Inhibition of Histone Deacetylase Activity Diminishes Pressure Overloaded Cardiac Hypertrophy in Mice

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ABSTRACT

To explore the role of histone deactylase (HDAC) activation in an *in vivo* model of hypertrophy, we studied the effects of Trichostatin A (TSA). TSA subjected to thoracic aortic banding (TAB)-induced pressure stress in mice. In histological observations, TAB in treated mice showed a significant hypertrophic response, whereas the sham operation remained nearly normal structure with partially blunted hypertrophy. TSA treatment had no effect (measured as HW/BW) on sham-operated animals. TAB animals treated with vehicle manifested a robust ~50% hypertrophic response ($p<0.05$ vs sham). TAB mice treated with 2 mg/kg/day TSA manifested a blunted growth responses, which was significantly diminished ($p<0.05$) compared with vehicle-treated TAB mice. TAB mice treated with a lower dose of TSA (0.5 mg/kg/day) manifested a similar blunting of hypertrophic growth (~25% increase in heart mass). Furthermore, to determine activity duration of TSA *in vitro*, 1 nM TSA was added to H9c2 cells. Histone acetylation was initiated at 4 hr after treatment, and it was peak up to 18 hr, then followed by significantly reduced to 30 hr. We also analyzed the expression of p53 following TSA treatment, wherein p53 expression was elevated at 4 hr, and it was maintained to 24 hr after treatment. ERK was activated at 8 hr, and maintained till 30 hr after treatment suggesting an intracellular signaling interaction between TSA and p53 expression. Taken together, it is suggested that HDAC activation is required for pressure-overload growth of the heart. Eventually, these data suggest that histone acetylation may be a novel target for therapeutic intervention in pressure-overloaded cardiac hypertrophy.

(Key words : Histone deacetylase, Thoracic aortic banding, Cardiac hypertrophy, Trichostatin A)

INTRODUCTION

Common to the care of all patients with cardiovascular disease is a data base on which sound diagnostic and therapeutic decisions can be made. Examination of the arterial pulse yields critically important information regarding the cardiovascular system (Goldman and Cook, 1984). Pulsus paradoxus refer to a decrease in systolic blood pressure of greater than 10 mmHg on inspiration and is a typical feature of pericardial tamponade (Gordon *et al.*, 1974). The carotid arteries provide the most direct reflection of cardiac activity because of their central location in proximity to the left ventricle and aorta. The amplitude of the carotid pulse is typically increased under circumstances associated with higher cardiac output, including fever, anemia, hyperthyroidism, and arteriovenous fistulas (Gordon *et al.*, 1974; Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure, 1984; Department of Health and Human Services, Public Health Services, 1989).

On basic functional anatomy of the heart, the right ventricle is thin walled (3~4 mm) and somewhat irregular in shape, with the interventricular septum being largely formed by the left ventricle. The right ventricle is more compliant than the left, the upper limit of normal for right ventricular end diastolic pressure being 6 mmHg (Department of Health, Education, and Welfare, 1979; Department of Health and Human Services, Public Health Services, 2002). The left ventricle has a thicker wall (8~9 mm), and the upper limit of normal for the left ventricular end-diastolic pressure is higher (12 mmHg). The left ventricle has an ellipsoidal shape, shortens more in its short axis, and normally empties about two thirds of its contents during ejection (Department of Health and Human Services, Public Health

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Service, 1988; World Health Organization).

In recent study, cardiac mechanical dysfunction poses a new therapeutic trial because the existing therapy are not effective. Heart failure represents that eventual pathway for reversible disorders intrinsic to or impinging on the myocardium, including myocardial infarction and hypertension-induced cardiac hypertrophy. Pathological hypertrophy comprises defects in myocytes performance, fibrosis, and myocyte loss, compounded by insufficient replacement (Olson and Schneider, 2003). Activation of multiple pathways is associated with cardiac hypertrophy and heart failure (Kook *et al.*, 2003). Cardiac hypertrophy can occur in response to physiological stress or/and may be maladaptive leading to cardiac dilatation (Braunwald, 2001). Pathological hypertrophy is accompanied by reactivation of fetal gene programs that may be stimulated by changes in cation homeostasis including calcium and mechanical stretch (McKinsey and Olson, 1999; Marks, 2003; Epstein and Davis, 2003). The genetic programs leading to cardiac hypertrophy are diverse and complex. Mutations in a number of genes encoding structural components of the contractile unit, the sarcomere, result in hypertrophic cardiomyopathy in animal models (Seidman and Seidman, 2001). The status of histone acetylation, at a given promoter, is determined by the balanced action of histone acetyltransferases (HATs) and HDACs. The essential role of a HAT protein in cardiac muscle was first proven by deletion of the coactivator p300, which perturbed heart development and cell proliferation (Yao *et al.*, 1998). So far, many of mammalian HDACs have been discovered then categorized into three classes that are based on sequence homology and domain organization. Class I HDACs including HDAC 1, 2, 3, and 8 are expressed ubiquitously and are composed largely of the catalytic domain. Class II HDACs including HDAC 4, 5, 6, and 7 are highly expressed in striated muscle and contain extensions at the N- or C-terminus (Zhang *et al.*, 2002). Class III HDACs is the proteins similar to the yeast NAD1-dependent deacetylase Sir2 (Imai *et al.*, 2000). Class I and II HDACs have been found to function as corepressors recruited for transcriptional repression, whereas the class III HDACs is important for gene silencing at telomeres. Over the past years, it has been found that the epigenetic silence of tumor suppressor genes induced by overexpression of HDACs plays an important role in carcinogenesis. Thus, HDAC inhibitors have emerged as the accessory therapeutic agents for multiple human cancers, since they can block the activity of specific HDACs, restore the expression of some tumor suppressor genes and induce cell differentiation, growth arrest and apoptosis.

A role in DNA damage response has not yet been described for the class I and II HDACs, but is suggested by the observation that treatment with the class I and II HDAC inhibitor TSA which is an antifungal antibiotic that is a potent and specific inhibitor of mammalian HDAC both *in vivo* and *in vitro* leads to significant radiosensitization of human cells. Moreover, TSA, a potent inhibitor of HDAC activity, imposes a dose-dependent blockade to hypertrophy and fetal gene activation in cultured cardiomyocytes.

Thus, based on this background, to explore the role of HDAC activation in an *in vivo* model of hypertrophy, we hypothesized that mechanical and/or hormonal stimuli alter cardiac remodeling such as cardiac hypertrophy which could be inhibited by TSA, a potent inhibitor of class I and class II HDAC. Finally, we studied the effects of TSA in mice subjected to TAB-induced pressure stress as well as cultivated H9c2 cardiomyoblast cells.

MATERIALS AND METHODS

Materials

The drugs used were as follows: Trichostatin A (TSA) (BioVision, USA) and demethyl sulfoxide (DMSO) (SigmaAldrich, St. Louis, MO, USA). Antibodies to acetyl histon H3, acetyl histone H4, p53 and ERK1/2 antibody as constitutively expressing protein in cardiac tissues were obtained from Santa Cruz Biotechnology (Santa cruz, CA, USA). All other materials were obtained from SigmaAldrich (St. Louis, MO, USA) and were of reagent grade quality. All solutions used were prepared in Milli-Q water and filtered (0.45 μm) before use.

Animal Surgery

The surgical procedures and pre- and post-operative care of the animals conformed to the Inje University Animal Care and Use Committee in accordance with Korean Department of Agriculture guidelines and all efforts were made to minimize animal suffering and to reduce the number of animal used. All of described procedures are based upon ethical principles which have been approved by the Institutional Ethics Committee according to the Helsinki Declaration. In detail, male mice (C57/BL6, 6~8 weeks old) were anesthetized with ketamine (100 mg/kg, IP) plus xylazine (5 mg/kg, IP). The mice were orally intubated with 20-gauge tubing and ventilated (Harvard Apparatus Rodent Ventilator, model 687) at 120 breaths per minute (0.1 ml tidal volume). A 3 mm left-sided thoracotomy was created at the second intercrostal space. The transverse aortic arch was ligated (7-0 Prolene) between the innominate and left common carotid arteries with an overlying 27-gage needle, and then the needle was removed, leaving a discrete region of stenosis. The chest was closed, and the left-sided pneumothorax was evacuated. Some mice were subjected to a sham-operation wherein the aorta is

visualized but not manipulated. On the first post- operative day, mice were randomized to daily subcutaneous treatment with TSA (1.0 or 2.0 mg/kg) dissolved into DMSO or vehicle (DMSO). All 4 treatment arms were well tolerated with 3-week mortality less than 40% in all groups.

Histological Staining

The whole hearts from 4 groups were subjected H-E staining to valuate the hypertrophic response by thoracic aortic banding induced cardiac hypertrophy. In detail description, heart tissues were embedded in optical cutting temperature (O.C.T.) compound, frozen, and 10 um sections were obtained by a cryostat microtome.

Immunoblot Analysis

Neonatal cardiomyocytes were collected upon specified treatments with/without TSA as indicated in the text, centrifuged, and washed with phosphate-buffered saline (PBS), and whole cell lysates were prepared by resuspending the pellets in RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 20 mM Tris-HCl, pH 7.2) containing protease inhibitors (1 μM phenylmethylsulfonyl fluoride, 0.01 μM benzamidine HCl, and 0.5 μg each of antipain, leupeptin, pepstatin A, and aprotinin). Equal amounts of cell lysates, typically 10~20 μg, were fractionated by SDS-polyacrylamide gel electrophoresis on 12.5% gels and transferred to methanol soaked polyvinylidene difluoride (PVDF) membranes at 200 mA for 90 min in a Bio-Rad mini Transblot apparatus. After staining (Ponceau S) to confirm equal loading, filters were incubated in PBS plus 0.1% (v/v) Tween 20 (TPBS) supplemented with 5% (w/v) nonfat dry milk for at least 1 hr before with antiserum probing antisera. First antibody incubations were performed in TPBS-milk (blocking buffer) at room temperature for 1 hr and subsequently incubated with specific antibodies. Antibody incubations were followed by washing three times for 30 min each. Antibody-antigen complexes were detected by using the ECL system (Amersham Pharmacia Biotech, NJ, USA). Antibodies for acetyl-histone H3, acetyl-histone H4, p53 and ERK1/2 were used as 1:2,000 dilution. Cultivated H9c2 cells were homogenized in 5 ml of buffer A (5 mM HEPES, pH 7.5, 250 mM saccharose, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 mM aprotinin) by 20 strokes in a Dounce Homogenizer. After centrifugation (100 × g for 10 min) the supernatant was sonicated for 20 sec. Sonicates were cleared in a microcentrifuge for 8 min at 800 × g and its protein concentration was measured. Total protein of H9c2 cells was resolved by SDS-PAGE, transferred to PVDF. The immunoblot was done with primary antibodies followed by HRP-conjugated anti-rabbit or anti-mouse secondary antibody. Protein was visualized using an ECL kit as described previously.

Statistical Analysis

Data are expressed as mean±S.E. "*n*" in Results refers to number of animal preparations on which observations were made. SPSS for window version 18.0 program (SPSS, Inc. Chicago, IL, USA) was used to analyze data. Differences between the means were analyzed by Student's unpaired *t*-test or 1-way ANOVA for comparison of two groups. *P* value of less than 0.05 was considered significant.

RESULTS

TSA Diminishes the TAB-Induced Cardiac Hypertrophy

As reported at previously, the molecular signaling pathways involved in the pathogenesis of hypertrophy and transition to heart failure are the subject of intense investigation (Frey *et al.*, 2000; Molkentin, 2000). Electrical remodeling, culminating in action potential prolongation, is a fundamental aspect of both ventricular hypertrophy (Wickenden *et al.*, 1998) and heart failure (Nabauer and Kaab, 1998; Nuss *et al.*, 1999; Tomaselli and Marban, 1999). Delayed repolarization leads to increased dispersion of refractoriness within the diseased ventricle thereby predisposing to arrhythmia, syncope, and sudden death (Brown *et al.*, 2000). Indeed, the clinical impact of cardiac hypertrophy stems largely from disordered electrical currents that predispose patients to devastating arrhythmias. As similar animal model for cardiac hypertrophy which is established, at 3 weeks after TAB microsurgery, when the hypertrophic response has reached steady state, animals were subjected to histological analysis. H-E staining shows that the TAB/Veh had a significant hypertrophic response, while the sham operation remained normal structure (Fig. 1). However, TSA partially blunted the hypertrophy (Fig. 1). Trichrome staining showed the collagen staining in TAB/Veh, but no fibrosis showed up in TAB/TSA suggesting hypertrophic cardiac remodeling in left ventricular septum due to accumulation of fibrotic materials such as fibronectin and collagen (data not shown).

HDAC Suppression by TSA Leads to Dose-Responsive Blunted of Pathologic Cardiac Growth

The heart weight-to-body weight ratio was increased approroximately 20~25%, and the ventricular walls were symmetrically thickened (Fig. 2) with hypertrophied heart induced by TAB. Introduction of of TSA (1, 2 mg) resulted in diminishing the cardiac hypertrophy measuring heart mass and tibia length ($p<0.05$). TSA treatment had no effect (measured as HW/BW ratio) on sham-operated animals (TSA 4.8±0.3 *vs* Vehicle 4.7±0.3).

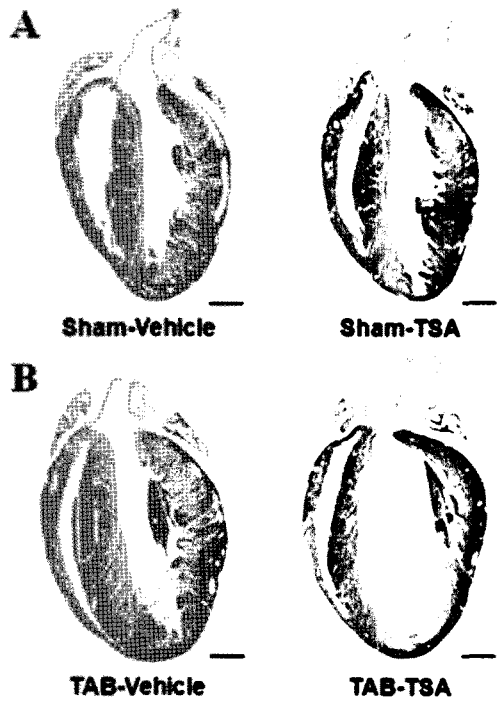

Fig. 1. Histological visualization of Hematoxil-Eosin (HE)-stained 4 chamber-hearts applied TAB with/without TSA. Representative 4-chamber histological sections of hearts treated (A) sham-vehicle *vs* sham-TSA and (B) TAB-vehicle *vs* TAB-TSA. Bars represent 1 mm.

TAB animals treated with vehicle manifested a robust ~ 50% hypertrophic response (HW/BW 6.8±0.4; $p<0.05$ *vs* Sham). TAB mice treated with 2 mg/kg/day TSA manifested a blunted growth response (HW/BW 6.1± 0.1), which was significantly diminished ($p<0.05$) compared with vehicle-treated TAB mice. TAB mice treated with a lower dose of TSA (0.5 mg/kg/day) manifested a similar blunting of hypertrophic growth (data not shown). Also, measurements of lung and liver weights

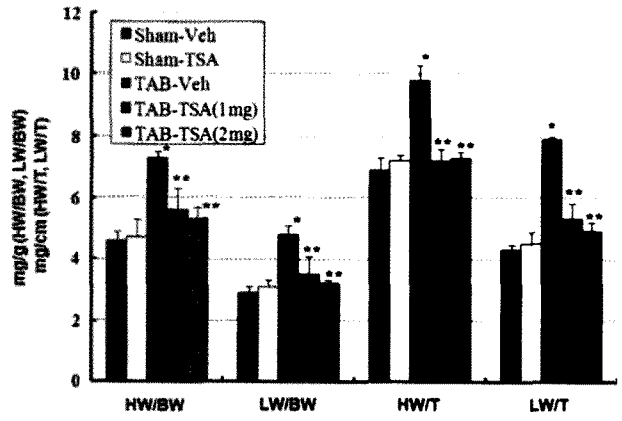

Fig. 2. HDAC suppression by TSA leads to dose-responsive blunting of pathologic cardiac growth. * $p<0.05$ *vs* Sham+Veh, ** $p<0.01$ *vs* TAB+Veh.

revealed no evidence of venous engorgement which is suggestive of heart failure (data not shown).

Immunoblot Analysis on Histone Acetylation

Although the effect of HDAC to promote cardiomyocyte hypertrophy *in vivo* and *in vitro* is established, its overall necessity as a hypertrophic mediator is an area of ongoing debate. To examine correlation of HDAC to the cardiac cell hypertrophy, immunoblot analysis was performed in cultivated H9c2 cells. As shown in Figure 3, TSA treated group was prominent of acetylated histone H3 using anti-acetyl H3, H4 antibodies in the H9c2 cells (Fig. 3), whereas very weak immunoreactive signals were visible throughout entire cardiac layers in the control of neonatal cardiac cells and TAB operated left ventricular layer (data not shown). These results indicate that change of expression level of HDAC might be involved in the development of cardiac hypertrophy as another related molecule to be regulated following the progression of pressure-dependent pathological cardiac hypertrophy. Most of all in this experiment, TSA treated H9c2 cells had an increase of global histone H3 and H4. Although the similar result was appeared in sham operation, opposite result was detected in TAB operation which showed a decrease of histone H3 acetylation (data not shown). These results define a new regulatory mechanism in the signaling pathway that couples hypertrophic signals at the cell membrane to changes in cardiac gene expression, suggesting opportunities for pharmacological intervention into cardiac hypertrophy and heart failure.

Duration of Histone H3 Activity by TSA in H9c2 Cells

A series of experiment were performed to investigate whether TSA-diminished histone H3 acetylation relied on class I HDACs activity. Undifferentiated H9c2 cardiomyoblast cells were treated with TSA, and expression of acetyl-histones and controlled genes were analyzed. 50 nM TSA was treated on the cells to analyze histone acetylation. Acetyl-histones were induced at 4 hr after second treatment, and H4 was more sensitive to acetylate for TSA as a time course (Fig. 3 A, B). To determine activity duration for TSA, 1nM TSA was added to H9c2 cells. Histone acetylation was initiated at 4 hour after treatment, and it was peak up to 18 hr. Thereafter, acetylation was significantly reduced to 30 hr (Fig. 3C). Numerous studies have been reported the cross-talk between histone acetylation and controlled genes, p53. Based on these studies, we analyzed the expression of p53 following TSA treatment. p53 was elevated at 4 hr, and it was maintained to 24 hr after treatment. Ras/extracellular signal-regulated kinase (ERK) was responsible to regulate gene expression including cell proliferation, growth and tumorigenesis. We also measure ERK protein level to determine connection bet-

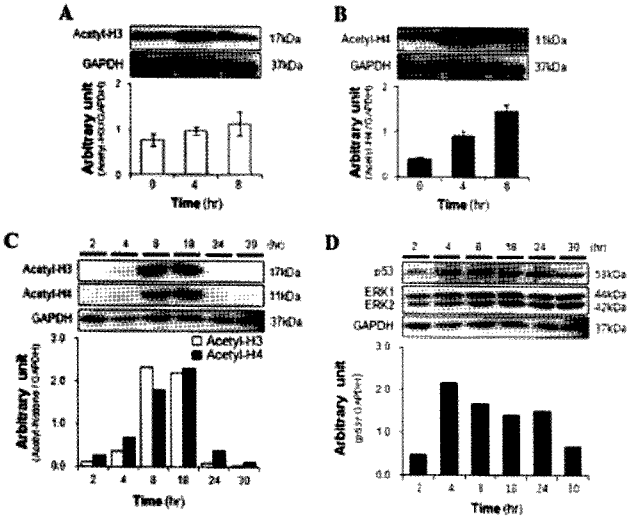

Fig. 3. Western analysis of the effect of TSA on hypertrophy *in vitro*. Upregulation of acetylated histones as a time-dependent manner. H9c2 cardiomyobalsts were treated with 50 nM TSA two times every 12 hr, and cells were harvested, then the expression of acetylated histones were analyzed. Not only (A) Acetyl-H3 but also (B) Acetyl-H4 was increased as a time-course. (C) Acetylated- histones were significantly upregulated at 8 hr, and it was maintained up to 18 hr after treatment. (D) p53, one of histone-controlled genes was increased from 4 hr after treatment, and ERK was also upregulated by 1 nM TSA.

ween TSA and p53 expression. ERK was activated at 8 hr, and it was maintained at 30 hours after intervention (Fig. 3D). It has been well known that HDAC inhibitors such as TSA were effective to treat heart disease. TSA treatment induced histone acetylation, thereby upregulating p53 expression via ERK pathway.

DISCUSSION

Cardiac hypertrophy is a common clinical problem arising in patients with hypertension, infarction, and valvular disease (Wang *et al.*, 2001; Hill *et al.*, 2002). Hypertrophy is associated with significantly increased risk of a number of cardiovascular complications including: heart failure stemming from systolic and/or diastolic dysfunction, malignant arrhythmia leading to sudden cardiac death. Despite elimination of myocyte hypertrophy, systolic function remains compensated revealing an underlying positive inotropic (Anrep) effect. As described above, many risk factors are involved in cardiac hypertrophy and heart failure in human. Upon this basis, various therapeutic trials including reduction of cardiac hypertrophy due to neurohumoral stress have been attempted (Wang *et al.*, 2001; Kong *et al.*, 2006).

Interestingly, histone proteins exist in a delicate balance between acetylated and deacetylated states. Acetylation leads to nucleosomal relaxation and increased expression of numerous genes. These enzymes remove an acetyl group from histones, which allows histones to bind DNA and inhibit gene transcription (Jenuwein and Allis, 2001). HDAC have been categorized into three classes (11 isoforms) that are based on sequence homology and domain organizationis (Zhang *et al.*, 2002). Inhibition of the HDAC results in multiple anti-cancer effects (Lau *et al.*, 2006; Pan *et al.*, 2007) such as Ⅰ) the inhibition of cancer cell proliferation, Ⅱ) the induction of apoptosis (cell death) of cancer cells, Ⅲ) cell cycle regulation, Ⅳ) the induction of tumour suppressor genes, Ⅴ) the blocking of tumour angiogenesis (development of new tumour blood vessels).

Recent work implicates this chromatin remodeling response might be involved in hypertrophic growth of the myocardium. TSA, a potent inhibitor of histone deactylase (HDAC) activity, imposes a dose-dependent blockade to hypertrophy and fetal gene activation in cultured cardiomyocytes. Thus, to explore the role of HDAC activation in an *in vivo* model of hypertrophy, we studied the effects of TSA in mice subjected to TAB-induced pressure stress.

Here, we demonstrate that HDAC inhibitor is 1) Capable of diminishes the pressure-stressed cardiac hypertrophy which is due to inhibit the fibrosis in the interventricular septum 2) Contribute to recover of cardiac function as well as systolic and diastolic function in echocardiography 3) Blunted cardiac hypertrophy by inhibit the acetylation of histone 3 which involved in class I HDAC. Response to the stress of neurohumoral activation, hypertension, or myocardial injury, the heart initially with an adaptive hypertrophic increase in LV mass are reported. Recent work has uncovered the importance of chromatin remodeling, especially histone acetylation, in the control of gene expression in heart disease (Kong *et al.*, 2006). HAT activity is antagonized by histone deacetylases (HDACs), which promote nucleosomal condensation and consequent transcriptional repression (Johnson and Turner, 1999). Recent studies have shown that both HAT and HDAC activities participate in regulating the hypertrophic response of the heart (Zhang *et al.*, 2002; Gusterson *et al.*, 2003; Lu *et al.*, 2000). A complex network of signaling cascades is activated during cardiac remodeling (Frey *et al.*, 2004).

As mentioned introductory section of this study uopn the fundamental orientation of hypertension induced by TAB, mice model for pressure overloaded hypertrophy are showed cardiac hypertrophy concomitant with enlargement of heart size, thicken cardiac wall. Given that cardiac muscle undergoes atrophy in a variety of disease states, the ability to promote cardiac hypertrophy would have important clinical implications. Thus, the present aimed to examine acetylation and deacetylation of histone subtypes in the neucleosome and their signaling pathways which might be encompassed by putative nuclear events, especially histone H3 in circula-

tory organs such as heart and capacitance arteries. The introduction of a concept of the regulatory function of histone H3 in the pressure/hemodynamic stress-induced signaling molecule such as calcium and calcineurin/Akt signaling pathway have suggested a regulatory role of cardiac hypertrophy in many, but not all, animal models of hypertrophy or cardiomyopathy. In order to disclose molecular mechanism of the cardiac hypertrophy in animal model, molecular and immunohistochemical experiment should be performed.

All taken together, these results define a new regulatory mechanism in the signaling pathway that couples hypertrophic signals at the cell membrane to changes in cardiac gene expression and strongly suggest opportunities for pharmacological intervention against cardiac hypertrophy and heart failure.

To date, the precise mechanisms by which HDAC inhibitors induce cell death have not yet been fully elucidated and the roles of individual HDAC inhibitors have not been identified. Moreover, the practical uses of HDAC inhibitors in cancer therapy, as well as their synergistic effects with other therapeutic strategies are yet to be evaluated. In this study, these data suggest that HDAC activation is required for pressure-overload growth of the heart. Activation of the fetal gene program is being studied presently and measurements of ventricular size and performance are underway for further study. While much work remains, these data point to the importance of chromatin remodeling in the cardiac stress response including pressure-overload or hemodynamic, and suggest that regulation of histone acetylation /deacetylation may be a novel target for therapeutic intervention.

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