Reactive Oxygen Species Suppress Cardiac Na\textsubscript{v}1.5 Expression through Foxo1

Weike Mao\textsuperscript{1}*, Tao You\textsuperscript{1,3}, Bo Ye\textsuperscript{1}, Xiang Li\textsuperscript{1}, Henry H. Dong\textsuperscript{4}, Joseph A. Hill\textsuperscript{5}, Fiqian Li\textsuperscript{1}, Haodong Xu\textsuperscript{1,2a}

\textsuperscript{1} Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, New York, United States of America, \textsuperscript{2} Aba Cardiovascular Research Institute, University of Rochester Medical Center, Rochester, New York, United States of America, \textsuperscript{3} Division of Cardiology, Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu, People’s Republic of China, \textsuperscript{4} Division of Immunogenetics, Rangoes Research Center, Children’s Hospital of Pittsburgh of UPMC, Pittsburgh, Pennsylvania, United States of America, \textsuperscript{5} Division of Cardiology, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America

Abstract

Na\textsubscript{v}1.5 is a cardiac voltage-gated Na\textsuperscript{+} channel subunit and is encoded by the SCN5a gene. The activity of this channel determines cardiac depolarization and electrical conduction. Channel defects, including mutations and decrease of channel protein levels, have been linked to the development of cardiac arrhythmias. The molecular mechanisms underlying the regulation of Na\textsubscript{v}1.5 expression are largely unknown. Forkhead box O (Foxo) proteins are transcriptional factors that bind the consensus DNA sequences in their target gene promoters and regulate the expression of these genes. Comparative analysis revealed conserved DNA sequences, 5'-CAAAACA-3' (insulin responsive element, IRE), in rat, mouse and human SCN5a promoters with the latter two containing two overlapping Foxo protein binding IREs, 5'-CAAAACAAAAAACA-3'. This finding led us to hypothesize that Foxo1 regulates Na\textsubscript{v}1.5 expression by directly binding the SCN5a promoter and affecting its transcriptional activity. In the present study, we determined whether Foxo1 regulates Na\textsubscript{v}1.5 expression at the transcriptional level and also defined the role of Foxo1 in hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-mediated Na\textsubscript{v}1.5 suppression in HL-1 cardiomyocytes using chromatin immunoprecipitation (ChIP), constitutively nuclear Foxo1 expression, and RNAi Foxo1 knockdown as well as whole cell voltage-clamp recordings. ChIP with anti-Foxo1 antibody and follow-up semi-quantitative PCR with primers flanking Foxo1 binding sites in the proximal SCN5a promoter region clearly demonstrated enrichment of DNA, confirming Foxo1 recruitment to this consensus sequence. Foxo1 mutant (T24A/S319A-GFP, Foxo1-AA-GFP) was retained in nuclei, leading to a decrease of Na\textsubscript{v}1.5 expression and Na\textsuperscript{+} current, while silencing of Foxo1 expression by RNAi resulted in the augmentation of Na\textsubscript{v}1.5 expression. H\textsubscript{2}O\textsubscript{2} significantly reduced Na\textsubscript{v}1.5 expression by promoting Foxo1 nuclear localization and this reduction was prevented by RNAi silencing Foxo1 expression. These studies indicate that Foxo1 negatively regulates Na\textsubscript{v}1.5 expression in cardiomyocytes and reactive oxygen species suppress Na\textsubscript{v}1.5 expression through Foxo1.

Introduction

Alterations of ion channel activity play an important role in the development of cardiac arrhythmias. Na\textsubscript{v}1.5 encoded by the SCN5a gene is a subunit of the voltage-gated cardiac Na\textsuperscript{+} channel, and this channel activity determines cardiac excitability and conduction. Reactive oxygen species (ROS) are generated during myocardial infarction [1–4] and contribute to the development of life-threatening ventricular arrhythmias [5,6]. It has been shown that alteration of cardiac Na\textsuperscript{+} channel activity immediately following ROS challenge requires activation of Ca\textsuperscript{2+}-dependent calmodulin II kinase (CaMKII) [7]. CaMKII regulates Na\textsuperscript{+} channel gating by phosphorylating Na\textsubscript{v}1.5 to increase late Na\textsuperscript{+} current, leading to the prolongation of the action potential duration and development of early afterdepolarization [8]. H\textsubscript{2}O\textsubscript{2}-chronically mediates downregulation of Na\textsuperscript{+} channel activity requires activation of NF-KappaB to decrease Na\textsubscript{v}1.5 expression by inhibiting SCN5a promoter activity [9]. Mutation of the NF-KappaB binding site in the SCN5a promoter eliminates 42.3% decrease of this promoter activity by H\textsubscript{2}O\textsubscript{2} in H9C2 myoblasts [9]. These findings indirectly support the notion that other signaling pathways are also significantly involved in the regulation of Na\textsubscript{v}1.5 expression.

Foxo transcription factors form a subclass of the large family of Forkhead proteins characterized by the presence of a “winged-helix” DNA-binding domain called Forkhead box O, which gave the name Foxo proteins to the family [10]. Post-translational modifications such as phosphorylation, acetylation and methyla-
tion affect Foxo subcellular localization and transcriptional activity [11–13]. Foxo proteins harbor conserved threonine and serines that can be phosphorylated by different kinases, leading to their nuclear retention or exclusion [11]. Akt/protein kinase B (PKB) [14,15], serum and glucocorticoid-induced kinase 1 (SGK1) [16], cyclin-dependent kinase 2 (CDK2) [17] and IKB kinase (IKK) [18] phosphorylate Foxo proteins, resulting in their nuclear exclusion while c-Jun-N-terminal kinase (JNK) [19] and mammalian sterile 20-like kinase 1 (MST1) [20] phosphorylate Foxo proteins, causing their nuclear retention. Foxo proteins also contain acetylated lysines that can be deacetylated by a NAD-dependent histone deacetylase, silent mating type information regulation 2 homolog (Sirt1), leading to their nuclear retention [12]. Protein arginine methyltransferase 1 (PRMT1) methylates Foxo1 primarily at Arg248 and Arg250 within the consensus Akt phosphorylation motif. These methylations directly abrogate Akt-induced phosphorylation of Foxo1 at Ser253, which in turn results in increased Foxo1 nuclear localization [13]. Accumulating evidence suggests that Foxo1, 3 and 4 are expressed in the heart and they are critical in maintaining cardiac function and mediating cardiac stresses. In the heart, the Foxo family is believed to be involved in diverse activities, including response to oxidative stress [21–23], regulation of metabolism [24], modulation of KATP channels [25], promotion of autophagy [26], cell cycle control [27,28], and commitment to apoptosis [29]. In the nucleus, Foxo proteins target a conserved DNA binding sequence, 5′-GTAAA(C/T)A-3′ [30] or insulin response element (IRE), 5′-CAAAA(C/T)A-3′ [30–32] in the promoters of their target genes to affect these gene expression. The end result of Foxo transcriptional activity is generally associated with countering oxidative stress and promoting cell cycle arrest and apoptosis [11]. Silico promoter analysis on a list of 354 of the most differentially expressed genes in the thymocyte lineage predicted that the promoter of SCN5a has Foxo binding elements [33].

In this study, we tested the hypothesis that H2O2 suppressing NaV1.5 requires the Foxo1 signaling pathway. Our results showed that Foxo1 negatively regulated NaV1.5 expression by directly binding the SCN5a promoter, and H2O2-mediated inhibition of NaV1.5 expression required Foxo1.

Materials and Methods

Ethics

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University Committee on Animal Resources (UCAR) of University of Rochester Medical Center (UCAR # 2007-060).

Cell Culture

HeLa cells (obtained from American Type Culture Collection) were maintained in 10 cm plates containing Dulbecco’s modified Eagle’s medium (high glucose) and 10% fetal bovine serum with 100 U/ml penicillin and 100 μg/ml streptomycin. A cardiomyocyte cell line, designated HL-1, from the AT-1 mouse atrial cardiomyocyte tumor lineage, was kindly provided by Dr. William C. Claycomb at Louisiana State University Health Sciences Center, New Orleans, USA. These cells were cultured as described previously [34]. The cells were cultured in Claycomb medium [JRH Biosciences, KS] supplemented with 10% fetal bovine serum [JRH Biosciences, KS], 2 mM L-glutamine, 100 μM norepinephrine, 100 U/ml penicillin and 100 μg/ml streptomycin in 10 cm plates pre-coated with fibronectin (BD Biosciences, PA). The medium was changed every 24–48 h. Mouse ventricular myocytes isolated from 10 week old FVB mice as previously described [35] were directly subjected to a chromatin immunoprecipitation (ChIP) assay.

Bioinformatics

The SCN5a genomic promoter sequences from mouse, rat, and human were downloaded from the University of California Santa Cruz genome browser [36] and comparative genomic analysis was done with the visualization tools for alignments (VISTA) algorithm (available from the VISTA web site). The Foxo protein binding sequences were compared.

Transfection and Luciferase Assays

Lipofectamine™ and plus™ reagents (Invitrogen) were used to transfect SV40 reporters (SCN5a promoter-Luc plasmid [37] generously provided by Dr. Hideko Kasahara in University of Florida College of Medicine, Florida, USA) and Foxo1 in the HeLa cells. Cells were seeded in 24-well plates and grown to 90% confluence. Transfections were performed according to the manufacturer’s instructions. A Renilla reporter gene (Promega) was included as an internal control. Cell lysates were prepared for the luciferase assay 24 hours after transfection as described by the manufacturer (Promega). Data were analyzed and expressed as the normalized-fold changes over controls.

RT-PCR

Total RNA was extracted from HL-1 and HeLa cells by RNeasy Mini kit (QIAGEN) and reversely transcribed to cDNA with a high capacity cDNA reverse transcription kit (AB Applied Biosystems). Semi-quantitative PCR was conducted to measure mRNA expression levels across different experimental conditions as indicated under “Results”. The primers used to amplify mouse(m) NaV1.5, mβ-actin, human(h) GAPDH, mGAPDH, hFoxo1, and mTR4 were summarized in table 1.

Western Blotting

HL-1 cells were rinsed with phosphate-buffered saline (PBS) twice, and protein was extracted in cold cell lysis buffer (Cell Signaling). Protein concentration was determined by a detergent-compatible protein assay (Bio-Rad). Equal amounts of protein were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, blocked with 5% non-fat milk for 1 hour, and then incubated with the indicated primary antibody overnight at 4°C. After 1 hour incubation with the appropriate secondary antibody, specific signals were revealed by enhanced chemiluminescence reagent (Pierce). The primary antibodies used were as follows: rabbit anti-Foxo1 polyclonal (Cell Signaling, 1:1000), rabbit anti-NaV1.5 polyclonal (Alomone Labs, 1:500), rabbit anti-GAPDH polyclonal (Millipore, 1:4000), and mouse anti-tubulin monoclonal (Sigma, 1:5000).

Fluorescence and Confocal Microscopy

HL-1 cells were dispersed on the glass in the center of the 32 mm dishes and grown to 80% confluence. HL-1 cells expressing GFP and Foxo1-GFP were fixed with 4% paraformaldehyde. Nuclear profiles of were revealed with a brief incubation in DAPI (Molecular Probes) prior to microscopic observation. Fluorescence was visualized with an inverted Olympus IX51 fluorescence confocal microscope and photographed for direct import into Image J. All images were processed in an identical manner to faithfully capture the real time images of each sample.
Table 1. Summary of primers for RT-PCR.

| Forward Primer | Reverse Primer |
|----------------|----------------|
| m1Na\(_{v,1.5}\) | 5'-AGGCGCTCTTAAAGTGACACCAGCA-3' |
| m2Na\(_{v,1.5}\) | 5'-GAGGCTTCTGTGCTGATGCTAACA-3' |
| mfl-actin      | 5'-AGGCGCTCTTAAAGTGACACCAGCA-3' |
| hGAPDH         | 5'-GAGGCTTCTGTGCTGATGCTAACA-3' |
| mGAPDH         | 5'-AGGCGCTCTTAAAGTGACACCAGCA-3' |
| hFoxo1         | 5'-GAGGCTTCTGTGCTGATGCTAACA-3' |
| mTR4           | 5'-GAGGCTTCTGTGCTGATGCTAACA-3' |

Note: m, mouse; h, human.

Adenovirus Vectors

The adenoviral vectors used were as follows: Adv–CMV-FoxO1-AA-GFP expressing constitutively nuclear FoxO1-AA-GFP and Adv–CMV-GFP expressing GFP. Adv–FoxO1-RNAi vector was constructed based on the Block-iT Adenoviral RNAi Expression System (Invitrogen). This FoxO1-RNAi vector encodes a 19-bp DNA (5'-CGGCCAGGTTGTTTGAGAAGC-3') that is complementary to the Foxo1 mRNA sequence (5'-GGGTCGTGTTGTGCCATGAACACA-3') (Adv-scramble-RNAi) under the control of the mouse U6 promoter. Likewise, a control adenovirus containing the scrambled RNAi (5'-GGGTCGTGTTGTGCCATGAACACA-3') (Adv-scramble-RNAi) under the control of mouse U6 promoter was constructed.

ChIP Assay

ChIP was used to study the interaction between Foxo1 and SCN5a promoter DNA in HL-1 cells, as previously described [38]. Approximately 1×10⁷ HL-1 cells were subjected to the ChIP assay using anti-Foxo1 antibody or rabbit control IgG as a control and the ChIP assay kit (Upstate Biotechnology), as previously described [38]. The immunoprecipitates were analyzed by immunoblot analysis using goat anti-Foxo1 (FKHR-C20; Santa Cruz Biotechnology Inc.), and by PCR assay to detect communoprecipitated DNA using two pairs of SCN5a promoter–specific primers (forward 5'-GGGTGACATTTTGCTGACAGCC-3' and reverse 5'-GCGACATCGATCGATCAGC-3') and reverse 5'-GCGACATCGATCGATCAGC-3') and reverse 5'-GCGACATCGATCGATCAGC-3').

Whole-cell Voltage Clamp Recording

Na⁺ currents were recorded from HL-cells using whole-cell patch-clamp techniques at room temperature (22–24°C). The bath solution for recording membrane currents contained (in mM): 145 NaCl, 4.5 CaCl₂, 1.5 MgCl₂, 1 CaCl₂, 5 HEPES, 0.1 CdCl₂ (pH 7.35 with CsOH); the pipette solution contained (in mM): 130 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES (pH 7.35 with CsOH). Electrode resistance ranged from 2.0 to 3.0 MΩ. Data acquisition was carried out using an Axopatch 200B amplifier and pClampex 9.6 software (Axon Instruments Inc., Foster, CA). Currents were acquired at 20–50 kHz (Digidata 1200 A/D converter; Axon Instruments Inc.) and low-pass-filtered at 5 kHz, and stored on a computer. In all recordings, 80% of the series resistance was compensated. Data analysis was accomplished by using pClampfit 9.6 software (Axon Instruments Inc., Foster City, CA).

Statistical Analysis

All values are expressed as Mean±SEM. Data from experimental groups were compared by student’s t-test or ANOVA one way analysis. A value of p<0.05 was considered statistically significant.

Results

The Proximal Region of SCN5a Promoter Contains Foxo1 Responsive Elements

Silico promoter analysis on a list of 354 of the most differentially expressed genes in the thymocyte lineage has revealed a putative direct target that is transcriptionally regulated by Foxo proteins in thymocytes, and that SCN5a expression is upregulated by Foxos deletion [33]. These findings suggest that transcription of SCN5a may be directly regulated by Foxos in cardiomocytes. Analysis of the SCN5a promoter region showed a highly conserved DNA sequence (5'-CAAAACA-3') as IRE in humans and rodents such as mouse and rat. The mouse and human SCN5a promoter regions harbor two overlapping Foxo1 binding IREs, 5'-CAAAACA-3' (Fig. 1A).

Foxo1 directly binds SCN5a promoter

To confirm the association of Foxo1 with the SCN5a promoter region including IREs, a ChIP assay was carried out in HL-1 cells and mouse ventricular myocytes, and it unveiled a clear enrichment of DNA amplified by PCR with two sets of primer pairs flanking Foxo1 binding IREs (5'-CAAAACA-3') in the proximal SCN5a promoter region following immunoprecipitation with Foxo1 antibody, but not with IgG control (Fig. 1B). Another pair of primers was designed to amplify a region away from Foxo1 binding site, 5'-CAAAACA-3'. As showed in Figure S1, there was no PCR product detected after chromatin immunoprecipitation with Foxo1 antibody.

Foxo1 Negatively Regulates SCN5a Promoter Activity

This conserved sequence is located from -1484 to -1473 in the mouse SCN5a promoter region. The proximal promoter region (-2308 to +119 bp) was examined in luciferase reporter assays in HeLa cells with and without expression of Foxo1 (Fig. 2A). Expression of Foxo1 significantly suppressed SCN5a promoter activity (p<0.01), and the suppression was Foxo1 dose dependent (Fig. 2B). Individual (CAAAAC to CCCAAC) or double mutations (CAAAAC to CCCAAC) in Foxo1 binding
sites significantly suppressed (p<0.01) Foxo1 responsiveness compared with wild type SCN5a, and double mutations of Foxo1 were significantly more effective (p<0.05) than mutation 1 on SCN5a promoter activity (Fig. 2C).

**Foxo1 Negatively Regulates NaV1.5 Expression in HL-cells**

HL-1 cells harbor robust, rapidly activated and inactivated Na\(^+\) currents [39], indicating that NaV1.5 is expressed in HL-1 cells. In order to test the hypothesis that Foxo1 regulates NaV1.5 expression, HL-1 cells were used in the study. The HL-1 cells were infected for 48 hours by 10 or 50 multiplicity of infection (MOI) adenoviruses (Adv)-CMV-Foxo1-AA-GFP [40] and 50 MOI Adv-CMV-GFP. Western blot analysis of the protein extracts from these infected HL-1 cells was performed, and showed that NaV1.5 protein was more significantly decreased (p<0.05 or p<0.01) in cells expressing constitutively nuclear Foxo-1-AA-GFP (AA: T24A/S319A) compared to cells expressing GFP (Fig. 3A and C). Interestingly, endogenous Foxo1 was significantly increased by overexpression of Foxo1-AA-GFP (Fig. 3A). This finding is consistent with a previous report that Foxo1 can be positively regulated by Foxo1 binding its own promoter [41]. Accordingly, NaV1.5 mRNA level determined by semi-quantitative RT-PCR analysis was also significantly decreased (p<0.01) in HL-1 cells expressing Foxo1-AA-GFP compared to HL-cells expressing GFP (Fig. 3B and D). This pair of primers (m1NaV1.5) for RT-PCR application was designed to detect two splicing variants, NaV1.5a and NaV1.5b [42]. In order to determine if Foxo1 affects the expression of these two splicing variants, the second pair of primers (m2NaV1.5) was designed to allow us to detect NaV1.5, NaV1.5a and NaV1.5b. Unfortunately, there was only one band of PCR product at 1300 bp detected, which consistently showed the inhibition of NaV1.5 expression by Foxo1-AA-GFP in HL-1 cells (Figure S2). This has prevented us from addressing if Foxo1 also affects the expression of other splicing variant(s).

**Figure 1. Foxo1 directly binds the insulin response elements in SCN5a promoter region.** A. Analysis of proximal region of the SCN5a promoter showed two overlapping conservative Foxo binding insulin response elements, 5’-CAAAAACA-3’; in rat, mouse and human. B. ChIP assay showed that Foxo1 bound SCN5a promoter region in both HL-1 cells (upper panel) and adult mouse ventricular myocytes (lower panel) using two sets of paired primers, respectively. Lane 1 and 2 were input and IgG, respectively. doi:10.1371/journal.pone.0032738.g001

**Figure 2. Foxo1 decreases SCN5a promoter activity.** A. Luciferase constructs contain proximal region of SCN5a promoter which has Foxo1 binding insulin response elements and mutations. B. Foxo1 significantly suppressed SCN5a promoter activity with dose dependence (***p<0.01, n = 3). C. Individual or double mutations of Foxo1 binding sites in SCN5a promoter region prevented Foxo1 inhibition (***p<0.01) compared with wild type SCN5a; Double mutation of Foxo1 binding sites had significantly more effective (*p<0.05) than mutation 1 (n = 6 in all individual groups). doi:10.1371/journal.pone.0032738.g002
As shown above, robust Na\textsubscript{V}1.5 expression was observed in the HL-1 cells (Fig. 3A and B). To test the hypothesis that a decrease of endogenous Foxo1 expression upregulates Na\textsubscript{V}1.5 expression, Adv–FoxO1-RNAi was used to infect HL-1 cells to express RNAi and silence Foxo1 expression (Fig. 4A). The downregulation of Foxo1 induced a significant increase in both Na\textsubscript{V}1.5 protein ($p<0.05$, at 100 MOI) (4A and C) and mRNA levels ($p<0.01$, at 100 MOI) in HL-1 cells (4B and D) when compared with those obtained from the cells infected with Adv-scramble-RNAi. This FoxO1-RNAi construct encodes a 19-bp oligonucleotide (5’-CGCCCCAGGTGGTGGAGAC-3’) that is complementary to the mouse Foxo1 mRNA sequence (10–29 nt). This sequence has two nucleotides different from that of human Foxo1 mRNA and one nucleotide at the most 5’ different from that of TR4 mRNA – encoded by Nrc2c gene. In order to determine the specificity of this RNAi, human HeLa cells expressing endogenous Foxo1 [43] and HL-1 cells were infected with Adv-Foxo-RNAi and Adv-scramble-RNAi, respectively. RT-PCR analysis showed that Foxo1 mRNA in HeLa cells and TR4 mRNA in HL-1 cells were not altered by expression of Adv-Foxo1-RNAi (Figure S3 and S4), indicating that RNAi specifically targeted mouse Foxo1 mRNA.

**Figure 3. Constitutively nuclear Foxo1 reduced Na\textsubscript{V}1.5 expression.** Western blots showed that overexpression of Foxo1-AA-GFP for 36 hours reduced Na\textsubscript{V}1.5 protein level while it increased endogenous Foxo1 expression (A); quantitation of Na\textsubscript{V}1.5 protein level relative to GAPDH revealed that expression of Foxo1-AA-GFP ($n=5$) significantly decreased (*$p<0.05$, at 10 MOI; **$p<0.01$ at 50 MOI) Na\textsubscript{V}1.5 expression compared with 50 MOI GFP ($n=5$) (C). RT-PCR showed that overexpression of 50 MOI Foxo1-AA-GFP for 36 hours decreased Na\textsubscript{V}1.5 mRNA level compared with that in cells expressing 50 MOI GFP (B) and the values of Na\textsubscript{V}1.5 mRNA relative to β-actin between GFP ($n=4$) and Foxo1-AA-GFP ($n=4$) groups were significantly different **($p<0.01$) (D).

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**H2O2 Promoting Foxo1 Nuclear Localization Reduces Na\textsubscript{V}1.5 Expression**

H\textsubscript{2}O\textsubscript{2} Promotes Foxo1 nuclear localization: HL-1 cells were infected with 50 MOI Adv-CMV-Foxo1-GFP and 50 MOI Adv-CMV-Foxo1-AA-GFP for 48 hours, respectively. Foxo1-AA-GFP was localized in the nuclei (Fig. 5B), while GFP was seen in both cytoplasm and nuclei (Fig. 5A). Recording traces of rapidly activating and inactivating Na\textsuperscript{+} currents from GFP and Foxo1-AA-GFP-expressing cells, respectively, 36 hours after adenovirus infection, are illustrated in Figure 5C and D. Na\textsuperscript{+} currents were significantly inhibited by expression of Foxo1-AA-GFP, and peak Na\textsuperscript{+} current density was significantly lower ($p<0.01$) in cells expressing Foxo1-AA-GFP (19.6±5.6 pA/pF, at $-25$ mV) than in cells expressing GFP (67.9±9.6 pA/pF, at $-25$ mV).

Foose1 Nuclear Localization Reduces Na\textsuperscript{+} Currents in HL-1 cells

To test the hypothesis that activation of Foxo1 reducing Na\textsubscript{V}1.5 expression decreases functional Na\textsuperscript{+} currents, whole-cell voltage-clamp Na\textsuperscript{+} current recordings were performed on HL-1 cells infected with 50 MOI Adv-CMV-GFP and 50 MOI Adv-CMV-Foxo1-AA-GFP for 48 hours, respectively. Foxo1-AA-GFP was localized in the nuclei (Fig. 5B), while GFP was seen in both cytoplasm and nuclei (Fig. 5A). Recording traces of rapidly activating and inactivating Na\textsuperscript{+} currents from GFP and Foxo1-AA-GFP-expressing cells, respectively, 36 hours after adenovirus infection, are illustrated in Figure 5C and D. Na\textsuperscript{+} currents were significantly inhibited by expression of Foxo1-AA-GFP, and peak Na\textsuperscript{+} current density was significantly lower ($p<0.01$) in cells expressing Foxo1-AA-GFP (19.6±5.6 pA/pF, at $-25$ mV) than in cells expressing GFP (67.9±9.6 pA/pF, at $-25$ mV).

H\textsubscript{2}O\textsubscript{2} inhibiting Na\textsubscript{V}1.5 expression is through the Foxo1 signaling pathway: Increase of Foxo1 nuclear localization by H\textsubscript{2}O\textsubscript{2} is expected to reduce Na\textsubscript{V}1.5 expression. In order to confirm this assumption, RNA and protein were extracted from HL-1 cells treated with 25 μM H\textsubscript{2}O\textsubscript{2} for 48 hours. Analyses of RT-PCR products and Western blot results showed that both Na\textsubscript{V}1.5 protein and mRNA levels were significantly decreased ($p<0.05$).
**Figure 4. Silencing expression of Foxo1 increases Na$_{\text{v}}$1.5 expression.** Western blots showed decrease of Foxo1 protein by 50 MOI (n = 4) and 100 MOI Adv-Foxo1 RNAi (n = 4) increased Na$_{\text{v}}$1.5 protein in HL-1 cells in comparison with 100 MOI Adv-scramble RNAi (n = 4) (A) and this inhibition reached the significant difference (*p < 0.05) at 100 MOI Adv-Foxo1-RNAi (C). RT-PCR showed that Na$_{\text{v}}$1.5 mRNA was increased by 50 MOI Foxo1-RNAi (n = 4) in comparison with that in cells expression scramble RNAi (n = 4) (B). The values of Na$_{\text{v}}$1.5 mRNA relative ß-actin between Foxo1-RNAi and scramble RNAi groups were significant different (***p < 0.005) (D).

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**Figure 5. Foxo1 inhibits Na$^+$ channel activity.** Foxo1-AA-GFP was localized in HL-1 cardiomyocyte nuclei (B) while GFP was seen in both nuclei and cytoplasm (A). Whole cell Na$^+$ currents were recorded during 80 ms depolarizing voltage steps to potentials between −60 and +60 mV from a holding potential of −100 mV in HL-1 cells expressing GFP or Foxo1-AA-GFP for 36 hours. The typical whole cell recording traces showed that robust Na$^+$ currents were present in HL-1 cells expressing GFP (C) while remarkable reduction of Na$^+$ currents in these cells expressing Foxo1-AA-GFP (D).

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Discussion

Foxo1 negatively regulates Na\textsubscript{v}1.5 expression

Foxo1 is known to function primarily as a transcription factor. The results presented here demonstrate that Foxo1 directly binds the IRE in the \textit{SCN5a} promoter region and inhibits its promoter activity. This inhibition leads to downregulation of Na\textsubscript{v}1.5 expression and a decrease of cardiac Na\textsuperscript{+} channel activity. In the nucleus, Foxo proteins bind a conserved DNA binding sequence, 5'GTAAA(C/T)A-3' [30] or IRE, 5'-CAAAA(C/T)A-3' [30–32] in the promoter regions of the targeted genes. Foxo recruitment to the promoter regions usually positively regulates expression of the target genes. For example, Foxo1 bound the consensus sequence in the Kir 6.1 gene promoter and increased the expression of KATP subunit Kir6.1 [25]. In the literature, there is one report in which the authors identified Foxo1 as a corepressor for androgen receptor (AR) [44]. Some corepressors possess intrinsic histone deacetylases (HDAC) activity and some repress gene expression through recruitment of HDAC proteins [45]. It has been shown that HDAC3, but not HDAC1 and HDAC2 associate with Foxo1, enhancing Foxo1-induced inhibition of AR function in LNCaP cells [46]. Whether Foxo1 recruits HADC3 to inhibit the \textit{SCN5a} promoter activity remains undetermined.

\textbf{H}\textsubscript{2}O\textsubscript{2} suppressing Na\textsubscript{v}1.5 expression requires Foxo1

ROS have been shown to acutely enhance late Na\textsuperscript{+} currents, leading to action potential prolongation and early afterdepolarizations [47–49] via activating CamKII [8,7] and phosphorylating Na\textsubscript{v}1.5. ROS have also been reported to acutely reduce fast transient outward K\textsuperscript{+} current, leading to the prolongation of cardiac repolarization by phosphorylating Kv4.3 [50]. The chronic effects of H\textsubscript{2}O\textsubscript{2} on Na\textsubscript{v}1.5 expression were also well studied [9]. Na\textsubscript{v}1.5 expression was suppressed by H\textsubscript{2}O\textsubscript{2} activating NF-KappaB and partially inhibiting \textit{SCN5a} promoter activity [9]. These findings indicate that other mechanisms are also involved in the regulation of Na\textsubscript{v}1.5 expression at the transcriptional level. As reflected in our studies, H\textsubscript{2}O\textsubscript{2} reduced Na\textsubscript{v}1.5 expression by promoting Foxo1 nuclear localization, and Foxo1 signaling is required for H\textsubscript{2}O\textsubscript{2} to affect Na\textsubscript{v}1.5 expression. ROS modulate Foxo1 subcellular localization through different pathways. H\textsubscript{2}O\textsubscript{2} inhibits phosphorylation of Foxo1 at Ser-253 and The-24 by reducing Akt kinase activity in response to insulin [12]. Under oxidative stress, the activated JNK pathway decreases the activity of Akt in HIT cells, leading to the decrease of phosphorylation of Foxo1 and promoting its nuclear localization [51]. Oxidative stress also augments PRMT1-induced Foxo1 methylolation, thereby leading to the inhibition of its phosphorylation by Akt and promotion of its nuclear localization [13]. Sirt1 has been reported to be activated by H\textsubscript{2}O\textsubscript{2} and the subsequently activated Sirt1 deacetylates and entraps Foxo1 in the nucleus, which is also independent of H\textsubscript{2}O\textsubscript{2}-induced dephosphorylation of Foxo1 [12]. It is likely that Sirt1, Akt, JNK and PRMT1 signaling pathways are involved in the regulation of Na\textsubscript{v}1.5 expression under oxidative stress. Foxo4 has been shown to interact with NF-KappaB and subsequently inhibit its transcriptional activity [52]. Whether Foxo1 suppressing Na\textsubscript{v}1.5 expression in cardiac myocytes is also partially through the NF-KappaB signaling pathway remains to be verified.

Novelty and Potential Significance

ROS including H\textsubscript{2}O\textsubscript{2} are significantly elevated in ischemic heart disease (IHD), [1–4] in which arrhythmias are frequently observed. A significant decrease in Na\textsuperscript{+} channel activity is considered to be one of mechanisms contributing to the development of arrhythmias in IHD [5,6]. The mechanisms of transcriptional regulation of Na\textsubscript{v}1.5 expression are largely uncertain. In this study, we demonstrated that Foxo1 directly targeted the \textit{SCN5a} promoter and inhibited its transcriptional activity. Furthermore, we confirmed that H\textsubscript{2}O\textsubscript{2}-mediated Na\textsubscript{v}1.5 downregulation. These findings indicate the important role of Foxo1 in cardiac ion channel regulation and in the development of arrhythmias.
Figure 7. H2O2 reduces NaV1.5 expression. Western blot analysis showed that NaV1.5 protein was significantly decreased (*p<0.05) in HL-1 cells treated with 25 μM H2O2 for 48 h hours (n = 3) compared with the control group (n = 3) (A, C). RT-PCR analysis showed that NaV1.5 mRNA significantly reduced (p*<0.05) in HL-1 cells treated with 25 μM H2O2 for 48 hours (n = 3) compared with the control group (n = 4) (B, D).

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Figure 8. H2O2-mediated downregulation of NaV1.5 requires Foxo1. Western blot analysis showed that NaV1.5 protein was significantly increased (*p<0.05) in HL-1 cells cultured in the medium with 25 μM H2O2 treatment for 48 hours after the cells expressed Foxo1-RNAi for 24 hours (n = 4) compared with scramble RNAi group (n = 4) (A, C). RT-PCR analysis showed that NaV1.5 significantly increased (**p<0.01) in HL-1 cells in the medium with 25 μM H2O2 treatment for 48 hours after the cells expressed Foxo1-RNAi for 24 hours (n = 4) compared with scramble RNAi group (n = 4) (B, D).

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Supporting Information

Figure S1 Foxo1 does not bind the SCN5a promoter region lacking the insulin responsive element. A pair of primers was designed to amplify a region far away from Foxo1 binding site, 5'-CAAAAACA-3'. There was no PCR product detected after chromatin immunoprecipitation (ChIP) by Foxo1 antibody (Lane 3). Lane 1 and 2 were input DNA and control IgG ChIP, respectively.

(TIF)

Figure S2 One isoform of NaV1.5 is detected in HL-1 cells. RT-PCR using a pair of m2NaV1.5 primers showed that there was only one DNA band representative of NaV1.5 mRNA after 40 cycles. Overexpression of 50 MOI Foxo1-AA-GFP for 36 hours decreased NaV1.5 mRNA level compared with that in cells expressing 50 MOI GFP and the amount of sample loading was equal as determined by GAPDH RT-PCR products.

(TIF)

Figure S3 RNAi targeting mouse Foxo1 mRNA does not affect human Foxo1 expression. RT-PCR using a pair of hFoxo1 primers showed that human Foxo1 mRNA expression was not altered in the HeLa cells infected with 50 MOI Adv-Foxo1-RNAi (n = 3) in comparison with the cells infected 50 MOI Adv-scramble-RNAi (n = 3) (A and B).

(TIF)

Figure S4 RNAi targeting mouse Foxo1 mRNA does not affect TR4 mRNA expression. RT-PCR using a pair of TR4 primers showed that TR4 mRNA expression was not altered in the HL-1 cells infected with Adv-Foxo1-RNAi (n = 3) in comparison with the cells infected Adv-scramble-RNAi (n = 3) (A and B).

(TIF)

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Author Contributions
Conceived and designed the experiments: HX FL WM TY. Performed the experiments: WM TY BY XI.HX. Analyzed the data: WM TY HX. Contributed reagents/materials/analysis tools: HHD JAH. Wrote the paper: WM TY FL HX.

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