Neurally Mediated Syncope as a Cause of Syncope in Patients With Brugada Electrocardiogram

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Neurally Mediated Syncope in Brugada Syndrome. ***Introduction:*** **Patients with type 1 Brugada electrocardiogram (ECG) and an episode of syncope are diagnosed as symptomatic Brugada syndrome; however, all episodes of syncope may not be due to ventricular tachyarrhythmia.**

Methods and Results: **Forty-six patients with type 1 Brugada ECG (all males, 51 ± 13 years, 29 spontaneous, 17 Ic-drug induced), 20 healthy control subjects (all males, 35 ± 11 years), and 15 patients with suspected neurally mediated syncope (NMS; 9 males, 54 ± 22 years) underwent the head-up tilt (HUT) test. During the HUT test, 12-lead ECGs were recorded in all patients, and the heart rate variability was investigated in some patients. Sixteen (35%) of 46 patients with Brugada ECG, 2 (10%) of 20 control subjects, and 10 (67%) of 15 patients with suspected NMS showed positive responses to the HUT test. Although no significant differences were observed in HUT-positive rate among Brugada patients with documented VT (7/14; 50%), syncope (5/19; 26%) and asymptomatic patients (4/13; 31%), the HUT-positive rate was significantly higher in patients with documented VT (50%) and those with VT or no symptoms (11/27, 41%) compared to that in control subjects (10%) ($P < 0.05$). Augmentation of ST-segment amplitude (≥0.05 mV) in leads V1-V3 was observed in 11 (69%) of 16 HUT-positive patients with Brugada ECG during vasovagal responses, and was associated with augmentation of parasympathetic tone following sympathetic withdrawal.**

Conclusion: **Thirty-five percent of patients with Brugada ECG showed vasovagal responses during the HUT test, suggesting that some Brugada patients have impaired balance of autonomic nervous system, which may relate to their syncopal episodes.** *(J Cardiovasc Electrophysiol, Vol. 21, pp. 186-192, February 2010)*

autonomic nervous system, Brugada syndrome, head-up tilt test, syncope, sudden death

Introduction

Brugada syndrome is characterized by ST-segment elevation in the right precordial leads V1 through V3 and an episode of ventricular tachyarrhythmia (VT) in the absence of structural heart disease.[1-3] In patients with Brugada syndrome, syncopal episodes are generally thought to be due to VT; however, all episodes of syncope may not be owing to VT events. Neurally mediated syncope (NMS) is 1 of the causes of syncope in general population, and it refers to a reflex response that some triggering factors give rise to arterial vasodilatation associated with relative or absolute bradycardia.[4] In general, the overall prognosis in patients with NMS is quite favorable.[4] On the other hand, the precise cause of syncope in patients with Brugada syndrome is difficult to determine. Therefore, the therapeutic strategy for Brugada patients with syncope is often problematic. The aim of this study was to evaluate the possibility of NMS as a cause of syncope in patients with Brugada electrocardiogram (ECG).

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Methods

Patients Population

The study population consisted of 46 consecutive patients with type 1 Brugada ECG who were admitted to the National Cardiovascular Center, Suita, Japan, between May 2004 and March 2006 (all males, ages 26 to 77; mean 51 ± 13 years, 29 spontaneous, 17 Ic-drug induced), 20 healthy control subjects (all males, 35 ± 11 years), and 15 patients suspected of NMS (9 males, 54 ± 22 years). Ethical approval was obtained from the Institutional Review Committee of our hospital, and all patients and control subjects gave their informed, written consent before participation. The control subjects and the patients with suspected NMS showed no structural heart diseases, normal physical examination results, and normal 12-lead ECGs, and received no drug treatment affecting the sympathetic nervous system. Type 1 Brugada ECG was defined as a coved type ST-segment elevation of ≥ 0.2 mV at

J point observed in more than 1 of the right precordial leads (V1 to V3) in the presence or absence of a sodium channel blocker.[2]

Head-Up Tilt Test

The HUT test was performed in the afternoon after 4 hours of fasting in a quiet and comfortable room equipped for cardiopulmonary resuscitation. All patients were allowed to lie on an electrically controlled tilt table an intravenous line containing 5% dextrose was inserted into 1 arm, and allowed to rest in supine position for at least 10 minutes. A positive HUT test was defined by the development of syncope or presyncope associated with relative bradycardia (≥20% decrease in heart rate compared with baseline) or hypotension (systolic blood pressure <80 mmHg). Presyncope was defined as the induction of symptoms of imminent syncope, and syncope was defined as sudden transient loss of consciousness. Positive response to the HUT test was classified into 3 types owing to hemodynamic status, such as vasodepressor type (hypotension without significant bradycardia), cardioinhibitory type (bradycardia without associated hypotention), and mixed type (hypotension followed by bradycardia).[4] At first, we performed passive tilt (Control-Tilt) at an angle of 70 degrees for 30 minutes. When Control-Tilt was negative, sublingual nitroglycerin (NTG) spray 0.3 mg was administered, and the test was continued for 15 minutes (NTG-Tilt). The endpoint of each tilt test was the time when patients showed positive responses or the completion of HUT-protocol.

Parameters Measured During the Head-Up Tilt Test

Heart rate and blood pressure

Heart rate was monitored, and cuff blood pressure was measured by electrosphygmomanometry with a microphone placed over the brachial artery to detect Korotkoff sounds every minute (STBP-780, Colin Electronics, Komaki, Japan) in all patients during the HUT test.

ST-segment amplitude in the right precordial leads

Twelve-lead ECGs were recorded every 1 minute during the HUT test, and the changes of ST-segment amplitude in the right precordial leads (V1-V3) were analyzed (ML-6500, Fukuda-denshi, Tokyo, Japan) in all patients during the HUT test.

Heart rate variability

Six-lead ECGs from the Task Force Monitor (CNSystem, Graz, Austria)[5-7] were measured for beat-to-beat heart rate and consecutive R-R intervals in 10 patients with Brugada ECG (4 documented VT, 5 syncopal episode only, and 1 asymptomatic), 9 control subjects, and 5 patients with suspected NMS. The heart rate variability (HRV) was investigated by a power spectral analysis delineating the low-frequency component (LF; 0.04–0.15 Hz) and the high-frequency component (HF; 0.15–0.40 Hz).[8] We analyzed the normalized unit of the HF components (%) calculated automatically (HF/power spectral density-very low-frequency component [0–0.04 Hz] × 100)[8,9] and the LF/HF ratio. The HF indicates the tone of the parasympathetic nervous system, and the LF/HF ratio indicates the sympathovagal balance.

Statistical Analysis

Numerical values were expressed as means ± SD unless otherwise indicated. Comparisons of parameters between 2 groups were made using the unpaired Student *t*-test. Comparisons of parameters among 3 groups were made with a one-way analysis of variance (ANOVA), followed by the Scheffe's multiple-comparison test. Categorical variables were compared using a chi-square analysis using the Yate's correction or Fisher exact test if necessary. An overall chi-square test for a 2 × n table was performed when comparisons involved >2 groups. A P-value < 0.05 was considered significant.

Results

Clinical Characteristics

The clinical characteristics of 46 patients with Brugada ECG and 15 patients with suspected NMS are shown in Table 1. The patients with Brugada ECG were divided into 3 groups: (1) 14 patients with documented VT; (2) 19 patients with syncopal episodes only; and (3) 13 asymptomatic patients. No significant differences were observed in age, incidence of spontaneous type 1 ECG, family history of sudden cardiac death (SCD), induced ventricular fibrillation during electrophysiologic study (EPS), and *SCN5A* mutation. Implantable cardioverter-defibrillator (ICD) was implanted more frequently in patients with documented VT. The triggers of VT and/or syncope are also shown in Table 1. Seventy-nine percent of VT episodes occurred during sleep or at rest in patients with documented VT ($P < 0.0001$ vs the patients with syncopal episodes only and suspected NMS). On the other hand, in patients with syncopal episodes only, 15% of syncopal episodes occurred after urination, 21% during standing, and 21% after drinking alcohol, which seemed to be similar patterns in patients with suspected NMS. Based on the clinical description of the syncopal events, 16 (84%) of 19 Brugada patients with syncopal episodes were suspected to have NMS. Syncopal episodes seemed to be due to VT in 1 of the remaining 3 patients.

Positive Response to the Head-Up Tilt Test

Comparison of the positive responses to the HUT test between 46 patients with Brugada ECG and 20 control subjects along with 15 patients with suspected NMS are shown in Table 2. Sixteen (35%) of 46 patients with Brugada ECG showed positive responses. Positive responses were developed in 1 (2%) of 46 patients during Control-Tilt and in 15 (33%) of 45 patients during NTG-Tilt, and the mixed type was predominant (94%). In patients with Brugada ECG, there were no significant differences in the incidence of positive responses among patients with documented VT (50%), those with syncopal episodes only (26%), and asymptomatic patients (31%). No significant differences were observed in the type of positive responses between the 3 groups. The mixed type was predominant (100%, 100%, and 75%, respectively), and cardioinhibitory type was not observed in all 3 groups. Two (10%) of 20 control subjects and 10 (67%) of 15 patients with suspected NMS showed positive responses. The HUT-positive rate was not significantly different between all 46 patients with Brugada ECG, 20 control subjects and 15 subjects with suspected NMS (35% vs 10% vs 67%);

TABLE 1

Clinical Characteristics of Patients with Brugada Electrocardiogram and Suspected NMS

	Documented VT (n = 14)	Syncopal Episodes only (n = 19)	Asymptomatic (n = 13)	Suspected NMS (n = 15)
Age (years)	50 ± 15	51 ± 12	52 ± 14	54 ± 22
Spontaneous type 1 ECG	10 (71)	9 (47)	10 (77)	–
Family history of SCD	4 (29)	4 (21)	4 (31)	–
Induced VF during EPS	10/12 (83)	15/18 (83)	8/11 (73)	–
SCN5A mutation	1 (7)	3 (16)	0 (0)	–
ICD implantation	14 (100)	13 (68)*	7 (54)*	–
Triggers of syncope				
During sleeping or at rest	11 (79)	1 (5)*	–	0*
After urination	0	3 (15)	–	1 (7)
Prolonged standing at attention	0	4 (21)	–	4 (27)
After drinking alcohol	0	4 (21)	–	6 (40)
After meal	1 (7)	0	–	0
After exertion	0	2 (11)	–	2 (13)
After sudden unexpected pain	0	2 (11)	–	0
During driving	0	1 (5)	–	0
Others	2 (14)	2 (11)	–	2 (13)

Values are mean ± SD for age, and expressed as frequency (%). *P < 0.05 vs documented VT group. ECG = electrocardiogram; EPS = electrophysiological study; ICD = implantable cardioverter-defibrillator; NMS = neurally mediated syncope; SCD = sudden cardiac death; VT = ventricular tachyarrhythmias; VF = ventricular fibrillation.

however, the HUT-positive rate was significantly higher in 14 patients with documented VT (50%) and 27 patients with VT or no symptoms (41%) compared to that in control subjects (10%) (P = 0.03, P = 0.04, respectively). The HUT-positive rate in 19 Brugada patients with syncopal episodes (26%) was significantly lower than that in 15 patients with suspected NMS (P = 0.04), although the syncopal episodes in 84% of the 19 patients were suspected to be due to NMS. Positive responses to the HUT test were more frequency observed in 15 patients with suspected NMS compared to those in 20 control subjects (10/15 vs 2/20; P < 0.001).

Comparison of the clinical characteristics between 16 HUT-positive patients and 30 HUT-negative patients with Brugada ECG were shown in Table 3. No significant differences were observed in cardiac events, such as documented VT or syncope. Furthermore, there were no significant differences in the clinical characteristics, such as age, spontaneous type 1 ECG, a family history of SCD, inducibility of ventricular fibrillation during EPS, *SCN5A* mutation, and ICD implantation.

Response of Heart Rate and ST-Segment Amplitude

In patients with Brugada ECG, the heart rate was increased by 12 ± 9 beats/min during Control-Tilt, and by 24 ± 14 beats/min during NTG-Tilt. As the heart rate was increased, decrease of ST-segment amplitude of ≥ 0.05 mV from baseline in the right precordial leads was observed in 11 (24%) of 46 patients during Control-Tilt (−0.14 ± 0.08 mV), and in 19 of 45 (42%) patients during NTG-Tilt (−0.15 ± 0.10 mV) (Fig. 1C). However, augmentation of ST-segment amplitude of ≥ 0.05 mV in the right precordial leads was observed just before and after positive responses to the HUT test in 11 (69%) of 16 HUT-positive patients (0.10 ± 0.06 mV) (Figs. 1D and E). These significant ST-segment augmentation was observed in 1 patient during Control-Tilt (documented VT), and 10 patients during NTG-Tilt (5 documented VT, 2 syncopal episodes only, 3 asymptomatic), respectively. On the other hand, augmentation of the ST-segment amplitude of ≥ 0.05 mV was 2 (7%) of 30 HUT-negative patients during NTG-Tilt (1 documented VT, 1 syncopal episodes only). As a result, the average ST-segment augmentation was

TABLE 2

Responses to Head-Up Tilt Test in Patients with Brugada Electrocardiogram, Control Subjects, and Patients with Suspected NMS

	All (n = 46)	Documented VT (n = 14)	Syncopal Episodes Only (n = 19)	Asymptomatic (n = 13)	Brugada ECG with VT or No Symptoms (n = 27)	Control Subjects (n = 20)	Suspected NMS (n = 15)
Age (years)	51 ± 13*	50 ± 15*	51 ± 12*	52 ± 14*	51 ± 14*	35 ± 11	54 ± 22*
Positive response	16 (35)	7 (50)*	5 (26)†	4 (31)	11 (41)*	2 (10)	10 (67)*
Control-tilt	1/46 (2)	1/14 (7)	0/19 (0)	0/13 (0)	1/27 (4)	0/20 (0)	0/15 (0)
NTG-tilt	15/45 (33)†	6/13 (46)*	5/19 (26)†	4/13 (31)	10/26 (38)	2/20 (10)	10/15 (67)*
Type of positive response							
Vasodepressive	1/16 (6)	0	0	1/4 (25)	1/11 (9)	0	1/10 (10)
Cardioinhibitory	0	0	0	0	0	0	0
Mixed	15/16 (94)	7/7 (100)	5/5 (100)	3/4 (75)	10/11 (91)	3 (100)	9/10 (90)

Values are expressed as frequency (%). *P < 0.05 vs control subjects, †P < 0.05 vs suspected NMS. ECG = electrocardiogram; NMS = neurally mediated syncope; NTG = nitroglycerin; VT = ventricular tachyarrhythmias.

TABLE 3
Comparison of Clinical Characteristics Between Head-up Tilt-Positive Patients and Head-up Tilt-Negative Patients

	HUT-Positive (n = 16)	HUT-Negative (n = 30)	P-value
Age (years)	52 ± 13	50 ± 14	0.58
Documented VT	7 (44)	7 (23)	0.15
Syncope only	5 (31)	14 (47)	0.49
Asymptomatic	4 (25)	9 (30)	0.99
Spontaneous type 1 ECG	11 (69)	18 (60)	0.79
Family history of SCD	4 (25)	8 (27)	1.0
Induced VF during EPS	13/15 (87)	20/26 (77)	0.72
SCN5A mutation	1 (6)	3 (10)	1.0
ICD implantation	14 (88)	24 (80)	0.82

Values are expressed as frequency (%). ECG = electrocardiogram; EPS = electrophysiological study; HUT = head-up tilt test; ICD = implantable cardioverter-defibrillator; SCD = sudden cardiac death; VT = ventricular tachyarrhythmias; VF = ventricular fibrillation.

significantly larger in 16 HUT-positive patients than in 30 HUT-negative patients at similar heart rate (0.06 ± 0.06 mV vs −0.04 ± 0.06 mV, P < 0.0001). No ventricular arrhythmias were induced during the HUT test in any patients with Brugada ECG. The ST-segment augmentation was not observed during the HUT test in any control subjects (−0.02 ± 0.02 mV, P < 0.0001 vs 16 HUT-positive Brugada patients) and patients with suspected NMS (−0.02 ± 0.04 mV, P < 0.001 vs 16 HUT-positive Brugada patients; Fig. 2).

Heart Rate Variability and ST-segment Amplitude

Positive responses during NTG-Tilt were observed in 4 (40%) of 10 patients with Brugada ECG, in 1 (11%) of 9 control subjects, and in 4 (80%) of 5 patients with suspected NMS in whom the HRV was monitored. The autonomic activities in a representative NTG-Tilt-positive patient with Brugada ECG and those with suspected NMS are shown in Figure 3A and B, respectively. Before positive responses to the HUT test, sympathetic activity (LF/HF ratio) dramatically increased; and then, sympathetic withdrawal occurred immediately. Thereafter, parasympathetic nerve activity (the normalized unit of the HF components) gradually increased. The similar pattern of augmented parasympathetic nerve activity following sympathetic withdrawal during positive responses to the HUT test was observed in all 9 HUT-positive patients. The patterns of HRV were not different among the HUT-positive patients with Brugada ECG, the HUT-positive control subjects, and the HUT-positive patients with suspected NMS. In 3 (75%) of 4 HUT-positive patients with Brugada ECG, the LF/HF ratio decreased and the HF component increased gradually toward the maximum ST-segment elevation just before and after positive response for the HUT test (Fig. 3A), but ST-segment was decreased in patients with NMS (Fig. 3B).

Discussion

In this study, 35% of patients with Brugada ECG showed vasovagal responses during the HUT test regardless of the presence VT or syncope. The HUT test was also positive in 41% among only Brugada patients with documented VT or no symptoms. During vasovagal response, ST-segment augmentation in the right precordial leads (V1-V3) was observed in 11 (69%) of 16 HUT-positive patients with Brugada ECG, but no ventricular arrhythmias were induced in any HUT-positive patients.

Neurally Mediated Syncope as a Cause of Syncope in Brugada Syndrome

Several case reports have described patients exhibiting clinical phenotype of both Brugada syndrome and NMS.[10-12]

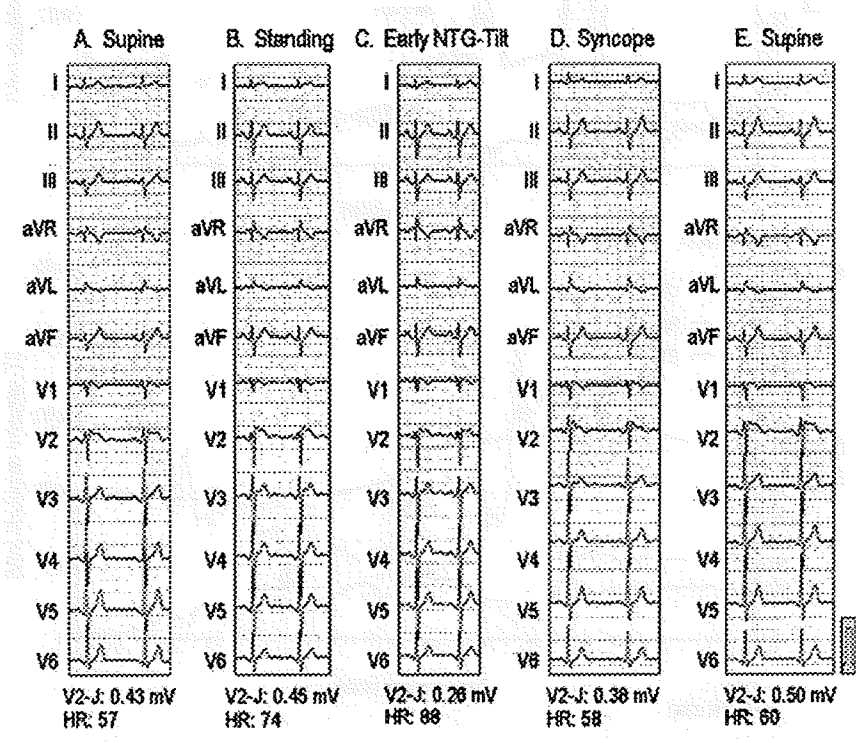

Figure 1. *The12-leads electrocardiogram (ECG) during head-up tilt test in a representative nitroglycerin (NTG)-Tilt-positive patient with type 1 Brugada ECG at supine position (A), at standing position (B), at early phase of NTG-Tilt (C), at syncope (D), and at supine position following syncope (E). The ST-segment elevation was decreased from 0.45 mV to 0.26 mV at early phase of NTG-Tilt as the heart rate was increased (C), while it was augmented to 0.38 mV at syncope (D), and to 0.50 mV at supine position following syncope (E).*

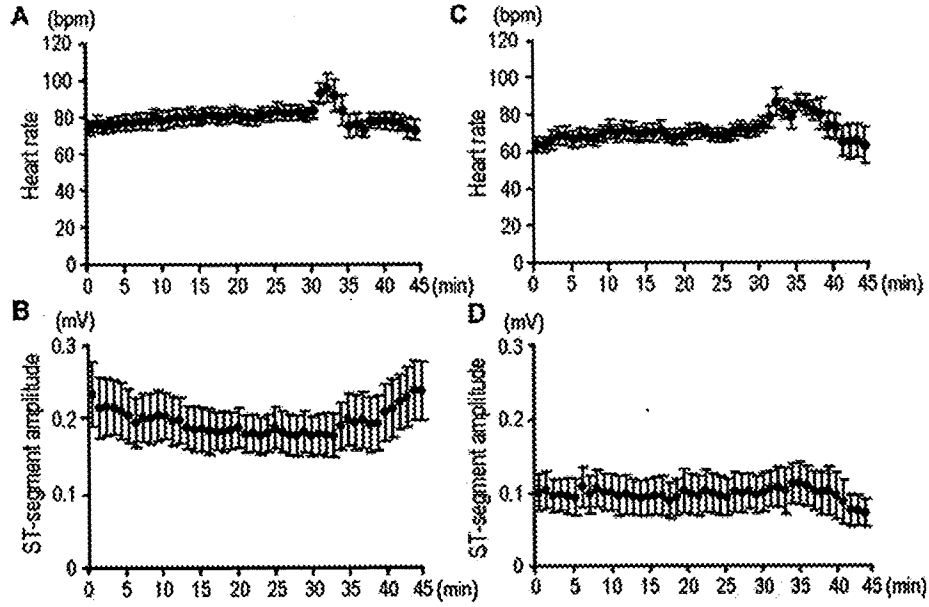

Figure 2. *Response of the heart rate and ST-segment amplitude during the head-up tilt (HUT) test in 16 HUT-positive patients with Brugada electrocardiogram (ECG) (A, B) and in 10 HUT-positive patients with suspected neurally mediated syncope (NMS) (C, D). At first, the passive tilt (Control-Tilt) was performed for 30 minutes (0–30 minutes). When Control-Tilt was negative, nitroglycerin tilt was continued for 15 minutes (30–45 minutes). The responses of heart rate during positive responses to the HUT test were similar in patients with Brugada ECG (A) to those in patients with suspected NMS (C). In patients with Brugada ECG, ST-segment in lead V2 was augmented before and after positive responses to the HUT test (B), but not in those with suspected NMS (D).*

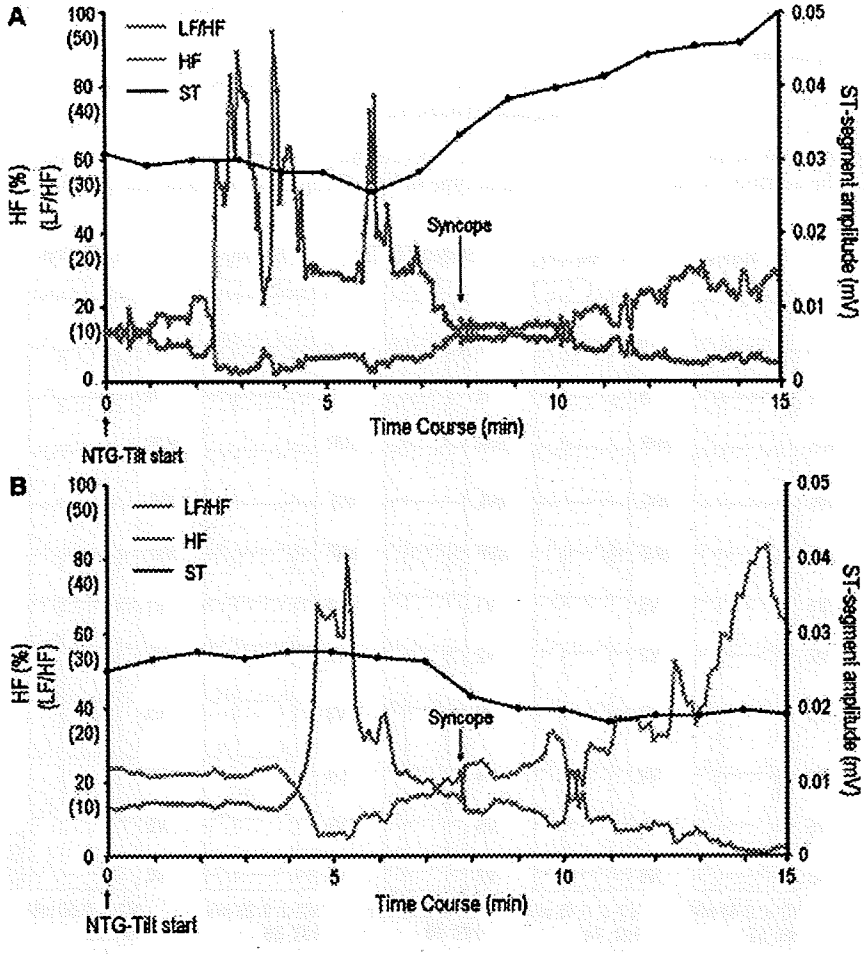

Figure 3. *Autonomic responses during head-up tilt (HUT) test. The autonomic activities in a representative nitroglycerin (NTG)-Tilt-positive patient with type 1 Brugada electrocardiogram (ECG) (A) and those in a representative NTG-Tilt-positive patient with suspected NMS (B). Before tilt-induced syncope, sympathetic activity (LF/HF ratio) dramatically increased; and then, sympathetic withdrawal occurred immediately. Thereafter, parasympathetic nerve activity (the normalized unit of the HF components) gradually increased. In the HUT-positive patient with Brugada ECG, ST-segment augmentation in lead V2 was observed just before and after positive responses, and the LF/HF ratio decreased and the HF component increased gradually toward the maximum ST-segment elevation (A). In contrast, in the HUT-positive patient with suspected NMS, ST-segment amplitude in lead V2 was decreased gradually after positive responses (B).*

It is well known that the autonomic nervous system plays an important role on the arrhythmogenesis of Brugada syndrome. Previous studies showed that the withdrawal of sympathetic activity and the sudden rise in vagal activity was an important triggering factor of ventricular fibrillation.[13-15] Similarly, it has been presumed that parasympathetic tone increase during NMS events in patients with Brugada ECG. Recent basic study showed that *SCN5A*, a major responsible gene in Brugada patients, is expressed not only in the myocardial cells but also in intracardiac ganglia.[16] Makita *et al.* also demonstrated a novel nonsense mutation in *SCN5A* gene in a patient with Brugada syndrome who had been diagnosed as NMS.[17] These results suggested that the abnormal regulation or imbalance of autonomic nervous system may exist regardless of the presence or absence of cardiac events in patients with Brugada ECG.

ST-Segment Elevation in the Precordial Leads During the HUT Test in Patients with Brugada ECG

In Brugada syndrome, spontaneous augmentation of ST-segment elevation occurred along with an increase in vagal activity, especially just before and after the occurrence of ventricular fibrillation.[14] The ST-segment elevation is also known to be modulated by exercise,[18] pharmacological interventions that interact with automatic nervous activities,[19] or taking meals associated with glucose-induced insulin levels.[20] In this study, ST-segment augmentation in the right precordial leads was observed just before and after positive responses to the HUT test in two-thirds (69%) of the HUT-positive patients with Brugada ECG but only in 7% of the HUT-negative patients. In patients with Brugada ECG, the preceding increase of sympathetic nerve activity during the HUT test may cause augmentation of ICa-L, resulting in attenuation of ST-segment elevation.[19] Subsequent augmentation of parasympathetic nerve activity during the HUT test may decrease of ICa-L, and increase Ito, thus augmenting ST-segment amplitude.

Clinical Implication

The second consensus report suggested that symptomatic patients displaying type 1 Brugada ECG (either spontaneous or after class Ic drugs) who present with aborted sudden death should undergo ICD implantation.[3] ICD implantation is also recommended in patients with syncope, seizure, or nocturnal agonal respiration, after noncardiac causes of these symptoms have been carefully ruled out.[3] Needless to say, the ECG recording during syncope is the only convincing way to rule in or out VT during syncope, and only clinical judgment can be used to guide diagnostic and therapeutic decisions. However, in patients with Brugada syndrome, there is an abnormal regulatory imbalance of the autonomic nervous system that may be a common denominator to both syncope and ventricular fibrillation.

Limitations

The control subjects were significantly younger than patients with Brugada ECG or those with suspected NMS. However, it is reported that the positive rate of NTG-Tilt in the elderly was comparable to that seen in younger subjects.[21] Therefore, lower incidence of positive rate of the HUT test in the control subjects than that in the other 2 groups was not due to the relevant difference of age. The incidence of spontaneous type 1 ECG and the positive rate of the HUT test are smaller in Brugada patients with syncope episodes only than in those with documented VT or asymptomatic patients; however, statistical significance was not observed between the 3 groups.

Conclusions

Thirty-five percent of patients with Brugada ECG showed vasovagal responses during the HUT test. The HUT test was also positive in 41% among only Brugada patients with documented VT or no symptoms. During vasovagal response, ST-segment augmentation in the right precordial leads was observed in 69% of the HUT-positive Brugada patients, but no ventricular arrhythmias were induced. These data suggest that some Brugada patients have impaired balance of autonomic nervous system, which may relate to their syncopal episodes. Additional studies including a large number of subjects are needed to validate our findings and possibly evaluate the role of the HUT test in risk stratification of patients with Brugada ECG.

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Voltage-Gated Sodium Channels Are Required for Heart Development in Zebrafish

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Rationale: Voltage-gated sodium channels initiate action potentials in excitable tissues. Mice in which *Scn5A* (the predominant sodium channel gene in heart) has been knocked out die early in development with cardiac malformations by mechanisms which have yet to be determined.

Objective: Here we addressed this question by investigating the role of cardiac sodium channels in zebrafish heart development.

Methods and Results: Transcripts of the functionally-conserved *Scn5a* homologs *scn5Laa* and *scn5Lab* were detected in the gastrulating zebrafish embryo and subsequently in the embryonic myocardium. Antisense knockdown of either channel resulted in marked cardiac chamber dysmorphogenesis and perturbed looping. These abnormalities were associated with decreased expression of the myocardial precursor genes *nkx2.5*, *gata4*, and *hand2* in anterior lateral mesoderm and significant deficits in the production of cardiomyocyte progenitors. These early defects did not appear to result from altered membrane electrophysiology, as prolonged pharmacological blockade of sodium current failed to phenocopy channel knockdown. Moreover, embryos grown in calcium channel blocker-containing medium had hearts that did not beat but developed normally.

Conclusions: These findings identify a novel, and possibly nonelectrogenic, role for cardiac sodium channels in heart development. (*Circ Res.* 2010;106:00-00.)

Key Words: ion channels ■ heart development ■ zebrafish ■ *scn5La*

The Na_v1 family of voltage-gated sodium channels are multi-protein complexes that account for the initial upstroke (phase 0) of the action potential in neurons, myocytes, and other excitable cells by permitting a rapid influx of Na^+ ions.[1] Ten distinct pore-forming (α) subunit genes (*SCNxA*) have been cloned,[1–3] and *SCN5A* encodes $Na_v1.5$, the predominant sodium channel isoform in myocardium.[4]

Perturbed expression or function of $Na_v1.5$ in patients can cause a range of phenotypes including the long QT syndrome, Brugada syndrome, progressive cardiac conduction system disease, and atrial arrhythmias.[5,6] Mice heterozygous for *Scn5a* deletion display slow conduction and susceptibility to ventricular tachycardia.[7] By contrast, $Scn5a^{-/-}$ homozygotes die between embryonic day (E)10 and E11 with abnormalities of ventricular morphogenesis, indicating that *Scn5a* is also required for normal development.[7] The mechanisms underlying these defects have not been determined.

Here, we used zebrafish to examine the role of sodium channels in the developing heart. Zebrafish embryos are optically transparent and externally fertilized, facilitating the study of early organ formation. Genetic manipulation is readily achieved using antisense morpholinos, and embryos are also permeable to small molecule drugs placed in their medium. The stages of zebrafish cardiac development have been well-delineated: cardiac precursors are located at the blastula margin at 5 hours postfertilization (hpf).[8] These bilateral precursors undergo a complex series of movements that result in the formation of a cardiac cone and subsequently a beating heart tube by 22 to 24 hpf.[9,10] The tube then loops to form a 2-chambered heart and the ventricular wall begins to thicken concentrically between 48 to 72 hpf.[9,10] Our findings identify a previously-unappreciated role for voltage-gated sodium channels in early cardiac development.

Methods

The following zebrafish strains were used in this study: AB, TuAB, *Tg(cmlc2:GFP)*, *Tg(cmlc2:DsRed2-nuc)*. Whole-mount in situ hybridization was performed using digoxigenin-labeled probes. Gene knockdown was achieved by morpholino antisense oligonucleotides (Gene Tools) designed to inhibit translation of mRNAs or disrupt splicing of premRNAs. Drugs and toxins were delivered to embryos by bath exposure or by pressure injection directly into the yoke sac, trunk circulation, sinus venosus, or pericardial sac, depending on the experi-

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Non-standard Abbreviations and Acronyms	
DsRed	Discosoma sp. red fluorescent protein
E	embryonic day
GFP	green fluorescent protein
hpf	hours postfertilization
$Na_v1.5$	sodium channel α subunit 1.5
nuc	nuclear
TTX	tetrodotoxin

ment and developmental stage. Transcript levels were quantified using Power SYBR Green (Applied Biosystems). Confocal images of live embryos were captured using a Zeiss LSM 510 Confocal Microscope System equipped with either ×20 or ×40 objective lenses. Image analysis and cell counts were performed using Image J software. Photographs of embryos were adjusted for brightness and contrast in Adobe Photoshop and/or Microsoft Powerpoint and identical adjustments were made for active treatment groups and controls. Statistical analyses were performed as indicated in the text and figure legends.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Cardiac Sodium Channels in Zebrafish

Two *SCN5A* orthologs have been cloned in zebrafish[11] (see also Online Figure I) and are termed *scn5Laa* and *scn5Lab*. These encode typical voltage-gated sodium channels sharing 60% to 65% amino acid identity with SCN5A (Online Figures II and III), with even greater conservation in important functional domains including the transmembrane segments, voltage-sensors, pore loops, inactivation gate, and C-terminus (Online Figure III). Expression of full-length *scn5Laa* and *scn5Lab* in CHO (Chinese hamster ovary) cells produced typical voltage-gated sodium currents (Figure 1A and 1B). Transcripts of both genes were detected in gastrulating embryos before the expression of early cardiogenic transcription factors and well in advance of myocardial differentiation (Figure 1C). Both sodium channel genes were expressed in the heart, brain, and spinal cord at 52 to 104 hpf by in situ hybridization (Figure 1D and 1E).

Knockdown of Zebrafish Cardiac Sodium Channels Results in Defects of Cardiac Morphogenesis

To investigate the role of *scn5Laa* and *scn5Lab* in development, we used multiple translation- and splice-blocking morpholino-antisense oligonucleotides (morpholinos) that specifically target each gene as well as 5-nucleotide mismatch control morpholinos (Online Figures IV and V; Online Table I). Knockdown of either *scn5Laa* or *scn5Lab* in *Tg(cmlc2:GFP)* zebrafish, a transgenic line expressing green fluorescent protein in the myocardium,[12] resulted in embryos with marked defects in cardiac development and function

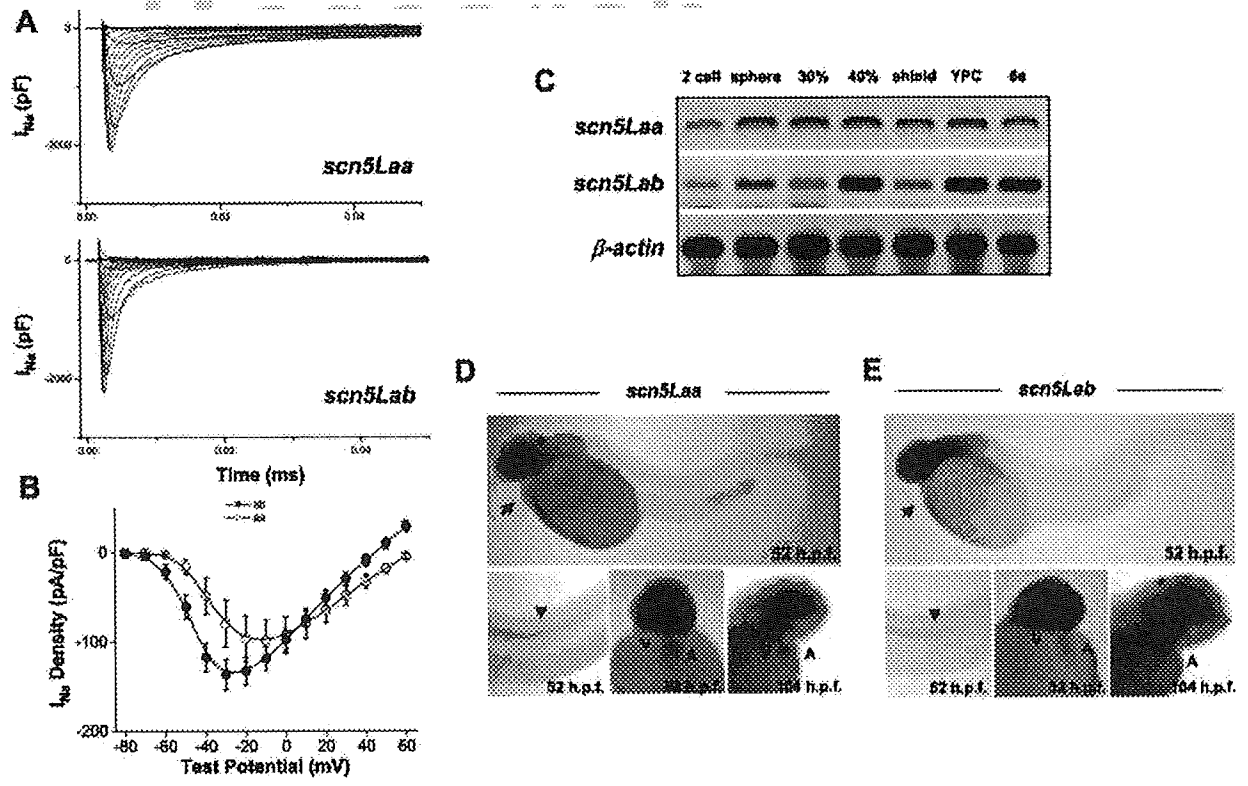

Figure 1. Function and developmental expression of zebrafish cardiac-type sodium channels. A and B, Whole cell sodium currents **(A)** and current–voltage relationships in CHO cells transfected with full-length *scn5Laa* and *scn5Lab* **(B). C,** Temporal expression of *scn5Laa* and *scn5Lab* in early embryos (0 to 12 hpf) by RT-PCR. s indicates somites; YPC, yoke plug closure stage. **D and E,** By whole-mount in situ hybridization, sodium channel gene expression was detected diffusely in both heart chambers before displaying predominately ventricular expression by 104 hpf. Transcripts were also detected in the brain and spinal cord. Developmental stages are as labeled. **Arrow** indicates heart; **arrowhead,** spinal cord. A, atrium; V, ventricle.

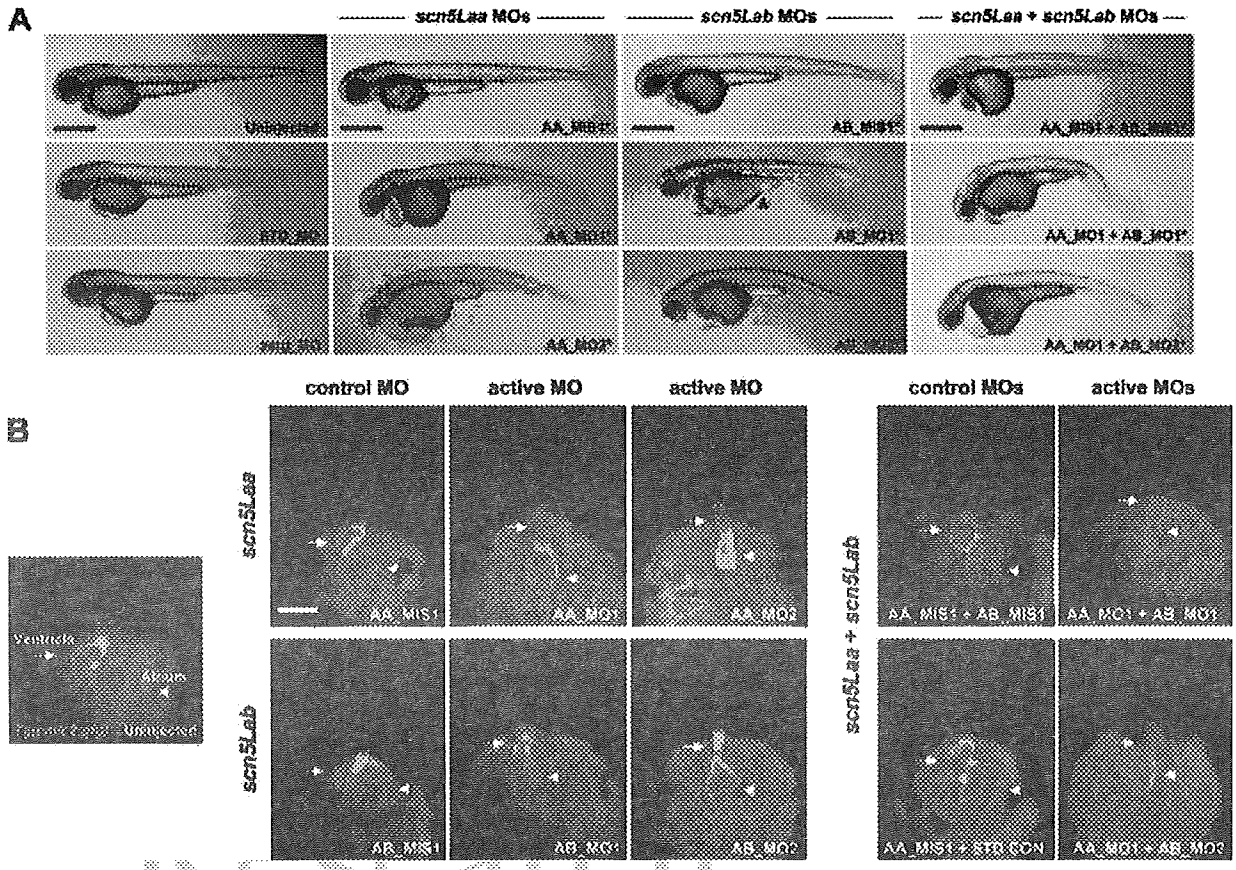

Figure 2. Zebrafish *scn5Laa* and *scn5Lab* are each required for normal cardiac development. A, Embryos at 58 to 62 hpf following treatment with active or control antisense morpholinos. All embryos were also treated with the p53 morpholino. Injection of p53 morpholino alone did not cause any identifiable phenotype (Online Figure VII). Note that head size is reduced in all sodium channel morphants and that many embryos injected with AB_MO1 display a yoke sac extension abnormality **(arrowhead). Scale bars:** 500 μmol/L. **B,** Cardiac developmental defects in morphant *Tg(cmlc2:GFP)* embryos. AA_MO1 and AB_MO2 caused the most severe cardiac phenotypes at the lowest doses. Knockdown of both *scn5Laa* and *scn5Lab* resulted in cardiac defects that were more severe than knockdown of either sodium channel alone, particularly with respect to ventricular morphogenesis. **Arrows** indicate ventricle; **arrowheads,** atrium. **Scale bar:** 150 μmol/L. Treatments are as labeled: STD_MO, standard control morpholino; *zerg*_MO, cardiac potassium channel morpholino; AA_MIS1, *scn5Laa* 5-mismatch control morpholino; AA_MO1, *scn5Laa* translation-blocking morpholino (ATG initiation site); AA_MO2, second *scn5Laa* translation-blocking morpholino (5′UTR); AB_MIS1, *scn5Lab* 5-mismatch control morpholino; AB_MO1, *scn5Lab* splice-blocking morpholino (E6I6); AB_MO2, second *scn5Lab* splice-blocking morpholino (E25I25).

compared to embryos injected with equivalent doses of control morpholinos (Figure 2 and Online Table II). Dual knockdown of *scn5Laa* and *scn5Lab* produced embryos with cardiac defects that appeared more severe than those resulting from knockdown of either gene alone, primarily with respect to ventricular development (Figure 2B). As an additional control, we knocked-down the cardiac potassium channel *zerg* which caused arrhythmias by 48 hpf but did not perturb development (Figure 2 and Online Table II).

Nonspecific morpholino toxicity or cell death can be minimized by concomitant knockdown of the zebrafish *p53* gene.[13] Coinjection of sodium channel and *p53* morpholinos resulted in embryos with increased head size and overall body length but equally severe defects in cardiac development compared to embryos injected with sodium channel morpholinos alone (Figure 2 and Online Figure VI). Acridine orange staining of morphant embryos identified no apoptotic cells in the dysmorphic, hypoplastic sodium channel morphant hearts (Online Figure VII).

Sodium Channels Are Required for the Production of Physiological Numbers of Embryonic Cardiomyocytes

Scn5Laa morphant hearts displayed marked abnormalities of both atrial and ventricular chamber morphogenesis and looping at 58 hpf (Figure 3A and 3B). At 104 hpf, when growth of the zebrafish heart is achieved primarily by cardiomyocyte proliferation, *scn5Laa* morphant cardiac chambers remained small (Figure 3C and 3D). Whereas control embryo hearts added cardiomyocytes to the interior of the ventricular wall to form trabeculae, *scn5Laa* morphant ventricles remained a single cell layer lacking trabeculated myocardium (Figure 3E through 3H). These findings indicate that sodium channel knockdown compromises both early chamber formation and normal patterned growth of the ventricle. Quantification of cardiomyocyte number in *Tg(cmlc2:DsRed2-nuc)* zebrafish revealed significant deficits in *scn5Laa* and *scn5Lab* morphants at 60 to 62 hpf (Figure 3I). Dual *scn5Laa/scn5Lab* morphants had the fewest number of cardiomyocytes (40% less than controls) (Figure 3I).

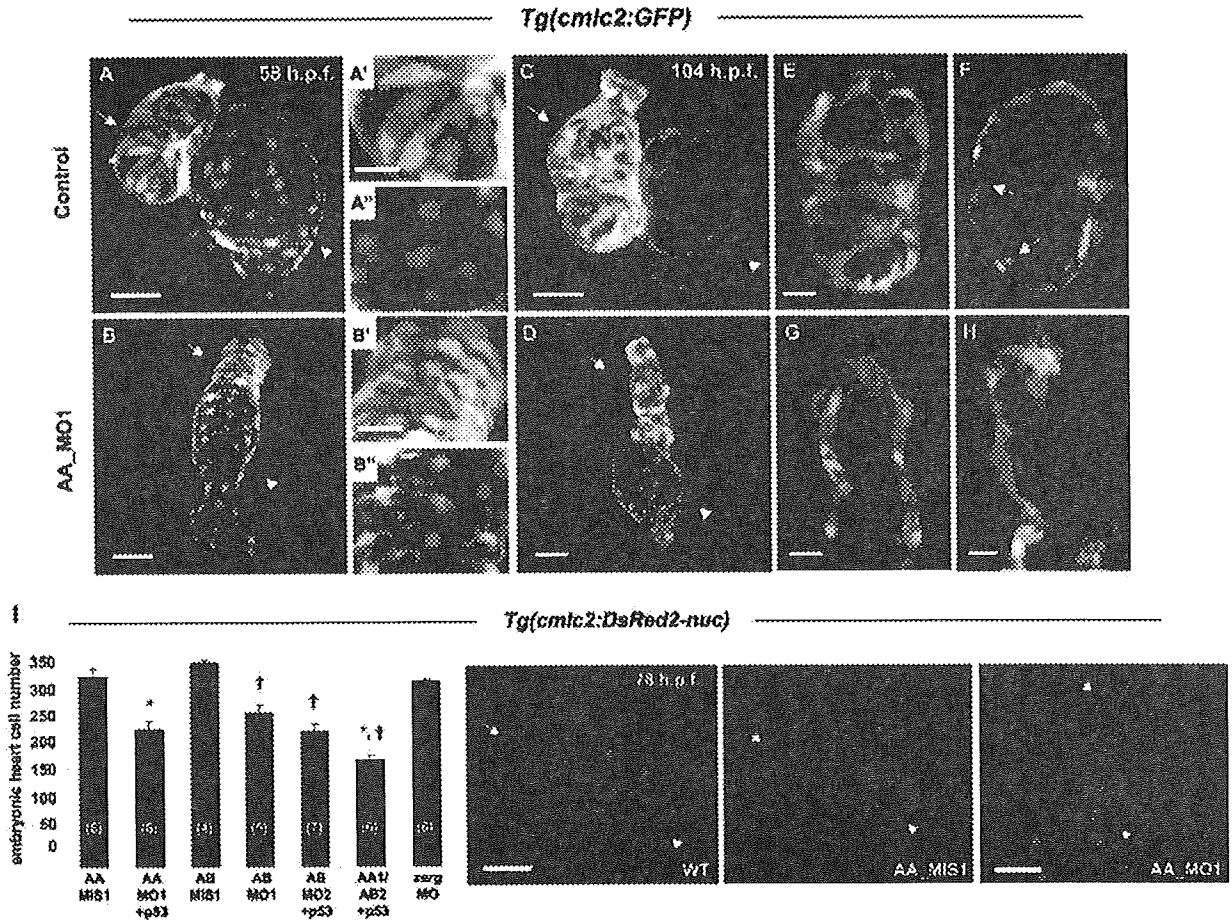

Figure 3. Sodium channel morphant embryos have reduced numbers of embryonic cardiomyocytes. A through H, Confocal reconstructions of hearts of control *Tg(cmlc2:GFP)* embryos **(A and C)** or clutchmates injected with the *scn5Laa* translation inhibitor morpholino (AA_MO1) **(B and D)** illustrate that sodium channels are required for normal numbers of embryonic cardiomyocytes. **Arrows** indicate ventricle; **arrowheads,** atrium at 58 hpf **(A and B)** and 104 hpf **(C and D)**, respectively. Higher magnification of ventricular and atrial chambers presented in **A′ and B′** and **A″ and B″**, respectively, illustrate similar chamber-specific cellular morphology in both control **(A′ and A″)** and morphant **(B′ and B″)** embryos. **Scale bars:** 50 μm **(A through D)** and 20 μm **(A′, A″, B′, and B″). E and F,** Serial confocal sections through the ventricular chamber of control embryos revealed trabeculation by 104 hpf **(arrows in F). Scale bar:** 20 μm. **G and H,** At the same time point, *scn5Laa* morphant ventricles remained a single layer (shown are ventricles from 2 different morphant embryos). **Scale bars:** 20 μm. **I,** Confocal reconstructions of the embryonic heart in *Tg(cmlc2:DsRed2-nuc)* embryos permitted quantification of deficits of embryonic cardiomyocytes in morphant embryos at 60 to 62 hpf. *Scn5Laa and scn5Lab* morphant hearts have significantly fewer embryonic cardiomyocytes than those of mismatch control morpholino-injected clutchmates and embryos injected with a morpholino targeting the *zerg* cardiac potassium channel. Results are means±SEM (N) in bar graph=number of hearts analyzed. **P*<0.01 vs AA_MIS1 and †*P*<0.01 vs AB_MIS1, ANOVA. Embryos injected with both AA_MO1 and AB_MO2 morpholinos (*scn5Laa*, *scn5Lab* double knockdown) had fewer cardiomyocytes than embryos injected with either AA_MO1 or AB_MO2 alone (177±8 vs 228±14 and 226±12, respectively), but the results were not statistically significant following ANOVA of all 7 groups. Where indicated, p53 morpholino was coinjected with active morpholinos as a control for nonspecific morpholino toxicity. Images are representative of wild type, *scn5Laa* control, and *scn5Laa* morphant embryo hearts at 78 hpf. **Arrows** indicate ventricle; **arrowheads,** atrium. **Scale bars:** 50 μm.

Knockdown of Sodium Channels Disrupts the Expression of Cardiogenic Transcription Factors in Anterior Lateral Mesoderm

The expression of scn5Laa and scn5Lab during gastrulation and the diminished production of cardiomyocytes in morphant embryos together suggested that sodium channels may be required for the specification of normal numbers of cardiac progenitor cells. To test this hypothesis, we used in situ hybridization to examine gene expression in the heart-forming region of anterior lateral plate mesoderm, a population of undifferentiated cells with cardiac potential demarcated by the expression of *nkx2.5* and the overlapping expression domains of *gata4* and *hand2*.[14] Compared to control embryos, *scn5Laa* morphants displayed decreased expression of all three transcription factors in anterior lateral plate mesoderm at the 6-somite stage (Figure 4A and 4C). Somite staging was used to confirm that the observed differences were not the result of developmental delay. Although *gata5* is a potent inducer of *nkx2.5* expression in zebrafish,[15] *gata5* expression levels were indistinguishable in *scn5Laa* morphant and control embryos (Figure 4A). These findings were independently corroborated by real-time RT-PCR (Figure 4B). Similar to *scn5Laa*, knockdown of *scn5Lab* also resulted in mispatterning of anterior lateral mesoderm with decreased expression of both *nkx2.5* and *gata4* (Figure 4D). The effect of *scn5Laa* and *scn5Lab* morpholinos on *nkx2.5* expression at 6 to 8 somites was dose-dependent (Figure 4E). These results indicate that zebrafish

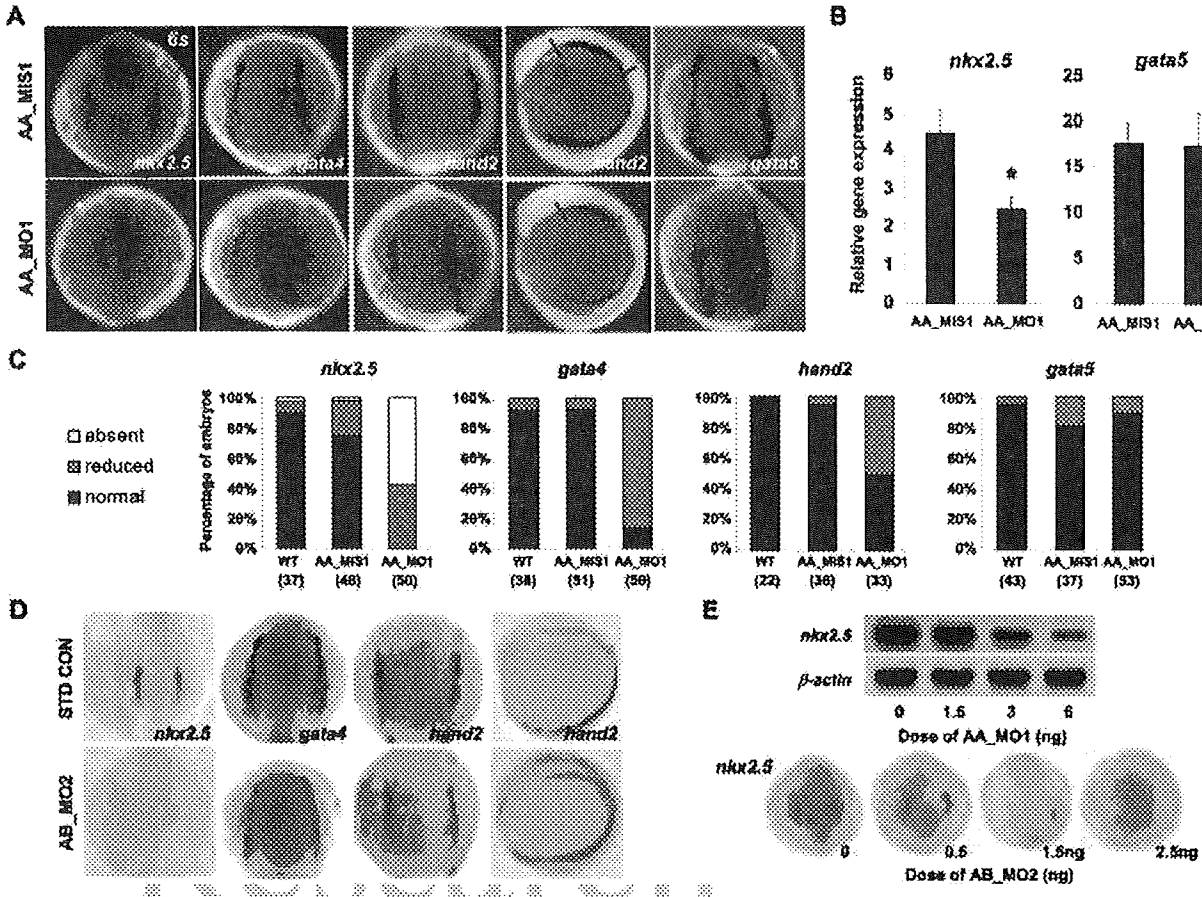

Figure 4. Sodium channel knockdown perturbs the expression of cardiac precursor genes in anterior lateral mesoderm. A, Analysis of gene expression in the early heart-forming region of anterior lateral plate mesoderm at the 6-somite stage by in situ hybridization. Embryos injected with *scn5Laa* translation inhibitor morpholino (AA_MO1) had reduced expression of *nkx2.5*, *gata4*, and *hand2* but no change in *gata5* expression compared to embryos injected with 5-mismatch control morpholino (AA_MIS1). All embryos shown are from the same injected clutch. **Scale bar:** 200 μm. **B,** Real-time quantitative RT-PCR performed using independent clutches of embryos also revealed significantly reduced *nkx2.5* expression but no change in *gata5* expression following injection of AA_MO1 compared to injection of AA_MIS1 control morpholino. Results are means±SEM for 3 independent experiments, each performed in triplicate. $P<0.05$ for *nkx2.5*, *t* test. **C,** Quantification of numbers (n) of *scn5Laa* morphant embryos displaying absent, reduced, or normal (comparable to wild type) expression levels of cardiac precursor genes as assessed by in situ hybridization. **D,** Knockdown of *scn5Lab* also resulted in markedly reduced expression of *nkx2.5* and *gata4* at 6 somites. However, no significant change in the expression of *hand2* was observed. **E,** Sodium channel morpholinos reduce *nkx2.5* expression in a dose-dependent manner as shown by RT-PCR (AA_MO1) and in situ hybridization at 6 somites (AB_MO2).

cardiac sodium channels are required for the normal expression of cardiac fate-determining genes in anterior lateral mesoderm and for specification of appropriate numbers cardiac progenitor cells during development.

Sodium Channel Knockdown Results in Reduced Numbers of Differentiating Cardiomyocytes

At the 16-somite stage, *scn5Laa* and *scn5Lab* morphants displayed reduced expression not only of *nkx2.5* but of the sarcomeric genes *cmlc2* and *vmhc* (Figure 5A). Moreover, examination of the number of somites (marked by *vmhc* expression) revealed that reductions in the size of the field of differentiating cardiomyocytes were not attributable to a global developmental delay. To assess the magnitude of the reduction in differentiating cardiomyocytes, we quantified total cardiac cell number in the cardiac cone of *Tg(cmlc2:GFP)* zebrafish at the 22-somite stage, the earliest time point when we could detect fluorescence in this line of transgenic zebrafish. At this stage, we found a significant reduction in the total number of newly differentiated myocytes in *scn5Laa* morphants compared to control embryos (Figure 5C).

Voltage-Gated Sodium Channels May Regulate Early Cardiac Development Independent of Membrane Electrophysiology

Prior studies in mice and chicks suggest that coordinated electric activity in the early heart does not require fast sodium current (I_{Na}).[16–18] Consistent with these prior observations, delivery of sodium channel blockers (eg, tetrodotoxin) and activators (eg, anemone toxin II) directly into the pericardial space of zebrafish embryos failed to perturb heart-beating at early stages but caused conduction abnormalities at later stages (Online Figure VIII; Online Table III; Online Movies I through IV). By contrast, the L-type calcium channel blocker nisoldipine silenced the heart at every stage examined. Therefore, we reared embryos in a high concentration of nisoldipine (10 μmol/L) from cleavage-stages

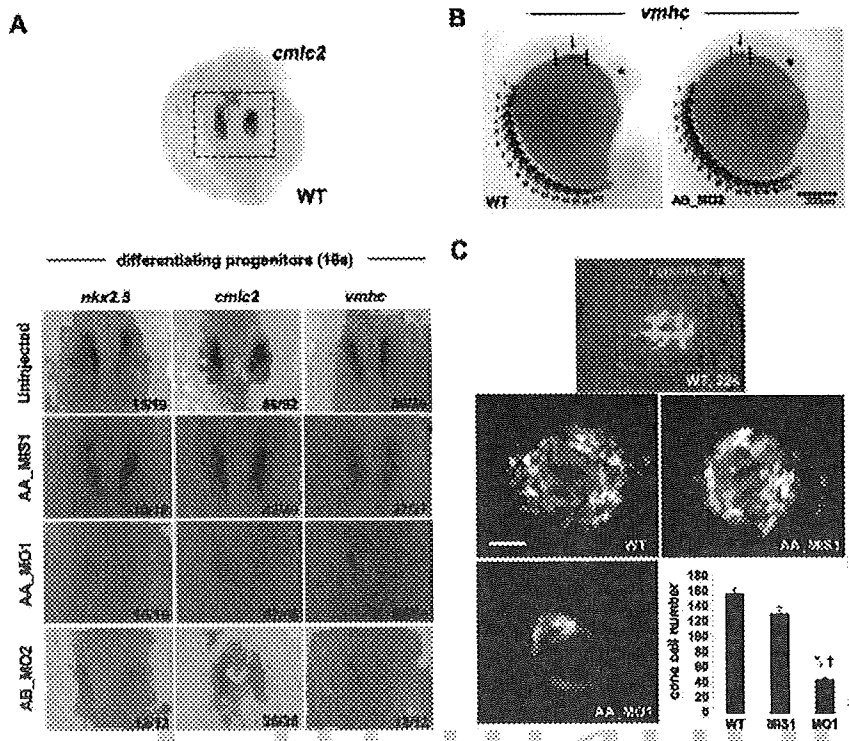

Figure 5. Sodium channel knockdown results in reduced numbers of differentiating cardiomyocytes. A, By in situ hybridization, *scn5Laa*, and *scn5Lab* morphant embryos at the 16-somite stage display markedly reduced expression of *nkx2.5* and of the myocardial sarcomeric genes *cmlc2* and *vmhc* compared to control embryos. Treatment groups are as labeled. The number of embryos displaying the phenotype/ the total number of embryos assessed is indicated in the **lower right corner of each image. B,** Deficiencies in cardiac differentiation are not attributable to developmental delay. Shown is an embryo injected with the AB_MO2 morpholino compared to an uninjected clutchmate. Despite dramatic differences in the size of the heart field and head, both embryos have equivalent numbers of somites. **C,** Confocal reconstructions of the developing heart cone in *Tg(cmlc2:GFP)* embryos at the 22-somite stage (embryo in **top image** is shown for orientation). **Scale bar:** 50 μm. *Scn5Laa* morphant heart cones (AA_MO1, n=4) have significantly fewer differentiating cardiomyocytes than the heart cones of wild type (WT) (n=6) and control-injected (AA_MIS1, n=3) clutchmates. Results are means±SEM. **P*<0.01 vs wild-type; †*P*<0.01 vs AA_MIS1-injected, ANOVA.

through 78 hpf. Nisoldipine-treated embryos failed to initiate heart beating at any stage examined but displayed normal cardiac morphogenesis through 60 hpf and normal cardiomyocyte number compared to vehicle-treated embryos (Figure 6A and 6B; Table). This result is similar to that previously observed in zebrafish harboring mutations in cardiac troponin T (*silent heart*) in which heart formation is unaffected despite an absence of both contractility and circulation.[19] These findings demonstrate that neither coordinated electric activity nor beating is required for the earliest steps of cardiac development.

We next hypothesized that sodium current (I_{Na}) may have an important role in early cardiac development before the onset of heart-beating. However, high concentrations of a range of drugs that activate or block I_{Na} had no effect on cardiac specification or differentiation when delivered to embryos in the bath solution or by microinjection (Figure 6C and the Table). Importantly, tetrodotoxin-treated embryos displayed complete neuromuscular paralysis at the end of the treatment period (≈28 hpf) (Table and Online Video V), and treatment with channel activators (eg, anemone toxin, veratridine) resulted in neuromuscular hyperexcitability (Table and Online Video VI). These findings indicate

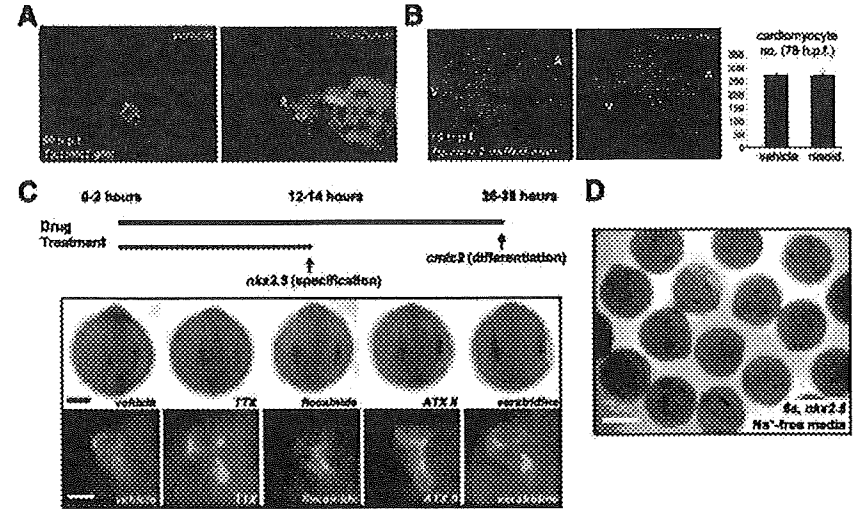

Figure 6. Voltage-gated sodium channels may regulate early cardiac development independent of membrane electrophysiology. A, Rearing of embryos in 10 μmol/L solution of the L-type calcium blocker nisoldipine inhibited heart-beating at all stages examined through 78 hpf but did not perturb early chamber formation or the initiation of looping. **B,** Analysis of vehicle-treated and nisoldipine-treated *Tg(cmlc2:DsRed2-nuc)* embryos indicated that L-type calcium channel-blockade did not affect embryonic cardiomyocyte number through 78 hpf. Results are means±SEM for vehicle-treated (n=2) and nisoldipine-treated (n=3) embryos, respectively. **C,** Prolonged exposure of developing embryos to pharmacological modulators of sodium channel function failed to disrupt either cardiac specification or differentiation. In situ hybridization for *nkx2.5* at the 6- to 8-somite stage **(top)** and fluorescent microscopy of the heart tubes of *Tg(cmlc2:GFP)* embryos at 26 to 28 hpf **(bottom)**, after the indicated treatments (see Table 2). ATX II indicates anemone toxin II; TTX, tetrodotoxin. **Scale bars:** 200 μm **(top)**; and 100 μm **(bottom). D,** In situ hybridization for *nkx2.5* at the 6-somite stage after incubation from the 1-cell stage in sodium-free media to inhibit inward sodium current (I_{Na}). Early heart development proceeded normally. Heart tubes also form normally (not shown). **Scale bar:** 500 μm.

Table. Developmental Effects of Pharmacological Modulators of Ion Channel Function

Target	Agent	Type	Delivery	[Drug]	n	Phenotypes
VGSC	Tetrodotoxin	Inhibitor	Bath solution	500 μm	74	• Normal heart tube formation (68/70) • Total neuromuscular paralysis (70/70)
			Bath solution	500 μm	26	• Normal *nkx2.5* expression (24/26)
			Microinjection	1 mmol/L	99	• Normal heart tube formation (99/99) • Total neuromuscular paralysis (93/99)
VGSC	Lidocaine	Inhibitor	Bath solution	500 μm	200	• Normal heart tube formation (200/200) • Epiboly defects at higher doses
VGSC	Anemone toxin II	Activator	Bath solution	10 μmol/L	25	• Normal *nkx2.5* expression (20/25)
			Microinjection	100 μmol/L	50	• Normal heart tube formation (50/50) • Convulsions, tonic contraction elicited with mechanical stimulation
VGSC	Veratridine	Activator	Bath solution	100 μmol/L	34	• Normal *nkx2.5* expression (26/34)
			Microinjection	1 mmol/L	50	• Normal heart tube formation (50/50) • Convulsions, tonic contraction elicited with mechanical stimulation
VGSC; ERG	Flecainide	Inhibitor	Bath solution	400 μm	46	• Normal heart tube formation (45/46) • Bradycardia, 2:1 heart block, less frequent silent ventricle or irregular atrial rhythm on day 2 in morphologically normal hearts, similar to ERG block alone (45/45)
			Bath solution	400 μm	29	• Normal *nkx2.5* expression (23/29)
LTCC	Nisoldipine	Inhibitor	Bath solution	10 μm	100	• Normal heart tube formation (100/100) • No heart beat, circulation at any stage • Atrium, ventricle, outflow tract morphologically normal through day 2.5 • Cardiac looping normal through day 2.5 • Markedly decreased *GFP* intensity (~*cmlc2* promoter activity) at all stages
LTCC/TTCC	Mibefradil	Inhibitor	Bath solution	250 μm	77	• Normal heart tube formation (77/77) • Bradycardia, weak contractility on day 2 in morphologically normal hearts (77/77)
ERG	E-4031	Inhibitor	Bath solution	250 μm	93	• Normal heart tube formation (93/93) • Arrhythmias on day 2 in morphologically normal hearts Bradycardia, silent ventricle (85/93) Irregular atrial rhythm, silent ventricle (7/93) silent heart (1/93)

ERG indicates ether-a-go-go related gene potassium channel; LTCC, L-type calcium channel; TTCC, T-type calcium channel; VGSC, voltage-gated sodium channel. For bath immersion, embryos were dechorionated and placed in drug solutions by the 64-cell stage. For microinjection, 3 nL of drug solution was injected in 2 different locations of the yoke sac of 1- to 4-cell-stage embryos. Cardiac lineage specification assessed by in situ hybridization for *nkx2.5* at 8 somites. Differentiation was assessed by heart tube formation and heart beating in *Tg(cmlc2:GFP)* embryos at 28–30 hours postfertilization. See Methods for additional details.

adequate compound penetration and confirmed that these toxins interact with other voltage-gated sodium channels in vivo, even at early stages. Early cardiogenesis also proceeded normally in embryos reared from the 1 cell stage in sodium-free media, another approach that inhibits inward I_{Na} (Figure 6D). Taken together, these results indicate that sodium channel expression but not sodium current is required for early cardiac development in zebrafish.

Discussion

Na_v1 voltage-gated sodium channels underlie the upstroke of the action potential and are thus the principal determinants of membrane excitability in the nervous system, skeletal muscle, heart and other tissues.[1] In the present study, we identified a previously-unappreciated role for cardiac-type sodium channels in the developing heart. We detected expression of *scn5Laa* and *scn5Lab* in the gastrulating embryo, before the differentiation of excitable tissues. Using both translation- and splice-blocking morpholino antisense oligonucleotides, we found that reduced expression of either isoform disrupted early cardiogenic gene expression in anterior lateral mesoderm and compromised the generation of sufficient numbers of progenitor cells for normal cardiac morphogenesis. We and others demonstrated that hearts that do not beat still display normal early development.[19] Moreover, pharmacological manipulation of sodium current (I_{Na}) in vivo generated neuromuscular phenotypes but did not affect cardiac gene expression or morphogenesis, suggesting that sodium channel expression but not I_{Na} is required for cardiac development. These findings indicate that voltage-gated sodium channels have important roles in vertebrate heart development and support the hypothesis that these roles are mediated by nonelectrogenic functions of the channel complex.

Voltage-Gated Sodium Channels Are Required for Early Cardiac Gene Expression

A key defect we observed in sodium channel morphant embryos was a deficiency in the expression of multiple transcription factors that contribute to the specification of cardiac progenitor cells from anterior lateral mesoderm. Although reduced in number, these progenitors appear to normally differentiate into nascent cardiomyocytes. Early cardiogenic mesoderm is distinguished by

the overlapping expression of a group of highly conserved regulatory genes belonging to the NK2, GATA, MEF2, T-box, and bHLH (basic helix–loop–helix) families of transcription factors.[20,21] The markedly reduced expression of *nkx2.5* following knockdown of either *scn5Laa* or *scn5lab* suggests a disruption of one or more of the molecular mechanisms that normally act to specify the cardiac cell fate during gastrulation.

Prior studies in zebrafish have demonstrated that *gata5* is a potent positive regulator of *nkx2.5* expression, potentially analogous to the role played by *gata4* or *gata6* in mammals.[15,22] Notably, we found that *scn5Laa* regulates *nkx2.5* expression without affecting levels of *gata5*, analogous to *fgf8* signaling.[23] This suggests that *scn5Laa* may act downstream of *gata5* or *fgf8* or in a parallel, previously-undefined pathway to regulate cardiac development. Knockdown of *scn5Laa* and *scn5Lab* also resulted in changes in the expression of *gata4* and *hand2* in the anterior lateral mesoderm. In zebrafish, both *gata4* and *hand2* are expressed in larger domains of anterior lateral mesoderm than *nkx2.5* and are important for normal cardiogenesis.[14,24,25] Reduced *gata4* expression was observed to cause defects in cardiac chamber growth and looping, whereas loss of *hand2* resulted in embryos with significantly fewer embryonic cardiomyocytes.[24,25] Moreover, the expression domain of *hand2* appears to more accurately mark the population of early myocardial progenitors in zebrafish than *nkx2.5*.[14] Rather than being required for the patterning or boundaries of the heart-forming region in mesoderm, *hand2* appears to have a direct, permissive role in promoting cardiac differentiation within this cell population.[14,25] Taken together, the diminished number of cardiac progenitor cells observed in zebrafish cardiac sodium channel morphants is likely to be a consequence of the reduced expression level and/or domain of *nkx2.5*, *gata4*, and *hand2*, leading to a reduction in the size of the heart-forming region in anterior lateral mesoderm and limiting overall myocardial potential.

Voltage-Gated Sodium Channels May Contribute to Early Cardiogenesis Independent of Membrane Electrophysiology

Supratherapeutic doses of tetrodotoxin or other sodium channel active drugs did not perturb cardiac development. Cardiogenesis also proceeded normally when sodium was withdrawn from the external media in which embryos developed, another method for blocking inward I_{Na}. These findings thus suggest that zebrafish cardiac sodium channels may act in development via mechanisms that are independent of membrane depolarization.

Mounting evidence indicates that voltage-gated sodium channels subserve multiple functions whose disruption may underlie the phenotypes we describe. For example, sodium channel complexes play a role in cell adhesion.[26] Pore-forming Na_v1 sodium channel α subunits are known to interact with one or more ancillary β subunits (encoded by *SCN1B-4B)* that modulate α subunit expression and function.[1,2] Through their extracellular immunoglobulin (IG) domains, β subunits also mediate interactions between the sodium channel complex and other cell adhesion molecules and extracellular matrix proteins.[26] Sodium channels are also known to interact with many other functionally-diverse proteins including 14-3-3, Nedd-4 type ubiquitinases, calmodulin, ankyrin G, syntrophins, plakophilin, and fibroblast growth factor homologous factors,[27,28] so it is possible that the sodium channel α subunit itself could act as a scaffold to facilitate intracellular signaling by these associated proteins within the channel complex.

Alternatively, sodium channel α subunits may generate nonelectrogenic intracellular signals via mechanisms that are intrinsic to the channel protein itself. The C-terminal tail of L-type calcium channels, for example, is cleaved from the pore-forming α subunit and translocates from the membrane to the nucleus where it regulates gene expression.[29] In this context, it is notable that zebrafish harboring a nonsense mutation in the cardiac L-type calcium channel α1c gene (*island beat)* display atrial arrhythmias and a small, noncontractile, hypoplastic ventricle by 72 hpf.[30] By contrast, we found that pharmacological blockade of calcium entry through L-type calcium channels over a similar time period did not significantly affect ventricular development. Future studies are required for additional insight into the possible nonelectrogenic functions of cardiac voltage-gated sodium and calcium channels.

Limitations

In these experiments, we cannot exclude the possibility that intracellular sodium conductance (eg, channels acting on the membrane of an organelle rather than at the cell surface) is required for normal development. Second, rescue of morphant phenotypes with full length *scn5Laa* or *scn5Lab* mRNA has not been possible to date. This is likely attributable to inadequate translation of the 6-kb transcripts, as we have been unable to detect an epitope tag engineered into the rescue construct. Finally, knockdown of either *scn5Laa* or *scn5Lab* resulted in a severe cardiac phenotype. It is possible that the two genes, which have significantly diverged in amino acid sequence, may subserve different functions during heart development that were not uncovered by our assays. Alternatively, it is possible that manipulating the expression of one gene altered the expression or function of the other.

In summary, the results presented here argue that sodium channels are required for heart development in addition to their canonical role as regulators of heart rhythm. Moreover, the data suggest that this developmental role is mediated by a nonelectrogenic function of the channel.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Activation of the voltage-gated sodium channels leads to the depolarization of excitable membranes in mature myocardium, skeletal muscle, and nerve.
- Mutations in *Scn5a*, the predominant cardiac sodium channel gene, are associated with multiple heritable disorders of heart rhythm.
- Whereas *Scn5a*$^{+/-}$ mice develop myocardial fibrosis in senescence, *Scn5a*$^{-/-}$ mice develop marked abnormalities of ventricular morphogenesis by embryonic day 10.5 and die by day 11.5.

What New Information Does This Article Contribute?

- In zebrafish, *scn5a* orthologs are expressed in the gastrulating embryo, before the differentiation of excitable tissues.
- Antisense knockdown of either zebrafish cardiac sodium channel resulted in reduced expression of early markers of cardiomyocyte fate and marked chamber dysmorphogenesis.
- These findings suggest that the zebrafish cardiac sodium channels affect heart development via a nonelectrogenic mechanism.

Voltage-gated sodium channels are the principal determinants of membrane excitability. In the heart, mutations in the cardiac sodium channel gene *Scn5a* are well-described in heritable arrhythmia syndromes. Whereas sodium channels are known to play a critical role in the mature heart, the function of *Scn5a* during cardiac development is less well understood. Here, we used zebrafish embryos to investigate the role of sodium channels in cardiogenesis. We observed that zebrafish cardiac sodium channel genes are expressed before myocardial differentiation. Embryos treated with sodium channel antisense displayed reduced expression of early cardiogenic transcription factors including *nkx2.5*, *gata4*, and *hand2*, leading to diminished numbers of cardiac progenitor cells, cardiac chamber dysmorphogenesis, and perturbed looping. Prolonged pharmacological blockade of sodium current failed to phenocopy channel knockdown, suggesting that sodium channels act in early development via mechanisms that are independent of membrane electrophysiology. These studies may contribute to an improved understanding of the spectrum of clinical phenotypes linked to mutations in *Scn5a*.

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RESEARCH ARTICLE

Metabolic syndrome and risk of development of chronic kidney disease: the Niigata preventive medicine study

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Abstract

Background The metabolic syndrome consists of a cluster of cardiovascular risk factors, many of which have also been implicated in the genesis of chronic kidney disease. We studied the impact of the metabolic syndrome on chronic kidney disease in general population.

Methods The Niigata Preventive Medicine Study was community-based prospective observational cohort study based upon the annual health examinations in Japan. We studied the association of the metabolic syndrome with a risk of development of kidney dysfunction and proteinuria in 34 986 participants without baseline kidney disease.

Results The metabolic syndrome was present in 3679 subjects (11%). During a follow-up of 5.8 years, kidney dysfunction developed in 184 subjects with metabolic syndrome (5.0%) and 746 subjects without metabolic syndrome (2.4%). The metabolic syndrome was associated with development of kidney dysfunction (hazard ratio [HR], 2.12). All of the metabolic syndrome components were associated with risk of kidney dysfunction. The risk of kidney dysfunction increased across a number of the fulfilled metabolic syndrome components. The association of metabolic syndrome with kidney dysfunction remained significant in subjects without hypertension, diabetes, or cardiovascular disease (HR, 1.99) and in those ≤60 years without hypertension, diabetes, or cardiovascular disease (HR, 2.11). The metabolic syndrome was similarly associated with the development of proteinuria in all subjects (HR, 1.67), in those without hypertension, diabetes, or cardiovascular disease (HR, 1.64) and in those ≤60 years without hypertension, diabetes, or cardiovascular disease (HR, 2.14).

Conclusions The metabolic syndrome was associated with kidney disease even in subjects without major classical risk factors for chronic kidney disease.

Keywords metabolic syndrome; kidney disease; proteinuria; kidney dysfunction

Introduction

Chronic kidney disease is a worldwide public health problem [1,2]. In addition to increasing size of the end-stage kidney disease population [1], chronic kidney disease often coexists with cardiovascular disease and is a risk factor for all-cause mortality and cardiovascular

events including coronary heart disease, heart failure, ischaemic stroke, and peripheral arterial disease [2]. Chronic kidney disease is generally irreversible, and thus identification of risk factors is important for development of therapeutic approaches to prevent kidney dysfunction.

The metabolic syndrome is characterized by a cluster of atherosclerotic risk factors including obesity, hypertension, insulin resistance, and dyslipidaemia [3]. Many of the metabolic syndrome components, especially for hypertension and impaired glucose tolerance, are also risk factors for the development of kidney disease [4,5]. Moreover, it has been implicated that the metabolic syndrome and chronic kidney disease share pathogenetic components such as inflammation and oxidative stress [6–9]. Since both of these conditions are associated with significant morbidity and mortality with an increasing health burden, it is important to assess the relationship between the two conditions [2,10,11]. Recently, it has been reported that the metabolic syndrome is associated with kidney disease in cross-sectional studies [12,13], and that the metabolic syndrome is a risk factor for kidney disease in longitudinal studies [14–18]. However, most of these prospective studies have been conducted in subdivided subjects (male subjects, subjects with diabetes, etc.), and one study has provided controversial results [18]. To study the impact of the metabolic syndrome on chronic kidney disease in general population, we studied the association of the metabolic syndrome with the development of kidney disease in relatively healthy subjects without diabetes, treated hypertension, or cardiovascular disease, all of which are classical risk factor for kidney disease, in the largest cohort to date.

Research design and methods

Study subjects

This community-based prospective observational cohort study was based upon the annual health examinations in Niigata Association for Comprehensive Health Promotion and Research, Niigata, Japan [19]. In the prefecture, annual health examinations supported by administration are available to residents aged ≥20 years. The population of the prefecture is about 2 400 000, and about 240 000 residents receive the examination during 1 year. The annual examination consists of a detailed medical history, physical examination, blood examination including blood cell count and biochemical markers, chest X-ray, urine test, and a 12-lead electrocardiogram. Urine protein level is measured using semiquantitative urine sticks (1+, 2+, and 3+ corresponding to protein levels of about ≥30, ≥100, and ≥300 mg/dL, respectively). The present report includes subjects who had at least one examination between 1996 and 1998 (designated the baseline examination) and subsequently were submitted to at least one annual examination through 2005. Detailed inclusion and exclusion criteria are provided below.

Definition of metabolic syndrome

The metabolic syndrome was defined according to the guidelines of the National Cholesterol Education Program Third Adult Treatment Panel with a modification for body size [3]. Based on the baseline examination, the metabolic syndrome was diagnosed when at least three of the following criteria were met: (1) elevated body mass index (BMI) (in lieu of waist measurement, which was not available in our database). BMI was calculated by dividing weight in kilograms by the square of the height in meters. The frequency of BMI ≥30 kg/m^2 is 2–3% in Japan and 20–30% in Western countries [2,20]. Because of the differences in BMI between Japanese and Western populations, values ≥25 kg/m^2 were considered elevated (in contrast to ≥30 kg/m^2 in Western populations) according to criteria of the Japan Society for the Study of Obesity [20,21]; (2) elevated triglycerides (≥150 mg/dL); (3) low HDL (high-density lipoprotein) cholesterol (<40 mg/dL in men, <50 mg/dL in women); (4) elevated blood pressure (systolic blood pressure ≥130 mmHg, diastolic blood pressure ≥85 mmHg, and/or the presence of treated hypertension); and (5) impaired glucose tolerance (fasting serum glucose ≥110 mg/dL and/or the presence of diabetes).

Definition of kidney disease

Estimated glomerular filtration rate (GFR) in each subject was calculated based on serum creatinine, sex, and age using the approach of the Japanese Society of Nephrology: GFR (mL/min per 1.73 m^2) = $0.881 \times 186 \times \text{age}^{-0.203} \times \text{serum creatinine}^{-1.154}$ (if female × 0.742) [22]. Subjects were included if they had a baseline examination including electrocardiogram, serum creatinine, and urine protein levels; and at least one follow-up consecutive examination including serum creatinine and urine protein evaluations. Subjects who had baseline kidney dysfunction (GFR <60 mL/min per 1.73 m^2) were excluded when the association of metabolic syndrome with kidney dysfunction was studied. The development of kidney dysfunction was defined by a baseline normal value for age (≥60 mL/min/1.73 m^2) and a decline by ≥10 mL/min/1.73 m^2, above the normal age-related decrease over 10 years [5], to <60 mL/min/1.73 m^2 in each annual follow-up. Proteinuria was defined as a urine stick result ≥1+. When the association of metabolic syndrome with development of proteinuria was studied, subjects who had proteinuria at baseline were excluded. Subjects who received antihyperlipidaemic drugs were excluded from all analyses because detailed data on individual drug regimens were not available.

Data analysis

Hazard ratio and 95% confidence interval were calculated from Cox proportional-hazards models to study contribution of age as a continuous value and sex. Cox models

were adjusted for age as a continuous value and sex to evaluate the contribution of the metabolic syndrome, the components of the metabolic syndrome, and the number of fulfilled metabolic syndrome components to development of kidney disease. All statistical analyses were performed with SPSS, version 12.0 (SPSS Inc., Chicago, IL). A two-sided $p < 0.05$ was considered statistically significant. Values are expressed as mean ± SD.

Results

Baseline characteristics

In the entire cohort including 34 986 subjects, the mean age was 59.0 ± 11.3 years and 66% of subjects were women (Table 1). Antihypertensive treatment was given in 19% of the subjects and diabetes was present in 6% of the subjects. The metabolic syndrome was present in 3679 subjects (11%). Prevalence of the metabolic syndrome components and number of fulfilled components are shown in Table 2.

Development of kidney dysfunction

During a follow-up of 5.8 ± 2.4 years, kidney dysfunction developed in 184 subjects with the metabolic syndrome (5.0%) (stage 3 chronic kidney disease [30 ≤ GFR <60], $N = 179$ [4.9%]; stage 4 [15 ≤ GFR <30], $N = 5$ [0.1%]) and 746 subjects without the metabolic syndrome (2.4%) (stage 3, $N = 732$ [2.3%]; stage 4, $N = 11$ [0.04%]; stage 5 [GFR <15], $N = 3$ [0.01%]). The changes in GFR from baseline were similar between subjects with the metabolic syndrome and those without the metabolic syndrome. The incidence of kidney dysfunction was 2.3 times higher in subjects with the metabolic syndrome than those without the metabolic syndrome (Table 3). In subjects without antihypertensive drug, diabetes, or cardiovascular disease and in those ≤60 years without antihypertensive drug, diabetes, or cardiovascular disease, the incidence of kidney dysfunction was also higher in the metabolic syndrome.

In univariate models, increasing age [hazard ratio (HR), 1.11 95% confidence interval (CI), 1.10–1.11 per year; $p < 0.001$] and male gender (HR, 1.38; 95% CI, 1.22–1.56; $p < 0.001$) were associated with new onset of kidney dysfunction. In multivariate models adjusted for age and sex, the presence of metabolic syndrome was associated with the development of kidney dysfunction (Table 4). The association of the metabolic syndrome with kidney dysfunction remained significant in subjects without antihypertensive drug, diabetes, or cardiovascular disease and in those ≤60 years without antihypertensive drug, diabetes, or cardiovascular disease.

We also evaluated the contribution of the metabolic syndrome components to development of kidney dysfunction. All of the five components of the metabolic syndrome were associated with development of kidney dysfunction. Among the components of the metabolic syndrome, elevated blood pressure showed the highest risk of development of kidney dysfunction in all subjects. However, impaired glucose tolerance showed the highest risk in subjects ≤60 years without antihypertensive drug, diabetes, or cardiovascular disease. The risk of development of kidney dysfunction increased across a number of the fulfilled components of the metabolic syndrome and the trend was significant.

Table 1. Baseline characteristics of study subjects with and without the metabolic syndrome

				Subjects without antihypertensive drug, diabetes, or CVD		Subjects ≤60 years without antihypertensive drug, diabetes, or CVD	
	All subjects $N = 34\,986$	No metabolic syndrome $N = 31\,307$	Metabolic syndrome $N = 3679$	No metabolic syndrome $N = 25\,100$	Metabolic syndrome $N = 2259$	No metabolic syndrome $N = 13\,807$	Metabolic syndrome $N = 1082$
Age, years	59.0 ± 11.3	58.7 ± 11.4	61.1 ± 10.3	57.3 ± 11.5	59.1 ± 10.9	48.9 ± 7.9	50.0 ± 7.8
Female sex, *N* (%)	23062 (66%)	20667 (66%)	2395 (65%)	16930 (67%)	1424 (63%)	20667 (66%)	2395 (65%)
Body mass index, kg/m^2	22.9 ± 3.0	22.5 ± 2.7	26.2 ± 2.9	22.4 ± 2.7	26.2 ± 2.9	22.3 ± 2.7	26.6 ± 3.0
Blood pressure, mmHg							
Systolic	129.1 ± 18.1	127.6 ± 17.6	142.4 ± 16.0	124.7 ± 16.2	139.2 ± 14.9	121.0 ± 15.1	136.8 ± 14.4
Diastolic	77.4 ± 11.1	76.6 ± 10.9	84.0 ± 10.3	75.3 ± 10.4	83.1 ± 10.0	74.5 ± 10.6	84.0 ± 10.2
HDL cholesterol, mg/dL							
Men	59.0 ± 15.6	60.6 ± 15.2	46.2 ± 12.2	60.4 ± 15.1	45.5 ± 11.8	66.6 ± 15.1	47.2 ± 10.2
Women	63.5 ± 15.2	65.3 ± 14.6	47.9 ± 10.4	65.5 ± 14.7	47.2 ± 9.9	60.1 ± 15.1	45.1 ± 11.6
Triglycerides, mg/dL	103.8 ± 69.9	93.3 ± 52.2	193.0 ± 120.0	92.4 ± 52.5	199.2 ± 125.8	91.0 ± 57.7	216.8 ± 154.6
Fasting glucose, mg/dL	94.3 ± 16.3	92.8 ± 13.6	106.8 ± 27.8	92.0 ± 12.0	103.4 ± 24.2	90.8 ± 11.0	101.9 ± 24.8
Serum creatinine, mg/dL	0.64 ± 0.18	0.64 ± 0.18	0.66 ± 0.17	0.63 ± 0.15	0.66 ± 0.17	0.62 ± 0.14	0.65 ± 0.16
Estimated GFR, mL/min/1.73 m^2	104.7 ± 24.6	105.1 ± 24.5	101.8 ± 25.7	105.9 ± 24.2	102.9 ± 24.3	109.1 ± 23.9	106.8 ± 23.0
Antihypertensive drug, *N* (%)	6609 (19%)	5370 (17%)	1239 (34%)	–	–	–	–
Diabetes, *N* (%)	2273 (6%)	1913 (6%)	360 (10%)	–	–	–	–
Cardiovascular disease, *N* (%)	2259 (6%)	1997 (6%)	262 (7%)	–	–	–	–

Values are expressed as mean ± SD or number when indicated. CVD denotes cardiovascular disease including heart diseases and stroke.

Table 2. Prevalence of the metabolic syndrome components and number of fulfilled components

	All subjects	Subjects without antihypertensive drug, diabetes, or CVD	Subjects ≤60 years without antihypertensive drug, diabetes, or CVD
Metabolic syndrome components			
Obesity	7499 (21%)	5280 (19%)	2837 (19%)
Elevated blood pressure	17919 (51%)	12118 (44%)	5210 (35%)
Impaired glucose tolerance	3229 (9%)	1883 (7%)	761 (5%)
Reduced HDL cholesterol	4932 (14%)	3666 (13%)	1926 (13%)
Elevated triglycerides	5250 (15%)	3837 (14%)	2045 (14%)
Number of metabolic syndrome component disorders			
0	11405 (32%)	10412 (38%)	6802 (46%)
1	13064 (37%)	9975 (36%)	4779 (32%)
2	6838 (20%)	4713 (17%)	2226 (15%)
≥3	3679 (11%)	2259 (8%)	1082 (7%)

HDL, high-density lipoprotein. Because of rounding, percentages do not always total 100%.

Table 3. Incidence of development of kidney dysfunction and proteinuria

	All subjects		Subjects without antihypertensive drug, diabetes, or CVD		Subjects ≤60 years without antihypertensive drug, diabetes, or CVD	
	No metabolic syndrome	Metabolic syndrome	No metabolic syndrome	Metabolic syndrome	No metabolic syndrome	Metabolic syndrome
Development of kidney dysfunction						
Number of events/person-years	746/179196	184/19193	437/146161	76/18433	102/82380	17/6053
Incidence per 1000 person-years (95% CI)	4.2 (3.9–4.5)	9.6 (8.2–11)	3.0 (2.7–3.3)	4.1 (3.2–5.0)	1.2 (1.0–1.5)	2.8 (1.5–4.1)
Development of proteinuria						
Number of events/person-years	1852/172985	354/17809	1245/141834	169/11362	508/79750	83/5644
Incidence per 1000 person-years (95% CI)	10.7 (10.2–11.2)	19.9 (17.8–21.9)	8.8 (8.3–9.3)	14.9 (12.6–17.1)	6.4 (5.8–6.9)	14.7 (11.6–17.8)

Kidney dysfunction was defined by occurrence of estimated glomerular filtration rate <60 mL/min/1.73 m^2.

Development of proteinuria

During the follow-up, proteinuria developed in 354 subjects with the metabolic syndrome (9.6%) and 1852 subjects without the metabolic syndrome (5.9%). The changes of GFR from baseline were similar between subjects who developed proteinuria and those who did not develop. The incidence of proteinuria was 1.9 times higher in subjects with the metabolic syndrome than those without the metabolic syndrome. In subjects without antihypertensive drug, diabetes, or cardiovascular disease and in those ≤60 years without antihypertensive drug, diabetes, or cardiovascular disease, the incidence of proteinuria was also higher in the metabolic syndrome.

In univariate models, increasing age (HR, 1.04; 95% CI, 1.03–1.04; $p < 0.001$) and male gender (HR, 1.77; 95% CI, 1.65–1.91; $p < 0.001$) were associated with new onset of proteinuria. In multivariate models adjusted for age and sex, the presence of metabolic syndrome was associated with the development of proteinuria (Table 5). The association of the metabolic syndrome with proteinuria remained significant in subjects without antihypertensive drug, diabetes, or cardiovascular disease and in those ≤60 years without antihypertensive drug, diabetes, or cardiovascular disease. All of the metabolic syndrome components were associated with development of proteinuria and the risk of development of proteinuria increased across a number of the fulfilled metabolic syndrome components.

Discussion

In this large general population of adults, we showed that the metabolic syndrome was associated with development of kidney dysfunction and proteinuria. The association remained significant in relatively healthy subjects who did not have diabetes, treated hypertension, or cardiovascular disease at baseline. Even subjects with one component of the metabolic syndrome were at risk of development of kidney disease and risk of kidney disease increased across a number of fulfilled components.

Association of the metabolic syndrome with development of chronic kidney disease has been reported in subdivided subjects [14–18], but one study also provided controversial results [18]. We showed the association of the metabolic syndrome with development of kidney disease using a large study cohort including about 35 000 subjects, and then found that the association was also significant in relatively healthy subjects without known risk factors for chronic kidney disease, strongly supporting the hypothesis. We also found that even subjects with one component of the metabolic syndrome were at higher risk for development of kidney dysfunction and proteinuria compared to those without any components, and the risk