Deletion of PDK1 Causes Cardiac Sodium Current Reduction in Mice

Zhonglin Han, Yu Jiang, Yuqing Yang, Xuehan Li, Zhongzhou Yang, Kejiang Cao, Dao W. Wang

1 Department of Cardiology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China, 2 Department of Geriatrics, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China, 3 Ministry of Education Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing University, Nanjing, China

* david37212@hotmail.com

Abstract

Background

The AGC protein kinase family regulates multiple cellular functions. 3-phosphoinositide-dependent protein kinase-1 (PDK1) is involved in the pathogenesis of arrhythmia, and its downstream factor, Forkhead box O1 (Foxo1), negatively regulates the expression of the cardiac sodium channel, Nav1.5. Mice are known to die suddenly after PDK1 deletion within 11 weeks, but the underlying electrophysiological bases are unclear. Thus, the aim of this study was to investigate the potential mechanisms between PDK1 signaling pathway and cardiac sodium current.

Methods and Results

Using patch clamp and western blotting techniques, we investigated the role of the PDK1-Foxo1 pathway in PDK1 knockout mice and cultured cardiomyocytes. We found that PDK1 knockout mice undergo slower heart rate, prolonged QRS and QTc intervals and abnormal conduction within the first few weeks of birth. Furthermore, the peak sodium current is decreased by 33% in cells lacking PDK1. The phosphorylation of Akt (308T) and Foxo1 (24T) and the expression of Nav1.5 in the myocardium of PDK1-knockout mice are decreased, while the nuclear localization of Foxo1 is increased. The role of the PDK1-Foxo1 pathway in regulating Nav1.5 levels and sodium current density was verified using selective PDK1, Akt and Foxo1 inhibitors and isolated neonatal rat cardiomyocytes.

Conclusion

These results indicate that PDK1 participates in the dysregulation of electrophysiological basis by regulating the PDK1-Foxo1 pathway, which in turn regulates the expression of Nav1.5 and cardiac sodium channel function.
Introduction
3-phosphoinositide-dependent protein kinase-1 (PDK1), a key member of the AGC (protein kinase A, protein kinase G and protein kinase C) protein kinase family, acts as an upstream protein kinase by phosphorylating and activating many other AGC-family members, including protein kinase B (PKB)/Akt [1], p70 ribosomal S6 kinase (S6K) [2], serum and glucocorticoid-induced protein kinase (SGK) [3], and Forkhead box O (Foxo) [4]. PDK1 has an established role in regulating physiological processes relevant to metabolism, growth, proliferation and survival [5]. Moreover, PDK1 [6] and its upstream or downstream factors, including phosphatidylinositol 3-kinase (PI3K) [7], Akt and mTOR [8], are involved in heart failure and pathologic heart remodeling. Mice with PDK1 deletion have markedly reduced myocardium, smaller cardiomyocytes, thinner ventricles and enlarged atria, which eventually leads to heart failure and sudden death within 11 weeks [9]; however, the underlying mechanisms of abnormal electrophysiological basis related deaths are not clear.

Recently, the involvement of the AGC protein kinase family in regulating arrhythmia has drawn considerable attention. PI3K signaling is associated with the alteration of ion channel function, which is established to play a role in the development of cardiac arrhythmia. Drug-induced increases in action potential and QT prolongation induce inhibition in multiple ion currents, including peak Na+ current, and are accompanied by decreased PI3K signaling [10]. Foxo1 transcription factor, one of the major PI3K Akt downstream effectors, binds to promoter sequences to regulate the expression of target genes, including the sodium channel gene SCN5A. Akt negatively regulates Foxo1 by phosphorylation on Thr24, Ser256 and Ser319, which induces Foxo1 to bind to 14-3-3, and consequently triggers the relocalization of Foxo1 from the nucleus to the cytoplasm, where it becomes inactivated [4]. Foxo1 inhibits the expression of Nav1.5, the protein encoded by SCN5A, upon treatment with hydrogen peroxide [11]. Furthermore, modulation of Foxo1 expression inhibits inward sodium currents [12]. Thus, the potential relationships between PDK1 signaling through Foxo1 and the function of Nav1.5 in the dysregulation of electrophysiological basis need further investigation.

In the clinic, arrhythmia is one of the primary cardiovascular events in patients with heart failure, and ventricular arrhythmias can lead to sudden death [13]. Specially, the cardiac sodium channel (SCN5A) mutations that reduce peak sodium current (such as mutations linked to Brugada syndrome) and QTc prolongation in patients with LQT3, or overlap syndrome. In animal experiments, PDK1 knockout mice die suddenly of heart failure; however, the potential electrophysiological basis lead to the sudden death has not been determined. Thus, the relationship between PDK1 and dysregulation of electrophysiology remains unclear. To understand the function of PDK1 in sodium channel activation and the dysregulation of electrophysiological basis, we prepared a conditional deletion of PDK1 in mouse cardiomyocytes through Cre-mediated excision. Using this conditional PDK1-deletion mouse, and using neonatal rat cardiomyocytes to exclude the possible impact of heart failure partly, we tested the hypothesis that PDK1 regulates sodium channel activation in cardiomyocytes via the PDK1-Foxo1 pathway, thus facilitating the potential mechanism in the development of dysregulation of electrophysiology.

Materials and Methods
Generation of Conditional PDK1 Knockout Mice
Standard tissue-specific “knockout” approaches using the “Cre-LoxP” system were used to delete PDK1 in the myocardium. PDK1-floxed mice were kindly provided by Dr. Zhongzhou Yang (Ministry of Education Key Laboratory of Model Animal for Disease Study, Nanjing
University, China) [14]. In brief, PDK1-floxed mice (PDK1\(^{F/F}\)) were crossed with αMHC (α-myosin heavy chain)-Cre mice to delete PDK1 in cardiomyocytes [9]. The deletion of PDK1 in the myocardium was confirmed by Western blot analysis (Fig. 1). PDK1\(^{F/F}\) αMHC-Cre mice were used in this study, and PDK1\(^{F/F}\) littermates without the αMHC-Cre transgene were housed as control. This study was approved by the ethical committee of the First Affiliated Hospital of Nanjing Medical University, and all animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institute of Health (No. 85-23, revised in 1996).

**ECG**

ECG (Lead II) was performed under anesthesia (ketamine hydrochloride, 10 mg/kg, intraperitoneally) and continuously recorded by the RM6240B multiple channel physiological signal collecting and processing system (Chengdu Instrument Factory, Chengdu, China). The heart rate (HR), QRS and QT durations were measured. The QTc was calculated by Bazett’s formula, where 

\[ QTc = QT / \sqrt{RR} \]

**Mouse Cardiomyocyte Isolation**

Single ventricular myocytes were obtained by enzymatic dissociation as previously described [15]. In brief, 8 weeks and 11 weeks of age mice were sacrificed by cervical dislocation, and the hearts were quickly placed in Tyrode’s solution containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM NaH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 5 mM HEPES, 20 mM taurine, 10 mM glucose, and 1.8 mM CaCl\(_2\) (pH 7.3) at 4°C. Then the aortas were quickly cannulated onto a modified Langendorff perfusion system for coronary perfusion. The hearts were perfused with Tyrode’s solution at 36°C for 1 min, followed by Ca\(^{2+}\)-free Tyrode’s solution at a reduced rate of 3 ml/min. After 5 minutes,
Ca²⁺ free Tyrode's solution containing 150 U/ml Collagenase Type II (Worthington, Lakewood, USA) was applied for another 12 minutes until the hearts were palpably flaccid. Next, the ventricles were minced and gently agitated, and the cardiomyocytes were placed in KB solution containing 70 mM L-glutamic acid, 50 mM KOH, 40 mM KCl, 20 mM taurine, 20 mM KH₂PO₄, 3 mM MgCl₂, 0.5 mM EGTA, 10 mM D-glucose, and 10 mM HEPES (pH 7.3) at room temperature for patch clamp experiments. All chemicals used above were purchased from Sigma (Sigma Chemical Co., St. Louis, USA), except Collagenase Type II.

**Ventricular Myocyte Culture**

Neonatal rat cardiomyocytes were prepared from 1–3 day old Sprague Dawley rats by enzymatic digestion. Hearts were excised and placed in sterile PBS solution. After connective tissue and blood were removed, the ventricles were minced and subjected to 8 min enzymatic digestion using serial 0.06% collagenase II and 0.08% pancreatin (both were purchased from Gibco, Thermo Fisher Scientific Inc., Waltham, USA) digestion. Cells were pre-plated on 10 cm petri dishes for 2 h to remove fibroblast cells and then cultured for 24 h in 10% (v/v) fetal bovine serum in Dulbecco’s modified eagle’s medium (high glucose) (Gibco) and 1% penicillin-streptomycin solution (Gibco) at 37°C in a humidified incubator with 5% CO₂. After 24 h the media was replaced with 3% fetal bovine serum containing media.

**Cardiac Sodium Current Recording**

In patch clamp experiment, 8 weeks and 11 weeks PDK1<sup>F/F</sup> αMHC-Cre mice were used as experimental group, and PDK1<sup>F/F</sup> mice in the same age were used as control group. Cells were transferred to a chamber (Warner Instrument Co, USA) and perfused with bath solution (5 mM NaCl, 130 mM choline chloride, 1.8 mM CaCl₂, 1.8 mM MgCl₂, 1 mM TEA-Cl, 10 mM HEPES, and 10 mM glucose, pH 7.4 with Tris base) at a constant rate of 1 ml/min. 200 μM NiCl₂ and 1 μM nisoldipine were applied to block T- and L- type calcium currents. Beside, low Na⁺ concentration is conducive to control Na⁺ current recording [16]. Pipettes (Sutter Instrument Co, Novato, USA) were pulled by the Pipette Puller Model P-1000 (Sutter instrument Co, Novato, USA). Current recording was performed with an Axopatch 200B amplifier (Molecular Devices, Union City, CA) and a Digidata 1440A (Molecular Devices, Union City, CA). Pipettes were filled with pipette solution (130 mM CsOH, 130 mM Aspartic acid, 2.5 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM EGTA, 10 mM TEA-Cl, and 10 mM HEPES, pH 7.2 with CsOH) and a resistance of 1–2 MΩ was applied. Data expressing large series resistance (> 10 MΩ) were rejected. All chemicals used above were purchased from Sigma (Sigma Chemical Co., St. Louis, USA).

Sodium currents were evoked by a voltage-clamp protocol [16]. Current recordings from adult mouse cardiomyocytes and neonatal rat cardiomyocytes were established using a whole-cell patch clamp technique at room temperature (20–22°C) for at least 5 min. The cell membrane capacitance was then calculated by integrating the capacitive transient current in response to a 5-mV depolarizing pulse. The ionic current density (pA/pF) was calculated from the current amplitude of cell capacitance. Gating kinetics of sodium currents were recorded.

**Western Blot Analysis**

Cardiac ventricular tissue and cultured cardiomyocytes were homogenized with a Kinamatica homogenizer (Bio-Gen Senes, Serial No.02-0145) in lysis buffer. After centrifugation for 15 min at 4°C at 14000g, the supernatants were collected and the protein concentrations were measured using the bicinchoninic acid (BCA) method. Nucleoprotein extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockfort, IL, USA).
total of 30–50 μg protein was electrophoresed in SDS–polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Roche Diagnostics GmbH, Germany). After blocking with 5% non-fat milk, membranes were incubated with specific primary antibodies overnight. Then the membranes were washed three times with TBST buffer, incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h and developed onto a Molecular Imager (BIO-RAD Laboratories, Hercules, USA) using the enhanced chemiluminescence (ECL) system. Primary antibodies were as follows: FOXO1 (#2880, Cell Signaling, Danvers, USA), Akt (pan) (#4691, Cell Signaling), phospho-FoxO1 (Thr24) (#9464, Cell Signaling), phospho-Akt (Thr308) (#13038, Cell Signaling), β-actin (#4967, Cell Signaling), anti-SCN5A (AV35542, Sigma Chemical Co., St. Louis, USA). Three bands for each protein represented ventricular tissue from three different hearts in experimental or control group respectively. The protocol of western blotting data analysis was previously described [17].

Drugs
For drugs used in patch clamping experiments, cells were maintained in culture media and final concentration of 100 nM of the PDK1 inhibitor GSK233447 (Sigma Chemical Co., St. Louis, USA) [18], or the Akt inhibitor MK2206 (Selleck, Houston, USA) [19], or the Foxo1 inhibitor AS1842856 (Merck Millipore, Billerica, USA) [20,21] or a combination of inhibitors were applied in cell culture media for 48 h. All these drugs mentioned above were dissolved in dimethyl sulfoxide (DMSO), with the final concentration of dimethyl sulfoxide is 0.2% in our experiments.

Statistics
Results are presented as mean ± SE. Statistical comparisons were made using the student’s t test. Differences were considered statistically significant if \( P < 0.05 \).

Results
The ECG Profile Is Altered in PDK1-deletion Mice
To examine the mechanism of dysregulated electrophysiology in PDK1 knockout mice, we performed ECGs of PDK1\(^{+/−}\) αMHC-Cre knockout mice (n = 6) and control PDK1\(^{+/+}\) littermates (n = 6) without the αMHC-Cre transgene. We measured the HR, QRS and QTc intervals, which are closely related to the onset of arrhythmias. After 8 weeks of deletion, HR was lower in PDK1 knock mice (362.22±12.69 vs. 422.31±20.10, \( P < 0.05 \)), and both the QRS intervals (12.8±0.30 ms vs. 18.93±1.17 ms) and the QTc duration (82.69±4.08 ms vs. 113.91±8.20 ms) were significantly longer in the PDK1-deletion mice than the control mice (\( P < 0.05 \); Fig. 2A and 2B). An abnormal conduction was found in 3 of 4 mice with PDK1 deletion at 11 weeks, but no anomalies occurred in the control PDK1\(^{+/+}\) mice (Fig. 2C). These results indicated the association between PDK1 and dysregulated electrophysiology.

PDK1 Deletion Reduces the Sodium Current Density and Mediates a Slight Change in the Sodium Channel Kinetics
To determine the underlying mechanisms of ECGs alteration, we recorded sodium currents using the whole-cell patch clamping technique. Current traces of cells from PDK1\(^{+/−}\) αMHC-Cre and PDK1\(^{+/+}\) mice were determined using 5 mV increment test pulse from −100 mV to 45 mV at a holding potential of −120 mV. At −30 mV, peak values of sodium currents were −23.86±1.10 pA/pF in PDK1\(^{+/−}\) αMHC-Cre cells (Fig. 3A) and −36.34±1.45 pA/pF in PDK1\(^{+/+}\) cells (\( P < 0.05 \), Fig. 3B), indicating a significant reduction of sodium current for PDK1\(^{+/−}\) αMHC-Cre cells. Similar results were observed in the 11 weeks olds mice (−24.11±
1.23 pA/pF in PDK1<sup>F/F</sup> αMHC-Cre cells vs. −36.76±2.07 pA/pF, *P* < 0.05) in PDK1<sup>F/F</sup> cells. (Fig. 3C-3F). These results suggest that the sodium current amplitude is decreased by about 33% in PDK1<sup>F/F</sup> αMHC-Cre mice.

Defects in cardiac sodium channels can disrupt channel gating and cause electrical abnormalities. To further investigate whether current kinetics are affected by PDK1 deletion, the activation, inactivation and recovery from inactivation curves were recorded. The steady-state activation and inactivation of sodium current were fitted by Boltzmann function, and the recovery from inactivation curves were fitted using a double exponential equation. As shown in Fig. 3G-3I and Table 1, fitting parameters of steady-state activation were similar in age-matched mice, but the midpoint of voltage dependence was slightly left-shifted for PDK1<sup>F/F</sup> αMHC-Cre mice at 11 weeks. For PDK1<sup>F/F</sup> αMHC-Cre knockout vs. PDK1<sup>F/F</sup> groups at 11 weeks, the V<sub>1/2</sub> for activation was −41.14±0.61 mV vs. −39.61±0.68 mV and the slope was 5.07±0.15 vs. 4.37±0.21. Slight changes were also observed in the inactivation curves at 11 weeks (−79.02±0.56 mV vs. −75.09±1.17 mV; *p* < 0.05); however, the slope of the Boltzmann curves did not change significantly. For recovery from inactivation, the same trend of variation between PDK1<sup>F/F</sup> αMHC-Cre and PDK1<sup>F/F</sup> groups was detected. The τ<sub>f</sub> and the τ<sub>s</sub> were shortened in the PDK1-deletion vs. control cells both at 8 weeks and at 11 weeks, with statistical differences in the τ<sub>f</sub> at week 8 and the τ<sub>s</sub> at week 11 (Table 1). These results demonstrate that PDK1-deletion causes a decrease in sodium current amplitude that may be explained in part by electrical abnormalities.
PDK1 Regulates Cardiac Sodium Current in Mice

Fig 3. Patch clamping in cardiomyocytes from mice lacking PDK1. (A-D) Sodium current recording in PDK1<sup>F/F</sup> control and PDK1<sup>F/F</sup> α-MHC-Cre knockout mice at 8 and 11 weeks. E) Current-voltage relationship for mice at different weeks. F) Suppression of sodium current in mice lacking PDK1. G) Voltage dependence of activation. H) Voltage dependence of inactivation. I) Time-dependent recovery for sodium channel. (For cardiomyocytes, n = 13 to 32; for cells from mice, n = 2 to 3 for each group) *P < 0.05
doi:10.1371/journal.pone.0122436.g003

Table 1. Biophysical parameters for sodium channel kinetics in mice with different age.

| Voltage dependence of activation | Voltage dependence of inactivation | Recovery from inactivation |
|----------------------------------|-----------------------------------|-----------------------------|
|                                  |                                   |                             |
|                                |                                |                             |

|                               |                               |                             |
|--------------------------------|--------------------------------|-----------------------------|
| 8W PDK1<sup>F/F</sup>         | –40.44±0.68                   | 4.82±0.14                   | 27   | –77.21±0.67                   | –6.14±0.07                   | 28   | 5.15±0.28                     | 51.51±2.40                   | 26   |
| 8W PDK1<sup>F/F</sup> α-MHC-Cre| –38.37±0.95                   | 4.61±0.21                   | 35   | –76.65±0.85                   | –5.61±0.05                   | 34   | 4.36±0.19*                    | 49.92±1.72                   | 32   |
| 11 W PDK1<sup>F/F</sup>       | –39.61±0.68                   | 4.37±0.21                   | 16   | –75.09±1.17                   | –6.19±0.08                   | 14   | 6.22±0.17                     | 69.32±1.47                   | 13   |
| 11W PDK1<sup>F/F</sup> α-MHC-Cre| –41.14±0.61                   | 5.07±0.15                   | 28   | –79.02±0.56*                  | –6.17±0.11                   | 20   | 5.98±0.44                     | 60.04±1.93*                  | 14   |

*P<0.05, compared with same week.
doi:10.1371/journal.pone.0122436.t001
Impaired PDK1-Foxo1 Pathway Activation Leads to Reduced Sodium Channel Expression in PDK1-Deletion Mice

To investigate the effects of PDK1 deletion on the Akt/Foxo1 pathway and the potential downstream effects on sodium channel expression, proteins were extracted from ventricle tissues of control PDK1^+/+ and PDK1^+/+ αMHC-Cre knockout mice. There were no obvious differences in the protein expression levels of Akt and Foxo1; however, increased levels of phosphorylation of Akt on Thr308 and Foxo1 on Thr24 were observed upon PDK1 deletion (Fig. 4A).

**Fig 4. Western blot analysis of the PDK1 signaling pathway.** A) Western blot analysis was performed to assess levels of the indicated of proteins and phosphoproteins in PDK1^+/+ control and PDK1^+/+ αMHC-Cre knockout mice. B) Expression of total and phosphorylated Akt and Foxo1 was assessed after treatment of cardiomyocytes from neonatal rats with vehicle control (DMSO) or PDK inhibitor (GSK 234470). C) The expression of Nav1.5 in rat cardiomyocytes was assessed after treatment with DMSO, GSK 234470 (GSK), the Foxo1 inhibitor AS 1842856 (AS) or a combination of the two drugs. D-E) Nuclear Foxo1 expression was assessed for cardiomyocytes isolated from control and PDK deletion mice (D) or neonatal rat cardiomyocytes before and after treatment with PDK inhibitor (E). β-actin was tested as a loading control for total cellular protein levels, and histone H3 was tested as a loading control for nuclear protein levels. The mean ±SD values from each group were normalized to 1.0 in the control mice. All drugs were dissolved in DMSO and added into cell culture media for 48h before the experiments. In each group, three bands of proteins represented three different hearts. *P <0.05.
Consistent with the results of patch clamping, markedly reduced expression of Nav1.5 in the PDK1 knockout group was also observed.

To verify the activation of Akt and Foxo1 phosphorylation by PDK1, we applied the PDK1 inhibitor GSK 2334470 to isolated cardiomyocytes from rats. Similar to the results from the PDK1 knockout mice, no obvious change in the total protein expression of Akt and Foxo1 was found between the GSK 2334470 group and the DMSO control group, but the levels of phosphorylation of these proteins were decreased in the GSK 2334470 group (Fig. 4B). GSK 2334470 also decreased the expression of Nav1.5, though the Foxo1 inhibitor AS 1842856 had the opposite effect on Nav1.5 expression and could counteract the effect of GSK 2334470 when the two drugs were used in combination (Fig. 4C). Because Foxo1 is an inhibitor of the PDK1 pathway that is inactivated by phosphorylation [12], these results are consistent with the positive role of the PDK1 pathway in regulating the expression of a sodium channel in cardiomyocytes.

Because phosphorylation affects the subcellular distribution of Foxo1 [11], we isolated nuclear proteins from control and PDK1 deletion cells and assessed the levels of Foxo1 relative to the nuclear protein. The nuclear Foxo1 levels were increased in PDK1 knockout cells compared to control cells (Fig. 4D). Furthermore, the levels of Foxo1 were increased by GSK 234470 (Fig. 4E). Collectively, these results support a model by which PDK1 deletion inhibits the activity of the Akt pathway, leading to increased Foxo1 activity and consequently, reduced Nav1.5 expression.

**PDK1 and Foxo1 Specific Inhibitors Regulate Sodium Channel Activation**

Lack of PDK1 in mouse cardiac muscle has been shown to be associated with less thickening of the ventricular wall and lead to heart failure [9]. To rule out an impact on chronic left ventricular function decline, neonatal rat cardiomyocytes were treated with the PDK1 inhibitor GSK 2334470, the Akt inhibitor MK2206, the Foxo1 inhibitor AS 1842856, or a combination of drugs, and the effects on sodium channels were investigated using the patch clamp technique. The amplitude of the current in untreated cells (−36.23±1.25 pA/pF) was suppressed by treatment with either GSK 2334470 (−27.52±1.49 pA/pF) or MK 2206 (25.48±2.06 pA/pF) (P<0.05), supporting the positive role of PDK1 and Akt (Fig. 5A-5C). Conversely, the amplitude of sodium current was increased after application of AS 1842856 (−42.85±1.51 pA/pF; P<0.05), which is consistent with the negative role of Foxo1. Furthermore, the sodium current had no obvious change when a combination of GSK 2334470 and AS 1842856 (−33.41±1.50 pA/pF) or MK 2206 and AS 1842856 (−35.09±4.29 pA/pF) were applied. These results verify the role of the PDK1 pathway on sodium channel activation.

As a control, there were no obvious changes in the biophysical parameters of sodium currents for activation and inactivation in the presence of GSK 2334470, MK 2206, AS 1842856 or a combination of drugs (Fig. 5D-5E and Table 2). However, the time of recovery was delayed when MK 2206 or a combination of drugs were applied. These results verify that the PDK1-Foxo1 pathway regulates the amplitude of the sodium current without consistent impact on the sodium channel kinetics.

**Discussion**

In the present study, we have demonstrated for the first time that PDK1 participates in the dysregulation of electrophysiological basis by regulating the expression of Nav1.5. This effect is likely to be mediated through the regulation of the phosphorylation and expression of the PDK1-Foxo1 pathway.
Previous studies have reported that Tamoxifen-inducible and heart-specific disruption of PDK1 in adult mice causes severe and lethal heart failure [6,9,14]. It is well known that arrhythmia is one of the most common phenomenons in patients with heart failure [22]. However, little is known about the dysregulation of electrophysiological basis by PDK1 knockout. In this study, differences in ECG and sodium channel parameters were found in PDK1-deletion mice, and the effects were shown to be induced via the PDK1-Foxo1 pathway. The finding may be the underlying mechanisms between PDK1 and the voltage-gated sodium channels are

Fig 5. Patch clamping results in neonatal rat cardiomyocytes. A) Sodium current recording in neonatal rat cardiomyocytes exposed to different inhibitors alone or in combination. B) Current-voltage relationship for cells incubated with drugs for 48 h. C) Changes in sodium current in cells after drug treatment. D) Voltage dependence of activation. E) Voltage dependence of inactivation. F) Time dependent recovery for sodium channel. (For cardiomyocytes, n = 6 to 24 per group of control experiments). GSK (GSK 234470) is a PDK1 inhibitor, MK (MK 2206) is an Akt inhibitor, and AS (AS 1842856) is a Foxo1 inhibitor. All drugs were dissolved in DMSO and added into cell culture media for 48h before the experiments, *P<0.05

Analyze: 1. What is the main conclusion of the study? 2. What were the key findings related to ECG and sodium channel parameters? 3. How were the effects induced? 4. What are the implications of these findings for understanding heart failure?
### Table 2. Biophysical parameters for sodium channel kinetics in the presence of different drugs.

|               | Voltage dependence of activation | Voltage dependence of inactivation | Recovery from inactivation |
|---------------|----------------------------------|-----------------------------------|---------------------------|
|               | $V_{1/2}$ (mV) | $K$ | $n$ | $V_{1/2}$ (mV) | $K$ | $n$ | $\tau_1$ (ms) | $\tau_s$ (ms) | $n$ |
| DMSO          | -42.25±0.85 | 4.20±0.14 | 19 | -79.02±0.62 | -6.06±0.12 | 21 | 4.86±0.27 | 62.23±2.72 | 19 |
| GSK 234470    | -41.23±0.96 | 4.75±0.19 | 21 | -77.37±0.85 | -6.03±0.11 | 23 | 5.39±0.36 | 70.21±3.66 | 21 |
| AS 1842856    | -41.91±1.06 | 4.46±0.15 | 24 | -78.24±0.88 | -6.13±0.12 | 25 | 5.61±0.39 | 66.85±3.41 | 24 |
| GSK 234470+AS 1842856 | -41.33±1.34 | 4.56±0.21 | 7  | -76.90±0.48 | -5.99±0.29 | 6  | 6.04±0.26* | 74.79±3.69* | 6  |
| MK 2206       | -40.88±1.37 | 4.29±0.28 | 13 | -79.36±1.16 | -6.09±0.22 | 14 | 7.58±0.88* | 70.12±6.33 | 11 |
| MK 2206+ AS 1842856 | -41.49±0.78 | 4.31±0.36 | 9  | -79.95±1.15 | -6.17±0.16 | 8  | 7.95±0.61* | 70.28±3.08 | 8  |

*P<0.05, compared with DMSO (dimethyl sulfoxide).

All drugs were dissolved in DMSO and added into cell culture media for 48h before the experiments.

doi:10.1371/journal.pone.0122436.t002

Responsible for electrophysiological abnormalities in the heart. Lu et al. [23] reported a mechanism for QT prolongation that involved an increase in persistent sodium current (late current) caused by defective PI3K signaling, however, their previous work [10] suggested that PI3K inhibitor decreased multiple ion current, including peak Na+ current. Much evidence has shown that a decline or dysfunction of sodium currents is responsible for QT interval prolongation or conduction blockade [24,25]. Especially, Remme et al. [26] and Korkmaz et al. [27] reported that downregulation of sodium current lead to lower heart rate and conduction dysfunction, but kinetics of voltage-dependent activation and inactivation had no obvious difference. In current study, we found that the QRS and QTc intervals are prolonged in PDK1-deletion mice. A change in ventricular conduction can be manifested as prolongation of the QRS interval on the ECG, and drug-induced effects on ventricular conduction are often associated with block of cardiac sodium current. Heath et al. [28] found that QRS interval prolongation (~10–20%) observed in either preclinical or clinical studies with sodium channel inhibitor. In addition, an earlier report by Cordes et al. [29] indicated that blocking sodium current was sufficient to produce QRS widening. Previous findings also predicted that small reductions in sodium current might affect intraventricular conduction [30]. While the relationship between changes in conduction velocity and QRS widening were not determined. Our findings demonstrated that reductions in cardiac sodium currents by PDK1 knockout might affect intraventricular conduction. In our study, the results of patch clamping experiments indicated that the density of sodium current was reduced by about 33% in PDK1 knockout mice. In addition, the steady-state inactivation and activation of the currents were minimally changed in the group with heart failure compared to the control group. The phenomenon is very similar to Remme’s [26] and Korkmaz’s [27] work. On the contrary, our results suggest that activation and inactivation curves are slightly left-shifted. Thus, further experiments to elucidate the relationship between the PDK1 signaling pathway and sodium channel are needed.

PDK1 phosphorylates Akt on Thr308, and phosphorylation of Thr308 is decreased in mice lacking the PDK1 gene [8]. Furthermore, the PI3K/PTEN signaling pathway, which is upstream of PDK1 and Akt, is involved in a wide variety of diseases including myocardial hypertrophy and contractility, heart failure, and preconditioning [31]. We have verified that Akt phosphorylation is reduced in PDK1 knockout mice and in isolated rat cardiomyocytes treated with GSK 2334470, a PDK1-specific inhibitor that does not suppress the activity of 93 other protein kinases including 13 AGC-kinases [18]. Additionally, our results show that Foxo1 phosphorylation is reduced by PDK1 deletion or inhibition. Similar reduction of Foxo1 phosphorylation was observed in the pancreatic islets of βPdk1-/- mice [32]. Furthermore, cells
lacking PTP non-receptor type 12 (PTPN12), which is an upstream mediator of \( PDK1 \), are defective in the activation of Foxo1/3a\[^{[33]}\]. Foxo1 binds the promoter region of \( SCN5A \) \[^{[34]}\], and exogenous Foxo1 expression decreases \( SCN5A \) promoter activity in HL-1 cells \[^{[11]}\]. Previous investigation has shown that Foxo1 phosphorylation promotes its translocation from nucleus to cytoplasm, which relieves its ability to negatively regulate \( SCN5A \) \[^{[12]}\]. Consistently, we observed increased nuclear Foxo1 expression levels in \( PDK1 \)-deleted cells, which may explain decreased Nav1.5 levels. Our results are consistent with the possibility that Nav1.5 expression is downregulated by \( PDK1 \) deletion via the suppression of phosphorylation of Akt and Foxo1.

In this study, the results suggested that a potential association of \( PDK1 \) down-regulation and decreased cardiac sodium current destiny, resulting in the sudden cardiac death in mice with \( PDK1 \) deletion. However, little is known on how reduction in peak sodium current caused death in these mice. But mutations in \( SCN5A \) have been previously linked to Brugada syndrome, conduction disturbances, sick sinus syndrome and dilated cardiomyopathy; most of these are associated with biophysical properties consistent with “loss of function” phenotype (reduced cardiac sodium current/function) \[^{[35]}\]. Clinically, patient with Brugada syndrome which reduces peak sodium current is well-known to cause cardiac arrhythmias and some of these mutations also cause dilated cardiomyopathy \[^{[36,37]}\]. Patients who carried those mutations had increased mortality and a high risk for sudden arrhythmic death \[^{[38]}\]. Therefore, the \( PDK1 \) deletion mice and Brugada syndrome had high similarity phenotypically and functionally, it is a reasonable assumption that \( PDK1 \) deletion mice shown a reduction in peak sodium current and that this may be a cause of sudden cardiac death, through mechanisms that have been described for the Brugada Syndrome. We would further speculate that \( PDK1 \) may be a candidate gene for the Brugada syndrome. These need to be verified genetically in patients with Brugada syndrome. Thus, PI3K-\( PDK1 \) signaling pathways may provide critical insights into arrhythmogenesis associated with “loss of function” phenotype.

On the other hand, it is not clear whether the decline occurs independently of heart failure. Zicha et al. reported a maximum density of sodium current reduction of about 33.6% in the dog with heart failure \[^{[39]}\]. To exclude the possible impact of heart failure and to support the association of the \( PDK1 \) pathway with sodium current and sodium channel expression, \( PDK1 \) signaling pathway specific inhibitors were applied to neonatal rat cardiomyocytes. In patch clamping assays, neonatal rat cardiomyocytes treated with \( PDK1 \) or the Akt inhibitor for 48 h decreased the sodium current from \(-36.23\pm1.25\) pA/pF to \(-27.52\pm1.49\) pA/pF \((P<0.05)\) or \(-25.48\pm2.06\) pA/pF \((P<0.05)\), which was similar to the in vivo results of \( PDK1 \) deletion. Conversely, the peak sodium current increased to \(-42.85\pm1.51\) mV \((P<0.05)\) when the Foxo1 inhibitor AS1842856 was applied, which is consistent with its inhibitory role in expression of Foxo1 \[^{[21]}\]. Furthermore, AS1842856 appeared to counteract the decrease promoted by the other two drugs, and no variations the channel activation or inactivation kinetics were detected, which suggests that Foxo1, one of the \( PDK1 \) downstream factors, involve in the regulation of sodium channel. Our results demonstrate that the expression of Nav1.5, the product of the \( SCN5A \) gene, is markedly reduced in \( PDK1 \) deletion mice via the \( PDK1 \)-Foxo1 pathway. These findings are supported by the use of \( PDK1 \), Akt and Foxo1 inhibitors.

\( PDK1 \) regulates phosphoinositide PI3K and activates a number of AGC kinases, including Akt, p70 ribosomal S6 kinase (p70S6K), and SGK1. Much evidence suggests that SGK1 stimulates a variety of ion channels \[^{[3]}\], including Nav1.5, and that the ubiquitin ligase Nedd4 may regulate SGK1 and its downstream effects on \( SCN5A \) \[^{[40]}\]. However, the effect of SGK1 is modest, and lack of SGK1 does not fully disrupt the downstream effects \[^{[41]}\]. In addition, small G proteins, molecular switches that control the activity of cellular and membrane proteins, regulate a wide variety of ion channels. In some cases, K-Ras and RhoA increase the activity of the epithelial sodium channel (ENaC) via PI3K and PI(4)P5-kinase signaling pathways\[^{[42]}\].
Furthermore, Rho inhibits ether-a-go-go–related gene channel via a protein serine/threonine (S/T) kinase, whereas Rac stimulates the channel via a protein S/T phosphatase [43]. Whether small G proteins regulate Nav1.5 via the PI3K signaling pathway needs further research.

In conclusion, through current study we have discovered that the peak Na+ is reduced in PDK1 KO mice and that this may be a cause of sudden death, through mechanisms that have been described for the Brugada Syndrome.

Limitations

First, heart failure is a complex pathophysiological process, and multiple influence factors, including other AGC protein kinase family, involve in cardiac structure and electrophysiological remodeling. Furthermore, action potential and QT duration are dependent on balance between depolarizing inward and repolarizing outward current. It is unclear if the changes are related to the reduction in PDK1 or due to some other changes associated with heart failure and outward potassium channels [10]. Second, we only made abnormal observations with body surface ECG, more reliable data, such as arrhythmias directly linked to the cause of sudden death, should be obtained using telemetry devices from PDK1F/F αMHC-Cre animals.

Acknowledgments

We are grateful to CongJia Shan at Model Animal Research Center, Nanjing University for providing αMHC-Cre mice.

Author Contributions

Conceived and designed the experiments: DWW ZZY KJC. Performed the experiments: ZLH YJ YQY XHL. Analyzed the data: ZLH YJ. Contributed reagents/materials/analysis tools: DWW ZZY KJC. Wrote the paper: ZLH DWW. Obtained permission for use of PDK1 deletion mice: ZZY.

References

1. Dummler B, Hemmings BA (2007) Physiological roles of PKB/Akt isoforms in development and disease. Biochem Soc Trans 35: 231–235. PMID: 17371246
2. Dann SG, Selvaraj A, Thomas G (2007) mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. Trends Mol Med 13: 252–259. PMID: 17452018
3. Lang F, Bohmer C, Palmada M, Seebohm G, Strutz-Seebohm N, Vallon V (2006) (Patho)physiological significance of the serum- and glucocorticoid-inducible kinase isoforms. Physiol Rev 86: 1151–1178. PMID: 17015487
4. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Joo P, Hu LS, et al. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96: 857–868. PMID: 10102273
5. Mora A, Komander D, van Aalten DM, Alessi DR (2004) PDK1, the master regulator of AGC kinase signal transduction. Semin Cell Dev Biol 15: 161–170. PMID: 15209375
6. Ito K, Akazawa H, Tamagawa M, Furukawa K, Ogawa W, Yasuda N, et al. (2009) PDK1 coordinates survival pathways and beta-adrenergic response in the heart. Proc Natl Acad Sci U S A 106: 8689–8694. doi: 10.1073/pnas.0900641106 PMID: 19429709
7. Pretorius L, Du XJ, Woodcock EA, Kiriazis H, Lin RC, Marasco S, et al. (2009) Reduced phosphoinositide 3-kinase (p110alpha) activation increases the susceptibility to atrial fibrillation. Am J Pathol 175: 998–1009. doi: 10.2353/ajpath.2009.090126 PMID: 19679877
8. Wu X, Cao Y, Nie J, Liu H, Lu S, Hu X, et al. (2013) Genetic and pharmacological inhibition of Rheb1-mTORC1 signaling exerts cardioprotection against adverse cardiac remodeling in mice. Am J Pathol 182: 2005–2014. doi: 10.1016/j.ajpath.2013.02.012 PMID: 23567640
9. Mora A, Davies AM, Bertrand L, Sharif I, Budas GR, Jovanovic S, et al. (2003) Deficiency of PDK1 in cardiac muscle results in heart failure and increased sensitivity to hypoxia. EMBO J 22: 4666–4676. PMID: 12970179
10. Lu Z, Wu CY, Jiang YP, Ballou LM, Clausen C, Cohen IS, et al. (2012) Suppression of phosphoinositide 3-kinase signaling and alteration of multiple ion currents in drug-induced long QT syndrome. Sci Transl Med 4:131ra150.

11. Mao W, You T, Ye B, Li X, Dong HH, Hill JA, et al. (2012) Reactive oxygen species suppress cardiac NaV1.5 expression through Foxo1. PLoS One 7: e32738. doi: 10.1371/journal.pone.0032738 PMID:22400069

12. Kaur K, Zarzoso M, Ponce-Balbuena D, Guerrero-Sema G, Hou L, Musa H, et al. (2013) TGF-beta1, released by myofibroblasts, differentially regulates transcription and function of sodium and potassium channels in adult rat ventricular myocytes. PLoS One 8: e55391. doi:10.1371/journal.pone.0055391 PMID:23393573

13. Farr MA, Basson CT (2004) Sparking the failing heart. N Engl J Med 351:185–187. PMID:15247360

14. Di RM, Feng QT, Chang Z, Luan Q, Zhang YY, Huang J, et al. (2010) PDK1 plays a critical role in regulating cardiac function in mice and human. Chin Med J (Engl) 123: 2358–2363. PMID:21034549

15. London B, Wang DW, Hill JA, Bennett PB (1998) The transient outward current in mice lacking the potassium channel gene Kv1.4. J Physiol 509 (Pt 1): 171–182. PMID:9547391

16. Yang T, Atack TC, Stroud DM, Zhang W, Hall L, Roden DM (2012) Blocking Scn10a channels in heart reduces late sodium current and is antiarrhythmic. Circ Res 111: 322–332. doi:10.1161/CIRCRESAHA.112.265173 PMID: 22723299

17. Jiang Q, Ni B, Shi J, Han Z, Qi R, Xu W, et al. (2014) Down-regulation of ATBF1 activates STAT3 signaling via PIAS3 in pacing-induced HL-1 atrial myocytes. Biochem Biophys Res Commun 449: 278–283. doi:10.1016/j.bbrc.2014.05.041 PMID:24857987

18. Najafov A, Sommer EM, Axten JM, Deyoung MP, Alessi DR (2011) Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. Biochem J 433: 357–369. doi:10.1042/Bj20101732 PMID: 21087210

19. Hirai H, Soothe H, Nakatsuru Y, Miyama K, Taguchi S, Tsujioka K, et al. (2010) MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther 9: 1956–1967. doi:10.1158/1535-7163.MCT-09-1012 PMID:20571069

20. Remme CA, Verkerk AO, Nuyens D, van Ginneken AC, van Brunschot S, Belterman CN, et al. (2006) Overlap syndrome of cardiac sodium channel disease in mice carrying the equivalent mutation of human SCN5A-1795insD. Circulation 114:2584–2594. PMID:17145985

21. Heath BM, Cui Y, Worton S, Lawton B, Ward G, Ballini E, et al. (2011) Translation of flecainide- and mexiletine-induced cardiac sodium channel inhibition and ventricular conduction slowing from nonclinical models to clinical. J Pharmacol Toxicol Methods 63: 258–268. doi:10.1016/j.vascn.2010.12.004 PMID:21194571

22. Cordes J, Li C, Dugas J, Austin-LaFrance R, Lightbown I, Engwall M, et al. (2009) Translation between in vitro inhibition of the cardiac Nav1.5 channel and pre-clinical and clinical QRS widening. Journal of Pharmacological and Toxicological Methods 60: 221.
30. Schott JJ, Alshinawi C, Kynadt F, Probst V, Hoornjte TM, Hulsbeek M, et al. (1999) Cardiac conduction defects associate with mutations in SCN5A. Nat Genet 23: 20–21. PMID: 10471492

31. Oudit GY, Penninger JM (2009) Cardiac regulation by phosphoinositide 3-kinases and PTEN. Cardiovasc Res 82: 250–260. doi: 10.1093/cvr/cvp014 PMID: 19147653

32. Hashimoto N, Kido Y, Uchida T, Asahara S, Shigeyama Y, Matsuda T, et al. (2006) Ablation of PDK1 in pancreatic beta cells induces diabetes as a result of loss of beta cell mass. Nat Genet 38: 589–593. PMID: 16642023

33. Harris IS, Blaser H, Moreno J, Treloar AE, Gorrini C, Sasaki M, et al. (2014) PTPN12 promotes resistance to oxidative stress and supports tumorigenesis by regulating FOXO signaling. Oncogene 33: 1047–1054. doi: 10.1038/onc.2013.24 PMID: 23435421

34. Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, et al. (2007) FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 128: 309–323. PMID: 17254969

35. Remme CA, Wilde AA (2008) SCN5A overlap syndromes: no end to disease complexity? Europace 10: 1253–1255. doi: 10.1093/europace/eun267 PMID: 18820249

36. Frigo G, Rampazzo A, Bauce B, Pilichou K, Beffagna G, Daniele GA, et al. (2007) Homozygous SCN5A mutation in Brugada syndrome with monomorphic ventricular tachycardia and structural heart abnormalities. Europace 9: 391–397. PMID: 17442746

37. Nguyen TP, Wang DW, Rhodes TH, George AL Jr (2008) Divergent biophysical defects caused by mutant sodium channels in dilated cardiomyopathy with arrhythmia. Circ Res 102: 364–371. PMID: 18048769

38. Antzelevitch C, Brugada P, Borggrefe M, Brugada J, Brugada R, Corrado D, et al. (2005) Brugada syndrome: report of the second consensus conference: endorsed by the Heart Rhythm Society and the European Heart Rhythm Association. Circulation 111: 659–670. PMID: 15655131

39. Zicha S, Maltsev VA, Nattel S, Sabbah HN, Undrovinas AI (2004) Post-transcriptional alterations in the expression of cardiac Na+ channel subunits in chronic heart failure. J Mol Cell Cardiol 37: 91–100. PMID: 15242739

40. Boehmer C, Wilhelm V, Palmada M, Wallisch S, Henke G, Brinkmeier H, et al. (2003) Serum and glucocorticoid inducible kinases in the regulation of the cardiac sodium channel SCN5A. Cardiovasc Res 57: 1079–1084. PMID: 12650886

41. Lang F, Artunc F, Vallon V (2009) The physiological impact of the serum and glucocorticoid-inducible kinase SGK1. Curr Opin Nephrol Hypertens 18: 439–448. doi: 10.1097/MNH.0b013e32832f125e PMID: 19584721

42. Pochynyuk O, Medina J, Gamper N, Genth H, Stockand JD, Staruschenko A (2006) Rapid translocation and insertion of the epithelial Na+ channel in response to RhoA signaling. J Biol Chem 281: 26520–26527. PMID: 16829523

43. Storey NM, O'Bryan JP, Armstrong DL (2002) Rac and Rho mediate opposing hormonal regulation of the ether-a-go-go-related potassium channel. Curr Biol 12: 27–33. PMID: 11790300