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Histone Deacetylase Adaptation in Single Ventricle Heart Disease and a Young Animal Model of Right Ventricular Hypertrophy

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Abstract

Background—Histone deacetylase (HDAC) inhibitors are promising therapeutics for various forms of cardiac disease. The purpose of this study was to assess cardiac HDAC catalytic activity and expression in children with single ventricle heart disease of right ventricular morphology (SV), as well as in a rodent model of right ventricular hypertrophy (RVH).

Methods—Homogenates of RV explants from non-failing controls and SV children were assayed for HDAC catalytic activity and HDAC isoform expression. Postnatal 1-day old rat pups were placed in hypoxic conditions and echocardiographic analysis, gene expression, HDAC catalytic activity and isoform expression studies of the RV were performed.

Results—Class I, IIa, and IIb HDAC catalytic activity and protein expression were elevated in hearts of SV children. Hypoxic neonatal rats demonstrated RVH, abnormal gene expression and

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elevated class I and class IIb HDAC catalytic activity and protein expression in the RV compared to control.

Conclusions—These data suggest that myocardial HDAC adaptations occur in the SV heart and could represent a novel therapeutic target. While further characterization of the hypoxic neonatal rat is needed, this animal model may be suitable for pre-clinical investigations of pediatric RV disease and could serve as a useful model for future mechanistic studies.

INTRODUCTION

Right ventricular hypertrophy (RVH) and pulmonary hypertension (PH) are common sequelae in pediatric patients with various forms of congenital heart disease. On the severe end of the spectrum are children with single ventricle heart disease (SV), especially those with a single right ventricle (RV), such as hypoplastic left heart syndrome. SV is a rare but important form of hypoxic congenital heart disease that is fatal without intervention(1). Current treatment strategies for infants born with SV include a 3-stage surgical palliation, or primary heart transplantation. Primary heart transplant is significantly limited by donor availability. Although there have been advancements in perioperative and surgical approaches for SV, in the largest prospective study to date, 32% of those managed with surgical palliation died or were transplanted prior to 1 year of age(2). While there are many causes of poor outcome in SV patients, systemic right ventricular dysfunction, for which there are no proven therapies, is a risk factor for death and listing for transplant.(3–6)

Histone deacetylases (HDACs) are epigenetic enzymes that function canonically through removal of acetyl groups from lysine residues within nucleosomal histone tails. The 18 mammalian HDACs are categorized into 4 classes: class I (HDACs 1, 2, 3, 8), class II (class IIa: HDACs 4, 5, 7, 9; class IIb: HDACs 6, 10), class III (SIRT1-7), and class IV (HDAC11). Class III HDACs are also known as sirtuins and use NAD^+ as a cofactor, while HDACs 1-11 employ Zn^{2+} as a cofactor for catalytic activity. Class IIa HDACs are thought to be protective in the setting of heart failure due to their ability to bind to and inhibit the transcriptional activity of myocyte enhancer factor 2 (MEF2)(7). Elevated catalytic activity of sirtuins appears to be beneficial in failing hearts(8), whereas elevated catalytic activity of class I and IIb HDACs is thought to be maladaptive(9, 10).

Two HDAC inhibitors, Vorinostat (suberoylanilide hydroxamic acid, Zolinza™) and Romidepsin (Istodax™), are FDA-approved to treat cutaneous T-cell lymphoma. These and other HDAC inhibitors are currently in clinical trials, investigating efficacy in a variety of oncologic and non-oncologic diseases. HDAC inhibition has beneficial effects in pre-clinical models of adult heart failure, reducing cardiac hypertrophy(11) and fibrosis(12), and suppressing the fetal gene program associated with adverse cardiac remodeling(13). These findings suggest that HDACs could be therapeutically targeted for the treatment of adult human heart failure(14). However, nothing is known about the roles of HDACs in pediatric heart failure.

The purpose of this study was to examine the catalytic activity and protein expression of HDACs in pediatric SV patients with a single RV, and characterize a neonatal animal model with potential to elucidate molecular mechanisms controlling pathological remodeling of the

pediatric RV. We hypothesized that pediatric SV patients would exhibit elevated HDAC catalytic activity, and that hypoxia would result in elevated HDAC catalytic activity coincident with adverse remodeling in neonatal rat RVs.

METHODS

This study used heart tissue samples from pediatric patients (<18 years of age) that donated their hearts to the University of Colorado Institutional Review Board-approved pediatric heart tissue bank (informed consent is obtained for all patients). Patients included males and females of all races and ethnic background undergoing heart transplantation at Children's Hospital Colorado. Nonfailing (NF) control hearts were obtained from pediatric donors (<18 years of age) with structurally normal hearts and normal heart function whose hearts could not be placed for technical reasons, usually size or blood type mismatch. SV tissue was obtained from explanted hearts of patients with single ventricle physiology and a morphologic single RV. Patients with a single LV or indeterminate morphology of the single ventricle were excluded. All heart tissue was rapidly flash frozen in the operating room immediately after removal from the subject. A detailed description of patients included in this study is outlined in Table 1.

Histone deacetylase catalytic activity assays

Measurement of HDAC catalytic activity was performed as previously described(15). Reagents were purchased from indicated vendors. HDAC inhibitor, Trichostatin A (TSA; Sigma) diluted in dimethylsulfoxide (DMSO). Synthetic HDAC substrates: class I HDAC substrate (custom synthesis by Genscript), class IIa HDAC substrate (I-1985; Bachem), class I/IIb HDAC substrate (I-1875; Bachem). Trypsin, Triton X-100, and DMSO were obtained from Sigma. 7-Amino-4-methylcoumarin (AMC; Alfa Aesar).

Frozen explants from human pediatric RV were prepared in PBS (pH 7.4) containing 0.5% Triton X-100, 300mM NaCl and HALT^{TM} protease/phosphatase inhibitor cocktail (ThermoFisher) using a Bullet Blender homogenizer (Next Advance). Protein concentrations were determined by BCA Protein Assay Kit (ThermoFisher). Incubation of tissue lysates with class selective HDAC substrates results in a cleavable AMC product. AMC fluorescence was measured using a BioTeK Synergy 2 plate reader, with excitation and emission filters of 360 nm and 460 nm. Background fluorescence from buffer blanks were subtracted from raw signals, and data were normalized as needed using appropriate controls.

Immunoblotting

Immunoblotting was performed as previously described(16). Briefly, pediatric human and neonatal rat RV homogenates were prepared and concentrations quantified as above for HDAC catalytic activity assay. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (BioRad) and probed with antibodies for HDAC1 (Cell Signaling Technology, 5356), HDAC2 (Cell Signaling Technology, 5113), HDAC3 (Cell Signaling Technology, 3949), HDAC4 (Cell Signaling Technology, 5392), HDAC5 (Cell Signaling Technology, 2082), HDAC6 (Santa Cruz Biotechnology, 11420), HDAC7 (Cell Signaling Technology, 2882), calnexin (Santa Cruz Biotechnology, 11397).

Animal model

The pediatric neonatal rat RV hypertrophy model was adapted from a previous study(17). Timed-pregnant E17 Sprague Dawley rats were obtained from Charles River Laboratories. Rat mothers birthed their litters at Denver altitude and equilibrated for one day. Post-natal day 1 normoxic animals were placed in a hypobaric chamber simulating sea level altitude (21% oxygen), while post-natal day 1 hypoxic animals were placed in a hypobaric chamber simulating 18,000 feet (~10% oxygen) for seven days. Neonatal rats were anesthetized with isoflurane and sacrificed by thoracotomy. Hearts were flushed with saline through the aorta, atria were removed, and right ventricles were carefully separated from left ventricle and septum prior to flash freezing in liquid nitrogen.

Cardiac Imaging

Echocardiographic analysis was performed on the neonatal rats using a Vevo2100 system equipped with a MS400 18–38 mHz transducer (VisualSonics), as previously described(9).

Real-time polymerase chain reaction

Real-time polymerase chain (RT-PCR) reactions were performed as previously described(18). Total RNA was extracted using a mirVana kit (Ambion, Austin, TX), RNA was reverse transcribed into complementary DNA using I-script (Bio-Rad, Hercules, CA), and Power SYBR Green PCR Master Mix was (Applied Biosystems/Life Technologies, Carlsbad, CA) were used in the RT-PCR reactions. Reactions were performed using the ABI 7300 system. Primer sequences are indicated below:

ANF: Forward 5′- gcgaaggtcaagctgctt; Reverse 5′- ctgggctccaatcctgtcaat

BNP: Forward 5′- ggtgctgccccagatgatt; Reverse 5′- ggtgctgccccagatgatt

Serca2a: Forward 5′- ggccagatcgcgctaca; Reverse 5′- gggccaattagagagcaggttt

Data analysis

GraphPad Prism software was used to generate graphs and analyze data. Student's T-test with Welch's correction or ANOVA with Bonferroni's post-test (*P*<0.05) was used to determine statistical differences between groups.

RESULTS

Patient characteristics

The age range of the NF controls was 1.4–14 years, with 17% female. The SV group was composed of infants less than 3 months of age, with 17% female. The indication for transplantation for the SV group is outlined in Table 1, and encompasses those undergoing primary transplant (listed for transplant shortly after birth prior to surgical palliation) and includes one patient that failed an attempt at interventional palliation.

Cardiac HDAC catalytic activity is increased in pediatric single-ventricle patients

Eleven zinc-dependent HDACs are grouped into four classes (Figure 1a). HDAC catalytic activity has yet to be quantified in the human heart. To investigate whether pediatric SV

patients have altered HDAC catalytic activity, RV homogenates from pediatric NF controls and pediatric SV patients were incubated with HDAC class-specific fluorescent substrates. A significant increase in class I, IIa and IIb HDAC catalytic activity was observed in RVs of SV patients relative to NF control RVs (Figure 1b). Class IIb HDAC catalytic activity was most dramatically increased (~3.5 fold), followed by a 2-fold increase in class I HDAC catalytic activity, and a modest, but statistically significant increase in class IIa HDAC catalytic activity. These observations are consistent with reported increases in HDAC catalytic activity in hearts of adult rats with RVH due to PH(15).

Protein expression of distinct HDAC isoforms is elevated in hearts of pediatric single-ventricle patients

Since all classes of HDACs measured in the enzymatic assay exhibited elevated catalytic activity in RVs of SV patients, we investigated whether protein expression of HDACs belonging to class I, IIa and IIb was concomitantly augmented. Protein expression of HDAC1-7 was measured by immunoblotting (Figure 2a), and quantified by normalization to a house-keeping protein, calnexin (Figure 2b). Expression of class I HDACs -1, -2, and -3 was elevated and reached statistical significance in SV patient RVs compared to NF controls. Of the class IIa HDACs, only HDAC5 expression was significantly elevated in SV patient RVs. Expression of class IIb HDAC6 was not increased in SV patient RVs.

Activation of cardiac HDACs in a hypoxic neonatal rat model

RVH and failure is a common phenotype in pediatric patients with PH and various congenital heart lesions, including SV. In an effort to re-create elements of pediatric RVH, neonatal rats were placed in a hypobaric chamber simulating 18,000 feet in elevation and 10% O_2 for seven days. Control animals were simultaneously housed at sea level altitude (21% O_2) for seven days (Figure 3a). The use of neonatal rats, as opposed to mice, facilitated echocardiographic assessment of RV hypertrophy and pulmonary artery blood flow.

In response to seven days of hypobaric hypoxia, thickness of the free wall of the neonatal rat RV was significantly increased in both systole and diastole compared to sea level controls, as quantified by M-mode echocardiography (Figure 3b and 3c). Assessment of heart chamber remodeling by B-mode parasternal short axis evaluation demonstrated septal wall flattening and chamber dilation in the RVs of hypoxic neonatal rats (Figure 3b, lower images). Septal wall flattening occurred in 100% of the hypoxic animals.

Pulmonary artery blood flow was assessed using color Doppler and pulse-wave Doppler. Hypoxic neonatal rats exhibited a significant decrease in pulmonary artery acceleration time (PAAT) compared to sea level controls. Pulmonary valve velocity-time integral (VTI) and pulmonary valve peak velocity (PVPV) were also significantly decreased in hypoxic rats (Figure 3d – f and data not shown); PAAT, PVPV and PV VTI are well-accepted measurements of PH in humans(19, 20). These findings suggest that PH exists in neonatal rats exposed to 7 days of hypobaric hypoxia.

Morphometric analysis performed at the time of necropsy confirmed significant RV hypertrophy in neonatal rats exposed to hypobaric hypoxia. After 7 days of hypobaric

hypoxia, the ratio of RV-to-left ventricle (LV) + septum (S) was 1.5-fold higher in hypoxic rats compared to sea level control animals (Figure 4a).

Myocardial failure in humans is characterized by activation of a "fetal" gene program, as embryonically-expressed genes are reactivated(7). This gene program is indicative of ventricular remodeling and is typified by increases in expression of B-type natriuretic peptide (BNP) and atrial natriuretic peptide (ANP), and a coordinate decrease in sarcoplasmic reticulum calcium-ATPase 2a (SERCA2a) expression. Quantitative PCR revealed that ANP and BNP mRNA levels were elevated 3-fold and 5-fold, respectively, while SERCA2a expression was significantly attenuated in the hypoxic neonatal rat RVs compared to sea level control RVs (Figure 4b – d).

HDAC catalytic activity was subsequently quantified in homogenates of RVs from sea level and hypoxic neonatal rats. Significant increases in class I HDAC and class IIb HDAC catalytic activity were observed in hypertrophic RVs, which was consistent with pediatric SVs. However, unlike pediatric SVs, there was no difference in class IIa HDAC catalytic activity in hypoxic RVs (Figure 5a). Increases in class I and class IIb HDAC activity correlated with the abundance of HDAC1, HDAC2, HDAC3 and HDAC6 in RVs of hypoxic neonatal rats (Figure 5b).

Discussion

Pharmacotherapy to prevent or treat myocardial failure in patients with single-ventricle physiology does not exist, underscoring the need for research to define the molecular mechanisms of this complex congenital heart lesion(4). Treatment of adult heart failure (HF) has dramatically advanced over the past several decades, yet many of the drugs used to treat adult HF have not been proven to be beneficial in treating pediatric HF(21). Part of the challenge lies in the fact that pediatric HF is a much more heterogeneous disease than in adults, with a low incidence of ischemic disease. Pediatric HF ranges from idiopathic dilated cardiomyopathy (IDC), which is the leading indication for heart transplant in children, to single ventricle heart disease, the leading indication for transplant in infants(22). There are inherent differences in myocardial adaptation in pediatric versus adult HF, including altered gene, protein, adrenergic receptor and microRNA expression, as well as protein phosphorylation and enzyme activity(18, 23–25). In addition, children are a vulnerable population, limiting the ability to perform the invasive studies that have been so useful in advancing the care of adults with HF. This study was designed to characterize the adaptations occurring in myocardial HDACs in the pediatric SV population, and to develop and perform initial characterization of an animal model that could serve to facilitate future mechanistic studies and drug development relevant to RV remodeling in the young.

We found that the catalytic activity of class I, IIa and IIb HDACs is elevated in RVs of children with SV disease compared to NF controls (Figure 1b). For class I and IIa HDACs, enhanced catalytic activity correlated with increased levels of HDAC isoform protein expression (class I HDACs -1, -2 and -3; class IIa HDAC5) (Figure 2). In contrast, activation of class IIb HDAC activity occurred independently of altered expression of the predominant isoform within this class, HDAC6. In this regard, multiple post-translational mechanisms for

regulation of HDAC6 activity have described in non-cardiac cells. For example, phosphorylation of HDAC6 by glycogen synthase kinase-3β was shown to activate HDAC6 catalytic activity in neurons(26). Elucidation of the mechanisms controlling HDAC catalytic activity in SV disease awaits further investigation.

The molecular mechanisms by which HDACs contribute to adult pressure overload-induced LV hypertrophy, myocardial interstitial fibrosis, cardiac inflammation, pulmonary hypertension and RV remodeling are only beginning to be understood(27–29). HDAC inhibition is effective in improving HF symptoms in animal models of spontaneously hypertensive rats(30), mouse models of myocardial infarction/ischemia-reperfusion(31) and an angiotensin II-infusion model of cardiac fibrosis(12). Consistent with our hypothesis, the class I HDAC inhibitor apicidin attenuated hypoxia-induced RVH and pulmonary vascular remodeling in neonatal mice(32). A recent proteomic study using 19 different HDAC inhibitors in three different human cell lines demonstrated the complexity of individual small molecule effects on the acetylome, with differential effects on the magnitude of acetylation signal and specific acetylation targets between compounds(33). Due to these findings, selection of the class of HDAC inhibitor and specific small molecule for therapeutic development requires careful consideration.

In combination with our previously described abnormalities in the beta-AR system(18), elevated HDAC catalytic activity and expression in the RVs of children with SV suggests that SV hearts are undergoing molecular remodeling. Consistent with these findings, clinically the single RV does not tolerate chronic pressure overload indefinitely, and patients remain at risk for the development of ventricular failure.

While prenatal models of SV exist(34–36), a postnatal animal model is not feasible due to complexities of maintaining the SV circulation after birth. Therefore, we developed a hypoxic neonatal rat model that reasonably recapitulates some of the echocardiographic and molecular signatures of pediatric SV. Children born with SV are hypoxic until the 3rd stage of surgical palliation (Fontan) is completed at 2–5 years of age and systemic afterload on the RV is present throughout their lifespan, with hypertrophy essentially a universal finding. Hypoxia in neonatal rats results in PH and RVH by echocardiographic and morphometric assessment, altered myocardial gene expression, with partial recapitulation of the fetal gene program, elevated HDAC class I and IIb activity, and elevated HDAC protein expression. From both a physiologic and molecular perspective, the hypoxic neonatal rat model has similarities to the human SV condition that support its use for future mechanistic studies of RV remodeling, as well as for therapeutic development. While HDAC IIa activity was not increased in the rat RV as it was in the pediatric SV, whether this difference is clinically significant is unknown. Interestingly, HDAC 4 is associated with the cardiac sarcomere and may play a role in regulating cardiac contractility(37), warranting future study. The use of echocardiography in this model not only provides translational utility, but also allows for longitudinal investigation. RVH and failure are not uncommon in the pediatric cardiovascular disease population, and this model has potential to affect a myriad of populations beyond SV. PH, intrinsic lung disease, various forms of biventricular congenital heart disease (e.g. tetralogy of Fallot, Ebstein's anomaly) also result in pediatric RV remodeling and failure.

There are important limitations to this study. The tissue bank-based aspect of this study is inherently cross-sectional, and proof of mechanistic associations based on our results is not possible. Due to the rarity of this form of congenital heart disease, and thus the limited tissue available for study, it is not possible for us to determine the influence of age, prior surgical procedures, medications, clinical status of the patient or the temporal relationship of gene expression changes on our findings. We recognize that we have not developed a rodent model of SV. However, many of the findings in this study show consistent changes in phenotype and molecular signatures of hypoxic neonatal rats and SV patients versus their respective controls. This model could be used as a platform for the study of mechanisms of pressure-overloaded RV failure in the young and determination of the influence of aging and development vs disease on myocardial adaptations.

In conclusion, though HDACs are ubiquitously expressed and are essential enzymes, HDAC inhibitors are surprisingly well tolerated(38–40). There is an evolving body of literature suggesting that HDAC-inhibition has value for the treatment of adult cardiovascular diseases(14). The results of this study demonstrate that HDACs are altered in the ventricle of young children with single ventricle heart disease. Given the challenges of studying a rare disease in a vulnerable population, the hypoxic neonatal rat represents a reasonable platform for extending pre-clinical studies to explore mechanisms of disease and investigate novel therapeutics, such as HDAC inhibitors, in preventing or limiting pathologic RV remodeling.

Acknowledgments

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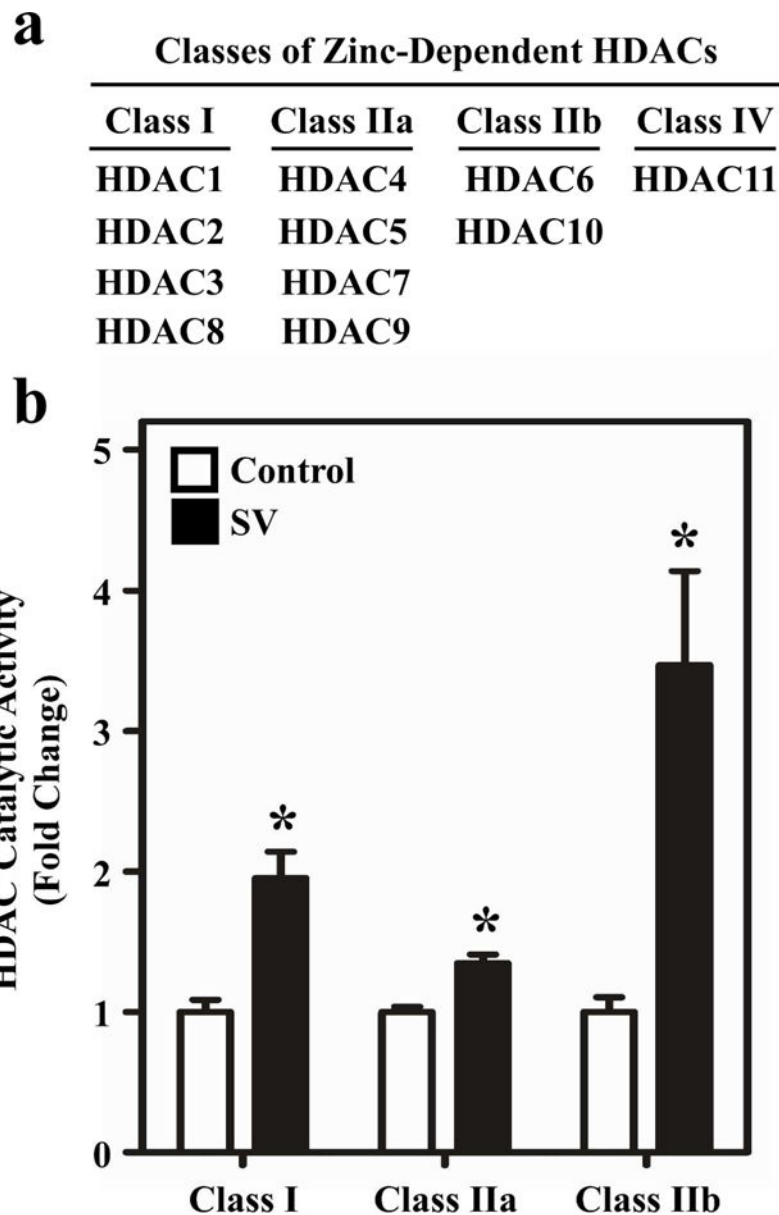
Figure 1.
HDAC catalytic activity is elevated in the RV of children with SV. (a) Classifications of zinc-dependent HDACs. (b) HDAC Catalytic Activity: RV tissue from non-failing pediatric and SV hearts was homogenized in a mild lysis buffer and incubated with HDAC class I, IIa, and IIb-specific substrates. All classes of HDACs displayed elevated catalytic activity relative to control. Results are displayed as the mean with standard error; N = 6 per group, *$P<0.05$ vs controls.
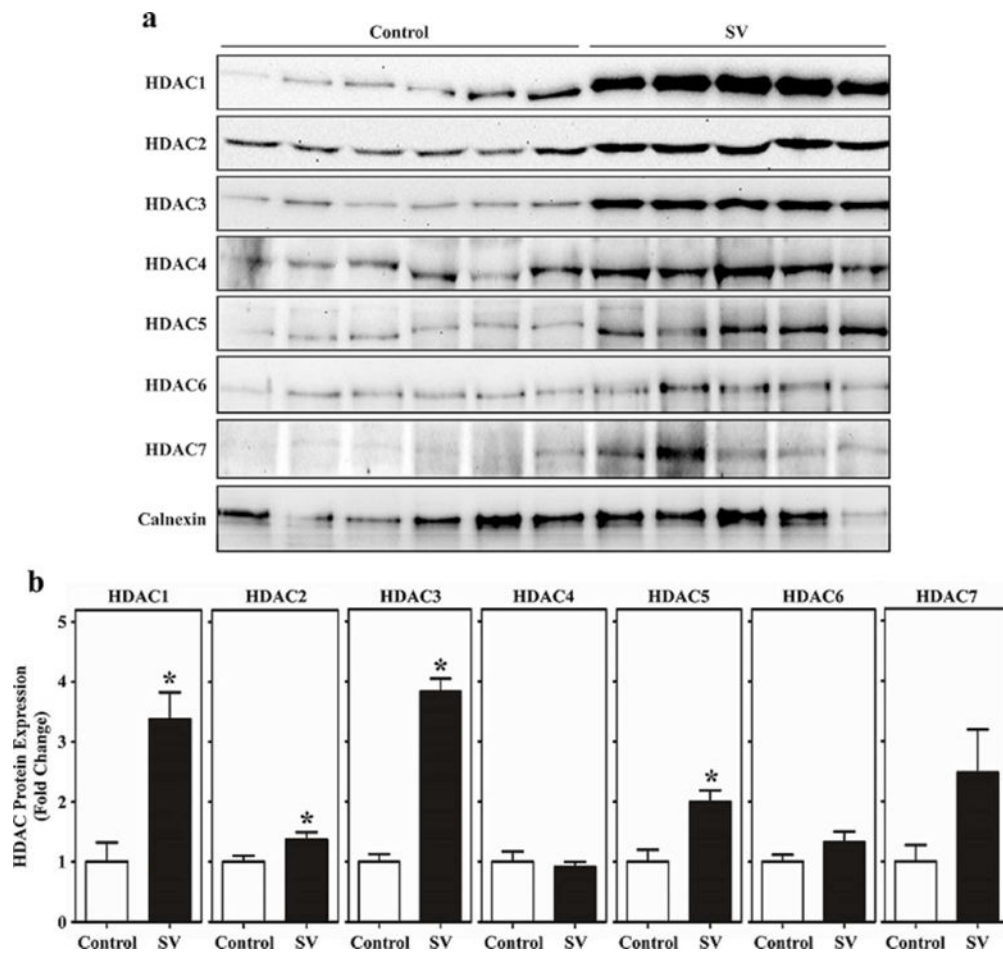
Figure 2.
HDAC protein expression is elevated in pediatric RVs from SV patients. (a) Immunoblot analysis was performed with the same homogenates used for the HDAC catalytic activity assays employing antibodies specific for class I HDACs (HDAC1-3), class IIa HDACs (HDAC4, 5, 7) and class IIb HDAC6. (b) Densitometry was used to quantify immunoblot signals; *P<0.05 vs. controls.
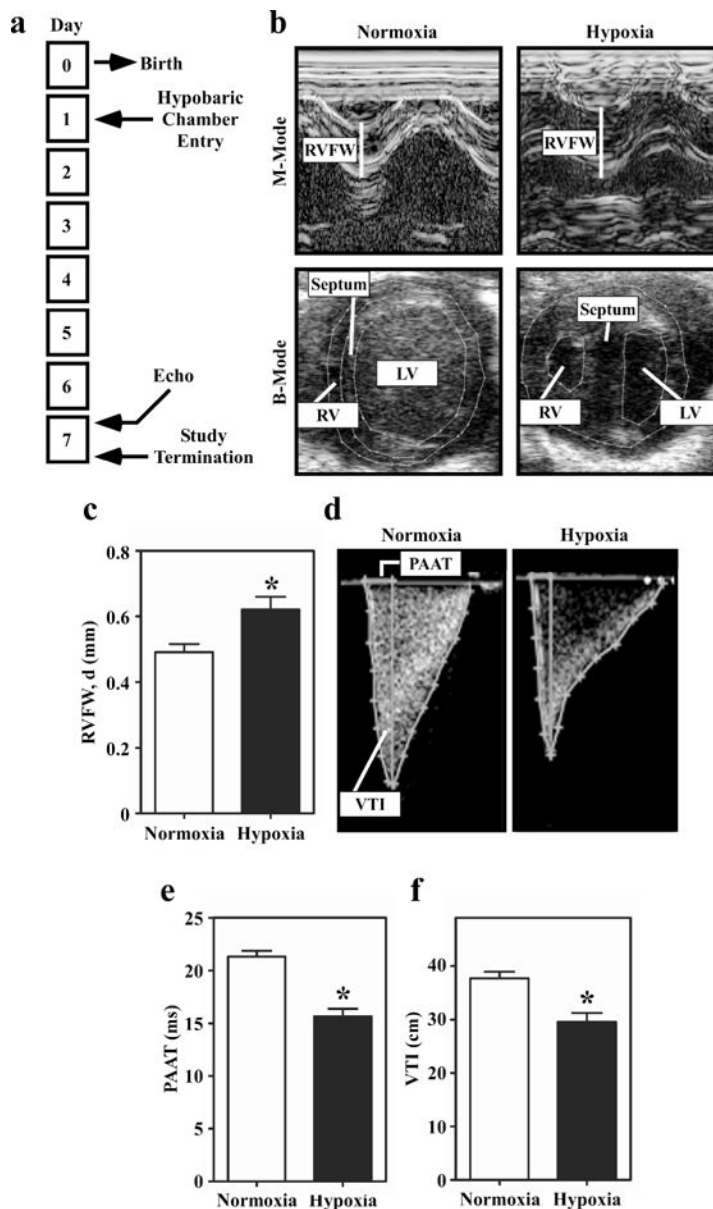
Figure 3.
Echocardiographic analyses of right ventricular hypertrophy and pulmonary hypertension in hypoxic neonatal rats. (a) Study design. (b) Representative M-mode and B-mode images of hearts from neonatal rats exposed to hypobaric hypoxia or normoxic conditions for 7 days. (c) RV Free Wall Thickness, Diastole: Quantitative assessment of RV free wall (RVFW) thickness demonstrates increased thickness in the hypoxic rats; *$P<0.05$ vs. controls. (d) Representative Doppler images of pulmonary blood flow in normoxic and hypoxic neonatal rats. (e) Pulmonary artery acceleration time (PAAT) is significantly decreased in the hypoxic neonatal rats compared to control. (f) Pulmonary valve velocity-time integral (VTI) is also significantly decreased in hypoxic neonatal rats compared to control. Results are displayed as the mean with standard error. N = 11 per condition; $P<0.05$ vs. controls.
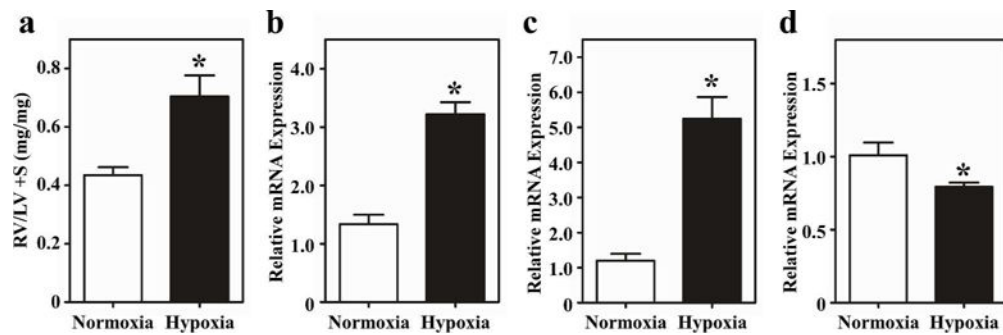
Figure 4.
Pathological RV remodeling in neonatal rats exposed to hypoxia. (a) RV hypertrophy was assessed by comparing weights of RV vs. LV + septum (S). The RV/LV+septum ratio was higher in the hypoxic rats. (b) Atrial natriuretic peptide, (c) B-type natriuretic peptide (BNP), and (d) sarcoplasmic reticulum calcium ATPase 2a (Serca2a) mRNA expression was determined by quantitative PCR. ANP and BNP expression was increased, while Serca2a expression was decreased in the hypoxic animals. Results are presented as the mean with standard error. N = 3 (normoxia), N = 6 (hypoxia); *P<0.05 vs. control.
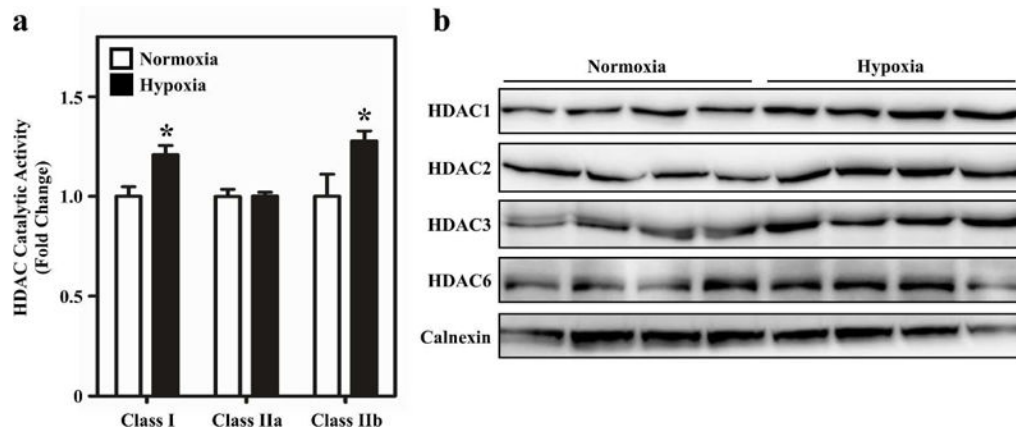
Figure 5.
Activation of HDACs during pathological RV remodeling in neonatal rats. (a) HDAC catalytic activity was quantified in RV homogenates from neonatal rats exposed to hypobaric hypoxia (N = 6) or normoxic (N = 3) conditions for 7 days. Class I and IIb activity was increased in the RV of the hypoxic rats. Results are displayed as the mean with standard error; *$P < 0.05$ vs. normoxic controls. (b) Immunoblot analysis was performed with the same rat RV homogenates used for the HDAC catalytic activity assays employing antibodies specific for class I HDACs (HDAC1-3) and class IIb HDAC6. HDAC 1, 2, 3 and 6 protein expression was increased in the RV of the hypoxic rats.

Table 1

Patient Demographics. NF- non-failing control. SV- single ventricle patients. M- male. F- female. Inotropes included dopamine and norepinephrine. PDE-phosphodiesterase. ACEI- Angiotensin converting enzyme inhibitor. BB- beta-blocker (the non-selective beta-blocker labetalol was used in NF1). PGE-prostaglandin E1, used to maintain ductus arteriosus patency. PDA- patent ductus arteriosus.

Study ID	Age (years)	Sex	HDAC Activity	HDAC Westerns	Inotrope	Digoxin	ACEI	BB	Diuretic	Palliation History/Indication for transplant
NF1	13	M	X	X	Y	N	N	Y	N	NA
NF2	8	F	X	X	N	N	N	N	N	NA
NF3	7	M	X	X	N	N	N	N	N	NA
NF4	1.4	M	X	X	N	N	N	N	N	NA
NF5	12	M	X	X	N	N	N	N	N	NA
NF6	14	M	X	X	NA	NA	NA	NA	NA	NA
SV1	0.1	F	X	X	N	N	N	N	Y	No palliation, on PGE/Primary transplant, RVH dilation
SV2	0.1	M	X	X	N	N	N	N	Y	Aortic valvuloplasty and atrial septal stent on PGE/Not a candidate for additional surgical palliation, ventilator dependent
SV3	0.2	M	X	X	N	N	Y	N	Y	PDA stent/Primary transplant, RV dilation
SV4	0.2	M	X	X	N	N	N	N	Y	PDA stent/Primary transplant, RVH
SV5	0.2	M	X		NA	NA	NA	NA	NA	No palliation, on PGE/Primary transplant, RVH
SV6	0.1	M	X	X	N	Y	Y	N	Y	No palliation, on PGE/Primary transplant, RVH and RV dilation