Altered ENaC Is Associated With Aortic Baroreceptor Dysfunction in Chronic Heart Failure

Yu-Long Li,1,2 Dongze Zhang,1 Huiyin Tu,1 and Robert L. Muelleman1

BACKGROUND
Abnormal baroreceptor function contributes to attenuated arterial baroreflex sensitivity in chronic heart failure (CHF). As a mechanosensor in mammalian nonepithelium, the epithelial sodium channel (ENaC) is an amiloride-sensitive and voltage-independent ion channel. The ENaC is thought to be a component of baroreceptor mechanosensitive ion channels in aortic baroreceptor cell bodies and nerve terminals. In this study, therefore, we measured the expression and activation of the ENaC in nodose neuronal cell bodies and aortic baroreceptor nerve terminals in sham and CHF rats.

METHODS AND RESULTS
CHF was induced by surgical ligation of left coronary artery. The development of CHF was confirmed by hemodynamic and morphological characteristics. The aortic baroreceptor sensitivity was blunted in anesthetized CHF rats, compared with that in sham rats. The data from immunostaining and western blot analysis showed that the protein of β- and γ-ENaC subunits was expressed in nodose neuronal cell bodies and aortic baroreceptor nerve terminals in sham and CHF rats.

Cardiovascular system is strictly under autonomic neural control, and arterial baroreflex is a key regulator of the circulation.12 The arterial baroreflex includes an afferent limb, a central neural component, and autonomic neuroeffector component. As a main afferent component of the arterial baroreflex arc, arterial baroreceptors have mechanosensitive afferent nerve terminals located in the adventitia of aortic arch and carotid sinus. Mechanical deformation of the arterial vascular wall elicited by an increase in blood pressure leads to baroreceptor excitation to regulate autonomic outflow and to maintain cardiovascular homeostasis through whole arterial baroreflex.34

Chronic heart failure (CHF) is a leading cause of death in the United States. Blunted arterial baroreflex sensitivity is observed in patients with CHF and experimental CHF animal models,5,7 which is thought to mediate autonomic imbalance and to increase morbidity and mortality in the CHF state.3,6 It has been shown that central autonomic component of the arterial baroreflex arc is altered and contributes to depressed arterial baroreflex in CHF.3,9 However, some previous studies7,10–12 have shown that reduced baroreceptor excitability also contributes to blunted arterial baroreflex sensitivity in CHF. Although Dr Zucker’s study suggests that depressed arterial baroreflex is most likely due to the desensitization of individual baroreceptors,12 the mechanisms responsible for reduced sensitivity of baroreceptor nerve terminals to blood pressure in CHF are still poorly understood.

The epithelial sodium channel (ENaC) is one of families in degenerin/ENaC superfamily identified in the mammalian species.13–15 The ENaC has 3 subunits (α-ENaC, β-ENaC, and γ-ENaC) and is found in various tissues including hypothalamus, brainstem, and aortic baroreceptors.16–21 Basic function of the ENaC in polarized epithelial cells is to allow vectorial transcellular transport of Na+ across tight epithelia.15 Besides served as Na+ transporter, the ENaC is also as a mechanosensor in nonepithelial cells. Genetic studies have suggested that ENaC protein may serve as mechanosensory channels in mechanosensory neurons.44 Additionally, an amiloride-sensitive Na+ current is recorded in cells expressed the ENaC protein when cells are stimulated with membrane stretch.18 Growing evidence demonstrates that the ENaC is a mechanosensitive channel and mediates mechanosensation in mechanosensitive cells.17,18,22–24 Furthermore, β- and γ-ENaC subunits are expressed in baroreceptor cell bodies and nerve...
To assess the correlation between altered ENaC and aortic baroreceptor dysfunction in coronary artery ligation-induced CHF rats, therefore, we investigated alteration of the ENaC in aortic baroreceptors from sham and CHF rats.

METHODS

All experimental procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were carried out in accordance with the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the American Physiological Society’s “Guides for the Care and Use of Laboratory Animals.” Detail description for methods is available in the Supplementary Methods.

CHF animal model

Male Sprague-Dawley rats (180–200 g) were randomly assigned to 1 of 2 groups: CHF (n = 24) and sham (n = 16). Rats were anesthetized for surgical ligation of the left coronary artery to produce CHF. On the day of the terminal experiment (6–8 weeks after surgical ligation of the left coronary artery), Left ventricular pressure and infarct size were measured. The rats with both left ventricular end-diastolic pressure >15 mm Hg and infarct size >30% of left ventricular area were considered as CHF. Eight rats in CHF group were excluded from study, in which 5 rats died within 2 days after surgical ligation, and 3 rats were not considered as CHF.

Recording of aortic depressor nerve activity

Under anesthetized condition, rat cervical region was exposed, and a 3–5 mm segment of the left or right aortic depressor nerve was isolated and placed on a pair of recording electrodes. The recording of aortic depressor nerve activity (ADNA) and blood pressure were recorded by LabChart 7. The baroreceptor firing range was assessed by changes in arterial blood pressure. Arterial blood pressure was decreased to about 50 mm Hg by sodium nitroprusside, and then increased by phentolamine. ADNA was normalized and plotted against mean arterial pressure to obtain mean arterial pressure–ADNA curves.

Labeling of aortic baroreceptor neurons

Aortic baroreceptor neurons in the nodose ganglia were selectively retrograde-labeled by a transported fluorescent dye, DiI (red color). Briefly, a thoracotomy at the 3rd intercostal space was performed, and DiI (2 µl) was injected into the adventitia of the aortic arch. At least 1 week was allowed for the tracer to diffuse to aortic baroreceptor neurons in the nodose ganglia.

Isolation of aortic baroreceptor neurons and recording of mechanosensitive currents

Nodose neurons were isolated by 2-step enzymatic digestion protocol. Nodose ganglia were incubated for 30 minute in an enzymatic Ringer’s solution containing 0.1% collagenase/0.1% trypsin and then transferred to a Ringer’s solution containing 0.2% collagenase and 0.5% bovine serum albumin for a 30-minute incubation. Nodose neurons including aortic baroreceptor neurons (DiI-labeled nodose neurons) were kept in culture medium and cultured at 37 °C in a humidified atmosphere of 95% air-5% CO2 for 4–7 h before the experiments.

Mechanosensitive currents in DiI-labeled nodose neurons (aortic baroreceptor neurons) were recorded by the whole cell patch-clamp technique using Axopatch 200B patch-clamp amplifier (Axon Instruments, Burlingame, CA). The patch pipette was filled with the following solution (in mM): 140 KCl, 0.1 EGTA, 10 HEPES, and 1 MgATP (pH 7.3). The extracellular (bath) solution consists of (in mM): 120 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 5 HEPES (pH 7.4), in which, osmolarity was adjusted by sucrose to normal osmotic solution (330 mOsm, with sucrose) or hypo-osmotic solution (255 mOsm, without sucrose). Current-voltage (I–V) relationships were elicited using a voltage-ramp protocol. Ion currents were recorded in normal bathing solution and hypo-osmotic solution. Difference between ion currents in normal osmotic medium and in hypo-osmotic medium served as mechanosensitive currents. Additionally, ion currents under both normal osmotic solution and hypo-osmotic solution were recorded before and after treatment of amiloride (a specific ENaC inhibitor). ENaC currents were obtained by subtracting ion currents after treatment of amiloride from ion currents before treatment of amiloride.

Western blot analysis

Nodose ganglia were rapidly removed, immediately frozen in liquid nitrogen, and stored at −80 °C until analyzed. After protein was extracted, total protein concentration was determined. Equal amounts of protein samples were loaded on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins of these samples were electrophoretically transferred onto PVDF membranes. Membranes were probed with primary antibodies against α-, β-, and γ-ENaC subunits, respectively. After washing, the membranes were incubated with appropriate secondary antibodies. The signal was detected and analyzed using UVP bioimaging system. The blot was reprobed with mouse anti-β-actin antibody, allowing normalization of target protein intensity to that of β-actin.

Localization of specific proteins by immunohistochemistry

Localization for specific proteins was measured in aortic baroreceptor cell bodies and nerve terminals using 2-step immunofluorescent staining method (primary antibodies including antibodies against α-ENaC, β-ENaC, γ-ENaC, PGP9.5, and fluorescence-conjugated secondary antibodies).

Statistical analysis

All data are presented as mean ± SE. SigmaPlot 12 was used for data analysis. Student’s unpaired t-test or 2-way
analysis of variance with post hoc Bonferroni test was used to determine statistical significance. Statistical significance was accepted when $P < 0.05$.

**RESULTS**

**Hemodynamic and morphological characteristics of sham and CHF rats**

As summarized in Table 1, there was an average myocardial infarct size over 30% of the left ventricular area in CHF rats. Heart weight-to-body weight ratio and left ventricular end-diastolic pressure were significantly increased in CHF rats, compared with sham rats ($P < 0.05$). LVSP, LV $dP/ dt_{\text{max}}$ (an index of myocardial contractility) and LV $dP/ dt_{\text{min}}$ (an index of diastolic relaxation) were markedly reduced in CHF rats. These data suggest the development of CHF. However, there was no significant difference in body weight, blood pressure, and heart rate between sham and CHF rats (Table 1).

**Expression and localization of ENaC protein in aortic baroreceptors from sham and CHF rats**

Using immunofluorescent staining, we found that the protein of β- and γ-ENaC subunits was expressed in aortic baroreceptor nerve terminals in the aortic arch (Figure 1) and nodose neuronal cell bodies (Figure 2). Additionally, the data from western blot analysis also confirmed the existence of β- and γ-ENaC proteins in nodose neuronal cell bodies (Figure 2). However, α-ENaC protein was not detectable in above tissues (Figures 1 and 2), which is same as that in other report. More importantly, β- and γ-ENaC proteins in aortic baroreceptor nerve terminals and nodose neuronal cell bodies from CHF rats were decreased, compared to those from sham rats (Figures 1 and 2).

**Table 1. Hemodynamic and morphological characteristics of sham and CHF rats**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sham ($n=16$)</th>
<th>CHF ($n=16$)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>412.6±5.9</td>
<td>416.3±7.2</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>98.4±1.2</td>
<td>101.1±1.5</td>
</tr>
<tr>
<td>HR (BPM)</td>
<td>348±9</td>
<td>361±11</td>
</tr>
<tr>
<td>Heart weight/body weight (mg/g)</td>
<td>3.54±0.12</td>
<td>5.49±0.14*</td>
</tr>
<tr>
<td>Infarct size (% of left ventricle)</td>
<td>0</td>
<td>36.6±1.2*</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>1.9±0.7</td>
<td>18.2±0.9*</td>
</tr>
<tr>
<td>LVSP (mm Hg)</td>
<td>129.3±2.8</td>
<td>96.8±3.1*</td>
</tr>
<tr>
<td>LV $dP/ dt_{\text{max}}$ (mm Hg/second)</td>
<td>7618±186</td>
<td>4745±205*</td>
</tr>
<tr>
<td>LV $dP/ dt_{\text{min}}$ (mm Hg/second)</td>
<td>5436±144</td>
<td>3237±156*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Abbreviations: BPM, beats per minute; CHF, chronic heart failure; HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure. $P < 0.05$ vs. sham.

**Mechanosensitive currents in aortic baroreceptor neurons from sham and CHF rats**

We used hypo-osmotic mechanical stress to induce mechanosensitive currents in aortic baroreceptor neurons (Dil-labeled nodose neurons) from sham and CHF rats. Current-voltage relationships in isolated baroreceptor neurons perfused with normal osmotic solution (330 mOsm) or hypo-osmotic solution (255 mOsm) were obtained by voltage-ramp protocol (Figure 3A). Hypo-osmotic solution-induced increase of ion currents (difference between ion currents in hypo-osmotic solution and in normal osmotic solution) served as mechanosensitive currents. Mechanosensitive currents in aortic baroreceptor neurons from CHF rats were lower than that in aortic baroreceptor neurons from sham rats (Figure 3C).

To measure ENaC currents, ion currents under both normal osmotic solution and hypo-osmotic solution were recorded before and after treatment of amiloride (1 µM, a specific ENaC inhibitor) in isolated aortic baroreceptor neurons from sham and CHF rats. Amiloride did not alter current in normal osmotic solution, whereas it totally abolished hypo-osmotic solution-induced mechanosensitive currents (Figure 3B). These results suggest that there is non-activation of ENaC channels in normal osmotic solution, and hypo-osmotic solution-induced mechanosensitive currents are fully dependent on the activation of ENaC channels. Additionally, CHF markedly decreased ENaC currents in aortic baroreceptor neurons, compared to the sham state (Figure 3D).

**ADNA in sham and CHF rats**

The function of aortic baroreceptor afferent is characterized by measuring ADNA over a wide range of arterial blood pressure. Blood pressure was decreased by sodium nitroprusside (30 µg, IV), and then increased by phenylephrine (10 µg, IV). The ADNA was gradually increased in response to the increase in arterial blood pressure (Figure 4). The response of the ADNA to the increase in arterial blood pressure was decreased in CHF rats, compared to sham rats (Figure 4B), which indicates that the function of aortic baroreceptor afferent is attenuated in CHF rats.

**DISCUSSION**

Clinical trials and animal experiments have confirmed that blunted arterial baroreflex contributes to the prognosis and mortality in CHF. As a primary component of the arterial baroreflex arc, the afferent limb comprised of arterial baroreceptor neurons is involved in attenuated arterial baroreflex sensitivity in the CHF condition. The mechanisms responsible for mediating afferent sensitivity of barosensitive neurons to the change in blood pressure are complex and not thoroughly understood. The generally acknowledged dogma states that the process of translating changes in arterial wall tension into impulse traffic to the nucleus tractus solitarius (the first site of baroreceptor neurons contacting with the central nervous system) involves 3 broad functional steps. The first step is vascular distension and deformation.
of baroreceptor nerve terminals. Sensory nerve terminals of the aortic and carotid baroreceptor neurons innervate the adventitia of the aortic arch at the origin of the right subclavian artery and the carotid sinus. The mechanical distension of arterial vascular walls during an increase in blood pressure stimulates the deformation of baroreceptor nerve terminals, which is viscoelastic coupling between elements in the arterial wall and baroreceptor nerve terminals. The second step is mechanoelectrical transduction. The deformation of baroreceptor nerve terminals activates the opening of mechanosensitive ion channels and resultant depolarization of the nerve terminals, which transduces mechanical deformation into membrane depolarization of baroreceptor nerve terminals. The third step is the translation of membrane depolarization into action potentials. The mechanically caused depolarization reaches a specific voltage level (voltage threshold) to induce the activation of membrane voltage-sensitive ion channels (including sodium, calcium, and potassium channels) and subsequent generation of action potentials at the baroreceptor nerve terminals (spike initiating zone). The excited signals (action potentials) in baroreceptors are conveyed to the dorsal medial nucleus tractus solitarii. The ENaC, a mechanosensitive channel, is expressed in baroreceptor neurons where it may mediate mechanosensation. In our present study, expression of ENaC isoforms (β- and γ-ENaCs) was decreased in aortic baroreceptor nerve terminals and nodose neuronal cell bodies from CHF rats (Figures 1 and 2). ENaC (mechanosensitive) currents in aortic baroreceptor neurons were reduced in CHF rats (Figure 3). Additionally, the aortic baroreceptor sensitivity (the response of the ADNA to the increase in arterial blood pressure) was also attenuated in CHF rats (Figure 4). These results indicate that reduced ENaC expression and activation might be involved in attenuated aortic baroreceptor sensitivity and resultant arterial baroreflex dysfunction in the CHF state.

The ENaC has been reported to have 3 subunits (α-ENaC, β-ENaC, and γ-ENaC) in various tissues. However, the data from immunofluorescent staining and western blot in the present study showed that β-ENaC and γ-ENaC but...
not α-ENaC were expressed in rat aortic baroreceptor nerve terminals and nodose neuronal cell bodies, which was also demonstrated by other study. Bonny et al. demonstrated that β- and γ-ENaCs generate mechanosensitive currents without requirement of α-ENaC subunit. Therefore, one possibility is that ENaC currents recorded in aortic baroreceptor neurons are generated by β- and γ-ENaCs-formed βγ-ENaC channels. The other possibility is that the undetectable α-ENaC subunit exists in aortic baroreceptor neurons and combines with β- and γ-ENaC subunits to form αβγ-ENaC channels for the generation of mechanosensitive currents.

Although our present study demonstrated that CHF decreased protein expression of the ENaC in nodose neurons and aortic baroreceptor nerve terminals (Figures 1 and 2), the mechanisms responsible for this phenomenon are not understood. It has been reported that aldosterone and vasopressin up-regulate ENaC expression in mouse cortical collecting duct cells through modulating gene transcription and protein translation of the ENaC. It is well known that plasma aldosterone and vasopressin are significantly elevated in CHF and exogenous aldosterone and vasopressin reduce the baroreceptor reflex sensitivity in normal animals. We can speculate that the elevation of endogenous aldosterone and vasopressin might down-regulate ENaC expression in nodose neurons and aortic baroreceptor nerve terminals from CHF rats because Drummond’s study has shown that exogenous aldosterone inhibits the expression of β-ENaC and γ-ENaC subunits in cultured sensory neurons, which is somewhat unusual compared to the more common overexpression of the ENaC in renal epithelial cells induced by aldosterone.

We clearly understand that ENaC subunits were expressed in aortic baroreceptor nerve terminals and CHF induced alteration of ENaC subunits in aortic baroreceptor nerve terminals (Figure 1). However, a study of ion...
channel electrophysiological changes (such as ENaC current recording) at the level of baroreceptor sensory terminals was not performed because it requires the development of advanced technique, not yet available. Nevertheless, we used an alternative preparation of aortic baroreceptor cell bodies for measurement of ENaC-mediated mechanosensitive currents because we found that CHF also decreased β- and γ-ENaCs expressed in aortic baroreceptor cell bodies. Therefore, it is acceptable for us to test the alteration of aortic baroreceptors by combining electrophysiological data of ENaC-mediated mechanosensitive currents in isolated aortic baroreceptor cell bodies with immunostaining data of ENaC subunits in aortic baroreceptor nerve terminals.

Amiloride or benzamil, as a specific ENaC channel blocker, can block mechanosensory transduction. In the present study, we used amiloride to confirm ENaC currents in aortic baroreceptor neurons from sham and CHF rats (Figure 3). However, we did not investigate the influence of amiloride in the aortic baroreceptor sensitivity in sham rats.

Figure 3. Original recordings of ion currents under a ramp patch-clamp protocol in the aortic baroreceptor neuron from a sham rat (A) and mean data of inward currents at −80 mV in aortic baroreceptor neurons from sham and CHF rats (B). Osmolarity means the osmolarity in the extracellular solution. *P < 0.05 vs. sham normal-osmolarity; †P < 0.05 vs. CHF normal-osmolarity; ‡P < 0.05 vs. sham hypo-osmolarity; §P < 0.05 vs. CHF hypo-osmolarity. (C) Mechanosensitive inward currents at −80 mV in aortic baroreceptor neurons from sham and CHF rats. Mechanosensitive currents were obtained by subtracting ion currents in normal osmotic solution from ion currents in hypo-osmotic solution. *P < 0.05 vs. sham. (D) ENaC currents at −80 mV in aortic baroreceptor neurons from sham and CHF rats. ENaC currents were obtained by subtracting ion currents after treatment of amiloride (1 µM, a specific ENaC inhibitor) from ion currents before treatment of amiloride. *P < 0.05 vs. sham. Data are means ± SE; n = 8 neurons from 6 rats in each group.

Figure 4. Segments of original traces illustrating the bursting pattern of aortic depressor nerve activity (ADNA) in phase with arterial blood pressure pulse at low, normal, and high arterial blood pressure in one sham rat (A) and mean data for arterial blood pressure-ADNA relationship (B) in sham and CHF rats. Data are mean ± SE; n = 6 rats in each group. *P < 0.05 vs. sham rats.
and CHF rats because the ENaC is also expressed in the smooth muscle of arterial vascular walls, and it is impossible to selectively block the ENaC in aortic baroreceptor terminals when amiloride is administered into the circulating blood. As we know, proteins are synthesized in the cell soma and transported to axon endings by axoplasmic flow. Therefore, role of the ENaC in the aortic baroreceptor sensitivity is further confirmed in future study by manipulating expression of ENaC channels in aortic baroreceptor nerve terminals through local transfection of lentiviral ENaC shRNAs or adeno viral ENaC genes into the nodose ganglia. Nevertheless, a further study about systemic blockade/stimulation of the ENaC is also needed to assess the possible overall relevance.

Although there are other mechanical stimuli to be used for measurement of the mechanosensory activity in the single neuron, such as puffing with physiologic solutions to deform the cell, it is difficult to hold the cell for the electrophysiological recording when directly mechanical stimulation is administered. Therefore, hypo-osmotic extracellular solution was used to activate mechanosensitive channels in the present study, which has already been used in other research groups.

In summary, our present study found that CHF not only attenuates the aortic baroreceptor sensitivity but also decreases the expression and activation of the ENaC in aortic baroreceptors. These results indicate that reduced expression and activation of the ENaC might be associated with blunted aortic baroreceptor sensitivity and resultant impairment of the arterial baroreflex in the CHF state. The findings provide a potential pharmacological or genetic target for improving arterial baroreflex function and reducing mortality in CHF.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at American Journal of Hypertension (http://ajh.oxfordjournals.org).

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DISCLOSURE

The authors declared no conflict of interest.

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