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Deletion of *FoxO1* Leads to Shortening of QRS by Increasing Na⁺ Channel Activity through Enhanced Expression of both Cardiac Na_v1.5 and β3 Subunit

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Abstract

Our *in vitro* studies revealed that a transcription factor, Forkhead box protein O1 (FoxO1), negatively regulates the expression of Na_v1.5, a main α subunit of the cardiac Na⁺ channel, by altering the promoter activity of *SCN5a* in HL-1 cardiomyocytes. The *in vivo* role of FoxO1 in the regulation of cardiac Na_v1.5 expression remains unknown. The present study aimed to define the role of FoxO1 in the regulation of Na_v1.5 expression and cardiac Na⁺ channel activity in mouse ventricular cardiomyocytes and assess the cardiac electrophysiological phenotype of mice with cardiac *FoxO1* deletion. Tamoxifen-induced and cardiac-specific *FoxO1* deletion was confirmed by polymerase chain reaction (PCR). Cardiac *FoxO1* deletion failed to result in either cardiac functional changes or hypertrophy as assessed by echocardiography and individual ventricular cell capacitances, respectively. Western blotting showed that FoxO1 was significantly decreased while Na_v1.5 protein level was significantly increased in mouse hearts with *FoxO1* deletion. Reverse transcription-PCR (RT-PCR) revealed that *FoxO1* deletion led to an increase in Na_v1.5 and Na⁺

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Conflict of interest

None declared

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channel subunit $\beta 3$ mRNA, but not $\beta 1$, 2, 4, or connexin 43. Whole patch-clamp recordings demonstrated that cardiac Na^+ currents were significantly augmented by *FoxO1* deletion without affecting the steady-state activation and inactivation, leading to accelerated depolarization of action potentials in mouse ventricular cardiomyocytes. Electrocardiogram recordings showed that the QRS complex was significantly shortened and P wave amplitude was significantly increased in conscious and unrestrained mice with cardiac *FoxO1* deletion. $\text{Na}_v1.5$ expression was decreased in the peri-infarct (border-zone) of mice with myocardial infarction and FoxO1 accumulated in the cardiomyocyte nuclei of chronic ischemic human hearts. Our findings indicate that FoxO1 plays an important role in the regulation of $\text{Na}_v1.5$ and $\beta 3$ subunit expression as well as Na^+ channel activity in the heart and that FoxO1 is involved in the modulation of $\text{Na}_v1.5$ expression in ischemic heart disease.

Keywords

FoxO1; $\text{Na}_v1.5$; $\beta 3$ subunit; Na^+ channel; cardiac depolarization

1. Introduction

The voltage-gated Na^+ channel plays an important role in the membrane excitability of cardiomyocytes [1-3]. As a major Na^+ channel isoform, the $\text{Na}_v1.5$ α subunit encoded by the *SCN5a* gene determines the cardiac rapid depolarization characteristic of ventricular myocytes and drives subsequent electrical propagation throughout the heart [1, 3]. Heterozygous deletion of *SCN5a* leads to prolongation of both the PR-interval and QRS complex as well as ventricular tachycardia in mice [4]. Numerous studies have shown that the dysfunction of Na^+ channels contributes to the development of life-threatening ventricular arrhythmias in both inherited ion channelopathies and acquired cardiac diseases [1-3, 5-8]. Mutations of *SCN5a* gene alter the function of the Na^+ channel and have been linked to Brugada syndrome, long QT syndromes (LQTS), cardiac conduction defects, and atrial fibrillation [1, 5, 9-10]. Dysregulation of $\text{Na}_v1.5$ expression has been widely reported in myocardial infarction, heart failure, and other heart diseases [2-3, 8]. Na^+ channel activity is finely regulated by complex molecular mechanisms [11-12]. Na^+ channel β subunits, ankyrin-G, fibroblast growth factor homologous factor 1B, caveolin-3, E3 ubiquitin-protein ligase Nedd4, glycerol-3-phosphate dehydrogenase 1-like protein, plakophilin-2, MOG1, a multiprotein complex composed of syntrophins and dystrophin [13] interact with $\text{Na}_v1.5$ to regulate Na^+ channel activity [14-21]. $\text{Na}_v1.5$ is directly phosphorylated by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and protein kinase A, resulting in changes of Na^+ channel activity [11]. $\text{Na}_v1.5$ requires ankyrin-G-binding for normal physiological function [22] and its localization at the intercalated discs is required for β (IV)-spectrin-dependent targeting of CaMKII to a critical phosphorylation site, S571, in $\text{Na}_v1.5$ [23]. At the transcription level, NF-KappaB and TBX5 directly affect the *SCN5a* promoter activity and alter $\text{Na}_v1.5$ expression [12, 24]. NF-KappaB functions as suppressor [12] while TBX5 functions as an activator of *SCN5a* gene transcription in cardiac cells [24].

Accumulating evidence indicates that Forkhead box O (FoxO) transcription factors are critical in maintaining cardiac function and mediating oxidative stress [25]. Although

FoxO1, 3, and 4 are all expressed in the heart, FoxO1 is the main isoform found in the adult heart [26]. The transcriptional activities of FoxOs largely depend upon their nuclear localization [27]. Deacetylation or phosphorylation of FoxO proteins typically determines their localization in either the nuclei or cytoplasm [27]. Generally, FoxO proteins directly bind to consensus DNA sequences and regulate the expression of target genes. The proximal regions of the mouse, rat, and human *SCN5a* promoters have FoxO-binding insulin response elements, indicating that FoxO1 may regulate $\text{Na}_v1.5$ expression [27]. Indeed, our previous studies have shown that FoxO1 reduces $\text{Na}_v1.5$ expression *in vitro* by binding to the *SCN5a* promoter and inhibiting its activity in HL-1 cardiomyocytes [27]. In our current study, we characterized the cardiac electrophysiological phenotype of mice with tamoxifen-induced and cardiac-specific deletion of *FoxO1* and defined the *in vivo* role of FoxO1 in the regulation of cardiac Na^+ channel activity.

2. Methods

2.1. Polymerase chain reaction (PCR)

DNA was extracted from mouse-tails or hearts using Genomic DNA kit (QIAGEN) following the manufacturer's instruction. Two pairs of primers (forward: 5'-CTA GGC CAC AGA ATT GAA AGA TCT -3', reverse: 5'-GTA GGT GGA AAT TCT AGC ATC ATC C -3'; forward: 5'-GCG GTC TGG CAG TAA AAA CTA TC -3', reverse: 5'-GTG AAA CAG CAT TGC TGT CAC TT -3') were used to determine the presence of *Cre*, while two additional pairs of primers (forward: 5'-GCT TAG AGC AGA GAT GTT CTC AGA TT -3', reverse 1: 5'-CCA GAG TCT TCG TAT CAG GCA AAT AA -3', and reverse 2: 5'-CAA GTC CAT TAA TTC AGC ACA TTG A -3') were employed to determine the presence of *LoxP* sites flanking *FoxO1* exon 2 and confirm deletion of exon 2.

2.2. Reverse transcription PCR (RT-PCR)

Using RNeasy Fibrosis Tissue kit (QIAGEN) and following the manufacturer's instruction, total RNA was extracted from adult mouse *FoxO1*^{+/+} and *FoxO1*^{-/-} ventricles as well as the peri-infarct (border-zone) region defined as the 2-mm area encircling the area of pathologic infarction of the mouse hearts with anterior descending artery ligation or ventricular tissue from the hearts with a sham procedure. The RNA sample was then reversely transcribed to first-strand cDNA using a high capacity cDNA reverse transcription kit (AB Applied Biosystems). Semi-quantitative RT-PCR was performed to measure mRNA expression levels of $\text{Na}_v1.5$, Na^+ channel β subunits, Connexin 43 (Cx43), and GAPDH in ventricular tissue. The primer sequences used to amplify the cDNA of these genes are provided in Supplemental Table 1.

2.3. Western blotting

Western blot analysis was performed on the proteins extracted from mouse ventricles with *FoxO1*^{+/+} and *FoxO1*^{-/-}. Tissue from the peri-infarct (border-zone) region, defined as the 2-mm area encircling the area of pathologic infarction of the wild mouse hearts with anterior descending artery ligation, or ventricular tissue from the mice with the sham procedure were used as well. Ventricular tissues were homogenized in cell lysis buffer (Cell Signaling Technology, Inc). Fifteen to thirty micrograms of protein were equally loaded on 10% SDS

polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 hour, and then incubated with the indicated primary antibody overnight at 4°C. After one-hour incubation with the appropriate secondary antibody, the specific signals were revealed by enhanced chemiluminescence reagent (Pierce). Primary antibodies used in this study include rabbit anti-FoxO1 polyclonal (Cell Signaling, 1:1000 for Western blot and 1:200 for immunohistochemistry), rabbit anti-Nav1.5 polyclonal (Alomone Labs, 1:500), rabbit anti-SCN3b polyclonal antibody (Abcam, 1:1000) and rabbit anti-GAPDH polyclonal (Millipore, 1:4000).

2.4. Whole-cell voltage clamp recording

Whole cell recording techniques were used to record Na⁺ currents in isolated ventricular myocytes from adult mice. The methods used to isolate cardiomyocytes were described previously [27]. Only Ca²⁺-tolerant ventricular cells with clear cross-striations and without spontaneous contractions or substantial granulation were selected for the experiments. All whole cell recordings were conducted at room temperature using a patch clamp amplifier (Axopatch 200B, Axon Instruments). The experiments were controlled by using *pClampex* 10.3 software (Axon Instruments Inc., Foster City, CA). The external solution for recording action potential (AP) contained (in mM): 136 NaCl, 4 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES, and 10 glucose (pH 7.35 with NaOH); the pipette solution for recording AP contained (in mM): 135 KCl, 1 MgCl₂, 10 EGTA, 10 HEPES, and 5 glucose (pH 7.2 with KOH); the external solution for recording Na⁺ currents contained (in mM): 20 NaCl, 1 MgCl₂, 1 CaCl₂, 55 CsCl, 10 CsOH, 10 Glucose, 20 HEPES, 2 4-AP, 50 TEACl and 0.5 CdCl₂ (pH 7.35 with CsOH); the pipette solution contained (in mM) 5 NaCl, 135 CsF, 10 EGTA, 5 MgATP, 5 HEPES and 20 TEACl (pH 7.2 with CsOH). In all recordings, 80% of the series resistance was compensated. Data analysis was accomplished by using *pClampfit* 10.3 software (Axon Instruments Inc., Foster City, CA).

2.5. Immunohistochemical staining

Formalin fixed and paraffin embedded tissues from the left ventricular free wall of human hearts, including 3 non-failing controls without a history of heart diseases (NFH) and 3 failing ones with ischemic heart disease (IHD) were used for immunohistochemical staining. The research protocol was approved by Institutional Review Boards at the University of Rochester. Four µm sections were cut from paraffin-embedded ventricular tissues and deparaffinized. Antigen retrieval was performed in sodium citrate buffer (pH 6.0) by heating to 99 °C for 20 minutes with PT Link system by Dako (Carpinteria, CA). After quenching endogenous peroxidase activity with 3% H₂O₂, the sections were blocked with 10% non-immune goat serum (Invitrogen, Carlsbad, CA) and then incubated overnight with primary anti-FoxO1 antibody (1:200) at 4 °C. The signals were amplified using the Histostain-SP Kit (Invitrogen) and detected with DAB substrate (Dako). Hematoxylin was used as a counterstain. Negative controls were incubated with serum instead of primary antibody under the same conditions.

2.6. Permanent left anterior descending coronary artery ligation surgery

Wild type mice were anesthetized with 2.0% isoflurane inhalation and placed onto a heating pad (half inch plexiglass between the animal and the heating pad). Oral tracheal intubation was performed with a PE 90 tube (o.d. 1.27 mm). Mechanical ventilation (tidal volume of 250 μ l at 130 breaths per minute) was started and 1.5% isoflurane was used for maintenance of anesthesia. After intubation, a left thoracotomy was performed in the fourth intercostal space and the pericardium was vertically opened. The left anterior coronary artery (LAD) was ligated with 9-0 polypropylene sutures. After observation of ventricle blanching and ST-segment elevation in the electrocardiograms (ECG), indicating successful occlusion of the artery, the chest was closed with 6-0 coated vicryl sutures. The skin was closed using 6-0 nylon, the anesthesia was stopped, and the mouse was allowed to recover for several minutes before the endotracheal tube was removed. Sham-operated animals served as surgical controls and were subjected to the same procedures as the experimental animals, except that the LAD was not ligated.

2.7. Electrocardiogram Recording

The method used to record electrocardiograms from conscious and unrestrained mice is as described in our previous study [28]. Standard lead II surface ECGs were recorded in adult (>25 weeks) mice. Two electrodes were placed on the right anterior and superior, and left anterior and inferior chest surfaces, respectively, and were connected to a Bioamplifier (AD instrument, CO) through an electrical swivel 6 channel (Promed-Tec Inc. Bellingham, MA). ECG recordings were obtained at a frequency response to 0.05 to 500 Hz. Signals were digitized at 2 kHz and recorded on a personal computer.

2.8. Echocardiography

Echocardiography was performed in conscious adult (>25 weeks) mice using a high-resolution mouse echo machine (Visualsonics Vevo 2100 ultrasound system with an 18-38 MHz MS-400 transducer). The following parameters were measured digitally in M-mode: LV dimensions at diastole (LVDd) and systole (LVDs), and anterior and posterior wall thickness at diastole and systole. All measurements were averaged over five consecutive cardiac cycles. The observer conducting the measurement was blind to the experiments and genotypes.

2.9. Statistics

Results are presented as means $\pm$ standard error; the statistical significance of differences between groups was assessed using a two-tailed Student t-test and the significance level was set at $p < 0.05$.

3. Results

3.1. Cardiac-specific *FoxO1* deletion mouse model

α -MHC-MerCreMer transgenic mice (mhy6-MCM^{+/+}) (Jackson Laboratory) were bred with mice containing *loxP*-flanked *FoxO1* exon 2 (*FoxO1*^{L/L}) (which were generated from *LoxP*-flanked *FoxO1*,3,4 mice kindly provided by Dr. Ronald A DePinho in the Department of

Cancer Biology, Division of Basic Science Research at the University of Texas MD Anderson Cancer Center, Houston, Texas) to produce mhy6-MCM^{+/-}/*FoxO1*^L, mhy6-MCM^{+/-}/*FoxO1*^{L/+}, mhy6-MCM^{+/-}/*FoxO1*^{L/L}, mhy6-MCM^{-/-}/*FoxO1*^{L/+}, and mhy6-MCM^{+/-}/*FoxO1*^{+/+} mice were generated from the further breeding. Tamoxifen was intraperitoneally injected in these mice for 5 consecutive days at a dose of 40 mg/kg/day. All mice treated with tamoxifen injection survived the process and remained healthy. PCR was performed on DNA extracted from the hearts of the tamoxifen treated mice one month after the injections. Tamoxifen-mediated cardiac-specific deletion of *FoxO1* (*FoxO1*^{-/-}) induced by Cre was confirmed by the results presented in Supplemental Fig. 1. *FoxO1*^{-/-} mice were healthy and did not exhibit any abnormal behaviors.

3.2. No cardiac hypertrophy identified in *FoxO1*^{-/-} heart

Global knockout of *FoxO3* has been reported to cause mouse cardiac hypertrophy [29]. To determine if *FoxO1* deletion was able to induce cardiac hypertrophy, mouse heart/weight ratios and individual ventricular cell capacitances were determined. There was no significant difference in heart/body weight ratio (mg/g) between *FoxO1*^{+/+} and *FoxO1*^{-/-} mice (3.8 ± 0.1 , n=7 versus 4.1 ± 0.1 , n=6) (Fig. 1A). After establishing whole-cell recordings, the currents were recorded to determine ventricular cell capacitance by depolarizing to -60 mV and hyperpolarizing to -80 mV from a holding potential (HP) of -70 mV. The capacitances of ventricular myocytes isolated from *FoxO1*^{-/-} and *FoxO1*^{+/+} mouse hearts were 117.1 ± 3.3 pF (n=26) and 133.2 ± 9.9 pF (n=18), respectively, and they were not statistically significantly different (Fig. 1B). These findings indicate that cardiac-specific *FoxO1* deletion does not result in cardiac hypertrophy in mice.

3.3. Heart function was not altered by *FoxO1* deletion

To clarify the influence of *FoxO1* deletion on mouse cardiac functions, echocardiography was performed. Representative M-mode images are shown in Fig. 1C. There were no significant differences in heart function parameters such as ejection fraction (EF) and fraction shortening (FS) between the *FoxO1*^{-/-} and *FoxO1*^{+/+} mice (Supplemental table 2). Furthermore, other echocardiography parameters such as LVPWs, LVPWd, LVIDs, and LVIDd did not significantly differ between *FoxO1*^{-/-} and *FoxO1*^{+/+} mice (Supplemental table 2). These findings indicate that *FoxO1* does not significantly affect the cardiac functions of nor cause hypertrophy in adult mice.

3.4. Deletion of *FoxO1* induced an increase in both cardiac Nav1.5 and $\beta 3$ subunit expression

FoxO1 negatively regulates Nav1.5 expression in HL-1 cardiomyocytes[27]. To test the hypothesis that *FoxO1* functions as a negative regulator of Nav1.5 expression in mouse hearts, Western blot was performed on the protein extracted from *FoxO1*^{+/+} and *FoxO1*^{-/-} mouse ventricles. As evidenced by Western blots (Fig. 2A) and quantitative analysis (Fig. 2B), Nav1.5 and $\beta 3$ protein levels were significantly ($p < 0.01$, $p < 0.05$) increased and *FoxO1* was significantly ($p < 0.01$) decreased in *FoxO1*^{-/-} hearts in comparison with *FoxO1*^{+/+} hearts. Densitometry analyses of *FoxO1*, Nav1.5, and $\beta 3$ levels compared to GAPDH level showed that Nav1.5 and $\beta 3$ expression were increased 162% and 21% while *FoxO1* expression was

decreased 84% in *FoxO1*^{-/-}, compared to *FoxO1*^{+/+} hearts (Fig. 2B). These findings indicate that FoxO1 is a suppressor of both Na_v1.5 and β3 expression in adult mouse hearts.

3.5. mRNA levels of Na_v1.5 and β3 subunit were increased in *FoxO1*^{-/-} mouse hearts

The QRS complex represents ventricular depolarization. Na⁺ channel and Cx43 channel activities determine cardiac depolarization. Na⁺ channel activity is primarily determined by a main α subunit, Na_v1.5; however, multiple β subunits also play roles in the regulation of this channel activity. Increased Na⁺ channel activity due to elevated expression of Na_v1.5 protein was found in *FoxO1*^{-/-} mouse hearts and this contributed to the shortening of the QRS complex. Other components related to the electrical conduction between ventricular myocytes, such as β1, 2, 3, and 4 subunits as well as Cx43 were assessed in these cardiac *FoxO1* deficient mice. RT-PCR analysis showed that the level of Na_v1.5 was significantly ($p < 0.01$) increased (Fig. 2C). Interestingly, mRNA level of the β3 subunit was also significantly ($p < 0.01$) increased, but β1, 2, 4, and Cx43 levels were not changed in mouse hearts with deletion of *FoxO1* in comparison with the control group (Fig. 2C and D).

3.6. Increase of I_{Na} in *FoxO1*^{-/-} mouse ventricular myocytes

To determine if *FoxO1* deletion and increased Na_v1.5 and β3 subunit expression were capable of inhibiting cardiac Na⁺ channel activity, Na⁺ currents were recorded from *FoxO1*^{+/+} and *FoxO1*^{-/-} left ventricular myocytes isolated from adult mice using the whole-cell voltage-clamp techniques. Typical traces of I_{Na} recorded from *FoxO1*^{+/+} and *FoxO1*^{-/-} ventricular cells are displayed in Fig. 3A. Peak Na⁺ current densities were obtained from the peak currents divided by individual cardiomyocyte capacitances. The average current densities, from test potentials of -35 mV to -5 mV, were significantly larger ($p < 0.05$) in *FoxO1*^{-/-} ventricular myocytes compared with *FoxO1*^{+/+} ventricular cells (Fig. 3B). At the test potential of -30 mV, the peak current density was 26.9 ± 1.4 pA/pF ($n=26$) for *FoxO1*^{-/-} and 21.3 ± 1.8 pA/pF ($n=18$) for *FoxO1*^{+/+}. There was no significant shift of the test potential for the peak Na⁺ current density between the two groups (Fig. 3B).

3.7. Inactivation but not activation of Na⁺ channel was accelerated by *FoxO1* deletion in ventricular myocytes

Time constants of activation ($\tau_{\text{activation}}$) and inactivation ($\tau_{\text{inactivation}}$) were analyzed by fitting the upstroke and decay traces of I_{Na} using a mono-exponential function, respectively. As shown in Fig. 4B, there was no statistical difference in the $\tau_{\text{activation}}$ of I_{Na} between *FoxO1*^{-/-} and *FoxO1*^{+/+} mouse ventricular myocytes, with average $\tau_{\text{activation}}$ values of 0.50 ± 0.05 for *FoxO1*^{-/-} and 0.53 ± 0.04 for *FoxO1*^{+/+} ventricular cells at -30 mV. (Fig. 4B). The $\tau_{\text{inactivation}}$ values, however, were significantly decreased ($p < 0.05$) at both -30 mV and -20 mV in *FoxO1*^{-/-} ventricular myocytes compared to *FoxO1*^{+/+} (Fig. 4C), indicating that deletion of *FoxO1* accelerated the Na⁺ channel inactivation. The values of $\tau_{\text{inactivation}}$ for the Na⁺ channel, for example, at -30 mV in ventricular myocytes from *FoxO1*^{-/-} and *FoxO1*^{+/+} mice were 1.91 ± 0.15 ms and 2.35 ± 0.16 ms, respectively.

3.8. Recovery from the inactivation but not steady-state activation and inactivation of Na⁺ channel was affected by *FoxO1* deletion in ventricular myocytes

In order to determine whether there were any differences in Na⁺ channel kinetics between *FoxO1*^{+/+} and *FoxO1*^{-/-} ventricular myocytes, the steady-state activation, steady-state inactivation, and recovery from inactivation of I_{Na} were recorded and analyzed. The voltage-dependent steady-state activation of the Na⁺ channel was determined by a series of test potentials ranging from -90 to +60 mV at 0.1 Hz, from a holding potential of -100 mV (Fig. 5A). These results showed that the voltage-dependent activation of I_{Na} was unchanged between *FoxO1*^{+/+} and *FoxO1*^{-/-} ventricular myocytes. The half-maximal activation voltage and slope factor were -45.03 ± 0.37 mV and 6.52 ± 0.34 in *FoxO1*^{-/-} ventricular myocytes (n=26), and -41.24 ± 0.27 mV and 5.32 ± 0.24 in control group (n=18), respectively, and they were not statistically significantly different. The voltage-dependent inactivation of I_{Na} was recorded by a two-pulse protocol with conditioning potentials from -130 mV to -10 mV, followed by a test potential of -30 mV (Fig. 5B). Similarly, there were no changes in the voltage-dependent inactivation curves of I_{Na} between the two groups. The half-maximal inactivation voltage and slope factor did not show a significant difference (-70.93 ± 0.37 mV and 7.20 ± 0.33 , n=18 in *FoxO1*^{-/-} versus -71.60 ± 0.61 mV and 7.60 ± 0.53 , n=16 in *FoxO1*^{+/+} ventricular myocytes). Recovery of I_{Na} from inactivation was recorded by a paired-pulse with a variable inter-pulse duration (from 0 to 230 ms) at holding potential of -100 mV. The curves of recovery from inactivation were obtained by normalizing the peak current from a test pulse (-30 mV, 80 ms) to a conditioning pulse (-30 mV, 300 ms) and then fitting the data with the mono-exponential equation. These results (Fig. 5C) showed that the recovery of I_{Na} from inactivation was significantly different between *FoxO1*^{-/-} and *FoxO1*^{+/+} ventricular myocytes (Tau=18.3 and 13.3; K slope factor = 0.075 ± 0.001 and 0.055 ± 0.000 in *FoxO1*^{-/-} versus in *FoxO1*^{+/+} mice; n=16 for each group; p<0.05). These findings indicate that the deletion of *FoxO1* leads to accelerated Na⁺ channel recovery from inactivation.

3.9. Deletion of *FoxO1* accelerated the depolarization of action potentials in ventricular myocytes

We further investigated whether *FoxO1* deletion influenced the ventricular myocyte action potential. Action potentials were recorded at 1 Hz from *FoxO1*^{-/-} and *FoxO1*^{+/+} ventricular myocytes. Typical action potential traces, as shown in Fig. 6A, exhibited that the depolarization of action potentials was faster in *FoxO1*^{-/-} than *FoxO1*^{+/+} ventricular myocytes (Fig. 6A). The values of maximal upstroke velocity (V_{max}) of the action potentials recorded from *FoxO1*^{-/-} and *FoxO1*^{+/+} ventricular myocytes (Fig. 6B) were 240 ± 13 mV/ms (n=18) and 147 ± 22 mV/ms (n=12), respectively, and were significantly different (p<0.05). The amplitude of action potentials was larger in *FoxO1*^{-/-} ventricular myocytes, but it was not significantly different from *FoxO1*^{+/+} (145 ± 4 mV, n=18 versus 127 ± 8 mV, n=12), and the resting membrane potential was also not statistically significant between *FoxO1*^{-/-} (-86.8 ± 3.1 mV, n = 18) and *FoxO1*^{+/+} (-89.0 ± 3.8 mV, n=12). Both action potential duration (APD)₅₀ and APD₉₀ were not significantly different (Fig. 6B) between *FoxO1*^{+/+} and *FoxO1*^{-/-} ventricular myocytes (APD₅₀: 3.7 ± 0.6 ms and 5.3 ± 0.9 ms; APD₉₀: 13.8 ± 1.6 ms and 16.0 ± 1.9 ms; n=18 and 12 in *FoxO1*^{-/-} and *FoxO1*^{+/+} ventricular myocytes,

respectively). These findings suggest that *FoxO1* deletion induces an increase in $\text{Na}_v1.5$ expression and Na^+ channel activity, which leads to faster depolarization of action potentials.

3.10. QRS shortening in mice with cardiac deletion of *FoxO1*

Electrocardiogram (ECG) recording was performed on conscious and unrestrained mice (Fig. 7A). No arrhythmias were observed in the mice with cardiac deletion of *FoxO1* or controls without this deletion. Although the PR-, QTc-, and RR-intervals were not prolonged in the mice with cardiac deletion of *FoxO1*, the QRS complex and P wave were markedly different from the *FoxO1*^{+/+} mice. The QRS complex was significantly shortened ($p < 0.05$) to 9.2 ± 0.3 ms ($n=5$) in mice with cardiac deletion of *FoxO1* from 10.2 ± 0.3 ms ($n=5$) in the control group (Fig. 7B); the P wave amplitude was significantly ($p < 0.05$) increased to 0.17 ± 0.01 mV ($n=5$) in mice with cardiac deletion of *FoxO1* from 0.13 ± 0.01 mV ($n=5$) in the control group.

3.11. Inverse relationship of nuclear FoxO1 to $\text{Na}_v1.5$ expression in ischemic hearts confirmed that $\text{Na}_v1.5$ is *in vivo* FoxO1 target during oxidative stress

In order to investigate the potential role of FoxO1 in the regulation of $\text{Na}_v1.5$ expression in the IHD, immunohistochemical staining was performed on ventricular tissue from human hearts with IHD to show more nuclear FoxO1 in cardiomyocytes from human IHD ($n=3$) than that from NFH ($n=3$) (Fig. 8A). Immunohistochemical staining was performed on sections taken from mouse hearts and no FoxO1 signaling was detected in our hands. RT-PCR was performed on the RNA extracts, and Western blot was done on the protein extracts from the peri-infarct (border zone) region of the mouse hearts (which was defined as the 2-mm area encircling the area of pathologic infarction) and ventricular tissue from the sham-control group. Both $\text{Na}_v1.5$ protein and mRNA levels were significantly ($p < 0.05$ or $p < 0.01$) decreased in the peri-infarct region ($n=3$), compared to those in the ventricular tissue from the control group ($n=3$) (Fig. 8B).

4. Discussion

This study uncovered that cardiac-specific deletion of *FoxO1* in adult mice enhances voltage-gated Na^+ channel activity by increasing $\text{Na}_v1.5$ and β_3 subunit expression, increases the speed of Na^+ channel inactivation, and increases the recovery of Na^+ channel from inactivation. This increase of Na^+ channel activity leads to an acceleration of cardiac depolarization as evidenced by the larger V_{max} of action potentials recorded from *FoxO1*^{-/-} ventricular myocytes as well as the shortening of the QRS complex and the increase in P wave amplitude in surface ECGs recorded from conscious and unrestrained mice with cardiac deletion of *FoxO1*. Cardiac function was not changed by *FoxO1* deletion. These *in vivo* findings support the conclusions from our previous *in vitro* studies that FoxO1 negatively regulates Na^+ channel activity by altering $\text{Na}_v1.5$ expression in HL-1 cardiomyocytes [27]. Furthermore, these studies showed that FoxO1 binds to the insulin response elements in the promoter of *SCN5a* and suppressed the promoter's activity, leading to the inhibition of $\text{Na}_v1.5$ expression [27]. In addition, our studies showed that accumulation of FoxO1 in the cardiomyocyte nuclei of human IHD and decrease of $\text{Na}_v1.5$

expression in the peri-infarct (border-zone) of mouse hearts with myocardial infarction. This further indicates that Nav1.5 is *in vivo* FoxO1 target during oxidative stress.

It has been reported that cardiac-specific overexpression of the *SCN5a* gene did not significantly increase the surface density of the Na⁺ channels in ventricular myocytes, indicating that Nav1.5 localization to the membrane and formation of functional channels are limited [30]. Na⁺ currents were increased in *FoxO1*^{-/-} ventricular myocytes, but our finding that *FoxO1* deletion led to a significant increase in Nav1.5 expression may not be able to explain the enhancement of the Na⁺ channel activity. It has been shown that the β3 subunit can increase Nav1.5 membrane expression and alter the channel kinetics in oocytes [31]. The subsequent promotion of Na⁺ channel β3 subunit expression following *FoxO1* deletion contributes to an increase in the Nav1.5 surface expression and densities of the functional cardiac Na⁺ channel. The findings are most consistent with the decrease in I_{Na} observed in cardiomyocytes of the β3 knockout mouse [32]. The acceleration of inactivation and the recovery from inactivation of the Na⁺ channel is likely due to increased β3 subunit expression, although steady-state activation and inactivation of this channel were not changed in *FoxO1*^{-/-} [31]. β3 regulating Nav1.5 may have similar mechanisms by which β3 increases both expression of the core-glycosylated form of Nav1.7 and Na⁺ channel activity [33].

Nav1.5, encoded by the *SCN5a* gene, is the main α subunit of the cardiac Na⁺ channel and its activity determines cardiac excitability and electrical conduction. The importance of the cardiac Na⁺ channel for normal cardiac electrical activity is emphasized by the occurrence of potentially lethal arrhythmias in the setting of inherited and acquired functional defects. Other extensive studies have been more focused on a large number of protein relationships, including β subunits, ankyrin-G, and MOG1 interactions with Nav1.5 [34] or concerned with post-translational modifications, such as the effects of phosphorylation, glycosylation, S-nitrosylation, ubiquitination, and methylation on the function of the Nav1.5 channel [35]. Knowledge of the mechanisms behind the regulation of Nav1.5 expression at the transcriptional level is largely limited. Currently, it has been shown that four transcriptional NF-KappaB and TBX5 directly affect the *SCN5a* promoter activity and alter Nav1.5 expression [12, 24]. *In vivo* studies showed that TBX5 is only expressed in the mouse cardiac conducting cells and that specific deletion of TBX5 in these cells led to a severe reduction in cardiac conduction [24]. Accumulating evidence suggests that FoxO1 is the main isoform among FoxOs in the heart [26] and that it plays an important role in the regulation of cardiac function under different stresses [36]. It typically functions as a transcription activator and binds to the DNA promoter consensus sequences of target genes to upregulate their expression in response to cardiac stresses [36]. Both our *in vitro* [27] and *in vivo* studies are the first to show that FoxO1 negatively regulates Nav1.5 expression. These findings strongly suggest that FoxO1 functions as a suppressor of *SCN5a* gene transcription. The mechanism of β3 subunit regulation by FoxO1 is unclear. FoxO proteins target a conserved DNA binding sequence, 5'-GTAAA(C/T)A-3', or insulin response element (IRE), 5'-CAAAA(C/T)A-3' in the promoters of their target genes to affect these gene expressions. Analysis of the β3 promoter revealed that there are IREs, CAAA(C/T)A, in mice and humans. It is possible that FoxO1 regulates β3 expression by affecting the

promoter activity. Additional studies including promoter activity and chromatin immunoprecipitation assays will be pursued to reach a definite conclusion in the future.

FoxO1 is a transcriptional factor and its nuclear localization is important for its transcriptional activity. The nuclear localization is determined by the status of FoxO1 phosphorylation [36] and acetylation [37]. Any physiological and pathological event may alter the FoxO1 nuclear localization, subsequently regulating $\text{Na}_V1.5$ expression. Previous studies showed that FoxO proteins were phosphorylated in thoracic aortic banding, induced cardiac hypertrophy, and by several hypertrophic ligands, and their transcriptional activity was changed through a mechanism involving the PI3K/Akt pathway [29]. Consistent with these findings, we previously reported that increased Na^+ current density was associated with higher $\text{Na}_V1.5$ protein levels and increased FoxO1 phosphorylation in mouse left ventricular hypertrophy and failure induced by transverse aortic constriction. Akt has been reported to phosphorylate FoxO1 at threonine 24, serine 256 and serine 319 and makes FoxO1 less localized in the nuclei [38]. This process potentially involves less FoxO1's suppressive effects on $\text{Na}_V1.5$ expression in mouse cardiac hypertrophy and failure [39].

Epidemiologic data indicate that ischemic heart disease (IHD), a leading cause of death worldwide, is responsible for 80% of fatal arrhythmias and includes conditions such as ventricular tachycardia and ventricular fibrillation [40-43]. Current therapies for cardiac arrhythmias include pharmacologic intervention, A-V node ablation, and electronic devices, which target ion channels with varying degrees of success and sometimes with severe side effects. It is crucial to identify the signaling pathways involved in cardiac ion channel regulations for IHD. Our studies revealed that FoxO1 is significantly increased in the nuclei of human cardiomyocytes during chronic ischemic cardiomyopathy and that both mRNA and protein levels of $\text{Na}_V1.5$ are significantly decreased in the peri-infarct (border-zone) of mouse ventricles. Elevated levels of reactive oxygen species (ROS) in IHD [44-46] induced the nuclear localization of FoxO1 in both HL-1 [27] and rat neonatal cardiomyocytes [26] potentially through acetylation [37] and phosphorylation at serine 212 by mammalian sterile 20-like kinase 1 [47] and suppresses $\text{Na}_V1.5$ expression through FoxO1 mediation of the *SCN5a* promoter activity [27]. Our findings indicate that FoxO1 plays an important role in the regulation of $\text{Na}_V1.5$ expression in IHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
LQTS	Long QT syndromes
LVPWs	Systolic left ventricular posterior wall thickness
LVPWd	Diastolic left ventricular posterior wall thickness
LVIDs	Systolic left ventricular internal diameter
LVIDd	Diastolic left ventricular internal diameter
EF	Ejection fraction
FS	Fraction shortening
APD	Action potential duration
IHD	Ischemic heart disease
ROS	Reactive oxygen species

Research highlights

- ➡ Cardiac deletion of FoxO1 increased Na_V1.5 and β3 subunit expression.
- ➡ Cardiac deletion of FoxO1 enhanced Na⁺ channel activity.
- ➡ Cardiac deletion of FoxO1 accelerated the cardiac depolarization.
- ➡ Cardiac deletion of FoxO1 led to the shortening of QRS wave and increasing of P wave amplitude.
- ➡ FoxO1 plays an important role in the regulation of Na⁺ channel expression in both physiological and pathological conditions.

Fig. 1. No cardiac hypertrophy and dysfunction were observed in *FoxO1*^{-/-} mouse hearts
 (A) Heart/body weight ratio was not significantly different between *FoxO1*^{+/+} and *FoxO1*^{-/-} mice. (B) Capacitances of ventricular myocytes were not significantly changed by cardiac deletion of *FoxO1*. (C) Echocardiographic M-mode images of *FoxO1*^{+/+} and *FoxO1*^{-/-} left ventricles. Cardiac function was not altered by *FoxO1* deletion (see parameters in Supplemental table 2).

Fig. 2. *FoxO1*^{-/-} induced increases of cardiac Nav1.5 protein and mRNA levels and β3 mRNA level

(A) Western blot showed that Nav_v1.5 protein was more expressed while FoxO1 was less expressed in *FoxO1*^{-/-} mouse ventricles as compared with *FoxO1*^{+/+} control group. (B) Quantitative analyses of Nav_v1.5, β3 and FoxO1 relative to GAPDH revealed that both Nav_v1.5 and β3 were significantly increased ($p < 0.01$ or $p < 0.05$) increased and FoxO1 was significantly ($p < 0.01$) decreased in *FoxO1*^{-/-} in comparison with *FoxO1*^{+/+} ventricles ($n = 3$ for each group). (C) RT-PCR was performed on the RNA extracts of mouse ventricles and the results showed that mRNA levels of Nav_v1.5 and β3 subunits but not β1, β2 and β4, and Cx43 were increased in *FoxO1*^{-/-} ventricles compared with *FoxO1*^{+/+} group. Quantitative analyses of Nav_v1.5, β subunits, and Cx43 mRNA levels relative to GAPDH revealed that Nav_v1.5 and β3 subunit were significantly increased ($p < 0.01$).

Fig. 3. I_{Na} was increased in *FoxO1*^{-/-} mouse ventricular myocytes

(A) Typical whole cell Na^+ currents were recorded during 80 ms depolarizing voltage steps to potentials between -90 and +15 mV from a holding potential of -100 mV in left ventricular myocytes isolated from *FoxO1*^{+/+} and *FoxO1*^{-/-} mouse hearts (n=18 and n=26, respectively). (B) The current-voltage relationship curve of Na^+ currents showed that the current densities obtained from the peak currents divided by individual cell capacitances were significantly increased ($p < 0.05$) from -35 mV to -5 mV in *FoxO1*^{-/-} as compared with *FoxO1*^{+/+} ventricular myocytes. No voltage-current relationship shift was observed between these two groups.

Fig. 4. Faster inactivation of Na^+ channel in $\text{FoxO1}^{-/-}$ ventricular myocytes

(A) Typical Na^+ current traces recorded from $\text{FoxO1}^{+/+}$ and $\text{FoxO1}^{-/-}$ ventricular myocytes at -30 mV from a holding potential of -100 mV. A monoexponential function was used to fit the activation and inactivation phases of I_{Na} from -40 mV to -10 mV and the activation and inactivation time constants were obtained. They were plotted with the different voltage levels. (B) $\tau_{\text{activation}}$ were not significantly different between $\text{FoxO1}^{+/+}$ and $\text{FoxO1}^{-/-}$ ventricular myocytes from -40 mV to -10 mV. (C) $\tau_{\text{inactivation}}$ of decay traces of I_{Na} were significantly decreased ($p < 0.01$ or $p < 0.05$) in $\text{FoxO1}^{-/-}$ in comparison with $\text{FoxO1}^{+/+}$ ventricular myocytes.

Fig. 5. *FoxO1*^{-/-} did not affect the voltage-dependent and steady-state activation and inactivation of Na⁺ channel but accelerated this channel recovery from inactivation

(A, B) Voltage-dependent and steady-state activation and inactivation of Na⁺ channel were not significantly different between *FoxO1*^{+/+} and *FoxO1*^{-/-} ventricular myocytes. (C) Representative *I*_{Na} recovery traces were recorded in left ventricular myocytes by using the stimulation protocol shown in the inset from *FoxO1*^{+/+} and *FoxO1*^{-/-} ventricular myocytes, respectively. Mean ± SEM normalized recovery data for peak *I*_{Na} were plotted and they are well described by a single exponential.

Fig. 6. Increased depolarization in *FoxO1*^{-/-} ventricular myocytes

(A) Representative action potentials were recorded from *FoxO1*^{+/+} and *FoxO1*^{-/-} left ventricular myocytes, respectively and the rising phase was faster in *FoxO1*^{-/-} cardiomyocytes as compared with *FoxO1*^{+/+} cells. (B) The amplitude of action potentials, resting membrane potential and duration of action potentials (APD₅₀ and APD₉₀) were not significantly different but the maximum upstroke velocity of the action potential (V_{max}) was significantly increased ($p < 0.05$) in *FoxO1*^{-/-} than in *FoxO1*^{+/+} ventricular myocytes.

Fig. 7. *FoxO1*^{-/-} led to shortening of QRS complex and increasing of P wave amplitude
Representative surface ECGs were recorded from conscious and unrestraint mice with or without cardiac-specific deletion of *FoxO1* in Lead II. (A) QRS complex was shortened and P wave amplitude was increased in *FoxO1*^{-/-} mice. (B) Surface ECG parameters, heart rate, QRS duration, P wave amplitude, QTc-, PR-, and RR-intervals were analyzed. The data showed that QRS duration was significantly ($p < 0.05$) decreased and P wave amplitude was significantly ($p < 0.05$) increased in *FoxO1*^{-/-} mice, compared with those in *FoxO1*^{+/+} mice but heart rate, QTc-, PR-, and RR- intervals were not changed.

Fig. 8. Inverse relationship of nuclear FoxO1 to Nav1.5 expression in ischemic hearts
 (A) Immunohistochemical staining revealed that FoxO1 accumulated in the cardiomyocyte nuclei in the human failing hearts with ischemic heart disease (IHD) (n=3) in comparison with non-failing controls without a history of heart diseases (NFH) (n=3). (B) Nav1.5 expression was decreased in the peri-infarct (border-zone) of mouse hearts with myocardial infarction. (a) RT-PCR analysis showed that mRNA level of Nav1.5 was significantly ($p<0.01$) decreased; (b) Western blot exhibited that protein level of Nav1.5 was significantly ($p<0.05$) downregulated as well.