Identification of N-terminal protein acetylation and arginine methylation of the voltage-gated sodium channel in end-stage heart failure human heart

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Abstract

The α subunit of the cardiac voltage-gated sodium channel, $Na_V1.5$, provides the rapid sodium inward current that initiates cardiomyocyte action potentials. Here, we analyzed for the first time the post-translational modifications of $Na_V1.5$ purified from end-stage heart failure human cardiac tissue. We identified R526 methylation as the major post-translational modification of any $Na_V1.5$ arginine or lysine residue. Unexpectedly, we found that the *N* terminus of $Na_V1.5$ was: 1) devoid of the initiation methionine, and 2) acetylated at the resulting initial alanine residue. This is the first evidence for N-terminal acetylation in any member of the voltage-gated ion channel superfamily. Our results open the door to explore $Na_V1.5$ N-terminal acetylation and arginine methylation levels as drivers or markers of end-stage heart failure.

Keywords

Voltage-gated sodium channel, proteomics, post-translational modification, N-terminal acetylation, arginine methylation, heart failure

1. Introduction

The cardiac voltage-gated sodium channel is responsible for the sodium inward current that initiates cardiomyocyte action potentials. The pore-forming α-subunit of the cardiac sodium channel ($Na_V1.5$) forms the core of the sodium channel [1]. $Na_V1.5$ is a large integral membrane protein that spans more than 2,000 residues, and includes 24 transmembrane segments. $Na_V1.5$ is predicted to be organized in four homologous domains, DI to DIV, joined by interdomain linkers. The *N* and *C* termini of the protein, as well as interdomain linkers, are cytosolic and accessible to post-translational modifications (PTMs).

Genetic mutations in $Na_V1.5$ increase the risk of sudden cardiac death due to *e.g*. Brugada syndrome [2]. Additionally, $Na_V1.5$ current has been shown to be altered in acquired diseases; for instance, due to reduction of $Na_V1.5$ protein expression in the setting of heart failure (HF) [3]. Mechanisms include the expression of non-functional $Na_V1.5$ splicing variants that may increase $Na_V1.5$ degradation [4, 5]. PTMs may also underlie the decrease in $Na_V1.5$ current documented in HF. For example, $Na_V1.5$ phosphorylation by Ca^{2+}/CaM-dependent protein kinase II (CaMKII), is thought to contribute to $Na_V1.5$ loss-of-function in HF [6].

The linker between domains DI and DII of $Na_V1.5$ is the *hot-spot* of PTMs, including phosphorylation and arginine methylation [7, 8]. Arginine methylation is emerging as an important novel PTM of voltage-gated ion channels. First, we detected methylation of R513, R526 and R680 in $Na_V1.5$ expressed in HEK293 cells [8], and showed that arginine methylation regulates $Na_V1.5$ plasma membrane expression [9]. The fact that R513H, R526H and R680H are $Na_V1.5$ mutations associated to sudden cardiac death syndromes suggests that $Na_V1.5$ arginine methylation is relevant to cardiac disease. In the same line, Baek *et al* have observed that levels of arginine methylation (including R513 methylation, $Na_V1.5$ numbering) of the brain voltage-gated sodium channel, $Na_V1.2$, increase after induction of seizure in rats [10].

Given the growing evidence that indicates that $Na_V1.5$ PTMs play a role in cardiac disease, the lack of proteomic studies of $Na_V1.5$ purified from human cardiac tissue, and the clinical relevance of $Na_V1.5$ current alterations in HF, we set ourselves to elucidate the PTMs of $Na_V1.5$ purified from end-stage HF human cardiac tissue. We identified R526 methylation as the major PTM of $Na_V1.5$ basic residues, and we found that the *N* terminus of $Na_V1.5$ was devoid of the initiation methionine, and acetylated at the resulting initial alanine residue. These results may provide a novel mechanism for the reduced $Na_V1.5$ current in end-stage HF.

2. Materials and Methods

2.1 $Na_V1.5$ purification

Human heart tissue samples were obtained from myocardial biopsies of four end-stage HF patients undergoing heart transplantation. Two patients had been diagnosed with idiopathic dilated cardiomyopathy, and two with ischemic cardiomyopathy. With the written consent of the patients, left and right ventricle samples were collected from the explanted heart, and frozen immediately in liquid N_2. Samples from the four patients were pooled, and the total amount of collected cardiac tissue was 126 g. All procedures were approved by the ethical committees of the Hospital Universitari de Girona Dr. Josep Trueta and the Hospital Universitari Clínic de Barcelona.

$Na_V1.5$ protein enrichment was performed by immunopurification from integral membrane protein preparations using $Na_V1.5$-specific antibodies (SC-271255, Santa Cruz Biotechnology). After SDS-PAGE separation, the putative $Na_V1.5$ band was excised from the gel, and digested. To maximize sequence coverage, we performed two independent digestions using either trypsin, or chymotrypsin. See Supplementary Material for detailed Methods.

2.2. Mass spectrometry

Previously digested samples were analyzed by liquid chromatography coupled to mass spectrometry. Briefly, peptide digests were chromatographically separated using a reverse phase C18 column, and eluting peptides were analyzed on-line with a LTQ-Orbitrap Velos Pro (Thermo) fitted with a nanospray source. Fragment ion spectra were produced either *via* collision induced dissociation (CID) and followed by acquisition in the ion trap mass analyzer, or by higher energy collisional dissociation (HCD) and acquired in the Orbitrap mass analyzer. For each survey scan, the twenty (CID) or ten (HCD) most intense ions were selected for fragmentation. All data were acquired with Xcalibur 2.1 software.

Protein identification was performed with Proteome Discoverer (v.1.4.0.288) using either Sequest-HT or Mascot (v2.4) search engines, against the reference Uniprot human FASTA database (version 2014_06, 88949 entries), allowing for 3 missed cleavages, and using 7 ppm for the MS peptide mass tolerance and 0.5 Da (CID) or 20 mmu (HCD) for the peptide mass tolerance for MS/MS spectra. Carbamidomethylation

of cysteines was set as static modification, and oxidation of methionine was set as variable modification. The following post-translational modifications were also added as variable modifications and searched for individually: mono- and dimethylation of arginine, mono-, di-, and trimethylation of lysine, lysine acetylation, palmitoylation, carbamylation, and formylation, protein *N* terminus acetylation. False discovery rates were set at 0.01 or 0.05 for high or medium confidence peptides, respectively. See Supplementary Material for detailed Methods.

3. Results and Discussion

Mass spectrometry analysis of $Na_V1.5$ purified from the human heart has not previously been reported. Here, we report the analysis of PTMs of $Na_V1.5$ purified from end-stage HF human cardiac tissue.

3.1. $Na_V1.5$ purification and mass spectrometric analysis

Initially, left and right ventricle samples were collected from myocardial biopsies of end-stage HF patients undergoing heart transplantation. Immunopurified $Na_V1.5$ (Supplementary Figure S1) from the explanted hearts was analyzed by mass spectrometry after trypsin or chymotrypsin-digestion, using either CID or HCD fragmentation. A total of 41 unique $Na_V1.5$ peptides were identified (Supplementary Tables 1 and 2). $Na_V1.5$ total coverage was *ca*. 26 % (Supplementary Figure S2), which was comparable to the 25 % sequence coverage reported by Marionneau *et al* after proteomic analysis of $Na_V1.5$ purified from mouse cardiac tissue [7]. Coverage of the linker between domains DI and DII (59 %) was improved compared to that obtained after $Na_V1.5$ FLAG-affinity purification from overexpressing HEK293 cells (40 %, [8]).

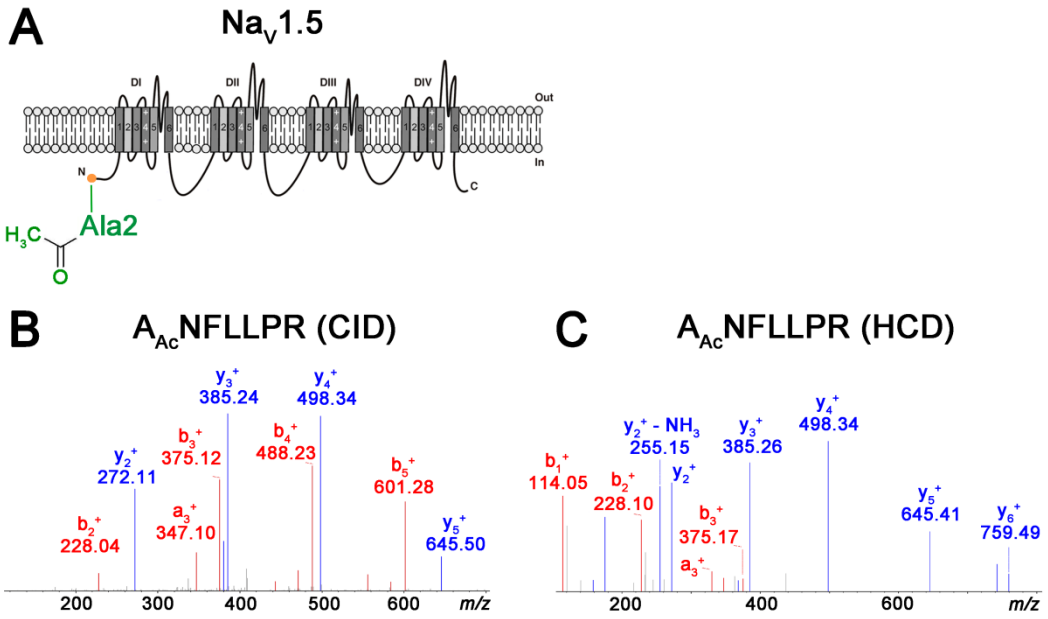
3.2. $Na_V1.5$ is N-terminally acetylated in end-stage HF

We searched our proteomic data to explore the existence of $Na_V1.5$ acetylation. Lysine acetylation was not observed. However, we found that the *N* terminus of $Na_V1.5$, which was devoid of the initiation methionine, was acetylated at the N-terminal Ala2 (Figure 1, Supplementary Figure S3 and Supplementary Tables 2 and 3). This is the first evidence for N-terminal acetylation in any member of the voltage-gated ion channel superfamily, which consists of more than 140 proteins including calcium, potassium and sodium channels.

N-terminal acetylation is catalyzed by N-terminal acetyltransferases, which transfer acetyl groups from acetyl-coenzyme A to the α-amino group of the N-terminal residue, either Met or the resulting initial residue after Met excision by aminopeptidases [11]. In general, protein N-terminal acetylation triggers protein degradation by the ubiquitin-dependent proteasome [12]. This is consistent with the demonstrated role of the ubiquitin ligase Nedd4-2 in $Na_V1.5$ internalization from cellular membranes [13]. The primary Nedd4-2 recognition site is the PY motif in $Na_V1.5$ *C* terminus. Nevertheless, a recent report has shown that $Na_V1.5$ lacking the *N* terminus domain was not targeted by the ubiquitin-dependent proteasome [14], supporting a role of the N-terminal domain in $Na_V1.5$ ubiquitin-dependent degradation.

Reduced $Na_V1.5$ expression is typical of HF [3, 6]. In our samples, N-terminally acetylated $Na_V1.5$ seemed to be the most abundant $Na_V1.5$ form, because the corresponding unmodified peptide was not detected (Supplementary Table 1). It is tempting to speculate that N-terminal processing of $Na_V1.5$ may be increased in end-stage HF. This may reflect a higher $Na_V1.5$ degradation rate during HF, perhaps as a cellular response to the expression of splicing variants, non-functional $Na_V1.5$ channels [4]. This hypothesis is supported by the observation that the *N* terminus of $Na_V1.5$ in hearts of healthy mice is intact (including the initiation methionine) [7]. Likewise, $Na_V1.5$ expressed in HEK293 cells includes Met1, and is not modified by acetylation [8], and $Na_V1.2$ purified from rat brain is not N-terminally acetylated [10]. Further testing of this idea would greatly benefit from the generation of antibodies specifically targeting $Na_V1.5$ N-terminal acetylation.

Figure 1. $Na_V1.5$ Ala2 is N-terminal acetylated. **A**. $Na_V1.5$ topology highlighting acetylation of Ala2. **B**. Mass spectrum of acetylated $Na_V1.5$ N-terminal tryptic peptide, using CID fragmentation. **C**. Mass spectrum of acetylated $Na_V1.5$ N-terminal tryptic peptide, using HCD fragmentation. For each peptide, identified *b* (red) and *y* (blue) series ions are shown. Not detected ions are in black.



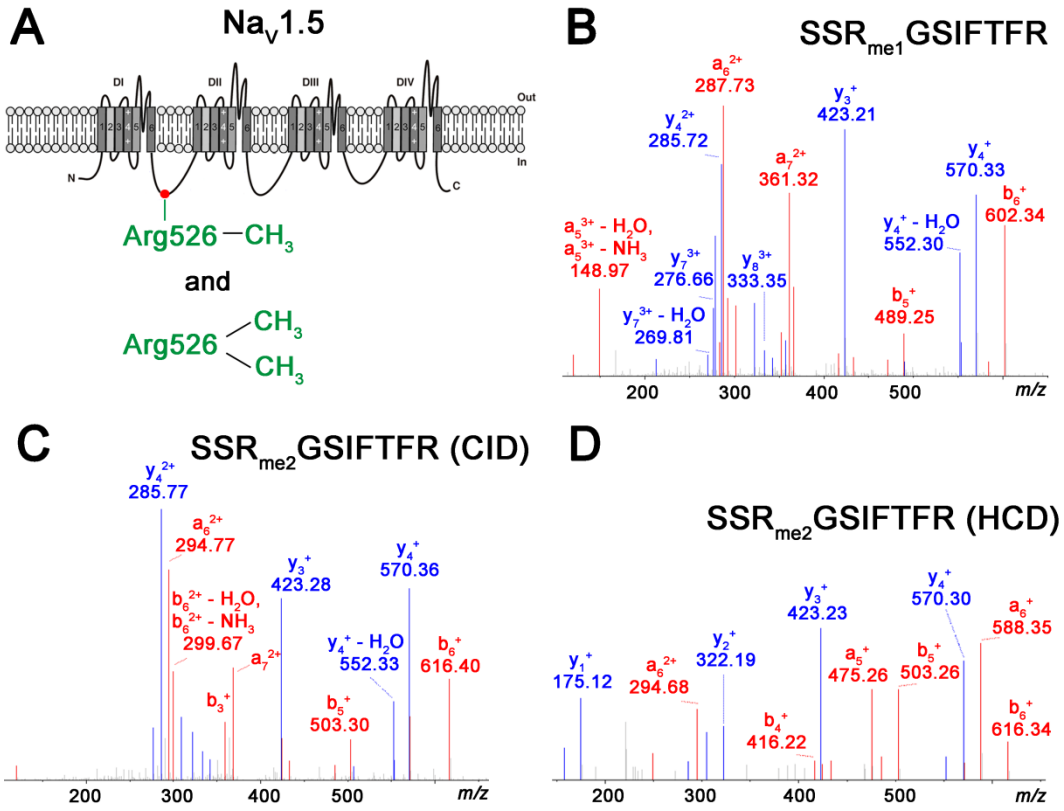
3.3. Na$_V$1.5 is arginine methylated in end-stage HF

Analysis of Na$_V$1.5 post-translational methylation identified mono- and dimethylation of R526 with medium and high confidence, respectively (Figure 2, Supplementary Figure S3, and Supplementary Tables 2 and 4). Peptides containing unmodified R526 were not detected, probably due to an enhanced trypsin performance in the absence of a methyl group at R526, which leads to a SSR peptide too small for detection by our LC-MS set up. Intriguingly, peptides containing unmodified R513 and R680 were observed, but not their methylated counterparts. Lysine methylation was not detected in any peptide.

We then performed a comprehensive search for Arg and Lys PTMs. First, we searched for palmitoylation, carbamylation or formylation of lysine, but we did not detect these PTMs. Then, we conducted error-tolerant searches to identify edited residues. We observed known Na$_V$1.5 PTMs and polymorphisms (Supplementary Table 5), but no new PTM was found.

Overall, our results suggest that R526 methylation could be the predominant form of Na$_V$1.5 Arg or Lys modification in end-stage HF. The fact that R526H is a known Na$_V$1.5 mutation associated with Brugada syndrome [15], suggests that R526 methylation levels are important for proper Na$_V$1.5 function. Further investigations should assess whether arginine methylation patterns differ in control and HF samples. In particular, it will be interesting to search for R513 and R680 methylation in control cardiac tissue.

Figure 2. $Na_V1.5$ R526 is mono- and dimethylated. **A**. $Na_V1.5$ topology highlighting mono- and dimethylation of R526. **B**. Mass spectrum of tryptic $Na_V1.5$ peptide containing monomethylated R526, using CID fragmentation. **C**. Mass spectrum of tryptic $Na_V1.5$ peptide containing dimethylated R526, using CID fragmentation. **D**. Mass spectrum of tryptic $Na_V1.5$ peptide containing dimethylated R526, using HCD fragmentation. We could not infer the methylation pattern (symmetric or asymmetric) of dimethylated R526 from the corresponding spectra. For each peptide, identified *b* (red) and *y* (blue) series ions are shown. Not detected ions are in black.



3.4 Conclusions

In this study we performed a characterization of PTMs of $Na_V1.5$ purified from end-stage HF human heart tissue. Our study led to 1) the discovery of N-terminal acetylation as a novel modification of voltage-gated ion channels; 2) the detection of $Na_V1.5$ N-terminal acetylation in end-stage HF; and 3) the identification of R526 methylation as the major $Na_V1.5$ methylation site in end-stage HF. Future studies should be addressed at investigating these PTMs specifically in the different etiologies linked to end-stage HF. Although these potential follow-up experiments are limited by

the unavailability of large amounts of control cardiac samples, and by the lack of specific antibodies targeting $Na_V1.5$ N-terminal acetylation, and arginine methylation, our current results uncover novel $Na_V1.5$ PTMs that can be explored as drivers or markers of end-stage HF.

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Disclosures

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Supplementary data

Supplementary data to this article can be found online.

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