Targeting proteostasis in atrial fibrillation
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Involvement of Class I and Class IIa HDACs in the derailment of gene expression in Atrial Fibrillation


Manuscript in preparation.
ABSTRACT

Background: Atrial Fibrillation (AF), the most common persistent clinical tachyarrhythmia, is associated with altered transcriptional changes leading to cardiomyocyte dysfunction and AF susceptibility and progression. Recent research showed class I and class IIa histone deacetylases (HDACs) to regulate pathological and fetal gene expression, which causes cardiac contractile dysfunction and hypertrophy. Whether class I and class IIa HDACs are involved in AF progression is unknown. Therefore, we investigated their role in tachypacing-induced contractile dysfunction and pathological fetal gene expression in experimental model systems for AF and in clinical AF.

Methods and results: Tachypacing (TP, 5Hz) of HL-1 cardiomyocytes resulted in a significant reduction in Ca\(^{2+}\) transient (CaT) amplitude. In control normal-paced (1Hz) cardiomyocytes, overexpression of class I HDACs, HDAC1 or HDAC3, caused a significant loss of CaT. TP aggravated the CaT loss in HDAC3, but not in HDAC1, overexpressing cardiomyocytes. Overexpression of class IIa HDACs, HDAC5 or HDAC7, protected against TP-induced CaT loss, while HDAC4 or HDAC9 did not. Notably, the protective effect of HDAC5 and HDAC7 was abolished in cardiomyocytes overexpressing a dominant negative HDAC5 or HDAC7 mutant, bearing a mutation in the binding domain for myosin enhancer factor 2 (MEF2). Furthermore, TP induced the phosphorylation of HDAC5, promoted its translocation from the nucleus to cytoplasm, and consequently increased MEF2-related fetal gene expression (β-MHC, BNP). In line with these experimental findings, patients with AF showed a significant increase in both phosphorylated HDAC5 and fetal gene expression (β-MHC, BNP).

Conclusion: Overexpression of class IIa HDAC5 and HDAC7 protects against tachypacing-induced CaT reduction in HL-1 cardiomyocytes. As HDAC5 is abundantly expressed in the heart, and AF induces its phosphorylation, nuclear export and MEF2 regulated fetal gene expression, HDAC5 is an interesting therapeutic target in clinical AF.
Involvement of Class I and Class IIa HDACs in Atrial Fibrillation

1. INTRODUCTION

Atrial fibrillation (AF) is the most common sustained and progressive clinical tachycardia and contributes to cardiovascular morbidity and mortality.1 AF is characterized by specific electrical, transcriptional and structural changes, commonly denoted as remodeling2, with the aim to identify novel druggable targets which attenuate remodeling and AF. Cardiomyocyte remodeling underlies contractile dysfunction and the progression of AF. Therefore, it is of great interest to dissect the molecular mechanisms underlying cardiomyocyte remodeling progression.

Epigenetic regulation has been identified as an important mechanism underlying the progression of AF. The role of epigenetic regulation originates from observations that (re)activation of the fetal gene program in cardiomyocytes promotes AF. Ausma et al. previously showed upregulation of two proteins of the fetal program in the goat model for AF, i.e. the slow-contracting beta-myosin heavy chain isoform (β-MHC) and smooth muscle α-actin (α-SMA).3-6 In persistent AF patients, numerous fetal-neonatal variants of the titin protein were observed in cardiac myofibrils, and atrial re-expression of TnI-skeletal-slow-twitch (ssTnI) was found in patients with paroxysmal AF.7 In addition, persistent AF was associated with higher cardiac mRNA expression of brain natriuretic peptide (BNP).8 Epigenetic regulation refers to processes that influence the packaging or processing of nuclear DNA, thus controlling the on/off states of multiple genes with discrete switches. The packaging of chromatin is largely dependent on the acetylation status of histones, which is controlled by histone acetyl transferases and histone deacetylases (HDACs). HDACs are an ancient family of enzymes that catalyse the removal of acetyl groups from the ε-amino group of specific acetyl lysine residues within their protein substrates. Deacetylation of histones in nucleosomes in general induces chromatin condensation, which represses transcription by preventing the binding of transcription factors and other components of the transcriptional machinery to gene promoter and enhancer regions and therefore serves as important regulators of gene expression. The zinc-dependent HDACs are classified into four groups based on their structure, complex formation, and expression pattern: class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), and class IV (HDAC11). We recently reported on a cytosolic member of HDAC class IIb, HDAC6, and its prominent role in AF progression. HDAC6 deacetylates α-tubulin, which causes disruption of microtubule structure, contractile dysfunction and AF progression.9 However, whether the other HDAC classes are involved in AF progression is unknown. Of the four classes, class I and IIa are well studied regarding their role in pathological gene expression, structural changes and the development of hypertrophy and heart failure. Class I HDACs reveal high HDAC activity in cardiomyocytes, but findings on their role in cardiac disease
development are conflicting. In recent years, class IIa HDACs, especially HDAC4, HDAC5 and HDAC9, have attracted considerable attention as regulators of transcriptional reprogramming. Since HDAC4 and HDAC5 are highly expressed in the heart, these two members are essential for transcriptional reprogramming in the heart. Under normal circumstances, class IIa HDACs localize in the nucleus and suppress cardiomyocyte hypertrophy by repressing the activity of pro-hypertrophic transcription factors, such as members of the myocyte enhancer factor-2 (MEF2) family. In response to stress signals, class IIa HDACs are phosphorylated and exported from the nucleus, thereby activating transcriptional reprogramming and the induction of hypertrophic gene expression. Although recent findings indicate a role for transcriptional remodeling in AF progression, the involvement of class I and class IIa HDACs in transcriptional reprogramming is unknown. Therefore, we examined the role of class I and IIa HDAC overexpression on contractile function in tachypaced HL-1 cardiomyocytes, followed by identification of downstream actors of protective HDACs. The experimental findings were confirmed in AF patients. In the current study, we found class IIa HDAC5 and HDAC7 to protect against tachypacing-induced contractile dysfunction, probably via prevention of MEF2 related fetal gene expression. Similar findings were observed in permanent AF patients compared to control patients in sinus rhythm. As HDAC5 is abundantly expressed in the heart, in contrast to HDAC7, HDAC5 is a promising therapeutic target in clinical AF.

2. MATERIALS AND METHODS

2.1. Tachypacing of HL-1 cardiomyocytes and calcium transient measurements

HL-1 cardiomyocytes were subjected to tachypacing as described before. To measure calcium transients (CaT), HL-1 cardiomyocytes were incubated with 2 µM of the Ca²⁺-sensitive Fluoro-4-AM dye (Invitrogen) for 30 min, followed by washing with DMEM (Gibco) and re-incubation with Claycomb medium (Sigma). Fluoro-4-AM loaded cardiomyocytes were detected with cardiomyocyte calcium and contractility recording system (IonOptix). The recording of CaT in HL-1 cardiomyocytes was performed at 1Hz stimulation at 37°C. The absolute value of fluorescent signals was determined utilizing the following calibration; Fcalc/F0, where F1 is the fluorescent dye signal at any given time and F0 is the fluorescent signal at rest.

2.2. Plasmids

Retroviral constructs of HDACs and HDACs mutants (HDACm) were generated in the lab of Dr. Miguel A. Esteban. To detect overexpression of the construct, the constructs contained a FLAG tag in the carboxyl terminal. DNA mutagenesis of HDAC5 and HDAC7
in the MEF2 binding domain was produced using suitable oligos and a PCR-based method. All new plasmids were verified by sequencing before use.\textsuperscript{15} GFP-HDAC5 constructs are generous gifts from Dr. Johannes Backs’ lab.\textsuperscript{10}

2.3. Retroviral infection

Recombinant retrovirus was generated by transfecting retrovirus constructs into HEK293T cells using Lipofectamin 2000 (Invitrogen). Medium was refreshed 12 hours after transfection. Virus in the medium were collected 48 hours after transfection and filtered through 0.45μm filter. Polybrene (Millipore) was added to the virus to obtain a final concentration of 5 μg/ml, followed by infection of the HL-1 cardiomyocytes. For control infection, a corresponding DsRED or GFP retrovirus was used. The infection efficiency was near 100% after 48 hours infection (based on the amount of control DsRED or GFP positive cardiomyocytes).

2.4. Real time PCR

Total RNA from cardiomyocytes and human tissue were isolated with using TRIzol reagent (Invitrogen) or using a kit (Machery-nagel) and cDNA was synthesized using Reverse Transcriptional kit (Invitrogen) following the manufacturer’s instructions. Relative gene expression was determined by quantitative real time PCR on a Bio-Rad CFX384 real time system using SYBR green dye. Gene expression values were normalized to internal reference gene values (GAPDH). Primer sequences are listed in Table S1.

2.5. Protein extraction and Western blot analysis

HL-1 cardiomyocytes or human tissue samples were lysed in radioimmunoprecipitation assay buffer as described before.\textsuperscript{9} In short, equal amounts of protein were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes, and probed with anti-HDAC5 (c-11, Santa Cruz), anti-phosphorylated HDAC5 (p-HDAC5) (Santa Cruz), anti-Flag (Invitrogen) or anti-GAPDH (Fitzgerald). Blots were subsequently incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Dako). Signals were detected by the ECL detection method (Amersham) and quantified by densitometry (Syngene, Genetools).

2.6. Immunofluorescence

To visualize cardiomyocytes transfected with GFP-HDAC5, HL-1 cardiomyocytes were grown on gelatin coated coverslips and tachypaced followed by three times rinsing in PBS, fixation in 4% formaldehyde for 15 min, three time rinsing in PBS, incubation in mounting media with DAPI (Vectashield) and finally sealing of slides with nail polish. To
visualize endogenous HDAC5, HL-1 cardiomyocytes were grown on coverslips till 80% confluence, followed by tachypacing, three times rinsing in PBS, fixation in 4% formaldehyde for 15 min, three times rinsing in PBS, permeabilization with 0.1% triton X-100 in PBS for 10 min and three times rinsing in PBS. Cardiomyocytes were blocked with blocking solution (0.5% BSA and 0.15% glycine in PBS) for 10 min and incubated with anti-HDAC5 (Cell signaling) overnight at 4 degree. After 3 times rinsing in PBS, the coverslips were incubated for 1 h at room temperature in the dark with Alexa Fluor® 488 Goat Anti-Rabbit IgG (Invitrogen) mixed with TOTO-3 iodide (Molecular probes) for nuclear staining. Finally, coverslips were rinsed three times in PBS, incubated with mounting media (Vectashield) and sealed with nail polish. Fluorescent signals were detected with confocal microscopy (Leica TCS SP2) by an investigator blinded for the groups, who scored: (1) cardiomyocytes with nuclear and/or cytosolic HDAC5 staining, (2) exclusively nuclear HDAC5 staining.

2.7. Patients

Before surgery, one investigator assessed patient characteristics (Table S2), as described before.16 Right atria (RA) and left atria (LA) tissue samples were obtained from patients with permanent AF (PeAF) and control patients in sinus rhythm (SR). Both, the PeAF and SR patients suffered from underlying mitral valve disease. After excision, atrial appendages were immediately snap-frozen in liquid nitrogen and stored at −80°C. The study conforms to the principles of the Declaration of Helsinki. The Institutional Review Board approved the study, and patients gave written informed consent. Tissues were used to perform real time PCR and western blot experiments.

2.8. Statistical Analysis

Results are expressed as mean ± SEM. Biochemical analyses were performed at least in duplicate. Individual group mean differences were evaluated with the Students t-test. Categorical data differences were evaluated with Pearson’s chi-squared test. All P values were 2 sided. Values of P<0.05 were considered statistically significant. SPSS version 22 was used for all statistical evaluations.
3. RESULTS

3.1. Class IIa HDACs protect against tachypacing-induced remodeling in HL-1 cardiomyocytes

To study the role of class I and IIa HDACs on contractile function in AF, various members of the HDAC classes were overexpressed in HL-1 cardiomyocytes by retroviral infection (Supplemental Figure S1A, B). In control DsRED retroviral infected HL-1 cardiomyocytes, tachypacing induced a significant loss in CaT (Figure 1A, B). Of the examined class I HDACs, HDAC1 or HDAC3 overexpression resulted in CaT loss in control cardiomyocytes, and tachypacing aggravated CaT loss in HDAC3 and not in HDAC1 overexpressing cardiomyocytes (Figure 1B). This result indicates a detrimental effect of class I HDACs overexpression on contractile function in normal and tachypaced HL-1 cardiomyocytes. Overexpression of class IIa HDACs, i.e. HDAC4, HDAC5, HDAC7 and HDAC9, rendered mixed results. None of the class IIa HDACs caused CaT changes in control HL-1 cardiomyocytes (Figure 1A, C). After tachypacing, overexpression of HDAC5 and HDAC7 significantly attenuated CaT loss in HL-1 cardiomyocytes, whereas overexpression of HDAC4 or HDAC9 were not protective (Figure 1A, C). Under normal circumstances, HDAC5 and HDAC7 are localized in the nucleus and bind to MEF2, via their MEF2 binding domain, resulting in repression MEF2 activity. In response to stress signals, HDAC5 and HDAC7 are phosphorylated and exported from the nucleus, thereby activating MEF2 and permitting the induction of pathological fetal gene expression. To test whether the protective effect of HDAC5 and HDAC7 is via binding to MEF2, HL-1 cardiomyocytes were transfected with HDAC5m or HDAC7m constructs bearing a mutation in the MEF2 binding domain. Notably, loss of MEF2 binding capacity did not rescue the significant loss of CaT after tachypacing (Figure 1D), suggesting that binding of HDAC5 and HDAC7 to MEF2 prevents cardiomyocyte remodeling. Our findings suggest that overexpression of class IIa HDAC5 and HDAC7 protect against tachypacing-induced CaT loss, probably via nuclear binding to MEF2 transcription factor and thus limiting downstream pathological reprogramming.

3.2. Tachypacing induces HDAC5 phosphorylation and its nuclear export in HL-1 cardiomyocytes

Of the two identified protective class IIa HDACs, HDAC5 represents an interesting candidate, because its expression is abundant in the heart, in contrast to HDAC7, and its function is regulated by calpain, which was previously found to induce structural remodeling in AF via degradation of HDAC6-deacetylated microtubules. Therefore, we determined the role of HDAC5 in cardiomyocytes in more detail. Upon stress, HDAC5 gets
phosphorylated, resulting in dissociation of HDAC5 from MEF2, and its nuclear export.\textsuperscript{17,19-21} Firstly, we studied whether tachypacing induces the activation of HDAC5 by measuring its phosphorylation levels in control and tachypaced HL-1 cardiomyocytes. As expected, tachypacing gradually and significantly increased phosphorylation of HDAC5 in HL-1 cardiomyocytes (Figure 2A, B). Secondly, we determined the localization of HDAC5 in the HL1 cardiomyocytes both by transfection of GFP-HDAC5 and immunostaining of endogenous HDAC5. In control cardiomyocytes (0h TP), over 50% of GFP-HDAC5 was found exclusively in the nucleus (Figure 2C, D). During a time course of tachypacing, the percentage of nuclear GFP-HDAC5 decreased gradually, and after 16 hrs tachypacing, <10% of the cardiomyocytes revealed exclusively nuclear staining of HDAC5 (Figure 2C, D). Similar findings were observed for endogenous HDAC5. Endogenous HDAC5 was localized in the nucleus in control conditions and tachypacing significantly induced the export to the cytoplasm (Supplemental Figure S2A, B). Together, these results demonstrate tachypacing to induce phosphorylation and nuclear export of both GFP-HDAC5 and endogenous HDAC5 in HL-1 cardiomyocytes.

Figure 1: HDAC5 and HDAC7 overexpression protects against CaT reduction in tachypaced HL-1 cardiomyocytes. A) Representative CaT traces of HL-1 cardiomyocytes, showing that cardiomyocytes overexpressing HDAC5 or HDAC7 are protected against tachypacing-induced CaT reduction. B), and C) Quantified data showing relative CaT amplitudes of non-paced (NP) and tachypaced (TP) cardiomyocytes, each from groups as indicated. Cardiomyocytes were infected with control plasmid DsRED retrovirus (CTL), HDAC1, HDAC3, HDAC4, HDAC5, HDAC7 or HDAC9 retrovirus. D) Quantified data showing relative CaT amplitudes of NP or TP cardiomyocytes infected with HDAC5 mutant retrovirus (HDAC5m) or HDAC7 mutant retrovirus (HDAC7m). HDAC5m, HDAC7m have mutations in MEF2 binding domains and therefore cannot find to MEF2. *\(P < 0.05\) vs CTL NP, ***\(P < 0.01\) vs CTL NP, #\(P < 0.01\) vs CTL TP. \(N \geq 8\) for each group.

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3.3. MEF2 regulates fetal gene expression in tachypaced HL-1 cardiomyocytes

Upon tachypacing, nuclear HDAC5 becomes phosphorylated, released from MEF2 and exported to the cytosol, which allows the histone acetyltransferase p300 to associate with MEF2 via the HDAC docking site, thereby converting MEF2 from a transcriptional repressor to a transcriptional activator of fetal genes, including β-MHC and BNP.\textsuperscript{17,19-21} To test whether tachypacing induces the expression of β-MHC and BNP, HL-1 cardiomyocytes were tachypaced and mRNA levels of β-MHC and α-MHC levels were determined by quantitative PCR with reverse transcription (RT–PCR). The ratio of β-MHC to α-MHC increased significantly after 12 hours tachypacing in HL-1 cardiomyocytes (Figure 3A). Comparable findings were observed for BNP. Tachypacing induced a gradual and significant induction of BNP mRNA in HL-1 cardiomyocytes (Figure 3B). In addition, tachypacing-induced BNP induction correlates significantly with the induction of phosphorylated HDAC5 (Figure 3C). These results reveal that tachypacing induces the
expression of fetal genes in HL-1 cardiomyocytes, which correlates with HDAC5 phosphorylation.

**Figure 3: Activation of fetal gene program in tachypaced HL-1 cardiomyocytes.** A) Time course of TP-induced increase in gene expression ratio of β-MHC (MHY7) to α-MHC (MHY6). *P<0.05, ***P<0.01 vs 0h. B) BNP gene expression is significantly increased during TP. ***P<0.01 vs 0h. C) BNP gene expression level correlates significantly with level of pHDAC5 during time-course of TP.

### 3.4. HDAC5 phosphorylation and fetal gene expression is induced in permanent AF patients

To investigate whether a similar modulating role of HDAC5 is found in patients with AF, the amount of phosphorylated HDAC5 was measured in left and/or right atrial appendages (LA and RA, respectively) of patients with permanent AF (PeAF) and controls in normal sinus rhythm (SR). A significant increase in the level of phosphorylated HDAC5 was observed in the LA of patients with PeAF compared to SR controls (Figure 4A, B). Moreover, the gene expression levels of β-MHC, α-MHC and BNP were determined. Comparable to tachypaced HL-1 cardiomyocytes, a significant induction in the ratio of β-MHC/α-MHC and increased expression of BNP was observed in patients with PeAF compared to control SR patients (Figure 4C, D). Also, the induction of BNP correlated significantly with phosphorylated HDAC5 (Figure 4E). Our findings in patients with PeAF indicate that activation of HDAC5 and subsequent activation of MEF2-related fetal gene expression underlies cardiomyocyte remodeling and AF progression.
Figure 4: Phosphorylated HDAC5 increased significantly and correlate with BNP gene expression levels in LA of AF patients. A) Representative Western blot of phosphorylated HDAC5 (pHDAC5), HDAC5 and GAPDH in patients with persistent AF (PeAF). B) Quantified ratio of pHDAC5 to HDAC5 in patients. N=5 from each group. ***P<0.01 AF vs SR. C) The gene expression ratio of β-MHC (MHY7) to α-MHC (MHY6) is significantly increased in AF patients. *P<0.05 vs SR RAA. N=6 for SR, N=7 for AF. D) BNP gene expression is significantly increased in AF patients. ***P<0.01 vs SR LAA. N=6 for SR, N=7 for AF. E) BNP gene expression level correlates significantly with pHDAC5 levels in patients (SR N=3 for LA/RA, AF, N=5 for LA/RA). Open circles: SR, filled circles: AF.

4. DISCUSSION

In the current study, we evaluated the role of class I and class IIa HDACs in tachypacing-induced cardiomyocyte remodeling. We found that overexpression of class I members, HDAC1 and HDAC3, results in detrimental effects on contractile function in HL-1 cardiomyocytes. In contrast, overexpression of class IIa HDAC5 and HDAC7 revealed protective effects. The protective effect was not observed in cardiomyocytes overexpressing MEF2-binding domain deficient HDAC5 or HDAC7, indicating that MEF2 acts as the downstream effector of HDAC5 and HDAC7. Moreover, tachypacing induced
phosphorylation of HDAC5, nuclear export and downstream fetal gene activation in HL-1 cardiomyocytes, including enhanced expression of β-MHC and BNP. Comparable findings were observed in permanent AF patients, which showed increased levels of phosphorylated HDAC5 and β-MHC and BNP. As HDAC5 is abundantly expressed in the heart, in contrast to HDAC7, this study identifies HDAC5 as a promising therapeutic target in clinical AF to attenuate pathological gene expression, contractile dysfunction and progression of the disease.

**Figure 5:** Schematic features of Class IIa HDACs and the repressive influence of Class IIa (5/7) on MEF2, which act with other transcription factors (TF) to control fetal gene program. A) Domain organization of class IIa HDACs. Each class IIa HDAC contains a conserved deacetylase domain and an amino-terminal myocyte enhancer factor 2 (MEF2) binding domain (marked by a blue square), a nuclear localization signal (NLS) and a carboxy-terminal nuclear export sequences (NES). Each class IIa HDAC can be phosphorylated at conserved serine residues (S). The binding sites for the 14-3-3 chaperone protein are also shown. B) The repressive influence of Class IIa (HDAC5 and HDAC7) on MEF2. MEF2 acts with other transcription factors (TF) to control fetal gene program. Signaling kinases such as PKC, PKD, CaMK, induce phosphorylation of class IIa HDACs, which creates docking sites for the 14-3-3 chaperone protein, resulting in nuclear export and, consequently, activation of fetal genes controlled by MEF2.

### 4.1. Key role for class IIa HDAC5 in AF

In the present study, we identified a key role for tachypacing-induced activation and translocation of HDAC5, which in turn mediates transcriptional reprogramming and AF progression in cardiomyocytes. Although all class IIa HDAC members are involved in
transcriptional reprogramming, only overexpression of HDAC5 and HDAC7 was found to protect. In general, class IIa HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) all have large N-terminal extensions with conserved binding sites for the transcription factor MEF2 and the chaperone protein 14-3-3 (Figure 5A, B) and thereby suppress transcriptional activity. Following phosphorylation by kinases, such as calcium/calmodulin-dependent protein kinase (CaMK), which was also found to be activated in AF, and protein kinase D (PKD), class IIa HDACs bind 14-3-3 and shuttle from the nucleus to the cytoplasm. The dissociation of class IIa HDACs from MEF2 allows the histone acetyltransferase p300 to associate with MEF2 via the HDAC docking site, thereby converting MEF2 from a transcriptional repressor (MEF2-HDAC complex) to a transcriptional activator (MEF2-p300 complex). In response to injury, activation of MEF2 is sufficient and necessary to drive pathological cardiac hypertrophy and heart failure.

Although all members of Class IIa HDACs can bind to MEF2 and suppress fetal gene expression in hypertrophy and heart failure, in the current study we observed a protective role for HDAC5 and HDAC7, but not HDAC4 and HDAC9, in tachypacing-induced remodeling. Different upstream regulating kinases and downstream targets of specific Class IIa HDACs might explain this discrepancy. The protective effect of HDAC5 may be caused by AF-induced activation of specific upstream kinases, which regulate individual HDAC members and/or individual HDACs that regulate specific downstream targets. Compared to HDAC5, a non-transcriptional function for HDAC4 in the heart was recently described, i.e., HDAC4 associated with cardiac sarcomeres and decreased myofilament calcium sensitivity. In our model system, the detrimental effect of HDAC4 overexpression on contractile function might therefore counteract the protective role of HDAC4 to suppress MEF2 related fetal gene expression. Moreover, HDAC4 binds constitutively to 14-3-3 in yeast and mammalian cells, whereas HDAC5 binding to 14-3-3 is dependent on CaMK signaling. In addition, 14-3-3 binding to HDAC5 is required for CaMK-dependent disruption of MEF2-HDAC complexes and nuclear export of HDAC5. Since CaMK signaling is involved in AF progression, the role of specific upstream kinases seems plausible. The involvement of HDAC7 in heart diseases has not yet been well studied. However, there are indications for HDAC7 to regulate the function of MEF2 proteins in heart and muscle tissue, suggesting a similar role as HDAC5.

HDAC5 and HDAC9 have redundant roles in the suppression of cardiac growth in response to stress signaling, and both are highly enriched in the heart compared to HDAC7. Despite sharing common upstream kinases, which stimulate the translocation of HDAC5 and HDAC9, only translocation of HDAC5 is activated by calpain. Interestingly, calpain was found to be activated in AF and underlies structural remodeling and contractile dysfunction in AF. Thus, calpain-induced HDAC5 activation may be involved in transcriptional reprogramming and cardiomyocyte remodeling as observed in
AF. Furthermore, we previously found a role for HDAC6 to deacetylate α-tubulin resulting in microtubule disruption, contractile dysfunction and AF progression.\textsuperscript{9} Interestingly, in injured neurons, HDAC5 has been found to deacetylate α-tubulin.\textsuperscript{31} Whether HDAC5 is able to deacetylate α-tubulin in cardiac tissue is unknown.

4.2. Role of class I HDACs in cardiac growth and diseases

In the present study, overexpression of HDAC1 and HDAC3, members of the class I HDAC family, had detrimental effects on the contractile function of HL-1 cardiomyocytes. Class I HDACs control expression of a vast array of genes involved in core cellular activities, such as cell proliferation and death.\textsuperscript{32} All class I HDAC members are ubiquitously expressed in the heart, localize predominantly in the nucleus and display high enzymatic activity toward histone substrates.\textsuperscript{32} Redundant functions of HDAC1 and HDAC2 have been found in Olson’s lab, i.e. cardiac deletion of either HDAC1 or HDAC2 sufficiently supports normal development, whereas cardiac deletion of both HDAC1 and HDAC2 alleles result in neonatal lethality, accompanied by cardiac arrhythmias, dilated cardiomyopathy, and upregulation of genes encoding skeletal muscle-specific contractile proteins and calcium channels in the heart.\textsuperscript{33} Trivedi et al. found that in HDAC2-deficient survivors exposed to hypertrophic stimuli, cardiac hypertrophy and fibrosis were attenuated and that cardiac-specific overexpression of HDAC2 resulted in cardiac hypertrophy.\textsuperscript{34} The recent finding that HDAC1 and HDAC2 play a major role in autophagy driven by α-adrenergic stimulation in cultured cardiomyocytes provides another indication that HDAC1 and HDAC2 may act as a driver of adverse cardiac remodeling.\textsuperscript{35} Furthermore, overexpression of HDAC3 in the heart leads to increased thickness of the myocardium, which is due to increased cardiomyocyte hyperplasia.\textsuperscript{36} HDAC3 is also present at myofilaments and directly modulate contractile function by modulating the acetylation of myosin heavy chain.\textsuperscript{37} Taken together, activation of class I HDACs has been associated with detrimental effects on the development and function of the heart. Our observation in tachypaced HL-1 cardiomyocytes are concordant with this analysis.

4.3. Therapeutic implications

In the current study, we observed a key role for class IIa HDAC5 to protect from AF remodeling via suppression of MEF2. This is evident by our findings showing that contractile dysfunction was prevented by overexpression of HDAC5, and abrogated by overexpression of a HDAC5 construct bearing a mutation in the MEF2 binding domain. Findings were expanded to patients and revealed increased phosphorylation levels of HDAC5 and induced expression of β-MHC and BNP in persistent AF patients compared to controls in sinus rhythm. Thus, the results suggest that HDAC5 plays a prominent role in
epigenetic regulation during AF and might be an interesting therapeutic target to prevent pathological fetal gene expression, functional loss and AF progression. Consequently, compounds inhibiting the release of HDAC5 from MEF2 might have a therapeutic potential for treatment of AF and it is of interest to test these compounds in experimental and clinical AF. One such compound is MC1568, since it inhibits the activity of HDAC4 and HDAC5, thereby leaving MEF2-HDAC complexes in a repressed state. Inhibitors of upstream kinases which phosphorylate HDAC5 and thus initiate de-repression of MEF2 may also be of interest. A more upstream approach would be to inhibit CaMK and PKC, since these represent two main kinases involved in HDAC5 phosphorylation and nuclear export in cardiomyocytes. Indeed, CaMK inhibitors have been reported to prevent AF. Furthermore, the PKC inhibitor Go6983 has been reported to block HDAC5 nuclear export and α-tubulin deacetylation after nerve injury. Given that deacetylation of α-tubulin is involved in AF structural remodeling, Go6983 may also be an interesting candidate to test in AF. As our study suggests HDAC5 repression of MEF2 responsive genes to be involved in AF, HDAC5 inhibitors that prevent stress-induced HDAC release from MEF2, such as MC1568 and PKC inhibitor Go6983, might therefore represent novel therapeutic approaches to attenuate AF progression.
REFERENCES


19. Zhang Y, Matkovich SJ, Duan X, Diwan A, Kang MY, Dorn GW, 2nd. Receptor-independent protein kinase C alpha (PKCalpha) signaling by calpain-generated free catalytic domains induces HDAC5 nuclear export and...


SUPPLEMENTAL MATERIALS

Supplemental Figures

Figure S1: Western blot showing overexpression of HDACs in retrovirus infected HL-1 cardiomyocytes. A) Representative Western blot image showing successfully infection of HL-1 cardiomyocytes by HDAC1, HDAC3, HDAC4, HDAC5, HDAC7 and HDAC9. All constructs contained a Flag-tag. B) Representative Western blot image showing successfully infection of HDAC5m, HDAC7m and GFP. DsRED without a flag-tag was used as a negative control.

Figure S2: Endogenous HDAC5 localization in HL-1 cardiomyocytes with a time course of tachypacing. A) Representative confocal images of cardiomyocytes showing the localization of HDAC5 in HL-1 cardiomyocytes tachypaced from 0 hours (0h) to 16 hours (16h). B) Quantified data of cellular localization of HDAC5, showing significant decrease in the amount of cardiomyocytes with exclusively nuclear HDAC5 and a significant increase in cytosolic localization of HDAC5 after 8 and 16 hours TP. ***p < 0.01 vs 0h.
**Supplemental Tables**

**Table S1: Primers used for PCR**

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m: mouse, h: human, MHY6: α-MHC, MHY7: β-MHC.

**Table S2: Baseline demographic and clinical characteristics of patients with permanent AF (PeAF) and control patients in sinus rhythm (SR).**

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**Underlying heart disease (n) / surgical procedure**

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**Medication (n)**

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<th>SR (43%)</th>
<th>PeAF (86%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE/ARB</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0 (0%)</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>0 (0%)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>2 (29%)</td>
<td>5 (71%)</td>
</tr>
<tr>
<td>Statin</td>
<td>2 (29%)</td>
<td>1 (14%)</td>
</tr>
</tbody>
</table>

MVD: mitral valve disease, ACE: angiotensin-converting enzyme, ARB: angiotensin receptor blocker, β-Blocker: beta-adrenergic antagonists, *P*<0.05 vs. SR.