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The biophysical characterization of the first *SCN5A* mutation R1512W identified in Chinese sudden unexplained nocturnal death syndrome

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Abstract

Increasing evidence observed in clinical phenotypes show that abrupt breathing disorders during sleep may play an important role in the pathogenesis of sudden unexplained nocturnal death syndrome (SUNDS). The reported Brugada syndrome causing mutation R1512W in cardiac sodium channel α subunit encoded gene *SCN5A*, without obvious loss of function of cardiac sodium channel in previous in vitro study, was identified as the first genetic cause of Chinese SUNDS by us. The R1512W carrier was a 38-year-old male SUNDS victim who died suddenly after tachypnea in nocturnal sleep without any structural heart disease. To test our hypothesis that slight acidosis conditions may contribute to the significant loss of function of mutant cardiac sodium channels underlying SUNDS, the biophysical characterization of *SCN5A* mutation R1512W was performed under both extracellular and intracellular slight acidosis at pH 7.0. The cDNA of R1512W was created using site-directed mutagenesis methods in the pcDNA3 plasmid vector. The wild type (WT) or mutant cardiac sodium channel R1512W was transiently transfected into HEK293 cells. Macroscopic voltage-gated sodium current (I_{Na}) was measured 24 hours after transfection with the whole-cell patch clamp method at room temperature in the HEK293 cells. Under the baseline conditions at pH 7.4, R1512W (-175 ± 15 pA/pF) showed about 30% of reduction in peak I_{Na} compared to WT (-254 ± 23 pA/pF, $P < 0.05$). Under the acidosis condition at pH 7.0, R1512W (-130 ± 17 pA/pF) significantly decreased the peak I_{Na} by nearly 50% compared to WT (-243 ± 23 pA/pF, $P < 0.005$). Compared to baseline condition at pH 7.4, the acidosis at pH 7.0 did not affect the peak I_{Na} in WT ($P > 0.05$) but decreased peak I_{Na} in R1512W ($P < 0.05$). This initial functional study for *SCN5A* mutation in the Chinese SUNDS victim revealed that the acidosis aggravated the loss of function of mutant channel R1512W and suggested that nocturnal sleep disorders-associated slight acidosis may trigger the lethal arrhythmia underlying the sudden death of SUNDS cases in the setting of genetic defect.

Abbreviations: ACMG = American College of Medical Genetics, BrS = Brugada syndrome, INa = voltage-gated sodium current, SUNDS = sudden unexplained nocturnal death syndrome, WT = wild type.

Keywords: Brugada syndrome, forensic pathology, mechanism of death, sudden unexplained nocturnal death syndrome

1. Introduction

Sudden unexplained nocturnal death syndrome (SUNDS), an entity with uncertain etiology, prevails predominantly in Southeast Asia and was named as Bangungut in the Philippines,[1] Lai Tai in Thailand,[2] Pokkuri in Japan,[3] and sudden manhood death syndrome in China.[4–6] SUNDS has unique clinical phenotype[1–5]: the vast majority of decedents were apparently healthy young males aged 20 to 40 years; death occurred during night sleep with typical symptoms of groaning, tachypnea, and abrupt tic of limbs; and comprehensive forensic autopsy examination revealed no morphological changes to explain the cause of death.

The clinical phenotype including ECG characteristics in SUNDS survivors indicated that SUNDS is similar to Brugada syndrome (BrS),[7] which is related to loss-of-function mutations in the *SCN5A*-encoded cardiac sodium channel α subunit.[8] Subsequently, with the identification of 3 *SCN5A* mutations in 3 of 10 probands with clinical evidence of SUNDS, SUNDS and BrS were thought most likely to be phenotypically, genetically, and functionally the same allelic disorder.[9] We initially performed molecular autopsy studies of *SCN5A* genes among a Chinese SUNDS cohort[4,10] and consequently confirmed the genetic association between BrS and SUNDS.

We formerly detected and determined the mutant R1512W in *SCN5A* to be the first genetic cause of Chinese SUNDS.[4,10] It

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was previously identified as a pathogenic *SCN5A* variant in 2 BrS patients from the Netherlands[11] and France,[12] respectively. However, the electrophysiological studies of this variant in hH1 (with Q1077 in SCN5A) background with heterologous expression systems did not show typical loss-of-function (decreased peak current) of sodium channels, and only manifested mild kinetics alterations (negative voltage shift of the steady-state activation and inactivation curves[11] or slow time constants of inactivation and recovery from inactivation[12]). Later, Ackerman et al[13] even found R1512W in 1 of 103 healthy Hispanics (unclear whether the variant carrier was truly healthy). All of these findings resulted in a conundrum and uncertainty in whether and how R1512W causes arrhythmia underlying BrS or SUNDS.

The abrupt breathing disorders in sleep (such as outbursts of tachypnea, strange groans or gasping, screams, and abnormal snores), observed to be the main clinical symptoms of SUNDS,[1–5] strongly suggest that sudden breathing abnormalities in nocturnal sleep may be a key trigger for SUNDS.[14,15] Sleep monitoring experiments revealed that nocturnal hypoxia might be the primary abnormality in SUNDS.[16] A population-based sleep investigation has indicated that the unique Hmong sleep disorders (a high prevalence of sleep apnea, sleep paralysis, and other rapid eye movement-related sleep abnormalities) may contribute to the high incidence of SUNDS in Hmong men in the United States.[17]

To address whether nocturnal breathing disorders associated with acidosis result in the significant loss-of-function of mutant cardiac sodium channel R1512W, which may account for sudden death of this SUNDS victim, we biophysically characterized this mutation under slight acidosis conditions in HEK293 cells.

2. Materials and methods

2.1. Mutation analysis

Genomic DNA was extracted from blood samples. All coding region exons and relevant exon–intron boundaries for corresponding candidate genes were PCR amplified. The PCR products were sequenced and analyzed as we previously described.[4,10,18–21] The principles outlined in the Declaration of Helsinki were followed. The project was approved for human research by the ethics committee of Sun Yat-sen University.

2.2. Plasmid constructions

R1512W was created in the SCN5A-Q1077del background (without Q1077, Genbank accession no. AY148488) using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) as we previously reported.[22–26] The clone was sequenced to confirm the presence of the R1512W mutation and the absence of other substitutions that might occur during PCR.

2.3. Mammalian cell transfection

The pMaxGFP, with either the wild type (WT) or mutant channel R1512W in *SCN5A*, were transiently transfected into HEK293 cells with FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions.

2.4. Electrophysiological measurements

Macroscopic voltage-gated sodium current (I_{Na}) was measured 24 hours after transfection with the whole-cell patch clamp method at room temperature (~22°C) in the HEK293 cells under both normal pH and slight acidosis (extracellular and intracellular pH 7.0) conditions. The intracellular solution contained the following (in millimoles per liter): CsF 120, $CsCl_2$ 20, EGTA 2, NaCl 5, and HEPES 5, and was adjusted to pH 7.4 or 7.0 with CsOH. The extracellular solution contained the following (in millimoles per liter): NaCl 140, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 0.75, and HEPES 5, and was adjusted to pH 7.4 or 7.0 with NaOH. Microelectrodes were manufactured from borosilicate glass using a puller (P-87, Sutter Instrument Co, Novato, CA). The resistances of microelectrodes ranged from 1.2 to 2.2 MΩ. Voltage clamp data were generated with pClampex 10.5 and analyzed using Clampfit 10.5 (Molecular devices corporation, Sunnyvale, CA). Membrane current data were digitalized at 100 kHz, low-pass filtered at 5 kHz, and then normalized to membrane capacitance. Standard voltage clamp protocols were used and data were measured and analyzed as described previously.[22–26]

The curves of activation were fit with a Boltzmann function: $G_{Na}=(1+\exp\ [V_{1/2}-V]/K)^{-1}$, wherein $V_{1/2}$ and K are the midpoint and slope factor, respectively. $G/G_{Na}=I_{Na}$ (norm)/(V−Vrev), wherein Vrev is the reversal potential and V is the membrane potential. Steady-state availability from inactivation was determined by fitting the data to the Boltzmann function: $I_{Na}=I_{Na\text{-}max}\ (1+\exp\ [Vc-V_{1/2}]/K)^{-1}$, wherein $V_{1/2}$ and k are the midpoint and the slope factor, respectively, and Vc is the membrane potential. Late I_{Na} was measured as the mean current between 600 and 700 ms after the initiation of the depolarization from −140 to −20 mV for 750 ms.

2.5. Statistical analysis

All data points are reported as the mean value and the standard error of the mean (SEM). Determinations of statistical significance were performed using a Student *t* test for comparisons of 2 means or using analysis of one-way analysis of variance followed by a Bonferroni test for comparisons of multiple groups. Statistical significance was determined by a value of $P<0.05$.

3. Results

3.1. Case report of the SUNDS victim

A 38-year-old male without cardiac disease history was witnessed by his roommate to have sudden tachypnea and abrupt tic of limbs during sleep at 4 >am in the morning. His roommate transported him immediately to a local hospital where he was identified and declared dead. The comprehensive gross and microscopic autopsies revealed no obvious pathological changes to explain the sudden unexpected death of this apparently healthy worker.[4] He did not have a family history of sudden cardiac death and was diagnosed as SUNDS.

3.2. Mutational analysis

Comprehensive open-reading frame/splice site mutational analysis for the *SCN5A* gene revealed a missense heterozygous variant (4534 C>T, R1512W). R1512W was absent in 230 reference alleles in the control group.[4] The subsequent mutational analysis for arrhythmia-associated genes (*SCN1B-4B*, *MOG1*, *GPD1-L*, *RyR2*, *PKP2*, *KCNQ1*, *KCNH2*, *KCNE1*, *KCNE2*, and *DSP*) did not show pathogenic rare variants in this case.[10,18–21] The follow-up genetic investigation for the parents of this case did not yield SCN5A-R1512W mutation.

3.3. Electrophysiology

Compared with Q1077 (hH1), the Q1077del cDNA reflects the most abundant alternatively spliced *SCN5A* transcript (~65%) in human hearts.[22] We tested R1512W in the SCN5A-Q1077del background in HEK293 cells as in our previous functional studies of SCN5A.[22–26]

We recorded the biophysical characteristics under both baseline pH (7.4) and acidosis conditions (pH 7.0). Under baseline pH, compared to WT sodium channels (-254 ± 23 pA/pF), R1512W channels (-175 ± 15 pA/pF, $P < 0.05$) showed around 30% of reduction in peak I_{Na} (Fig. 1, Table 1). There were no obvious differences between WT and R1512W in late I_{Na} (Table 1). Under the acidosis conditions at pH 7.0 and compared with WT (-243 ± 23 pA/pF), R1512W (-130 ± 17 pA/pF, $P < 0.005$) showed significantly decreased peak I_{Na} of approximately 50% (Fig. 1, Table 1). Again, the late I_{Na} showed no significant differences between WT and R1512W (Table 1). The summary data for peak I_{Na} density (Table 1 and Fig. 2) showed that the acidosis at pH 7.0 did not affect the peak I_{Na} in WT ($P > 0.05$), but decreased the peak I_{Na} in R1512W ($P < 0.05$), compared to baseline conditions at pH 7.4.

We analyzed the kinetic parameters of activation and inactivation at both baseline pH and pH 7.0 for WT and R1512W. There were no significant differences between any 2 groups in both activation and inactivation (Table 1). For recovery from inactivation, R1512W at pH 7.0 exhibited slower recovery from inactivation and had significantly larger fast time constants (τ_f) values compared with any other group (Table 2), suggesting that acidosis conditions interrupted the recovery from inactivation of R1512W channels. No significant difference of the fractional amplitudes of fast (Af) and slow (AS) components was observed between any 2 groups (Table 2, Af values not shown).

4. Discussion

Although the *SCN5A* mutation R1512W was genetically linked with 2 separate BrS patients and 1 SUNDS victim, the reported absence of significant loss-of-function of this mutant sodium channel and the existence of this mutation in a "healthy" individual make it uncertain whether or not the mutation is truly pathogenic.

In this investigation, we provide the first evidences that R1512W resulted in statistically significant loss-of-function of sodium channels by decreasing peak I_{Na} by 30%. These biophysical findings definitely show that R1512W by itself exerts mild but actual effects on sodium channel function in the most abundant Q1077del-*SCN5A* background. There exists mRNA for 2 splice variants of *SCN5A* (containing or lacking a glutamine at position 1077) in the human heart at a ratio of about 1:2 (Q1077:Q1077del).[22] We previously identified that the function of I_{Na} for arrhythmia- and cardiomyopathy-associated variants[23–26] depends largely on the Q1077 or Q1077del splice variant background. Our data again suggest the utmost importance of characterizing putative arrhythmia variants in both backgrounds to determine their possible pathogenicity. Moreover, the significant dependence of electrophysiological characteristics on the Q1077/Q1077del backgrounds indicates possible splice variants-based regulatory mechanisms by which cardiac sodium channels maintain normal function.[25,26]

Plant et al,[27] Wang et al,[28] and we[25] have previously investigated the effects of intracellular acidosis on the late I_{Na} at pH 6.7 for *SCN5A* variants S1103Y, R680H, and S1103Y/R680H, respectively, and identified acidosis as an important etiologic factor by significantly increasing late I_{Na}. Considering that the SUNDS victim carrying R1512W might have involved slight acidosis because of his sleep breathing disorder before sudden death,[4] we characterized the biophysical phenotype of

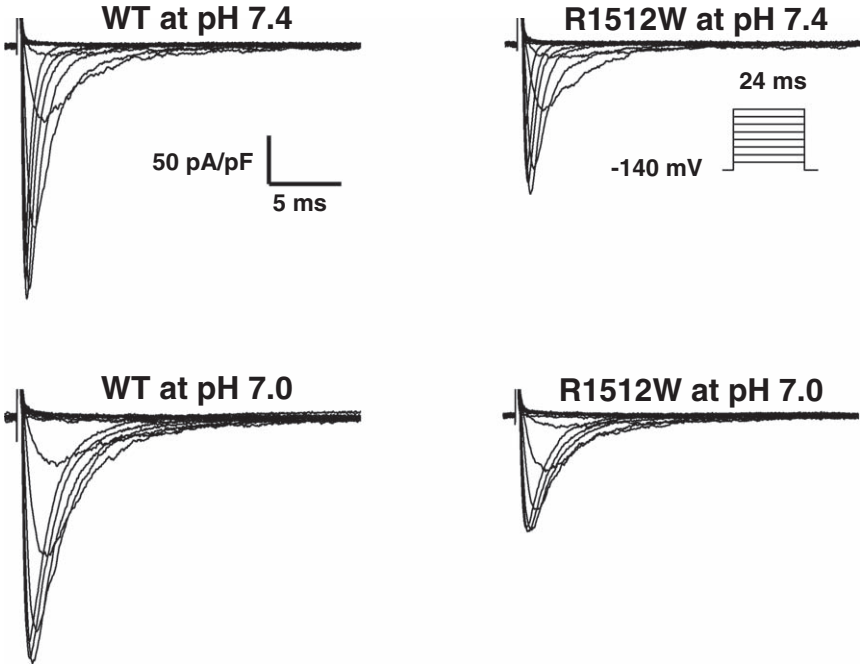

Figure 1. Representative whole-cell current traces in HEK 293 cells. Representative whole-cell current traces showing peak sodium current under both baseline (pH 7.4) and acidosis (pH 7.0) conditions in HEK293 cells expressing wild-type or mutant R1512W- SCN5A.

Table 1

Biophysical properties of WT or variant sodium channel R1512W in HEK293 cells.

Samples	Peak I_{Na}		Activation			Inactivation			Late I_{Na}	
	pA/pF	n	$V_{1/2}$, mV	K	N	$V_{1/2}$, mV	K	n	%	n
WT at pH 7.4	−254±23	24	−38.0±0.5	4.4	16	−70.8±0.4	5.1	24	0.10±0.02	20
R1512W at pH 7.4	−175±15*	18	−36.7±0.5	4.0	18	−72.3±0.5	5.0	22	0.14±0.03	18
WT at pH 7.0	−243±23	19	−37.8±0.6	4.1	15	−72.8±0.7	5.0	17	0.22±0.06	10
R1512W at pH 7.0	−130±17†	18	−33.9±0.9†	4.3	13	−73.8±0.7	5.0	15	0.20±0.09	6

I_{Na}, sodium current; K, slope factor; pA/pF, current density; $V_{1/2}$, voltage of half-maximal activation/inactivation. Values are mean ± SE for n experiments. The late I_{Na} level was described as a percentage of peak I_{Na}.

* $P < 0.05$ versus WT at pH7.4.

† $P < 0.005$ versus WT at pH 7.0. All parameters were analyzed using one-way ANOVA followed by a Bonferroni test.

R1512W under both intracellular and extracellular acidosis at pH 7.0. Notably, the acidosis significantly aggravated the loss-of-function of R1512W channels (~50% reduction in peak I_{Na} compared to WT at normal pH), whereas the WT channels remained intact under these conditions. These findings confirm the key role of environmental risk factors (such as acidosis, medications, hypokalemia, etc) in the occurrence of arrhythmia. The hypothesis that SUNDS may be a sleep and/or respiratory disorder-associated arrhythmia syndrome was conceived[4,14,15] and investigated[16,17] for decades in epidemiological studies. The significant different biophysical phenotype of R1512W between baseline and slight acidosis conditions strongly support the idea that nocturnal sleep breathing abnormalities-related acidosis may be a trigger for fatal arrhythmias in patients with pathogenic mutations in arrhythmia susceptibility genes.

The *SCN5A* gene is so far the most common BrS-susceptibility gene responsible for 20% to 30% of the disorder. Vatta et al[9] proposed SUNDS and BrS as the same allelic disorder based on genetic and functional studies of SCN5A mutations found in 3 of 10 SUNDS cases. In a much larger cohort of 123 SUNDS cases, we identified 10 putative pathogenic SCN5A variants and some arrhythmia-susceptible polymorphisms.[10] These studies implicated the important role of *SCN5A* in the pathogenesis of both BrS and SUNDS. However, according to regular biophysical study and/or the strict American College of Medical Genetics (ACMG) guideline definition,[29] many *SCN5A* rare variants or polymorphisms are not considered to be "pathogenic" or likely "pathogenic." If environmental risk factors (such as acidosis) are involved in assessing the pathogenicity of SCN5A variants in specific individuals, there will probably be more *SCN5A* variants that would account for BrS and SUNDS.

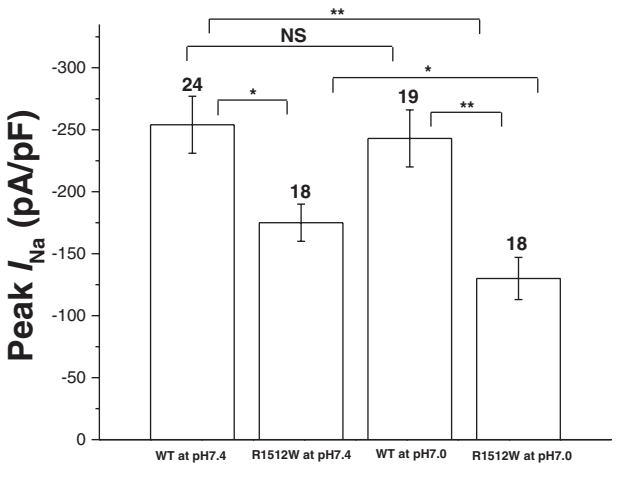

Figure 2. Summary data for peak sodium current in HEK293 cells. Summary data of peak sodium current densities from each group. The number of tested cells is indicated above the bar. NS, no significant difference. *$P < 0.05$. **$P < 0.005$ (one-way analysis of variance followed by a Bonferroni test).

The abnormal biophysical phenotype of R1512W identified in this study is a complimentary finding for previous studies, which confirmed R1512W as a BrS-causing variant. However, there were no distinct significant kinetic changes to explain the electrophysiological modifications of this variant. The mechanisms by which acidosis remarkably decreased peak I_{Na} may be related to slower recovery from inactivation. The limitations of this functional investigation include the in vitro nature of the study using "forced" expression of mutant sodium channel in noncardiac myocytes and the absence of genetic background for each specific patient. Thus, the detailed biophysical mechanisms for R1512W to cause BrS or SUNDS remain to be determined in mammalian cardiomyocytes and patient-specific inducible pluripotent stem cell-derived cardiomyocytes.

Under the condition of 60-minute no-flow ischemia, extracellular and intracellular pH in cardiac tissue have been reported to decrease below pH 6.0.[30] Sudden infant death syndrome is associated with sleep respiratory disorders wherein the arterial blood pH is found to be less than pH 7.0.[31] Although the clinical phenotype of breathing disorders (such as sleep apnea and nocturnal hypoxia) is presumed to be a critical trigger for SUNDS[14–16] and the biophysical phenotype in vitro study suggests low pH can significantly change the function of mutant cardiac sodium channel underlying BrS,[31] the mechanism of how pH in vivo changes and how it affects life-threatening arrhythmia needs further investigation in both SUNDS and BrS patients.

In summary, we characterized in this study the first SCN5A variant in Chinese SUNDS victims. We revealed that this R1512W variant caused statistically decreased peak I_{Na} and that acidosis aggravated the loss of function of this mutant channel in the most common SCN5A background (Q1077del). Our data provide the biophysical evidence for the hypothesis that sleep breathing disorder-associated acidosis may be a trigger for the lethal arrhythmia underlying sudden cardiac death of SUNDS cases in the setting of genetic defect.

Table 2

Recovery of WT or variant sodium channel R1512W in HEK293 cells.

Samples	Recovery at pH 7.4				Recovery at pH 7.0			
	τ_f, ms	τ_S, ms	A_S, %	N	τ_f, ms	τ_S, ms	A_S, %	n
WT	1.31 ± 0.20	24.03 ± 5.65	21.30 ± 5.20	24	1.36 ± 0.37	32.46 ± 13.33	21.50 ± 6.30	19
R1512W	1.29 ± 0.13	23.75 ± 7.98	20.70 ± 1.90	20	1.67 ± 0.48*	32.88 ± 12.73	22.40 ± 6.00	17

Time course of recovery from inactivation was analyzed by fitting data with a two-exponential (exp) function: normalized $I_{Na}(t) = A_f(1 - \exp[-t/\tau f]) + A_S(1 - \exp[-t/\tau_S])$, where t is time, A_f and A_S are fractional amplitudes of fast and slow components, respectively, and τ_f and τ_S are fast and slow time constant, respectively.

* $P < 0.05$ versus wild-type (one-way analysis of variance followed by a Bonferroni test).

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