by the ability of the HDAC agonist theophylline to completely block the beneficial effect of attenuated cardiac hypertrophy. Indeed, our results were compatible with recent findings of Kee et al. (15) showing that HDAC inhibition can prevent cardiac hypertrophy and interstitial collagen formation. Although their structures are dissimilar, VPA and tributyrin appear to share a common mediator, HDAC, and translation levels of HDAC may play a role in the signal transduction pathway. Our results were consistent with the notion that deacetylation of histones increases the winding of DNA around histone residues, resulting in a dense chromatin structure and reduced access of transcription factors to their binding sites, thereby leading to repressed transcription of hypertrophic genes. Also, administration of HDAC inhibitors ameliorates the function of chronically infarcted hearts. LVEDD was significantly smaller in the VPA- and tributyrin-treated groups than that in the vehicle-treated group. HDAC inhibition resulted in downregulation of β -myosin heavy chain expression, with a concomitant increase in the levels of α -myosin heavy chain (26). Thus HDAC inhibitors increased myofibrillar ATPase activity and improved contractility in the failing heart, consistent with our echocardiographically determined FS results. The extent of the improvement in FS is important, in that it may affect mortality.

It appears from our study that the attenuated ventricular remodeling is related to HDAC inhibition in response to treatment. The addition of theophylline to VPA- and tributyrin-treated rats impaired their ability to attenuate ventricular remodeling, implying that this effect is not a nonspecific action. The mechanisms by which HDAC inhibitors attenuate ventricular remodeling remain to be defined. However, several factors

Table 3. Echocardiographic findings

Parameters	Sham	Infarction					
		Vehicle	VPA	Tri	Theo	VPA + Theo	Tri + Theo
IVS, mm	1.8 $\pm$ 0.2	1.0 $\pm$ 0.2*	1.1 $\pm$ 0.2*	1.1 $\pm$ 0.3*	1.0 $\pm$ 0.3*	1.0 $\pm$ 0.2*	1.1 $\pm$ 0.2*
LVEDD, mm	6.2 $\pm$ 0.3	8.9 $\pm$ 0.4*	7.5 $\pm$ 0.3*†	7.3 $\pm$ 0.3*‡	9.2 $\pm$ 0.3*	9.0 $\pm$ 0.3*	9.2 $\pm$ 0.3*
LVESD, mm	3.9 $\pm$ 0.2	7.2 $\pm$ 0.3*	5.6 $\pm$ 0.4*†	5.3 $\pm$ 0.2*‡	7.3 $\pm$ 0.2*	7.2 $\pm$ 0.3*	7.4 $\pm$ 0.2*
LV posterior wall, mm	1.8 $\pm$ 0.2	2.1 $\pm$ 0.2*	1.7 $\pm$ 0.2†	1.8 $\pm$ 0.2‡	2.2 $\pm$ 0.3*	2.1 $\pm$ 0.3*	2.2 $\pm$ 0.2*
FS, %	37 $\pm$ 4	19 $\pm$ 4*	25 $\pm$ 4*†	27 $\pm$ 3*‡	21 $\pm$ 3*	20 $\pm$ 3*	21 $\pm$ 3*

Values are means $\pm$ SD. IVS, interventricular septum; LVEDD and LVESD, LV end-diastolic and end-systolic diameter; FS, fractional shortening. * $P < 0.05$ vs. sham. † $P < 0.05$ vs. infarction with vehicle and VPA + Theo. ‡ $P < 0.05$ vs. infarction with vehicle and Tri + Theo.

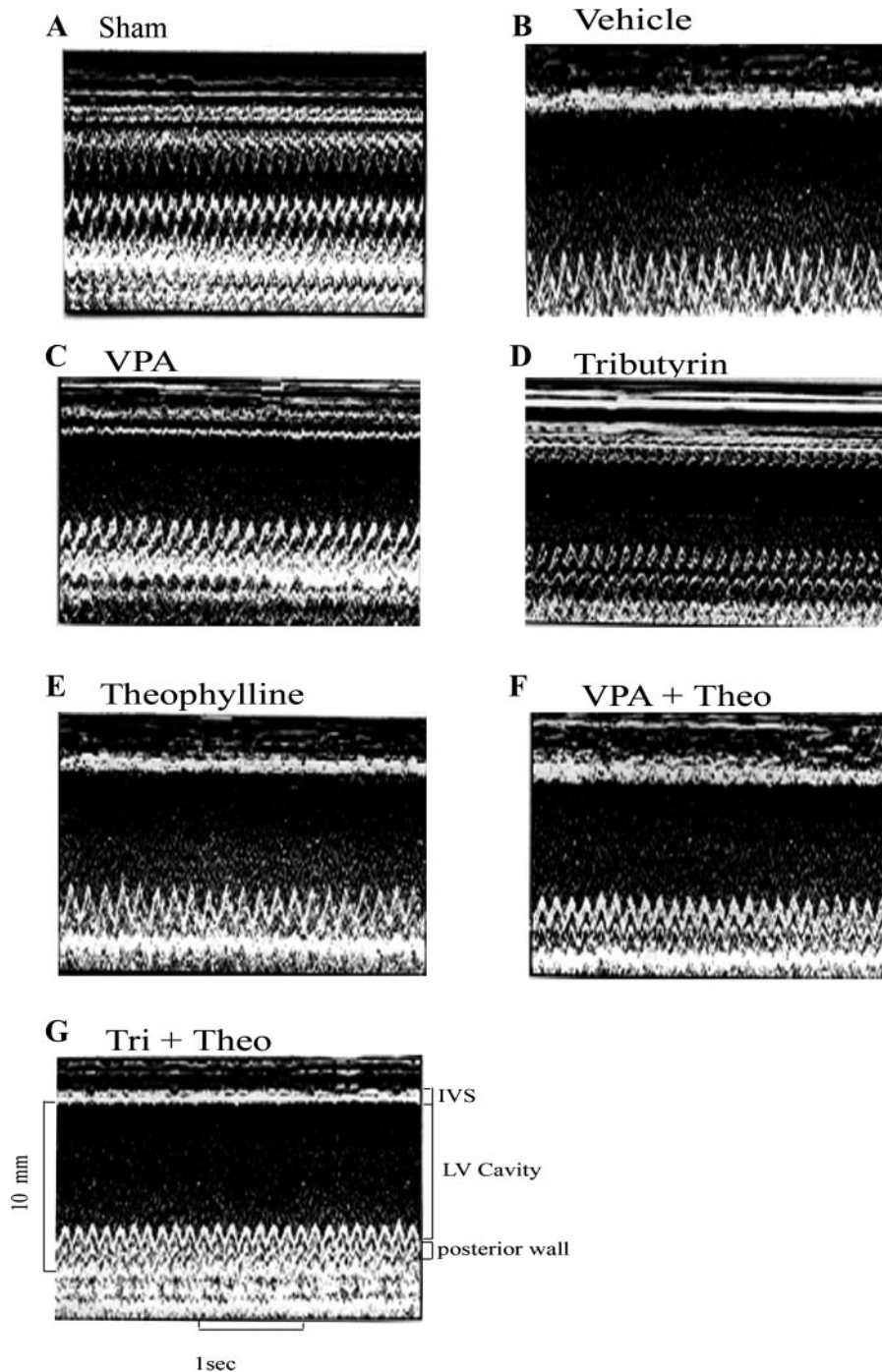


Fig. 4. Representative M-mode images reveal hypokinetic-to-akinetic anterior wall and LV dilation in infarcted hearts (B–G), in contrast to normal anterior wall motion in sham-operated heart (A). LV end-diastolic and end-systolic diameters were markedly dilated in groups treated with vehicle (B), theophylline (E), VPA + theophylline (F), and tributyrin + theophylline (G) compared with groups treated with VPA (C) and tributyrin (D). IVS, inter-ventricular septum.

can be excluded. 1) Because none of the treatments exerted hemodynamic effects in this study, the blockade of the hypertrophy might have been caused by direct cardiac effects, rather than secondarily by reduction of afterload. Although we demonstrated that ventricular remodeling after infarction was attenuated by HDAC inhibitors, the velocity of relaxation as assessed by maximum $-dP/dt$ and LV end-diastolic pressure did not improve. It is noteworthy that subtle changes of relaxation function do not affect maximum $-dP/dt$ (2). Furthermore, LV end-diastolic pressure was largely dependent on loading conditions (34). 2) Although MI size is an important

determinant of LV remodeling (11), it was excluded in this study because of similar infarct sizes among the groups.

The present findings contrast with a report (9) that ANP expression was induced by trichostatin A, an HDAC inhibitor. In that study, the induction of ANP expression was shown to be dependent on the inactivation of a repressive element known as a neuron-restrictive silencer element in the 3'-untranslated region of the gene. However, we have found that HDAC inhibitors can attenuate expression of ANP after infarction. The reason for the discrepancy is not clear. However, our finding was consistent with that of Antos et al. (3), who showed

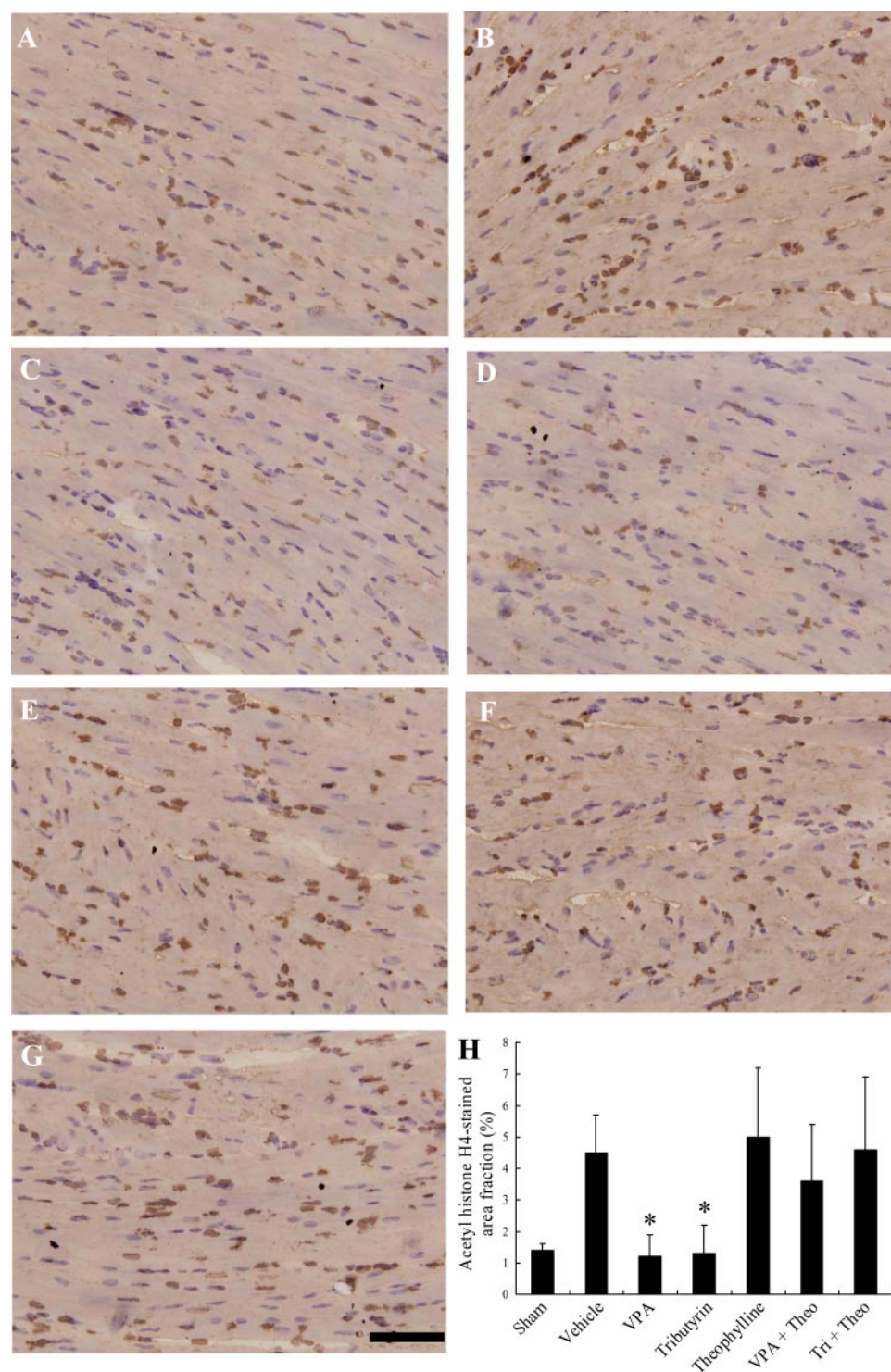


Fig. 5. A–G: immunohistochemical microscopic images of acetyl histone H4 (magnification $\times 400$) at the border zone (BZ) in sham-operated rats (A) and rats treated with vehicle (B), VPA (C), tributyrin (D), theophylline (E), VPA + theophylline (F), and tributyrin + theophylline (G). Positive staining for acetyl histone H4 (brown) in myocytes was significantly more intense in rats treated with vehicle, theophylline, VPA + theophylline, and tributyrin + theophylline than in sham-operated, VPA-treated, and tributyrin-treated rats. Scale bar, 30 μ m. H: area of immunoreactive acetyl histone H4 at the border zone. * $P < 0.05$ vs. vehicle, theophylline, VPA + Theo-, and Tri + Theo.

that HDAC inhibitors can block activation of the ANP promoter by hypertrophic agonists, independent of inactivation of a repressive element.

In contrast to our results, theophylline as a nonselective phosphodiesterase inhibitor has been shown to enhance ventricular contractility. Phosphodiesterase inhibitors can maintain systolic function when used in combination with a β -adrenoceptor blocker in patients with advanced heart failure (35). Despite the well-documented therapeutic benefits of these

agents, there is, nevertheless, substantial evidence that heart failure results in an attenuated phosphodiesterase inhibitor-induced contractile response (6). Reduced inotropic responses to phosphodiesterase inhibitors have been attributed to abnormalities in signal transduction between cAMP and Ca^{2+} -induced Ca^{2+} release (30). Our results were consistent with the notion that, under the β -adrenergic stimulatory condition, such as after MI, inotropic responses to phosphodiesterase inhibitors are markedly impaired (30).

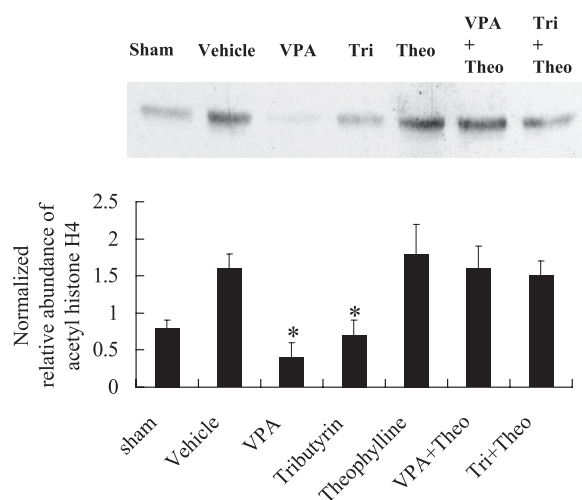


Fig. 6. *Top*: Western blot of acetyl histone H4 in homogenates of the LV from the border zone. Ventricular remodeling was associated with a marked increase of acetyl histone H4. Acetyl histone H4 was significantly reduced in groups treated with VPA or tributyrin compared with control. *Bottom*: densitometric quantification of blot band intensities for relative acetyl histone H4 normalized to β -actin. Values are means $\pm$ SD. * $P < 0.05$ vs. vehicle, theophylline, VPA + Theo, and Tri + Theo.

Other mechanisms. Although the present study suggests that the mechanisms of HDAC inhibitor-induced cardioprotection may be related to inhibition of acetyl histone H4, other potential mechanisms need to be studied. 1) Previous reports have shown that HDAC inhibitors induce apoptosis of transformed cells (25). It is possible that apoptosis in cardiomyocytes may occur in our animal models, leading to reduction of cardiac mass. However, Park et al. (31) clearly showed that untransformed epithelial cells are much more resistant to HDAC inhibitor-induced apoptosis than are cancer cells. Indeed, previous reports have shown that HDAC inhibitors do not significantly affect the survival of primary cultured rat cardiomyocytes and increase apoptosis in cardiomyocytes *in vivo* (9). 2) HDAC inhibitors have been shown to block tumor cell proliferation, in part, by causing cell death (7). We cannot formally rule out the possibility that suppression of hypertrophy by HDAC inhibitors is a nonspecific consequence of cytotoxicity. However, our results argue against this interpretation, because the addition of theophylline to HDAC inhibitor-treated rats impaired their ability to attenuate cellular hypertrophy. Thus, although the heart appears to possess numerous hypertrophic mechanisms, why one antagonist can essentially block hypertrophy remains unknown. A more complete understanding of the heart's hypertrophic mechanisms may ultimately lead to the design of new therapies for the treatment of ventricular remodeling.

Clinical implications. The development of cardiac hypertrophy after infarction is a multigenic, integrative response involving signal integration of multiple pathways. HDAC inhibitors have shown efficacy as anticancer agents in humans and animal models (12, 28). The cardiac effects of these agents are of interest, given the increasing likelihood of more widespread clinical use for noncardiac indications. Our data extended previous studies and suggest that LV remodeling, including cardiac structural and functional consequences, improved after administration of HDAC inhibitors. Previous studies have

demonstrated that regional myocyte hypertrophy parallels regional myocardial dysfunction during postinfarct remodeling (17). Thus attenuation of LV remodeling prevents the transition from compensatory dilation to ventricular dysfunction and failure. The HDAC inhibitors may have important biological effects that prevent postinfarct heart failure. Also, in this study, we have noted a negative correlation between cardiac hypertrophy and cardiac function, indicating that the detrimental effect of theophylline on cardiovascular mortality at least partially should be attributed to its effect on myocardial hypertrophy. Our results were consistent with findings of a large epidemiological study showing that theophylline was independently related to increased cardiovascular death in patients with coronary artery disease (37).

Study limitations. Some limitations in the present study must be acknowledged. 1) VPA or tributyrin inhibits class I and II HDACs (24). There are at least 17 HDACs (40). The relative contributions of each HDAC to the control of cardiac hypertrophy remain to be defined. HDAC5 and HDAC9, both class II HDACs, are highly expressed in myocardium, and inactivation of these genes promotes cardiac hypertrophy (40). These results might suggest that HDAC inhibition would promote hypertrophy. However, treatment of cardiomyocytes with non-selective HDAC inhibitors paradoxically repressed the increase in cell size and protein synthesis (3). Thus one or more HDACs have a positive role in the control of cardiac hypertrophy. Indeed, HDAC4 phosphorylation has been shown to be associated with cardiomyocyte hypertrophy (5). Because the inhibitors used in this study antagonize the action of multiple HDACs, the identity of the putative antihypertrophic HDACs remains unclear. 2) A potential problem with the present study is the use of theophylline as an agonist of HDAC when there are many potential nonspecific targets of theophylline, including phosphodiesterase inhibition or adenosine receptor antagonism. Theophylline has been used for many years in the treatment of obstructive airway diseases, although the molecular mechanisms underlying its therapeutic benefits remain unclear. Sustained stimulation of adenosine receptors is bene-

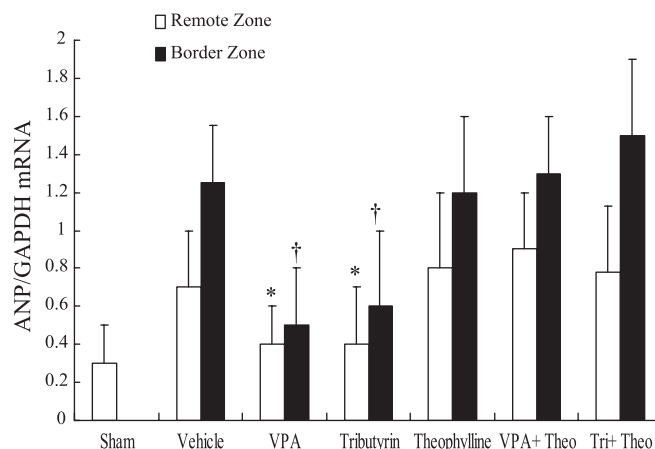


Fig. 7. LV atrial natriuretic peptide (ANP) in sham-operated rats and vehicle-, VPA-, tributyrin-, theophylline-, VPA + theophylline-, and tributyrin + theophylline-treated rats at remote and border zones. Each ANP mRNA value was normalized to GAPDH mRNA. Values are means $\pm$ SD. * $P < 0.05$ vs. vehicle, theophylline, VPA + Theo, and Tri + Theo at the remote zone. † $P < 0.05$ vs. vehicle, theophylline, VPA + Theo, and Tri + Theo at the border zone.

ficial for attenuation of LV mass (22), an effect unrelated to HDAC blockade. We cannot rule out the possibility that 8-(*p*-sulphophenyl)-theophylline, a nonselective adenosine antagonist, enhanced LV mass by acting as an adenosine receptor antagonist. However, this is unlikely, because theophylline as an adenosine antagonist required higher therapeutic concentrations (the therapeutic range is 44–110 μ M) than that reported in this study (10). Previous studies have shown that the direct activation of theophylline on HDAC activity occurs at therapeutic concentrations (1–10 μ M) but is lost at a higher concentration (100 μ M) (13).

Conclusions. These findings are consistent with a pathogenic role of HDAC in cardiac remodeling and myocardial function after infarction. This may be a new beneficial role of HDAC inhibitors in decreasing cardiovascular mortality. The pharmacological profile of HDAC antagonists gives new perspectives in the early treatment of acute infarction.

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