PARP1 inhibition attenuates NAD$^+$ depletion and protects against experimental and human Atrial Fibrillation

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*Manuscript in preparation*
ABSTRACT

Background: Progression of Atrial Fibrillation (AF), the most common persistent clinical tachyarrhythmia, is driven by structural and metabolic remodeling of cardiomyocytes. Activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) is implicated in metabolic remodeling in various forms of cardiomyopathies. During excessive PARP activation, nicotinamide adenine dinucleotide (NAD$^+$) is consumed, resulting in ATP depletion and energy loss of the cardiomyocyte. To determine whether PARP activation and NAD$^+$ depletion contribute to AF progression, we examined their role in experimental model systems for AF and in clinical AF.

Methods and Results: Tachypacing of HL-1 atrial cardiomyocytes induces DNA damage, resulting in PARP activation, NAD$^+$ depletion and calcium transients (CaT) loss. Accordingly, replenishment of NAD$^+$ or depletion of PARP1, but not PARP2, protects against tachypacing-induced contractile dysfunction. Moreover, inhibition of PARP, by the broad PARP inhibitor 3-AB or the specific PARP1/2 inhibitor ABT-888, protects against tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes and Drosophila. Consistent with these findings, PARP is also activated in atrial tissue of tachypaced dogs and of permanent AF patients, and PARP activation correlates with the level of DNA damage in patients.

Conclusion: AF induces DNA damage, PARP1 activation and consequently depletion of NAD$^+$ levels, which contribute to the loss of contractile function. Inhibition of PARP1 by 3-AB or ABT-888 protects against tachypacing-induced contractile dysfunction. Our results suggest a prominent role for PARP1 in AF-induced metabolic and functional remodeling and consequently disease progression. Inhibition of PARP1 may serve as a novel therapeutic target in AF by conserving the cardiomyocyte metabolism.
1. INTRODUCTION

Atrial Fibrillation (AF) is the most common human tachyarrhythmia. Over the past years, considerable progress has been made in understanding the causes of persistent AF, which may facilitate more effective treatment of AF. The current insight is that progression of AF is driven by the interplay between electrical, structural and functional remodeling of cardiomyocytes. In turn, principle factors grounding cardiomyocyte remodeling include derailment of calcium homeostasis, proteostasis and protein quality control system. To these, we recently added a key role for the activation of histone deacetylase 6 (HDAC6), which induces structural and functional remodeling of cardiomyocytes via deacetylation of the cytoskeletal protein α-tubulin. In the course of the studies, we observed that nicotinamide (vitamin B₃), an inhibitor of the HDAC class III (sirtuins), offers complete protection against cardiomyocyte remodeling in tachypaced cardiomyocytes and Drosophila pupae, yet unrelated to its inhibition of sirtuins. Thus, we set out to disclose nicotinamine’s mechanisms of action.

Poly(ADP-ribose) polymerases (PARPs) are inhibited by nicotinamide and represent a possible drug target to mitigate tachypacing-induced remodeling. PARPs constitute a family of 6 nuclear enzymes whose activation is precipitated by single and double strand breaks (SSBs and DSBs, respectively) of the DNA, which serve to recruit the DNA repair machinery by synthesis of poly(ADP-ribose) chains (PAR). Synthesis of the PAR chain consumes nicotinamide adenine dinucleotide (NAD⁺) up to an extent that it depletes the mitochondrial stores of NAD⁺, leading to progressive decline in ATP levels, metabolic remodeling and cell death in case of strong PARP activation. Moreover, PARP activation, especially of PARP1, was previously found involved in various cardiovascular diseases other than AF. Accumulating evidence reveals a role for PARP1 activation via reactive oxygen and nitrogen species-induced DNA damage in cardiomyocytes during myocardial ischemia/reperfusion injury, various forms of heart failure or cardiomyopathies, cardiac aging and myocardial hypertrophy. Moreover, pharmacological and/or genetic inhibition of PARP1 provides significant benefits in animal models of such cardiovascular disorders.

In the current study, we determined whether nicotinamide protects from tachypacing-induced cardiomyocyte remodeling through inhibition of PARP, preservation of NAD⁺ levels and consequently maintenance of cellular metabolism and contractility. We characterized the pathways involved and examined the therapeutic effect of PARP inhibitors.
2. MATERIALS AND METHODS

2.1. HL-1 cardiomyocyte model, calcium transient measurements and drug treatment

HL-1 cardiomyocytes derived from adult mouse atria were obtained from Dr. William Claycomb (Louisiana State University, New Orleans) and cultured as previously described.4,20 The cardiomyocytes were tachypaced (TP, 5 Hz) with a C-Pace100 culture pacer (IonOptix) for 12 hours. Ca^{2+} transient (CaT) measurements were performed as previously described.5,20

Prior to tachypacing, HL-1 cardiomyocytes were treated for 12 hours with the PARP inhibitors, 3-aminobenzamide (3-AB, Sigma-Aldrich), ABT-888 (Selleckchem), beta-nicotinamide adenine dinucleotide hydrate (NAD^+, Sigma-Aldrich) or transfected with scrambled siRNA (control), PARP1 siRNA (Ambion), PARP2 siRNA (Santa Cruz) to study the specific role of PARP1 and PARP2 respectively.

2.2. Drosophila stocks, tachypacing, and heart wall contraction assays

The wild-type W1118 strain was used for all experiments. The Drosophila prepupa were pre-treated with compounds, subjected to tachypacing and heart wall contraction was measured as previously described.9 See Table 1 for the applied doses of 3-AB, ABT-888 and NAD^+ in the Drosophila experiments.

2.3. Dogs

Left and right atrial tissue from sham dogs and atrial tachypaced dogs were used for Western blot analyses as described previously.9

2.4. Patients

Before surgery, one investigator assessed patient characteristics (Table 2), as described before.21,22 Right atria (RA) and left atria (LA) tissue samples were obtained from patients with underlying mitral valve disease displaying permanent AF (PeAF) or sinus rhythm (SR). 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.

2.5. Protein extraction and Western blot analysis

HL-1 cardiomyocytes or human tissue samples were lysed in radioimmunoprecipitation assay buffer as described before.4,9 In short, equal amounts of protein homogenates were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes, and probed with anti-poly (ADP-Ribose) (PAR, BD bioscience), anti-PARP1
PARP1 inhibition protects against AF

(Santa Cruz), anti-YH2AX (Millipore), anti-β-actin (Santa Cruz), 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. NAD Assay

NAD/NADH levels, in which NAD represents the sum of NAD⁺ and NADH, were measured according to the manufacturer’s instructions of the assay kit (Abcam, ab65348). In short, HL-1 cardiomyocytes were lysed in NAD extraction buffer, and the protein concentration was determined (BioRad). 50µl of each extracted protein sample was transferred into 96-well plates and mixed with 100 µl NAD cycling buffer. The plate was incubated at room temperature for 5 min to convert NAD⁺ to NADH, followed by the addition of 10 µl NADH developer buffer and 2 hours incubation at room temperature. NAD/NADH levels were measured at 450 nm (BioTek Synergy 4 plate reader). Notably, in accord with other findings, the NADH amount in cultured cardiomyocytes and tissue was below the detection limit. Therefore, the NAD⁺ per µg of protein was used as the final endpoint.

2.7. Comet assay

To evaluate DNA damage in cardiomyocytes, alkaline comet assay kit (Trevigen) was utilized according the manufacturer’s instructions with minor changes. HL-1 cardiomyocytes were trypsinized, harvested by centrifugation, suspended at 200,000 cells/ml in PBS, combined with 45 µl melted LAM agarose at ratio of 1:10 (v/v) and immediately pipetted onto CometSlides. Slides were dried for 30 minutes at 4 °C, immersed firstly in lysis solution for 1 h and then in freshly prepared alkaline unwinding solution (pH>13) for 1 hour. After placing the slides in 4 °C alkaline electrophoresis solution, electrophoresis at 21 volts for 30 minutes was started, followed by immersion (twice) in dH₂O for 5 minutes and 70% ethanol (once) for 5 minutes. Thereafter, slides were dried at 37 °C, stained with SYBR Gold for 30 minutes at room temperature in the dark, rinsed in water, and dried at 37 °C. Finally, slides were visualized at an excitation of 496 nm and emission of 522 nm by fluorescence microscopy (Leica). DNA damage was quantified by scoring the percentage of DNA in the tail, calculated by the Image J Marco “Comet_Assay” based on an NIH Image Comet Assay developed by Herbert M. Geller in 1997.

2.8. Quantitative RT-PCR
Total RNA was isolated from HL-1 cardiomyocytes utilizing the nucleospin RNA isolation kit (Machery-nagel). First strand cDNA was generated by M-MLV reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen). Relative changes in transcription level were determined using the CFX384 Real-time system C1000 Thermocycler (BioRad) in combination with SYBR green supermix (Bio-rad). Calculations were performed using the comparative CT method according to User Bulletin 2 (Applied Biosystems). Fold inductions were adjusted for GAPDH levels. Primer pairs used included; PARP1 F: CACCTTCCAGAAGCAGGAGA and R: GCAAGAAATGCAGCGAGAGT; PARP2 F: TCCTCTGGGCAATCATCTTTCT and R: AAGCTGGGAAAGGCTCATGT.

2.9. Immunofluorescence

HL-1 cardiomyocytes were grown on coverslips until 80% confluent, and subjected to TP or NP for various time periods, with or without drug treatment. Immediately after pacing, cardiomyocytes were rinsed in PBS and fixed with 4% formaldehyde for 15 minutes, rinsed twice with PBS, permeabilized with 0.1% triton X-100 in PBS for 10 minutes, rinsed twice in PBS and blocked with blocking solution (0.5% BSA and 0.15% glycine in PBS) for 10 minutes. After blocking, cardiomyocytes were incubated with primary antibodies for 2 hours at room temperature. After rinsing the cardiomyocytes three times with blocking solution, cardiomyocytes were incubated with secondary antibodies for 45 minutes at room temperature shielded from light, followed by rinsing with blocking solution three times and PBS twice. Lastly, cardiomyocytes were incubated with mounting media containing DAPI (Vectashield) and sealed with nail polish. Antibodies used were: anti-γH2AX (1:100, Millipore), anti-PAR (1:200, BD Bioscience), anti-PARP1 (1:200, Santa Cruz), goat anti rabbit FITC (1:200, Invitrogen), goat anti mouse TRITC (1:200, Southern Biotech).

2.10. Statistical Analysis

Results are expressed as mean ± SEM. Biochemical analyses were performed at least in duplicate. Individual group mean differences were evaluated with the Student’s t-test. Correlation was determined with the Spearman correlation test. To compare continuous variables with a skewed distribution, the Mann-Whitney test was applied. All P values were 2 sided. Values of P<0.05 were considered statistically significant. SPSS version 20 was used for all statistical evaluations.
PARP1 inhibition protects against AF

Table 1: Overview of protective effects of PARP inhibitors and NAD+ replenishment in tachypaced HL-1 cardiomyocytes and Drosophila melanogaster.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>targets</th>
<th>IC50</th>
<th>Concentration in HL-1</th>
<th>Protection in HL-1</th>
<th>Concentration in Drosophila</th>
<th>Protection in Drosophila</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-AB</td>
<td>PARPs</td>
<td>-</td>
<td>3 mM</td>
<td>+</td>
<td>30 mM</td>
<td>-</td>
</tr>
<tr>
<td>ABT-888</td>
<td>PARP1</td>
<td>5.2 nM</td>
<td>5-40 μM</td>
<td>+</td>
<td>200, 400 μM</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PARP2</td>
<td>2.9 nM</td>
<td>24 μM</td>
<td>+</td>
<td>5,10 mM</td>
<td>+</td>
</tr>
<tr>
<td>NAD+</td>
<td>-</td>
<td>-</td>
<td>0.25-1 mM</td>
<td>+</td>
<td>5,10 mM</td>
<td>+</td>
</tr>
</tbody>
</table>

-, no significant protective effect; +P<0.01 vs control TP.

Table 2: Baseline demographic and clinical characteristics of patients with permanent AF (PeAF) and control patients in sinus rhythm (SR).

<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>PeAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>RA (n)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>LA (n)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Age (mean, std)</td>
<td>56 ± 15</td>
<td>68 ± 4</td>
</tr>
<tr>
<td>Months of AF (median, range)</td>
<td>–</td>
<td>14.6 (8.3-36)</td>
</tr>
<tr>
<td>Underlying heart disease (n) / surgical procedure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVD/MV replacement or repair</td>
<td>5(100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Medication (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE/ARB</td>
<td>2 (40%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0 (0%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>0 (0%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>2 (40%)</td>
<td>4 (80%)</td>
</tr>
</tbody>
</table>


3. RESULTS

3.1. Tachypacing causes DNA damage, PARP activation and NAD+ depletion

Previously, we observed nicotinamide to protect against contractile dysfunction in tachypaced cardiomyocytes and Drosophila and the protective effect was independent on inhibition of sirtuin activity. As nicotinamide is also known to inhibit the activation of PARP, we tested the level of PARP activity by measuring PAR levels in control normal and tachypaced cardiomyocytes. A gradual increase in PAR levels was observed upon tachypacing, which reached significance after 8 hours of tachypacing and remained increased until 16 hours of tachypacing (Figure 1A-D, Figure S1A), while PARP expression was unchanged by tachypacing (Figure 1A, Figure S1B, C). This observation
indicates that tachypacing induces PARP activation. Since PARP gets activated by SSB and DSB in the DNA\textsuperscript{25}, the level of DNA damage was determined by comet assay (single-cell gel electrophoresis)\textsuperscript{26}, and phosphorylation of the Ser-139 residue of the histone variant H2AX, forming γH2AX. Four hours of tachypacing significantly increased both the percentage of DNA in the comet tail (Figure 1E, F) and γH2AX levels of cardiomyocytes (Figure 1G-J) after 4 hours tachypacing. This result shows that tachypacing induces DNA damage and PARP activation in HL-1 cardiomyocytes.

Upon activation, PARP consumes NAD\textsuperscript{+} to synthesize PAR. Therefore, progressive and excessive activation of PARP results in exhaustion of NAD\textsuperscript{+} levels, which finally results in the metabolic and functional impairment of cardiomyocytes.\textsuperscript{16} Therefore, we studied whether tachypacing-induced PARP activation depleted NAD\textsuperscript{+} levels in HL-1 cardiomyocytes. Hereto, NAD\textsuperscript{+} levels were measured in control and tachypaced cardiomyocytes at different time points. Eight hours of tachypacing induced a significant reduction in NAD\textsuperscript{+} levels (Figure 1K).

Together, these findings reveal that tachypacing induces DNA damage and consequently the activation of PARP, resulting in depletion of NAD\textsuperscript{+} levels in cardiomyocytes.

3.2. PARP1 is the key enzyme instigating tachypacing-induced contractile dysfunction in cardiomyocytes

To substantiate that the decline in NAD\textsuperscript{+} levels is a driving mechanism for metabolic remodeling and functional loss, the effect of replenishment of NAD\textsuperscript{+} on contractile function in tachypaced HL-1 cardiomyocytes was studied. Tachypacing resulted in a significant CaT loss, which was abrogated by preserving cellular NAD\textsuperscript{+} levels via exogenous, dose-dependent supplementation of NAD\textsuperscript{+} (Figure 2A, B). This observation was confirmed in tachypaced \textit{Drosophila} prepupae, where tachypacing resulted in loss of heart wall contractions, which was prevented by replenishment of NAD\textsuperscript{+} dose dependently (Figure 2C, D). Next, we examined whether PARP mediates the NAD\textsuperscript{+} depletion, since especially PARP1, and to a lesser extent PARP2 isoforms, consume NAD\textsuperscript{+}.\textsuperscript{12} Hereto, HL-1 cardiomyocytes were transfected with siRNA of PARP1 or PARP2. Western blot and qPCR revealed specific and effective suppression of PARP1 by PARP1 siRNA and PARP2 by PARP2 siRNA (Figure 3A, B). Suppression of PARP1 significantly protected cardiomyocytes against tachypacing-induced CaT loss, whereas suppression of PARP2 did not (Figure 3C, D). These results demonstrate that PARP1 is the key PARP enzyme instigating tachypacing-induced metabolic and contractile dysfunction in cardiomyocytes.
PARP1 inhibition protects against AF

Figure 1: Tachypacing induces PARP activation, DNA damage and NAD+ depletion in HL-1 cardiomyocytes. A) Representative Western blot of PAR and PARP1 levels in control non-paced tachypaced (TP) HL-1 cardiomyocytes for durations as indicated. B) Quantified data of PAR expression in A. C) and D) Immunofluorescent staining and quantified data of PAR levels in control (0 h), and in 12 h TP of HL-1 cardiomyocytes. ***P<0.01 vs 0 h, N≥7 images. E) Representative immunofluorescence images of HL-1 cardiomyocytes with time course TP (0 h-12 h), showing tail DNA. F) Quantified percentage of tail DNA in HL-1 cardiomyocytes from E). Boxplot of percentage of tail DNA of median, 25 and 75 percentile; whiskers represent the 10 and 90 percentile and dots the outliers. ***P<0.001 vs 0 h, N=10-50 cardiomyocytes. G) and H) Representative western blot of γH2AX, H2A and quantified data of γH2AX during time course of TP in HL-1 cardiomyocytes. ***P<0.01 vs 0 h, I) and J) Representative immunofluorescent staining and quantified data of γH2AX Levels in NP (0 h) and TP (12 h) HL-1 cardiomyocytes, ***P<0.01 vs 0h, N≥6 images. K) Relative NAD+ levels in HL-1 cardiomyocytes during time course of TP (2 h-8 h) compared to control (0 h). *P<0.05.
Figure 2: Repletion of NAD\(^+\) dose-dependently attenuates contractile dysfunction in cardiomyocytes and Drosophila. A) and B) Representative CaT traces and quantified CaT amplitude data of control non-paced (NP) and tachypaced (TP) HL-1 cardiomyocytes pretreated with or without different doses of NAD\(^+\) (0.25mM, 0.5mM, 1mM). ***\(P<0.01\) vs Control NP #\(P<0.01\) vs Control TP, N=20–40. C) and D) Representative heart wall motions and quantified data of relative heart rate to control NP Drosophila. Drosophila were treated with or without NAD\(^+\) (5mM or 10mM). ***\(P<0.01\) vs control NP #\(P<0.01\) vs control TP, N≥6 for each group.

3.3. Inhibition of PARP1 protects against NAD\(^+\) depletion and contractile function

To explore whether PARP1 represents a drug target to mitigate tachypacing-induced remodeling, the action of PARP inhibitors was examined in HL-1 cardiomyocytes. PARP inhibitors comprised the general inhibitors, nicotinamide and 3-AB, and the specific PARP1/2 inhibitor ABT-888. Both general and specific inhibition of PARP1/2 precluded tachypacing-induced parylation of proteins and decrease in NAD\(^+\) levels (Figure 4A, B, and Figure S3). Furthermore, 3-AB and ABT-888 also significantly attenuated tachypacing-induced contractile dysfunction in cardiomyocytes and Drosophila (Figure 5A-F), as previously observed for nicotinamide.\(^9\) These findings demonstrate that both general and
PARP1/2 specific PARP inhibitors attenuate tachypacing-induced PARP activation, NAD⁺ depletion and calcium transient loss.

**Figure 3:** PARP1, not PARP2, is the key enzyme mediating tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes. A) Representative Western blot showing significant knockdown of PARP1 in HL-1 cardiomyocytes transfected with specific PARP1 siRNA (PARP1i) compared to HL-1 cardiomyocytes transfected with scrambled siRNA (Control). *P<0.05 vs control. B) Representative qPCR showing significant knockdown of PARP2 in HL-1 cardiomyocytes transfected with specific PARP2 siRNA (PARP2i) compared to HL-1 cardiomyocytes transfected with scrambled siRNA (Control). ***P<0.01 vs control. C) and D) Representative CaT traces and quantified CaT amplitude data in control non-paced (NP) or tachypaced (TP) HL-1 cardiomyocytes transfected with scrambled siRNA (Control), PARP1 siRNA (PARP1i), PARP2 siRNA (PARP2i). ***P<0.01 vs control NP, ###P<0.001 vs control TP. N≥20 for each group.

**Figure 4:** PARP inhibitors inhibit parylation and NAD⁺ depletion in tachypaced HL-1 cardiomyocytes. A) Representative Western blot showing that the PARP inhibitors 3-AB (3 mM) and ABT-888 (40 µM) inhibit TP-induced PAR formation, which is an indicator of PARP activity. B) 3-AB (3 mM) and ABT-888 (40 µM) conserved NAD⁺ after TP. The average value of 4 independent experiments is shown. ***P<0.01 vs control NP, #P<0.05 vs control TP, ###P<0.01 vs control TP.
Figure 5: PARP inhibitors dose-dependently protect against contractile dysfunction in cardiomyocytes and Drosophila. A) and B) Representative CaT traces and quantified CaT amplitude in control non-paced (NP) or tachypaced (TP) HL-1 cardiomyocytes with pretreated with 3-AB (3 mM) or vehicle (CTL). ***P<0.01 vs control NP, #P<0.01 vs control TP, N≥40. C) and D) Representative CaT and quantified CaT amplitude of non-paced (NP) and tachypaced (TP) HL-1 cardiomyocytes pretreated with ABT-888 at different doses (5-40 µM) or vehicle (CTL). ***P<0.01 vs control NP, #P<0.01 vs control TP, N≥20. E) and F) Representative heart wall motions and quantified relative heart rate of control NP or TP Drosophila pretreated with 3-AB (30 mM), ABT-888 (0.2 mM, 0.4 mM), or vehicle (CTL). ***P<0.01 vs control NP, #P<0.05 vs control TP, N≥7.

3.4. PARP is activated in a dog model for AF and in human AF, and correlates with DNA damage

To extend our findings to an in vivo animal model for clinical AF, we measured PARP activation by determining PAR formation in both right atrial tissue (RA) and left atrial tissue (LA) of atrially tachypaced dogs and non-paced control dogs. Tachypacing in dogs
significantly increased PAR levels while PARP1 protein expression remained unchanged, indicating that atrial tachypacing of dogs induced activation of PARP (Figure 6A-C). Comparable findings were observed in heart tissue of permanent AF (PeAF) patients compared to control patients in sinus rhythm (SR). PeAF patients demonstrated a significant increase in PAR formation in both RA appendages and LA appendages, while no difference in PARP1 protein expression was observed compared to SR (Figure 6D-F). Whereas no significant difference in γH2AX levels was found between PeAF and SR patients (Figure 6G, H), PAR levels in patients correlated significantly with γH2AX levels (Figure 6I). Thus, patients with PeAF show increased levels of PAR, indicative for PARP activation, and PAR levels correlated with levels of the DNA damage marker γH2AX.

Taken together, these findings indicate that PARP is activated by DNA damage in human AF, which may contribute to metabolic remodeling, functional loss and AF progression.
Figure 6: PARP is activated in a dog model for AF and in human AF, which correlates with DNA damage. A-C) Western blot images and quantified data of PAR and PARP1 in control non-paced dogs (CTL) and in atrial tachypaced dogs (TP). ***P<0.01 vs CTL, N=5 for CTL, N=6 for TP. D-F) Representative Western blot of PAR and PARP levels in right atrial tissue (RA) and left atrial (LA) tissue of SR and AF patients, showing significant increase in PAR levels in AF patients compared to SR. PARP1 expression levels remain unchanged between AF and SR patients. N=5 for SR, N=5 for AF *P<0.05 SR RA vs AF RA, SR LA vs AF LA. G) and H) Representative Western blot and quantified data of γH2AX in right atrial tissue (RA) and left atrial (LA) tissue of SR and AF patients. N=3 for SR, N=5 for AF. I) Correlation of PAR and γH2AX levels, showing significant correlation between PARP activity (PAR levels) and DNA damage (γH2AX levels). SR: open circle and AF: filled circle.
4. DISCUSSION

In the current study, we identified PARP1 as a key enzyme in AF progression. Our results show that AF induces DNA damage and subsequent PARP1 activation. Active PARP1, in turn, consumes NAD⁺, resulting in metabolic remodeling and functional loss in tachypaced cardiomyocytes and Drosophila. Accordingly, replenishment of NAD⁺ protects against tachypacing-induced contractile dysfunction. Moreover, inhibition of PARP, by the broad PARP inhibitor 3-AB or the specific PARP1/2 inhibitor ABT-888, protects against tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes and Drosophila. Consistent with these findings, PARP is also activated in atrial tissue of tachypaced dogs and of permanent AF patients, and PARP activation correlates with the level of DNA damage. Taken together, these findings suggest a dominant role of PARP1 in AF-induced metabolic and functional remodeling and consequently in disease progression.

4.1. PARP1-induced NAD⁺ depletion: link to cardiovascular disease progression

In the current study, we found PARP, especially PARP1, to have a prominent role in AF progression. This is similar with other cardiovascular disease models, including heart failure models in mice, dogs and rats, where activation of PARP1 induced endothelial dysfunction, myocardial hypertrophy and remodeling. In addition, cardiac function in mouse models of diabetic cardiomyopathies showed marked improvement by the knockout of PARP1. Importantly, recent studies utilizing biopsy material from patients with heart failure reported overexpression of PARP1 and increased PARP1 activity to contribute to disease progression. Thus, the findings from the current study expand the importance of PARP1 activation in cardiovascular diseases.

Under physiological conditions, PARP1 is an abundant nuclear chromatin-bound DNA repair enzyme, catalysing the transfer of ADP-ribose moieties from NAD⁺ to acceptor DNA binding proteins. In general, PARP1 is activated by SSB in the DNA resulting from free radical and oxidant cell injury and/or nuclear accumulation of Ca²⁺. Under pathophysiological conditions, such as during AF, excessive activation of PARP1 leads to the depletion of cellular NAD⁺, a key coenzyme in cell metabolism, and in turn, results in the depletion of ATP content, further damage to the DNA, and ultimately, results in structural damage and functional loss. NAD is involved in redox reactions, carrying electrons from one reaction to another. Therefore, NAD is present in two forms in cells, i.e. the oxidized NAD⁺ and the reduced NADH. In addition, NAD⁺ is also used during the process of post-translational modification. During this process, NAD⁺ acts as a substrate for enzymes, such as PARP1, which is one of the main enzymes that consume NAD⁺. Therefore, maintaining the intracellular level of NAD⁺, by replenishment of NAD⁺ or inhibition of PARP1, is crucial for the management of stress conditions such as AF. This
notion is reflected in heart failure and hypertrophy studies, describing PARP1-induced cardiomyocyte dysfunction and cell death to be mediated by NAD$^+$ depletion$^{13,32}$ In addition, preservation of the NAD$^+$ levels protects against myocardial injury in ischemia and reperfusion$^{23}$ and also ventricular tachycardia in SCN5A overexpressing mice.$^{33}$ Mouse and human cardiomyopathic hearts are also associated with elevated NADH levels, mitochondrial ROS overproduction, a concomitant decrease in cardiac Na$^+$ current $I_{Na}$ and, consequently, reduction in conduction velocity, which were normalized by restoration of NAD$^+$. Notably, SCN5A encodes for Nav1.5, the $\alpha$-subunit of the sodium current ($I_{Na}$). $I_{Na}$ is responsible for the early fast depolarization upstroke of the cardiac action potential. Mutations in SCN5A have recently been shown to play a central role in lone AF. In a cohort of 117 lone AF patients, four different SCN5A mutations were found, supporting the potential role of sodium current disturbances in the development of lone AF.$^{35}$ Given these findings, it is possible that depletion of NAD+, caused by PARP overactivation, impairs INa in AF and thus results in the development and/or progression of AF.

4.2. PARP activation and DNA damage

In the current study, we observed DNA damage in tachypaced HL-1 cardiomyocytes and in patients with persistent AF. A unifying concept exists that cells exposed to DNA damaging conditions enter three major pathways based on the intensity of the trigger.$^{36}$ Extensive stress conditions can induce DNA damage, excessive activation of PARP1, and consequently depletion NAD$^+$ levels resulting in metabolic remodeling and functional loss.$^{37}$ Whereas moderate stress facilitates PARP1 activation and DNA repair, and more severe conditions trigger PARP1 cleavage and cell death. Importantly, both stress conditions are not accompanied with cellular NAD$^+$ depletion. In the current study, we observe that experimental and clinical permanent AF are accompanied by DNA damage, activation of PARP1, depletion of NAD$^+$, and functional loss, indicating that permanent AF is an extensive stress condition. Interestingly, no sign of PARP cleavage, and therefore initiation of cell death, by apoptosis or necrosis was observed.$^{38}$ This is in line with the observation that AF induces hibernation (myolysis) of the cardiomyocyte instead of cell death.$^{39}$ Consequently, in heart conditions associated with excessive PARP1 activation and NAD$^+$ depletion, as observed in AF, the pharmacological inhibition of PARP1 may offer substantial therapeutic benefits.

4.3. Clinical relevance of PARP inhibitors

Recently, novel PARP inhibitors have entered the clinical development for various cardiovascular indications.$^{16,40}$ Most of the PARP inhibitors, such as 3-AB and nicotinamide, are designed to compete with NAD$^+$ at the active site of the enzyme. They
PARP1 inhibition protects against AF

universally inhibit PARP1 and other members of the PARP family, as well as mono-ADP-ribosyl-transferases and sirtuins. The novel PARP inhibitors exhibit increased potency and specificity relative to earlier inhibitors. ABT-888 directly inhibits PARP1 and PARP2 with high potency, and is now in phase I and II clinical studies in cancer. Our study identifies PARP1 activation in experimental models and in human permanent AF, and demonstrates the protective effect of ABT-888 in HL-1 cardiomyocytes and Drosophila. Consequently, our findings call for the exploration of the action of ABT-888 in large animal AF models and in human AF.

In summary, this study documents the induction of DNA damage, excessive activation of PARP1, and subsequent NAD$^+$ depletion, as key events in AF progression. Importantly, inhibition of excessive PARP1 activation prevents NAD$^+$ depletion and conserves cardiomyocyte function, thereby attenuating AF progression. Our findings indicate that inhibition of PARP1 may serve as a novel therapeutic target in AF by conserving the cardiomyocyte metabolism.
REFERENCES


SUPPLEMENTAL MATERIALS

Supplemental Figures

Figure S1: Tachypacing induces PARP activation (PAR) and not PARP1 overexpression in HL-1 cardiomyocytes. A) Representative Western blot showing gradual PAR induction during tachypacing (TP) for the time periods as indicated. B) and C) Immunofluorescent staining and quantified data of PARP1 in control (non-paced, 0 h), and in 12 h TP HL-1 cardiomyocytes. No significant difference was found in the amount of PARP1.
Figure S2: NAD⁺ and PARP inhibitors do not influence baseline CaT in HL-1 cardiomyocytes. A) and B) Representative CaT traces and quantified CaT data of HL-1 cardiomyocytes treated with NAD⁺ (1 mM) or vehicle (Control), showing similar baseline CaT between the groups. C) and D) Representative CaT traces and quantified CaT data in HL-1 cardiomyocytes treated with 3 AB (3 mM) or vehicle (Control), showing similar baseline CaT between the groups. E) and F) Representative CaT traces and quantified CaT data demonstrate slight increase in HL-1 cardiomyocytes treated with ABT888 (40 µM) compared to vehicle (Control).

Figure S3: The PARP inhibitor ABT-888 dose dependently protects tachypaced HL-1 cardiomyocytes from NAD⁺ depletion. NAD⁺ was measured in control (NP) and tachypaced (TP) HL-1 cardiomyocytes pretreated with different doses of ABT-888 (5–40 µM) or vehicle (CTL). ABT-888 in, a dose dependent manner, prevented TP-induced reduction in NAD⁺ levels. ***P<0.01 vs control NP, #P<0.01 vs control TP.