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FULL PAPER

Pharmacology

A single injection of periostin decreases cardiac voltage-gated Na^+ channel in rat ventricles

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ABSTRACT. Changes in electrophysiological properties, such as ion channel expression and activity, are closely related to arrhythmogenesis during heart failure (HF). However, a causative factor for the electrical remodeling in HF has not been determined. Periostin (POSTN), a matricellular protein, is increased in heart tissues of patients with HF. In the present study, we investigated whether a single injection of POSTN affects the electrophysiological properties in rat ventricles. After male Wistar rats were intravenously injected with recombinant rat POSTN (64 µg/kg, 24 hr), electrocardiogram (ECG) was recorded. Whole-cell patch clamp was performed to measure action potential (AP) and Na^+ current (I_{Na}) in isolated ventricular myocytes. Protein expression of cardiac voltage-gated Na^+ channel ($Na_V1.5$) in isolated ventricles was examined by Western blotting. In ECG, POSTN-injection significantly increased RS height. POSTN-injection significantly delayed time to peak in AP and decreased I_{Na} in the isolated ventricular myocytes. POSTN-injection decreased $Na_V1.5$ expression in the isolated ventricles. It was confirmed that POSTN (1 µg/ml, 24 hr) decreased I_{Na} and $Na_V1.5$ protein expression in neonatal rat ventricular myocytes. This study for the first time demonstrated that a single injection of POSTN in rats decreased I_{Na} by suppressing $Na_V1.5$ expression in the ventricular myocytes, which was accompanied by a prolongation of time to peak in AP and an increase of RS height in ECG.

KEY WORDS: action potential, cardiac voltage-gated Na^+ channel, electrocardiogram, Na^+ current, periostin

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Heart failure (HF) is a clinical syndrome characterized by a disability of pump function in hearts. Changes in electrophysiological properties, such as ion channel expression and function, during HF are closely related to arrhythmogenesis [14, 17, 31]. Cardiac voltage-gated Na^+ channel ($Na_V1.5$) expression and/or its transient Na^+ current (I_{Na}), which are responsible for membrane depolarization, were decreased in heart of patients and animal models with HF [18, 26, 27, 29, 32]. However, a causative factor has not been determined.

Matricellular proteins are non-structural extracellular matrix proteins which regulate cell functions through cell-cell and cell-matrix interactions [3, 7]. Periostin (POSTN), a matricellular protein, is an approximately 90 kDa glycoprotein of fasciclin I family [2, 7, 25]. Several researches clarified that the expression level of POSTN was increased in heart tissues of patients and animal models with HF [2, 7]. Cell membrane receptors including integrins and proteoglycans, which interact with POSTN, are reported to regulate certain ion channels, such as L-type Ca^{2+} channel, K^+ channels and transient receptor potential channels [13, 15, 19]. However, effects of POSTN on expression and function of $Na_V1.5$ and related electrophysiological properties, such as action potential (AP) and electrocardiogram (ECG), in heart remain to be clarified. The present study investigated whether a single injection of POSTN induces electrical remodeling in rat ventricles.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant rat POSTN was produced by *Escherichia coli* expression system as described previously [12]. Anti-$Na_V1.5$ antibodies (#14421 for Fig. 2C, 2D; Cell Signaling Technology, Danvers, MA, USA or #sc-271255 for Fig. 3C, 3D; Santa Cruz

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Biotechnology, Santa Cruz, CA, USA) were used for Western blotting.

Animals

All animal studies were approved by Institutional Animal Care and Use Committee of Kitasato University (Approved No. 19-127, 20-014) and conducted in accordance with the guidelines of the Kitasato University. The animals were fed a standard laboratory diets and tap water and maintained in a 12 hr/12 hr light-dark cycle at 23 ± 3°C. Seven to nine-week-old male Wistar rats (Clea Japan, Tokyo, Japan) were intravenously injected with recombinant rat POSTN (64 µg/kg) or vehicle (500 mM L-arginine in phosphate buffered saline) via right jugular vein after making an incision in the neck skin under isoflurane inhalation (flow rate: 2 l/min, maintenance: 2.5–3.0%). After the skin was sutured, the rats were awakened and ECG examination was performed at 24 hr after the injection.

ECG

ECG in rat hearts was measured and recorded using FE132 Bio Amp (AD Instruments, Colorado Springs, CO, USA) and ML825 PowerLab 2/25 system (AD Instruments) as described previously [11]. Rats anesthetized with isoflurane inhalation (flow rate: 2 l/min, maintenance: 2.0–4.5%) were connected to MLA2340 3 Lead Shielded Bio Amp Cable (AD Instruments)-equipped MLA1210 Spring Clip Electrodes (AD Instruments) in lead II configuration. Heart rate (HR), RR interval, PR interval, QRS duration, QT interval, and RS height were measured by Lab Chart Pro (AD Instruments). QTc was calculated by a modified Bazett's formula [QT interval/(square root of RR/150)] [16].

Isolation of rat ventricular myocytes

Rat ventricular myocytes were isolated by enzymatic digestion as described previously [11]. Rats were anesthetized with an intraperitoneal injection of urethane (1.5 g/kg). The heart was excised and suspended in modified Langendorff apparatus via aorta. After normal HEPES-Tyrode solution [(in mM): NaCl 143, KCl 5.4, $NaH_2PO_4 \cdot 2H_2O$ 0.33, $MgCl_2 \cdot 6H_2O$ 0.5, glucose 5.5, HEPES 5, $CaCl_2$ 1.8 adjusted to pH 7.4 with NaOH] was perfused (5–10 min, 37°C), Ca^{2+}-free normal HEPES-Tyrode solution was perfused (5–10 min, 37°C). Then, 0.02% collagenase (Wako, Osaka, Japan) was applied for 25−50 min. After washing the heart with modified Kraft-Bruhe (KB) solution [(in mM): KOH 70, L-glutamic acid 50, KCl 40, taurine 20, $MgCl_2 \cdot 6H_2O$ 3, glucose 10, HEPES 10, EGTA 1 adjusted to pH 7.4 with KOH], the ventricle was isolated and minced in the modified KB solution. Then, ventricular myocytes were isolated from the ventricle.

Isolation of neonatal rat ventricular myocytes (NRVMs)

NRVMs were isolated by an enzymatic digestion as described previously [24]. The hearts were excised from neonatal Wistar rats (1−3-day-old). After the hearts were washed in phosphate buffered saline (without Ca^{2+} and Mg^{2+}) supplemented with 20 mM butanedione monoxime (BDM) (Cayman Chemical Co., Ann Arbor, MI, USA) on ice, the ventricles were minced into small pieces in isolation solution [Hanks' Balanced Salt Solution without Ca^{2+} and Mg^{2+} supplemented with 20 mM BDM and 0.32% trypsin]. The tissue fragments were predigested for 2 hr at 4°C by stirring and were incubated in a collagenase solution [Leibovitz's L-15 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20 mM BDM and 0.15% collagenase] for 30 min at 37°C with gentle agitation. After tissue fragments were removed by filtration, the remaining tissue was centrifuged (100 g, 5 min, 4°C). The tissue pellet was resuspended in Dulbecco-Modified Eagle's Medium (DMEM) containing 10% fatal bovine serum (FBS) and incubated for 90 min at 37°C to allow attachment of non-cardiomyocytes such as fibroblasts. The non-attached cells were collected and seeded on cultured dishes in high glucose DMEM containing 10% FBS.

Patch clamp technique

Electrophysiological properties of the isolated ventricular myocytes and NRVMs were measured and recorded by whole-cell patch clamp technique with a Patch/Whole Cell Clamp Amplifier CEZ-2400 (Nihon Kohden, Tokyo, Japan) and a Clampex 10 software (Molecular Devices/Axon Instruments, Union City, CA, USA) as described previously [11].

AP was measured by a current-clamp mode as described previously [10]. The solutions for AP measurement were composed of the followings; 1) the bath solution (mM): NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.52, glucose 10, HEPES 5 and L-arginine 1 adjusted to pH 7.4 with NaOH and 2) the pipette solution (mM): NaCl 8, KCl 10, potassium aspartate 140, HEPES 5 and Mg-ATP 2 adjusted to pH 7.2 with KOH. AP was elicited by 3–5 msec short pulse of 1–2 nA square current.

I_{Na} was recorded by a voltage-clamp mode as described previously [5, 28, 32]. The solutions for I_{Na} measurement in isolated ventricular myocytes were composed of the followings; 1) the bath solution 1 (mM): NaCl 120, CsCl 133, $MgCl_2$ 2, $CaCl_2$ 1.8, HEPES 5 and verapamil 0.02 adjusted to pH 7.3 with CsOH, 2) the bath solution 2 (mM): NaCl 5, CsCl 133, $MgCl_2$ 2, $CaCl_2$ 1.8, HEPES 5 and verapamil 0.02 adjusted to pH 7.3 with CsOH and 3) the pipette solution (mM): CsCl 133, NaCl 5, tetraethylammonium (TEA)-Cl 20, EGTA 10, Mg-ATP 5 and HEPES 5 adjusted to pH 7.3 with CsOH. Before recording I_{Na}, ventricular myocytes were perfused with the bath solution 1. Then, the bath solution 2 was perfused during I_{Na} recording. I_{Na} in the isolated ventricular myocytes was elicited by depolarization pulses from a holding potential of −120 mV to the test potentials ranging −80−0 mV in 5 mV increment.

The solutions for I_{Na} measurement in NRVMs were composed of the followings; 1) the bath solution (mM): NaCl 20, CsCl 5, $MgCl_2$ 1, glucose 10, HEPES 10 and TEA-Cl 115 adjusted to pH 7.35 with NaOH and 2) the pipette solution (mM): CsCl 135, NaCl 5, HEPES 10 and EGTA 10 adjusted to pH 7.3 with CsOH. I_{Na} in NRVMs was elicited by depolarization pulses from a

holding potential of −100 mV to the test potentials ranging −80−40 mV in 5 mV increment.

All data analyses were performed by using a Clampfit 10 software (Molecular Devices/Axon Instruments). Resting membrane potential (RMP), peak amplitude, time to peak of AP and AP duration at 20%, 50% and 90% repolarization from the peak (APD_{20}, APD_{50} and APD_{90}) were measured. Current-voltage curve for the peak amplitudes of I_{Na} to each test potential normalized by cellular membrane capacitance (pF) was made.

Western blotting

Western blotting was performed as described previously [12]. The ventricular tissues or NRVMs were mixed with a lysis buffer (Cell Signaling technology) and 0.1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). After these samples were centrifuged (13,000−16,200 g, 4°C, 10 min), the supernatant containing soluble proteins was collected. The protein concentration was determined by a bicinchoninic acid method (Pierce, Rockford, IL, USA). Equal amount of sample (20 µg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 0.5% skim milk, the membranes were incubated with primary antibody (overnight, 4°C). After the membrane was incubated with HRP-conjugated secondary antibody (1 hr, room temperature), the chemiluminescent signal was detected by using an EZ-ECL system (Biological Industries, Kibbutz Beit-Haemek, Israel) or Trident femto western HRP substrate (GeneTex, Irvine, CA, USA). ATTO light capture system (AE-6972, ATTO, Tokyo, Japan) was used for signal detection and the data were analyzed by using CS analyzer 3.0 software (ATTO).

Statistical analysis

All data were presented as means ± standard error of the mean. "N" represents the number of rats and "n" represents the number of cells. Statistical analyses were performed using unpaired two-tailed Student's *t*-test (Table 1, Figs. 1B–D, 2B, 2D, 3B) [4] or one-way analysis of variance followed by Bonferroni's *post hoc* test (Fig. 3D, 3F). A value of $P<0.05$ was judged as statistically significant.

RESULTS

A single injection of POSTN increases RS height in rats

We first investigated whether a single injection of POSTN (64 µg/kg, 24 hr) affects ECG parameters in rats. POSTN-injection increased RS height but not HR, RR interval, PR interval, QRS interval, QT interval and QTc compared with vehicle-injected rats (Table 1, $P<0.05$, Vehicle: N=19; POSTN: N=11).

A single injection of POSTN decreases peak amplitude, delays time to peak and prolongs repolarization of AP in rat ventricular myocytes

We next investigated AP in ventricular myocytes isolated from POSTN (64 µg/kg, 24 hr)- or vehicle-injected rats. The shift of AP to lower-right direction was observed in the ventricular myocytes from POSTN-injected rats (Fig. 1A). POSTN-injection showed a tendency to decrease the peak amplitude of AP but not RMP compared with vehicle-injected rats [Fig. 1B, Vehicle: n=11 (N=6); POSTN: n=12 (N=5)]. POSTN-injection delayed the time to peak of AP [Fig. 1C, $P<0.01$, Vehicle: n=11 (N=6); POSTN: n=12 (N=5)] and prolonged the APD_{20} but not APD_{50} and APD_{90} [Fig. 1D, $P<0.05$, Vehicle: n=11 (N=6); POSTN: n=12 (N=5)].

A single injection of POSTN decreases I_{Na} in rat ventricular myocytes

We next examined whether POSTN-injection affects I_{Na} in rat ventricular myocytes. POSTN (64 µg/kg, 24 hr)-injection significantly decreased the peak amplitude of I_{Na} at −55 to −35 and −20 to 0 mV [Fig. 2A, 2B, $P<0.05$, Vehicle: n=10 (N=6); POSTN: n=10 (N=3)].

A single injection of POSTN decreases $Na_V1.5$ protein expression in rat ventricles

We investigated whether POSTN decreases I_{Na} by suppressing the expression of $Na_V1.5$. POSTN (64 µg/kg, 24 hr)-injection significantly decreased the protein expression of $Na_V1.5$ in ventricles compared with vehicle-injected rats (Fig. 2C, 2D, $P<0.05$, Vehicle: N=12; POSTN: N=11).

Table 1. Effects of periostin on electrocardiogram (ECG) in rats

	HR (bpm)	RR interval (msec)	PR interval (msec)	QRS duration (msec)	QT interval (msec)	QTc	RS height (mV)
Vehicle (N=19)	400 ± 13	150 ± 1	45.2 ± 1.4	15.8 ± 0.7	49.1 ± 1.7	49.0 ± 1.6	1.12 ± 0.11
POSTN (N=11)	399 ± 11	151 ± 4	47.0 ± 0.7	16.2 ± 0.5	48.4 ± 4.3	48.4 ± 1.7	1.54 ± 0.15*

Periostin (POSTN, 64 µg/kg) or vehicle (Vehicle) was intravenously injected to rats. Twenty-four hr after the injection, the rats were anesthetized by an isoflurane inhalation. ECG was recorded in lead II configuration. Heart rate (HR), RR interval, PR interval, QRS duration, QT interval and RS height were measured. Data were presented as means ± standard error of the mean. bpm: beats per minute, QTc: [QT interval/(square root of RR/150)] (a modified Bazett's formula) [16], *$P<0.05$ vs. Vehicle (unpaired two-tailed Student's *t*-test).

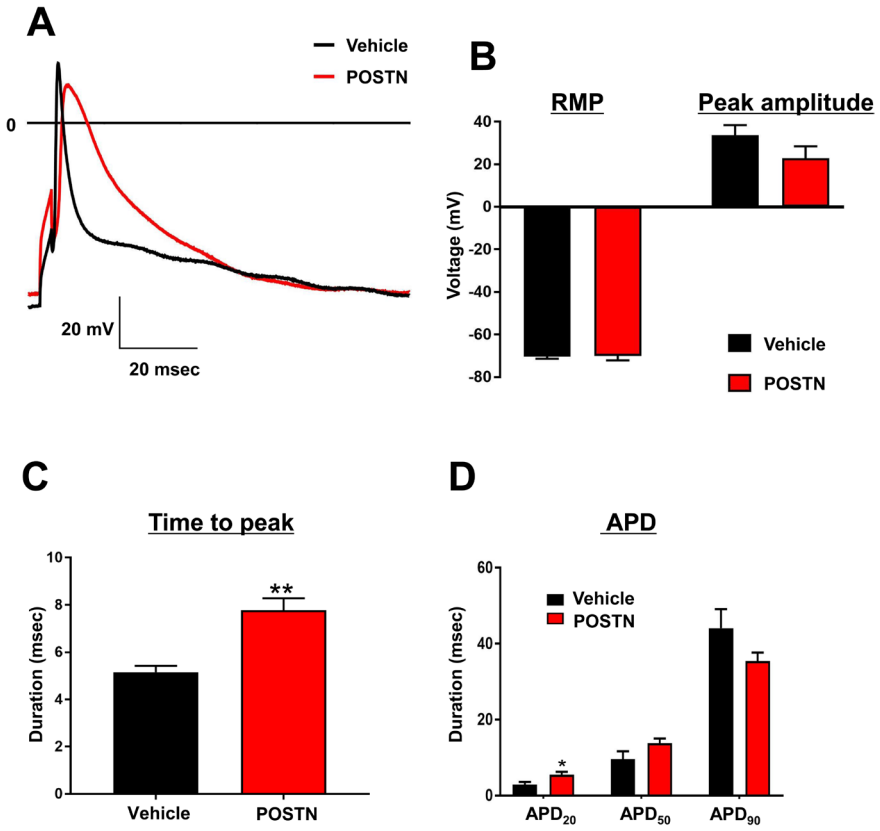

Fig. 1. A single injection of periostin (POSTN) decreases peak amplitude, delays time to peak and prolongs repolarization of action potential (AP) in rat ventricular myocytes. POSTN (64 μg/kg) or vehicle (Vehicle) was intravenously injected to rats via right jugular vein. Twenty-four hr after the injection, ventricular myocytes were isolated. Whole-cell patch clamp (current-clamp mode) was performed to measure AP. (A) Representative AP waveform in the ventricular myocytes from POSTN (red) or Vehicle (black) was shown. (B) The resting membrane potential (RMP) and the peak amplitude of AP were measured and shown as mean ± standard error of the mean (S.E.M.). (C) A time to peak of AP was measured and shown as means ± S.E.M. (D) The AP duration at 20%, 50% and 90% repolarization from the peak (APD_{20}, APD_{50} and APD_{90}) was measured and shown as means ± S.E.M. Vehicle: n=11 (N=6); POSTN: n=12 (N=5). *,**P<0.05, 0.01 vs. Vehicle (unpaired two-tailed Student's t-test).

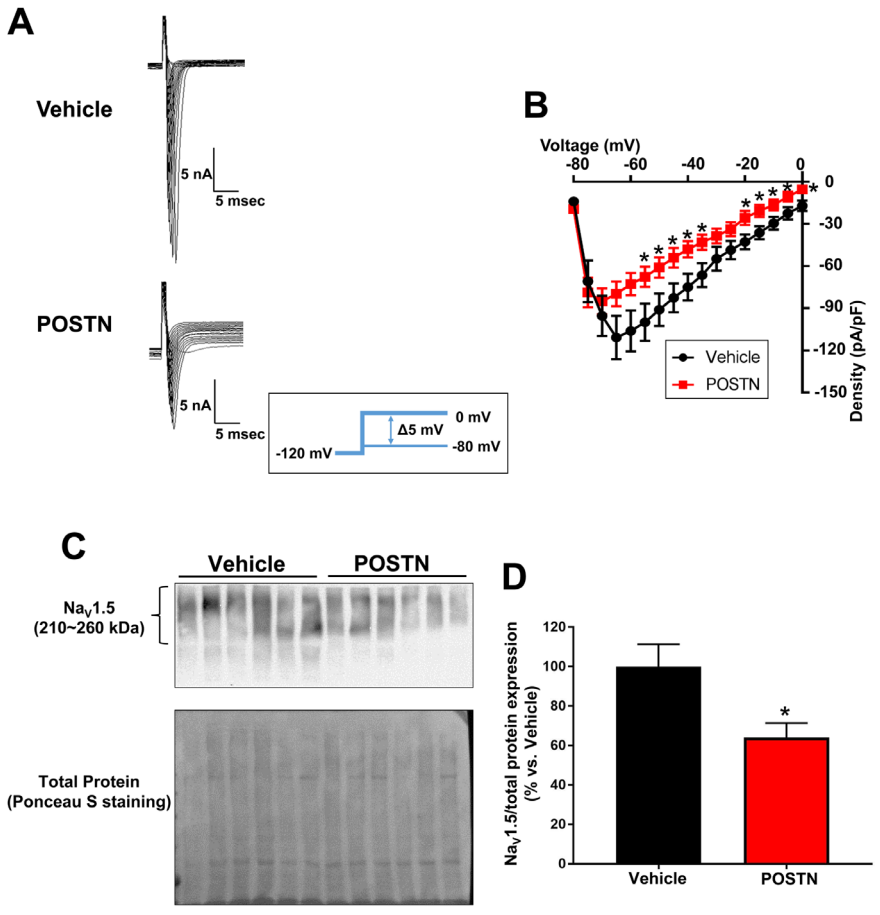

Fig. 2. A single injection of periostin (POSTN) decreases Na^+ current (I_{Na}) and protein expression of cardiac voltage-gated Na^+ channel ($Na_V1.5$) in rat ventricular myocytes. POSTN (64 μg/kg) or vehicle (Vehicle) were intravenously injected to rats for 24 hr. (A, B) Whole-cell patch clamp (voltage-clamp mode) was performed to measure I_{Na} in ventricular myocytes isolated from the rats. (A) Representative trances of I_{Na} in the ventricular myocytes from POSTN (lower) or Vehicle (upper) were shown. Inset: depolarization pulse protocol. (B) Current-voltage curve for peak amplitudes of I_{Na} was shown as means ± S.E.M. Vehicle: n=10 (N=6); POSTN: n=10 (N=3). The current (pA) was normalized by cellular membrane capacitance (pF). *P<0.05 vs. Vehicle [unpaired two-tailed Student's t-test]. (C, D) Western blotting was performed to examine the protein expression of $Na_V1.5$ in ventricles isolated from the rats. (C) Representative blots for $Na_V1.5$ and ponceau S-stained total protein in nitrocellulose membrane were shown. (D) The expression level of $Na_V1.5$ was corrected by ponceau S-stained total protein level, and the normalized expression relative to Vehicle was shown as mean ± S.E.M. Vehicle: N=12; POSTN: N=11. *P<0.05 vs. Vehicle (unpaired two-tailed Student's t-test).

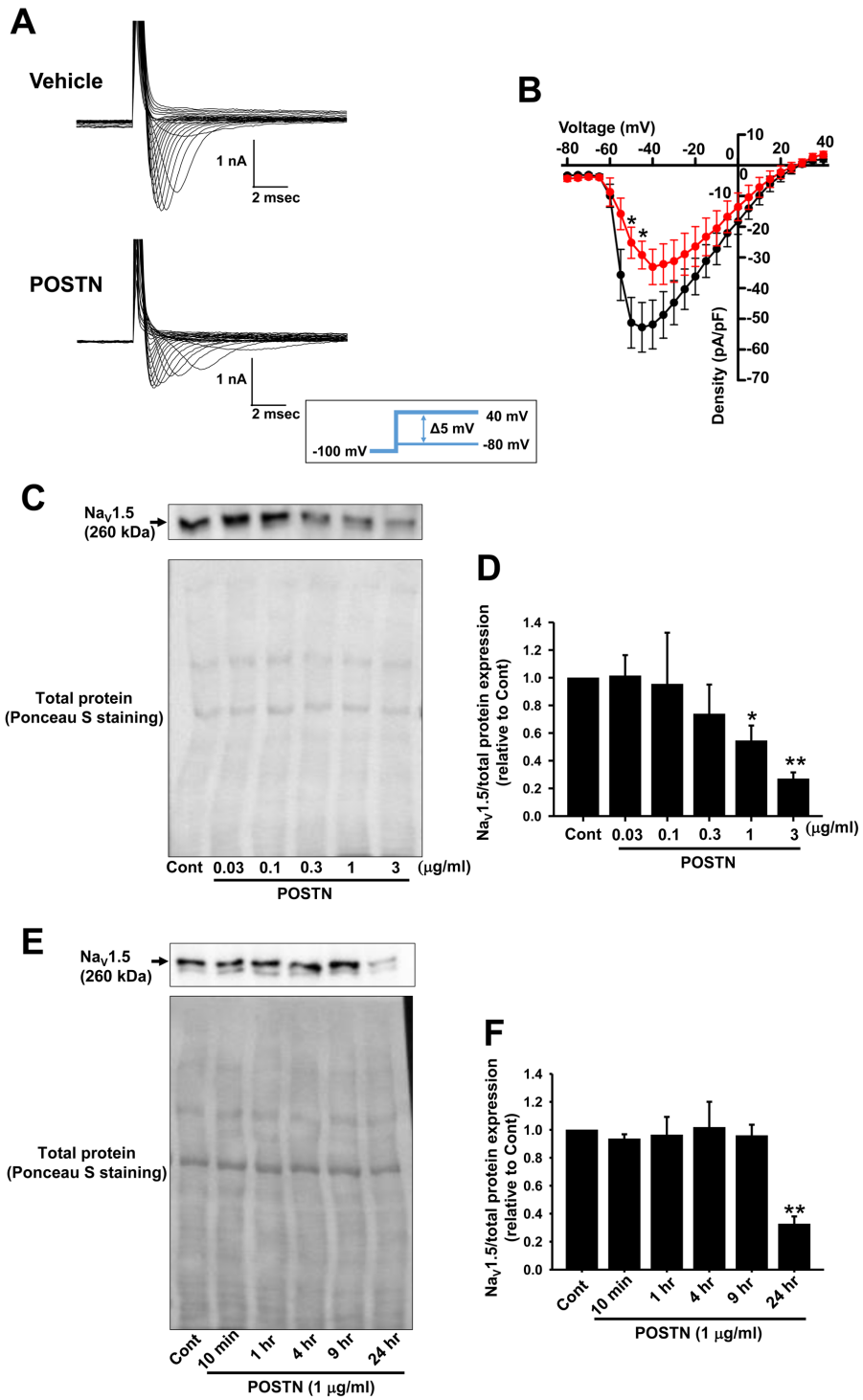

Fig. 3. Periostin (POSTN)-treatment decreases Na^+ current (I_{Na}) and protein expression of cardiac voltage-gated Na^+ channel ($Na_V1.5$) in neonatal rat ventricular myocytes (NRVMs). (A, B) NRVMs were stimulated with POSTN (1 μg/ml) or vehicle (Vehicle) for 24 hr. Whole-cell patch clamp (voltage-clamp mode) was performed to measure I_{Na}. (A) Representative trances of I_{Na} in POSTN (lower) or Vehicle (upper) were shown. Inset: depolarization pulse protocol. (B) Current-voltage curve for the peak amplitudes of I_{Na} was shown as means ± S.E.M. [Vehicle (black): n=16; POSTN (red): n=16]. The current (pA) was normalized by cellular membrane capacitance (pF). *P<0.05 vs. Vehicle (unpaired two-tailed Student's t-test). (C-F) NRVMs were stimulated with POSTN (0.03–3 μg/ml) or vehicle (Cont) for 10 min-24 hr. After the total protein was extracted, Western blotting was performed to examine expression of $Na_V1.5$. (C, E) Representative blots for $Na_V1.5$ and ponceau S-stained total protein in nitrocellulose membrane were shown. (D, F) The expression level of $Na_V1.5$ was corrected by ponceau S-stained total protein level, and the normalized expression relative to Cont was shown as mean ± S.E.M. (n=3). *, **P<0.05, 0.01 vs. Cont (one-way analysis of variance followed by Bonferroni's *post hoc* test).

POSTN-treatment decreases $Na_V1.5$ protein expression and I_{Na} in NRVMs

We performed *in vitro* experiment to investigate whether POSTN directly decreases I_{Na} by suppressing $Na_V1.5$ protein expression using NRVMs. We confirmed that POSTN (1 μg/ml, 24 hr)-treatment significantly decreased the peak amplitude of I_{Na} at −50 and −45 mV (Fig. 3A and 3B, P<0.05, n=16). POSTN (0.03–3 μg/ml, 24 hr)-treatment significantly decreased protein expression of $Na_V1.5$ in a concentration-dependent (Fig. 3C and 3D, P<0.05, 0.01, n=3). In addition, POSTN (1 μg/ml, 24 hr) significantly decreased the protein expression at 24 hr but not at 10 min-9 hr (Fig. 3E and 3F, P<0.01, n=3).

DISCUSSION

This study demonstrated for the first time that a single injection of POSTN in rats decreased I_{Na} in isolated ventricular myocytes by suppressing $Na_V1.5$ expression in ventricular tissues, which was accompanied by a prolongation of time to peak in APs and an increase of RS height in ECG (Fig. 4).

In the present study, POSTN-injection had no effect on HR, PR interval, RR interval, QRS interval, QT interval and QTc in the ECG of rats, but significantly increased RS height (Table 1). Penz *et al.* reported that RS height was a sensitive indicator which increased before QRS by a treatment with Na^+ channel blocker, such as lidocaine, quinidine and flecainide [21]. It is thus suggested that POSTN-induced increase in RS height might be mediated via inhibiting expression and/or activity of Na channel in the rat heart. Therefore, we isolated ventricular myocytes from POSTN-injected rats and measured ion channel activity, the result of which showed a significant decrease in I_{Na} compared with vehicle-injected rats (Fig. 2). Na^+ channel blockers delay the onset of AP depolarization phase (phase 0) by decreasing or delaying Na^+ influx into the cells [6]. In the present study, POSTN-injection tended to decrease the peak amplitude (Fig. 1B) and prolonged the time to peak (Fig. 1C) of AP in isolated rat ventricular myocytes. Thus, these changes induced by POSTN might be mediated by the inhibition of I_{Na}. We also found that POSTN-injection significantly prolonged APD_{20} but not APD_{50} and APD_{90} in isolated rat ventricular myocytes (Fig. 1D). It seems likely that the prolongation of APD_{20} by POSTN-injection was due to the delay of time to peak associated with I_{Na} suppression, while POSTN has little effect on overall length of the repolarization phase. In addition, we cannot deny the possibility that POSTN-injection affects the expression and activity of other channels involved in the AP repolarization phase, such as Ca^{2+} and K^+ channels. Thus, further investigation is needed to clarify the detailed mechanism of POSTN-injection on the prolongation of APD.

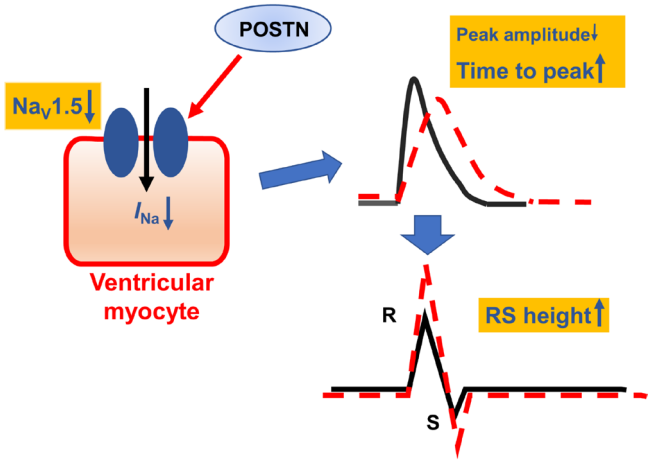

Fig. 4. Proposed model. A single injection of periostin (POSTN) decreased Na^+ current (I_{Na}) in isolated ventricular myocytes by suppressing cardiac voltage-gated Na^+ channel ($Na_V1.5$) protein expression in rats, which was accompanied by a decrease of peak amplitude and a prolongation of time to peak in action potential (AP) and increase in RS height in electrocardiogram.

We demonstrated that POSTN (64 µg/kg, 24 hr)-injection decreased the protein expression of $Na_V1.5$ in the isolated ventricles (Fig. 2C, 2D). We also confirmed that POSTN-treatment decreased the protein expression of $Na_V1.5$ concomitant with a suppression of I_{Na} in NRVMs at 24 hr (Fig. 3). Our preliminary data showed that POSTN did not decrease mRNA expression of $Na_V1.5$ in the isolated ventricles and NRVMs (Ventricles: 2.63 ± 1.94 fold relative to Vehicle, P=0.43, N=4; NRVMs: 1.87 ± 1.46 fold relative to Vehicle, P=0.58, n=3). The protein expression of $Na_V1.5$ is known to be regulated by post-translational mechanisms including ubiquitin/proteasome system (UPS) [1]. The UPS-dependent degradation of $Na_V1.5$ was promoted by calcium-dependent upregulation of Nedd4-2, a ubiquitin ligase, in ventricular myocytes from HF model rats [18]. Nedd4-2-dependent ubiquitination of several transporters is regulated by protein kinase C (PKC) [8, 30]. Integrin $\alpha_v\beta_3$, a receptor for POSTN [9, 23], is known to activate PKC [20, 22]. Thus, POSTN might decrease $Na_V1.5$ protein expression by an Nedd4-2-UPS-dependent degradation through activating PKC via binding to integrin $\alpha_v\beta_3$.

In conclusion, we for the first time demonstrated that a single injection of POSTN in rats prolonged time to peak in AP and increased RS height in ECG perhaps in part through the inhibition of I_{Na} via decreasing $Na_V1.5$ expression in ventricular myocytes. These results indicate that POSTN could be a novel molecule regulating electrical remodeling in HF.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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