The Loss of p21-Activated Kinase (Pak1) Promotes Atrial Arrhythmic Activity

The Role of Pak1 in Atrial Arrhythmia

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Abstract

Background: Atrial fibrillation (AF) is initiated through arrhythmic atrial excitation from outside the sinus node or remodeling of atrial tissue that allows reentry of excitation. Angiotensin II (AngII) has been implicated in initiation and maintenance of AF through changes in Ca^{2+} handling and production of reactive oxygen species (ROS).

Objective: We aimed to determine the role of Pak1, a downstream target in the AngII signaling cascade, in atrial electrophysiology and arrhythmia.

Method: WT and $Pak1^{-/-}$ mice were used to determine atrial function in vivo, on the organ and cellular level based on the quantification of electrophysiological and Ca^{2+}-handling properties.

Results: We demonstrate that reduced Pak1 activity increases the inducibility of atrial arrhythmia in vivo and in vitro. On the cellular level, $Pak1^{-/-}$ AMs exhibit increased basal and AngII (1 μM)-induced ROS production, sensitive to the NOX inhibitor apocynin (1 μM), and enhanced membrane translocation of Rac1 that is part of the multi-molecular NOX2 complex. Upon stimulation with AngII, $Pak1^{-/-}$ AMs exhibit an exaggerated increase in $[Ca^{2+}]_i$, and arrhythmic events that were sensitive to the NCX inhibitor KB-R7943 (1 μM) and suppressed in AMs from NOX2 deficient ($gp91^{phox-/-}$) mice. Pak1 stimulation (FTY720: 200 nM) in WT AMs and AMs from a canine model of ventricular tachypacing-induced AF prevented AngII-induced arrhythmic Ca^{2+} overload, by attenuating NCX activity in a NOX2 dependent manner.

Conclusion: Overall the experiments support that during AF Pak1 stimulation can attenuate NCX dependent Ca^{2+} overload and trigger activity by suppressing NOX2 dependent ROS production.

Keywords: p21-activated kinase, atrial fibrillation, NADPH-oxidase 2, excitation-contraction coupling, reactive oxygen species

Introduction

The pre-disposition for atrial fibrillation (AF) increases with age and in the presence of cardiovascular morbidities such as hypertension and hypertrophy. This makes AF the most common sustained arrhythmia with a high morbidity and mortality based on thromboembolic events and stroke[1]. AF occurs when non-pacemaker cells focally induce rapid, triggered, propagating electrical impulses, or when ectopic beats trigger re-entry[2]. Repeated AF episodes promote further electrical and structural remodeling of the atrial tissue creating a 'substrate' with increased propensity for recurrence and prolonged AF episodes[1].

Angiotensin II (AngII) has been identified as one prominent signaling component in the induction of AF as well as tissue remodeling. Increased plasma concentrations and expression levels of AngII in atrial tissue were detected in animal models with increased propensity for AF and patients with persistent AF[3,4]. Increase of AngII concentrations through cardiac specific overexpression of angiotensin converting enzyme (ACE)[5] or increased activation of the AngII signal transduction cascade e.g. by Rac1 overexpression, lead to enlarged atria with increased propensity for AF[2,6].

ROS production is a prominent effector of the AngII-induced signaling cascade. Models of AngII and pacing-induced heart failure depend on AngII receptor type 1 (AT1R) mediated ROS production[7] and in models of AF, ROS scavengers reduce arrhythmic trigger formation[8]. In the heart NOX2 is implicated in AngII-induced apoptosis and hypertrophy[9]. In atrial tissue a connection between AngII and NOX2 dependent ROS production has been proposed[10] and NOX2 expression was shown to be increased in humans with persistent and post-operative AF[11].

A downstream target of the AngII signaling cascade is Rac1 and the p21-activated kinase (Pak1). Pak1 is a serine/threonine kinase that is abundantly expressed in the cardiac muscle where its deficiency exaggerates the cardiac hypertrophic response and tissue damage after ischemia reperfusion injury[12,13]. On the other hand Pak1 stimulation was shown to antagonize AngII-induced hypertrophic remodeling by promoting MKK7 and JNK phosphorylation[14] and in

ventricular myocytes (VMs) we demonstrated that Pak1 signaling is critically important for the maintenance of the T-tubular structure and the integrity of the Ca^{2+} release unit composed of L-type Ca^{2+} channel (LTCC) and ryanodine receptor (RYR)[15]. While under basal conditions no increase in arrhythmic activity was identified in $Pak1^{-/-}$ VMs, the cells were highly sensitive to pacing, ischemia-reperfusion, and glycoside- induced Ca^{2+} overload[15,16]. In atrial tissue the role of Pak1 has not yet been examined but due to its position in the AngII/Rac1 signaling cascade, we aimed to test the hypothesis that modulation of Pak1 activity could critically influence the occurrence of arrhythmic events in atrial tissue.

Method

Animals and Cell Isolation

Male WT and Pak1 knockout mice (Pak1$^{-/-}$) 3-6 month old were used for this study and atrial myocytes were isolated as previously described[16]. The canine model of ventricular tachypacing-induced atrial fibrillation was established in Dr. Arora's laboratory and AMs were isolated as previously described[17,18]. All cells were plated on laminin (1 mg/ml, Sigma Aldrich) coated glass coverslips in standard tyrode solution (in mmol/L: NaCl 130, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 1.5, Glucose 10, HEPES 5; pH 7.4). Animal procedures were performed with the approval of the IACUC of Rush University and Northwestern University and in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

ECG Recordings and Burst Pacing-Induced Atrial Arrhythmia

ECG recordings were performed on mice using the ECGenie (Mouse Specifics, Inc.) recording system. Baseline ECGs were recorded after 10 min adaptation to the recording platform. Recordings were obtained before and after intraperitoneal injection of 150 ng/g of carbachol (CCh).

Bipolar left atrial electrograms were recorded from spontaneously beating mouse hearts (WT, Pak1$^{-/-}$) in the Langendorff configuration, during perfusion with Ctrl Krebs-Henseleit solution (in mmol/L: NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 2, glucose 10, KCl 4.0, $CaCl_2$ 1.8, Na-pyruvate 2, gassed with 95 % O_2/5 % CO_2). Sinus rhythm (SR) was interrupted by 10 episodes of burst pacing (1 s, 50 Hz) applied to the right atria in 10 s intervals. After burst pacing recovery to SR or occurrence and duration of arrhythmic activity was quantified.

Subcellular Fractionation of HL-1 cells.

HL-1 cells were maintained for 48 h after infection with adenovirus encoding for either Pak1-RNAi or LacZ [15] and harvested as previously described. Cell lysate samples were separated using pre-cast 4-20 % Novex tris-glycine gels (Invitrogen) following standard electrophoresis protocols for SDS-PAGE and

immunoblotting [15,16] with 25-45 µg of protein loaded per well. Primary antibodies were directed against Rac1 (Cytoskeleton, #ARC03) and Na^+/K^+-ATPase (NKA: ThermoSci, PA5-17251). Densiometric analysis of was performed using ImageJ. The data are presented as relative density adjusted to the respective loading controls (NKA).

Intracellular Ca^{2+} and ROS Measurements

To visualize changes of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), AMs were incubated (15 min) at room temperature with fluo-4 acetoxymethyl ester (10 µmol/L; fluo-4/AM; Invitrogen) for ROS measurements cells were loaded with DCFH (10 µmol/L for 30 min at 37°C). $[Ca^{2+}]_i$ and ROS measurements were performed and analyzed as previously described [15,16]. Ca^{2+}-transients are presented as background-subtracted fluorescence normalized to the diastolic fluorescence (F_0) at the beginning of the recording ($\Delta F/F_0$). AMs were field stimulated at a frequency of 0.5 Hz for the duration of the experiment.

All averaged data are presented as means ± S.E.M., the number of experiments (n) refers to the number of cells examined and is indicated in the text. For each experimental group, cells from at least 2 different cell isolations/animals were used. Significance was evaluated by paired and unpaired *t* test.

Results

A Role for Pak1 Regulation in Atrial Excitability

NOX2 derived ROS production has been described in humans with persistent and post-operative AF[11]. In ventricular myocytes we demonstrated that decreased Pak1 expression increases the propensity for arrhythmic events[16]. To determine the consequences of reduced Pak1 activity on atrial electrophysiology we obtained baseline ECG recordings from conscious WT and $Pak1^{-/-}$ mice. Under control conditions ECGs of these mice did not reveal a difference in heart rate, atrial and ventricular excitation or repolarization. Intraperitoneal injection of

carbachol (CCh 150 ng/g) which facilitates the inducibility of atrial arrhythmia[19], resulted in a comparable, transient decrease of heart rate (HR) in WT and Pak1$^{-/-}$ mice (Fig. 1A). In Pak1$^{-/-}$ mice injected with CCh however, the coefficient of variation of the R-R interval (CV: Fig. 1B), which is used to identify AF episodes[20], increased significantly compared to baseline and compared to Ctrl and CCh treated WT mice (Fig. 1B). Analysis of ECG traces showed the occurrence of spontaneous arrhythmic trigger events only in Pak1$^{-/-}$ atria, as indicated by the presence of a P-wave (Fig. 1CD).

Increased arrhythmicity was also observed in isolated Langendorff perfused Pak1$^{-/-}$ hearts during recordings of left-atrial electrograms (Fig. 2AB). Atrial burst pacing during sinus rhythm (SR) arrhythmic activity in 6 out of 6 Pak1$^{-/-}$ hearts, compared to 1 out of 5 WT hearts (χ^2 = 5.76; p = 0.01). Arrhythmic episodes in Pak1$^{-/-}$ occurred after 26.7 ± 7.2 % of the burst pacing episodes compared to only 1.9 ± 1.9 % in WT mice (Fig. 2D). In all other cases WT and Pak1$^{-/-}$ hearts went back to SR after burst pacing.

To determine if the increased arrhythmicity of Pak1$^{-/-}$ hearts can be mimicked by acute Pak1 inhibition, WT hearts were perfused with IPA-3 (10 μmol/L) an allosteric Pak1 inhibitor[21]. IPA-3 resulted in a significant increase of arrhythmic events in WT hearts. Arrhythmia after burst-pacing were now determined in 69% of WT+IPA-3 treated hearts (χ^2 = 6.24; p = 0.01; Fig. 2C), after 16.57 ± 6.5 %, (n=4) of the burst pacing episodes (Fig. 2D). This increase could be attenuated by perfusion of WT hearts with FTY720 (1 μmol/L) a sphingosine-1 phosphate receptor (S1PR) agonist known to stimulate Pak1 activity[22]. In the presence of IPA3+FTY720 WT atria showed arrhythmia after only 6.36 ± 3.4 % of the burst pacing events (n = 3). In Pak1$^{-/-}$ hearts FTY720 failed to attenuate arrhythmic activity (Pak1$^{-/-}_{FTY}$: 28.2 ± 14 %; n = 3; Fig. 2D) supporting that Pak1 expression is required for the anti-arrhythmic benefit of FTY720.

Arrhythmic episodes in Pak1$^{-/-}$ atria were short in duration, with 80% shorter than 1s. Those with durations >1 s ranged between 2 s and 45 s. The

experimental results support that in vivo decreased Pak1 activity increases the propensity for arrhythmic atrial trigger events.

Contribution of ROS to arrhythmic activity.

We have previously shown in ventricular myocytes that decreased Pak1 activity enhances ROS production[16]. To determine the impact of attenuated Pak1 activity on atrial ROS production, DCF fluorescence was monitored in WT and Pak1$^{-/-}$ AMs at basal conditions and during AngII superfusion (1 μmol/L)[16]. Under basal conditions DCF fluorescence was significantly elevated in Pak1$^{-/-}$ compared to WT AMs (Fig. 3B) and exaggerated ROS production was determined in response to AngII superfusion (Fig. 3AC). Increased basal ROS levels in Pak1$^{-/-}$ AM as well as AngII ROS production were attenuated by apocynin, a NADPH oxidase inhibitor (Fig. 3BC)[16]. Pre-treatment of WT and Pak1$^{-/-}$ AMs with the Pak1 stimulator FTY720 (200 nmol/L) did not affect basal ROS levels in WT AMs but significantly suppressed the rate of AngII ROS production (Fig. 3ABC). In Pak1$^{-/-}$ AMs FTY720 remained without effect indicating the necessity of Pak1 protein for FTY720 to attenuate ROS production.

A prerequisite for NOX2 activation is the association of Rac1 with p67^{phox} at the plasma membrane[30]. After AngII stimulation (30 min) or Pak1-RNAi treatment (24 h) of HL-1cells, Western blots showed significantly increased levels of Rac1 in the plasma membrane fraction compared to control or LacZ (Fig. 4A,B). The total amount of Rac1 protein isolated from whole cell lysate of HL-1 cells treated with LacZ or Pak1-RNAi in the presence and absence of AngII (Fig. 4C) remained constant. Overall the results support that Pak1 is a negative regulator of Rac1 membrane translocation and ROS production, likely through NOX2.

Basal Ca^{2+} handling properties of Pak1$^{-/-}$ AMs

In myocytes enhanced ROS production can lead to the occurrence of arrhythmic events due to the deregulation of the Ca^{2+} handling properties[10]. Despite higher basal ROS in Pak1$^{-/-}$, under control conditions Ca^{2+} transient

amplitudes in WT and Pak1$^{-/-}$ AMs were not significantly different (Fig. 5AB); however, the Ca^{2+} transient decay constant (τ_{Ca}) was prolonged in Pak1$^{-/-}$ AMs (Fig. 5C). Caffeine (10 mmol/L) Ca^{2+} release from the intracellular stores revealed no difference in the Ca^{2+} load of the sarcoplasmic reticulum between WT and Pak1$^{-/-}$ AMs. However, Ca^{2+} removal in the presence of caffeine (τ_{caff}), which predominantly reflects the activity of NCX, was accelerated in Pak1$^{-/-}$ compared to WT AMs (Fig. 5DE). The data indicate an increased contribution of NCX to Ca^{2+} extrusion in Pak1$^{-/-}$ AMs, which is consistent with the increased number of after depolarizations in Pak1$^{-/-}$ AMs (Fig. 6H). The increase in NCX activity occurred independent of an increase of NCX mRNA expression as determined by qPCR (not shown).

Superfusion of WT and Pak1$^{-/-}$ AMs with AngII (1 μmol/L)-induced an increase in diastolic $[Ca^{2+}]_i$ and Ca^{2+} transient amplitude that was significantly more pronounced in Pak1$^{-/-}$ AMs (Fig. 6A-C). Also the load of the sarcoplasmic reticulum was increased and τ_{caff} was accelerated in Pak1$^{-/-}$ AMs with arrhythmic events remaining significantly enhanced in Pak1$^{-/-}$ compared to WT AMs (Fig. 6E-G). In mice deficient for NOX2 expression (gp91$^{phox-/-}$), AngII failed to increase $[Ca^{2+}]_i$ and no increase in after-depolarizations was determined (Fig. 6E-H). Overall the data support the hypothesis that attenuation of Pak1 signaling enhances AngII-induced Ca^{2+} deregulation by increasing NOX2/ROS production.

In the presence of the NCX inhibitor KB-R7943 (1 μmol/L) the AngII dependent increase in diastolic $[Ca^{2+}]_i$ and Ca^{2+} transient amplitude in Pak1$^{-/-}$ cells was significantly attenuated (Fig. 6B-D) and the occurrence of after-depolarizations was almost eliminated (Fig. 6H). The data support that loss of Pak1 increases NOX2/ROS production leading to increased arrhythmic events in combination with the increased NCX activity.

Pak1 Attenuates AngII/ROS Production and Arrhythmic Events

Atrial arrhythmias are often linked to enhanced ROS production and increased NOX2 derived ROS was described in animal models of rapid atrial pacing-induced AF and tissue from AF patients [10, 23]. Our experiments identify

Pak1 as a negative regulator of NOX2/ROS. We therefore aimed to test the hypothesis that Pak1 stimulation can attenuate ROS-induced Ca^{2+} deregulation in AMs from a canine model of ventricular tachypacing-induced AF (VT-AMs) [17,18]. Diastolic $[Ca^{2+}]_i$ and Ca^{2+} transient amplitude were increased in isolated canine AMs during AngII superfusion (Fig. 7A-C), accompanied by a significant increase in arrhythmic after-depolarizations (Fig. 7E). Pak1 stimulation (FTY720: 200 nM) in VT-AMs, attenuated AngII-induced $[Ca^{2+}]_i$ increase as well as arrhythmic events (Fig. 7A-E).

Increased NCX activity was previously reported in these canine models of ventricular tachypacing-induced AF[24]. To determine the impact of Pak1 stimulation on NCX activity, VT-AMs were treated with FTY720. Pak1 stimulation did not impact the load of the sarcoplasmic reticulum however, it significantly prolonged τ_{caff} supporting a Pak1 mediated decrease in NCX activity (Fig. 8A-C). Our prior experiments showed that FTY720 also attenuates NOX2/ROS production. To determine the role of ROS in FTY720 signaling VT-AMs were treated with the NOX2 inhibitor gp91ds-tat (1 μM) and attenuated NCX activity (Fig. 8A-C) comparable to FTY720.

Overall our data support the hypothesis that Pak1 stimulation in atrial tissue with an increased propensity to triggered events could be a pharmacological tool to attenuate NOX2 dependent ROS production and prevent Ca^{2+} overload through attenuation of NCX activity.

Discussion

Our data demonstrate for the first time that regulation of Pak1 activity significantly influences the propensity for arrhythmic trigger events in the atrial muscle and that stimulation of Pak1 activity can prevent cellular Ca^{2+} overload by suppressing the NOX2/ROS dependent stimulation of NCX activity.

Physiological Consequences of Changed Pak1 Expression

Previous experiments in $Pak1^{-/-}$ mice have shown that cardiac contractile function is maintained under basal conditions[13] but that hearts exhibit an

increased sensitivity for hypertrophic remodeling when challenged by banding, β-adrenergic, or AngII stimulation[13,14]. Under control conditions the atria of Pak1$^{-/-}$ mice do not exhibit signs of an AF phenotype given that their ECGs don't exhibit prolongations of the P-wave, PQ- and QRS durations as described in other mouse models of AF[25]. Pak1$^{-/-}$ atria also did not exhibit increased tissue fibrosis that would support the formation of an arrhythmic substrate[14]. AF in animal models is characterized as an irregular rhythm after burst pacing that persists for more than 1 s. The arrhythmic episodes observed in Pak1$^{-/-}$ mice, were predominantly shorter than 1 s supporting that Pak1 atria exhibit increased trigger activity but have not developed an AF substrate that sustains reentry [2].

Comparable to ventricular myocytes, AMs of Pak1$^{-/-}$ mice exhibit increased basal ROS production that is sensitive to NOX2 inhibitors[16]. Increased ROS production upon Pak1 inhibition has now been demonstrated in atrial and ventricular myocytes, HL-1 cells[16] and microglia[26]. Nox2 activation requires the assembly of cytosolic (Rac1, p47^{phox}, p67^{phox}) and membrane bound (p22^{phox}, gp91^{phox}) proteins into a multi-cellular complex[27]. A critical step in this process is the translocation of Rac1 to the plasma membrane and its interaction with p67^{phox} [27]. The increased membrane bound Rac1 levels upon AngII stimulation or the loss of Pak1, support that while AngII stimulates, Pak1 antagonizes Rac1 activation. The mechanism by which Pak1 negatively regulates Rac1 and NOX2 activation remains yet to be determined[27]. Since Pak1 stimulation by FTY720 can attenuate ROS production, the Pak1 dependent regulation of NOX2 seems to be at least in part mediated by Pak1 kinase activity e.g. reduced Pak1 dependent PP2A activation could enhance p47^{phox} phosphorylation and NOX2 activation [26].

Mechanism of Arrhythmicity

Arrhythmic activity in the atrial muscle is often related to a deregulation of the Ca^{2+} handling properties and often coincides with increased NCX activity as it has been reported in animal models and patients with AF[28]. NCX whose activity depends on the membrane potential and the ion gradient for Na^+ and Ca^{2+}, can promote the occurrence of arrhythmic events. During diastole NCX removes Ca^{2+}

from the cytoplasm (forward mode), which conjoins with a depolarization of the membrane potential that can trigger after-depolarizations. However, during increased $[Na^+]_i$ NCX can operate in reverse mode where, during the upstroke and plateau of the action potential, it can contribute to Ca^{2+} influx and potentially Ca^{2+} over-load[29].

In Pak1$^{-/-}$ myocytes under resting conditions, Ca^{2+} removal is shifted from reuptake, to extrusion from the cell via NCX[16]. SR load did not change despite increased NCX activity consistent with reports from mouse myocytes overexpressing NCX[30]. Increased NCX activity there coincided with maintained and even increased load of the sarcoplasmic reticulum because the increased NCX dependent Ca^{2+} extrusion was counter-balanced by the enhanced Ca^{2+} entry through NCX reverse mode activity[30]. In Pak1$^{-/-}$ AMs NCX dependent Ca^{2+} entry is reflected in the sensitivity of the exaggerated increase in diastolic and systolic $[Ca^{2+}]_i$ in response to AngII, to the NCX inhibitor KB-R 7943.

Increased NCX activity has been described under conditions of AF but, similar to Pak1$^{-/-}$ myocytes, increased NCX activity was not always linked to increased protein expression. NCX activity can be regulated through changes in $[Na^+]_i$ and $[Ca^{2+}]_i$. This way increased $[Na^+]_i$ attenuates Ca^{2+} removal from the cytoplasm while it facilitates Ca^{2+} entry through reverse mode activity. Increasing $[Ca^{2+}]_i$ on the other hand enhances NCX forward mode[29]. We did not directly rule out an increase in $[Na^+]_i$ in Pak1$^{-/-}$ atrial myocytes, but NCX forward as well as reverse mode activity were enhanced, excluding a regulation by increased $[Na^+]_i$ only. An additional regulation by increased $[Ca^{2+}]_i$ is not supported by experimental data that show stable SR load.

An alternative explanation for enhanced NCX activity is its stimulation through ROS. Stimulation of NCX through ROS has been previously reported [31, 32]. Thiol-modifications as well as protein phosphorylation have been proposed as mediators of increased NCX activity. In Pak1$^{-/-}$ ventricular myocytes we demonstrated elevated levels of ROS and increased NCX activity that was reversible with ROS scavengers and NOX2 inhibitors[16]. Here we further demonstrate that increased NCX activity in Pak1$^{-/-}$ AMs correlates with increased

basal and AngII-induced ROS production. In addition we present evidence that in VT-AMs stimulation of Pak1, and NOX2 inhibition, attenuated basal and AngII induced ROS production together with NCX dependent Ca^{2+} extrusion and arrhythmic events. Overall we therefore propose that in AMs increased levels of ROS contribute to enhanced NCX activity. If ROS has a direct effect on NCX or the response is mediated through changes in local ion concentrations, membrane phospholipid composition or post-translational protein modifications remains to be determined.

Pak1 Stimulation Attenuates Arrhythmic Activity in AF

Modulation of the AngII signaling pathway was shown to impact arrhythmic activity in AF animal models. AngII receptor blocker, enhanced AngII degradation, or depressed AngII synthesis all attenuated tissue remodeling and arrhythmic activity[6]. Overexpression of Rac1 itself creates an AF phenotype, while Rac1 inhibitors can suppress it[6]. NOX2 is the downstream target of the AngII/Rac1 signaling cascade. Its activity is increased in patients with AF and suppression of NOX2 in AF patient's tissue decreased arrhythmic activity[10]. To stimulate Pak1 activity and antagonize NOX2/ROS production we used the sphingosine analog and S1P receptor agonist FTY720. FTY720 activates Pak1 and was shown to promote cardio-protective signaling[22]. We demonstrate on a cellular level that FTY720 prevents and reverses AngII-induced ROS production in AMs. This is in contrast to prior reports showing FTY720-induced ROS production[33]. One reason for this discrepancy could be that higher concentrations and longer treatment times were used. The latter leads to constitutive S1P receptor activation and subsequent internalization, which could attenuate downstream Pak1 signaling[33]. Future prolonged treatments have to address if the observed benefit of FTY720 is biphasic.

FTY720 activates a variety of signaling pathways[33,22]. However, we did not observe significant changes in atrial electrophysiology and excitation contraction coupling through FTY720 in vivo and in vitro, respectively. This, together with the lack of effect of FTY720 in $Pak1^{-/-}$ myocytes and hearts, leads us to propose that

the primary mechanisms by which FTY720 attenuates arrhythmicity in our model, is through the Pak1 dependent suppression of NOX2/ROS production.

We used isolated myocytes from a canine model of ventricular tachypacing-induced AF. The arrhythmic activity in this model was previously reported to base on the remodeling of the atrial substrate that facilitates the occurrence of reentry[17,18]. The enhanced NCX activity and ROS production described in this model however, made it a good target to identify the benefit of Pak1 stimulation. In vivo, Pak1 dependent attenuation of NOX2 and NCX activity might be insufficient to reverse the arrhythmia under conditions where AF is maintained by atrial substrate remodeling. Under conditions of paroxysmal trigger-induced AF, Pak1 stimulation could reduce arrhythmic events and prevent further atrial remodeling and fibrosis.

Conclusion

We have demonstrated for the first time that a negative regulation of the protein kinase Pak1 results in an increased chance for atrial arrhythmic trigger events. Pak1 stimulation on the other hand attenuates Rac1 mediated NOX2/ROS production and thereby NCX dependent Ca^{2+} overload. The anti-arrhythmic effect of Pak1 therefore makes it an interesting pharmacological target to attenuate baseline as well as AngII-induced ROS production and thereby arrhythmic activity. If Pak1 signaling is able to attenuate AngII-induced fibrotic remodeling and AF substrate formation remains to be determined.

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Disclosures

None

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Figure Captions:

Figure 1: Reduced Pak1 activity enhances the propensity for atrial arrhythmic events. (A) Heart rate (HR, beats per minute: bpm) and **(B)** Coefficient of variation (CV) recorded in conscious WT and Pak1$^{-/-}$ mice after intraperitoneal injection of carbachol (CCh: 150 ng/g). Representative ECG recording in WT (**C**) and Pak1$^{-/-}$ (**D**) mice show spontaneous arrhythmic events originating in the atria of Pak1$^{-/-}$ mice (arrows indicate P-wave). (*p < 0.05 compared to t = 0 min; #p < 0.05 compared to WT)

Figure 2: Acute Pak1 inhibition increases atrial arrhythmic events. Representative examples of left atrial electrograms showing spontaneous activity (SR: sinus rhythm) and arrhythmic episodes induced by burst pacing (burst) in WT (**A**), Pak1$^{-/-}$ (**B**) and WT hearts perfused with IPA-3 (10 μmol/L) (**C**). (**D**) Percent of burst pacing episodes that induced arrhythmic events in Pak1$^{-/-}$ and WT hearts during Ctrl conditions, or treatment with IPA-3, or FTY720 (1 μmol/L). (*p < 0.05).

Fig. 3: Attenuated Pak1 activity increases NOX2-dependent ROS production. (**A**) Change in fluorescence recorded in WT (black) and Pak1$^{-/-}$ (grey) AMs loaded with DCF during stimulation with AngII in the absence or presence of FTY720 (open symbols). Bar graphs show DCF fluorescence at baseline (**B**) and (**C**) 20 min after AngII, AngII+FTY720, or AngII+Apo (Apo: 1 μmol/L) superfusion in WT and Pak1$^{-/-}$ cells. (*: p < 0.05 compared to WT; #:p < 0.05 compared to own Ctrl)

Fig. 4: Attenuated Pak1 promotes Rac1 translocation to the plasma membrane. Western blotting results from lysates of HL-1 membrane fractions treated with AngII (**A**) or Pak1-RNAi (**B**) and (**C**) HL-1 whole cell lysate of cells treated with LacZ or Pak1-RNAi after stimulation with AngII. Densiometric quantification underneath the blots are presented as relative density adjusted to the Na^+/K^+-ATPase (NKA) loading control. (*: p < 0.05 compared to Ctrl or LacZ, respectively).

Figure 5: Ca^{2+} removal shifts from SERCA to NCX in Pak1$^{-/-}$ myocytes. (**A**) Representative field stimulation-induced Ca^{2+} transients recorded in WT (black) and Pak1$^{-/-}$ (grey) AMs under control conditions. Bar graphs show (**B**) the Ca^{2+} transient amplitude, (**C**) the Ca^{2+} transient decay constant (τ_{Ca}), (**D**) the caffeine

transient amplitude and (**E**) the caffeine transient decay constant (τ_{Ca}). (#: p < 0.05 compared to WT).

Fig. 6. Pak1$^{-/-}$ AMs exhibit exaggerated AngII-induced arrhythmic events. (**A**) Representative field stimulation-induced Ca^{2+} transients recorded in WT and Pak1$^{-/-}$ (grey) AMs. Bar graphs represent the percent change after 15 min of AngII superfusion of the (**B**) diastolic Ca^{2+}, (**C**) Ca^{2+} transient amplitude, and (**D**) Ca^{2+} transient decay constant (τ_{caff}) in AMs from WT, Pak1$^{-/-}$, gp91$^{phox-/-}$, and Pak1$^{-/-}$ AMs treated with NCX inhibitor KB-R7943 (KBR: 1 μmol/L). (**E**) Representative, normalized caffeine transients (10 mmol/L) after 15 min of AngII superfusion. Bar graphs show (**F**) caffeine transient amplitude and (**G**) decay constant. (**H**) Mean number of spontaneous arrhythmic events per second in AMs before and after AngII superfusion for the cells and treatment conditions described in A-D. (*: p < 0.05)

Fig. 7: Pak1 stimulation prevents arrhythmic events in AMs from a canine AF model. (**A**) Representative field stimulation-induced Ca^{2+} transients in VT-AMs before and 20 min after stimulation with AngII under Ctrl conditions (black) and in presence of Pak1 stimulation (FTY720: 200 nmol/L; grey). Bar graphs show the percent change in (**B**) diastolic $[Ca^{2+}]_i$, (**C**) Ca^{2+} transient amplitude and (**D**) Ca^{2+} transient decay constant (τ_{Ca}) after AngII stimulation under Ctrl conditions and in presence of FTY720. (**E**) Mean number of arrhythmic events per second in VT-AMs under the indicated treatment conditions (*: p < 0.05 compared to own Ctrl, #: p< 0.05 compared to VT-AMs+AngII).

Fig. 8: Pak1 stimulation and NOX2 inhibition attenuate NCX activity in VT-AMs. (**A**) Representative normalized caffeine-induced Ca^{2+} transients before and 20 min after stimulation with AngII under Ctrl conditions (black), in presence of gp91ds-tat (1 μmol/L, grey) or FTY720 (200 nmol/L, light-grey). Bar graphs show the (**B**) caffeine transient decay constant (τ_{caff}) and (**C**) caffeine transient amplitude under Ctrl conditions and in presence of gp91ds-tat or FTY720. (*: p < 0.05 compared to Ctrl).