Mitochondria-derived superoxide and voltage-gated sodium channels in baroreceptor neurons from chronic heart-failure rats

Huiyin Tu, Jinxu Liu, Zhen Zhu, Libin Zhang, Iraklis I. Pipinos, and Yu-Long Li

Departments of 1Emergency Medicine, 2Surgery, and 3Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska

Submitted 16 August 2011; accepted in final form 3 November 2011

Tu H, Liu J, Zhu Z, Zhang L, Pipinos I, Li YL. Mitochondria-derived superoxide and voltage-gated sodium channels in baroreceptor neurons from chronic heart-failure rats. J Neurophysiol 107: 591–602, 2012. First published November 9, 2011; doi:10.1152/jn.00754.2011.—Our previous study has shown that chronic heart failure (CHF) reduces expression and activation of voltage-gated sodium (Na+) channels in baroreceptor neurons, which are involved in the blunted baroreceptor neuron excitability and contribute to the impairment of baroreflex in the CHF state. The present study examined the role of mitochondria-derived superoxide in the reduced Na+ channel function in coronary artery ligation-induced CHF rats. CHF decreased the protein expression and activity of mitochondrial complex enzymes and manganese SOD (MnSOD) and elevated the mitochondria-derived superoxide level in the nodose neurons compared with those in sham nodose neurons. Adenoviral MnSOD (Ad.MnSOD) gene transfection (50 multiplicity of infection) into the nodose neurons normalized the MnSOD expression and reduced the elevation of mitochondrial superoxide in the nodose neurons from CHF rats. Ad.MnSOD also partially reversed the reduced protein expression and current density of the Na+ channels and the suppressed cell excitability (the number of action potential and the current threshold for inducing action potential) in aortic baroreceptor neurons from CHF rats. Data from the present study indicate that mitochondrial dysfunction, including decreased protein expression and activity of mitochondrial complex enzymes and MnSOD and elevated mitochondria-derived superoxide, contributes to the reduced Na+ channel activation and cell excitability in the aortic baroreceptor neurons in CHF rats.

CHRONIC HEART FAILURE (CHF) is hallmarked by decreased parasympathetic and increased sympathetic nerve activity (Creager et al. 1986; Porter et al. 1990; Saul et al. 1988). Impairment of baroreceptor reflex sensitivity could be directly related to this autonomic dysfunction (Creager 1992). Many studies have documented that baroreceptor reflex sensitivity is attenuated in both clinical (Creager and Creager 1994; Ellenbogen et al. 1989; Ferguson et al. 1992; Kassis et al. 1986) and experimental CHF (Chen et al. 1991; Dibner-Dunlap and Thames 1989; Wang et al. 1990, 1991a, 1991b). Dysfunction of primary afferent baroreceptor neurons is likely to be involved in the blunted baroreflex sensitivity observed in CHF patients and rats. One recent study has shown that enhancing input of baroreceptor afferent by chronic baroreceptor activation significantly increases the survival rate in dogs with pacing-induced CHF (Zucker et al. 2007). Our previous study has clearly confirmed that reduced expression and activation of the voltage-gated sodium (Na+) channels are involved in the attenuation of the baroreceptor neuron excitability and subsequently contribute to the impairment of baroreflex sensitivity in the CHF state (Tu et al. 2010). However, the mechanisms responsible for the reduced Na+ channel activity in the baroreceptor neurons from CHF animals are not understood.

A low level of superoxide is produced in a normal cellular metabolism under physiological conditions (McCord 1993), which is essential for proper cell functions (Fattman et al. 2003). Much evidence has accumulated to implicate the overproduction of reactive oxygen species (ROS), including superoxide in animals and patients with CHF (Giordano 2005; Kang et al. 2008; Li et al. 2007; Ungvari et al. 2005). An elevated superoxide level can modulate the activity of the Na+ channels (Liu et al. 2010; Takeuchi and Yoshii 2008; Zona et al. 2006). Mitochondria are thought to be an important source of the superoxide production in the CHF state, because the mitochondria-derived superoxide level increases (Ide et al. 1999), and the antioxidant reserve decreases (Kim et al. 2010; Miller et al. 2010). Thus mitochondria-derived superoxide may play an important role in the reduced Na+ channel activity in the baroreceptor neurons in CHF. In the present study, we measured the protein expressions and activities of mitochondrial complex enzymes (mitochondrial oxidative system) and manganese SOD (MnSOD; mitochondrial antioxidant system) in sham and CHF rats as the molecular bases for mitochondria-derived superoxide production. We also investigated the role of mitochondria-derived superoxide and the effect of adenoviral MnSOD (Ad.MnSOD) gene transfection on the Na+ channel activity and cell excitability in the baroreceptor neurons in 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.

CHF animal model. Male Sprague-Dawley rats, weighing 180–200 g (6–8 wk old), were assigned randomly to one of two groups: CHF (n = 70) and sham (n = 66). CHF was produced by surgical ligation of the left coronary artery, as described previously (Tu et al. 2010). Sham rats underwent the same surgery, but the coronary artery was not tied (Tu et al. 2010; Zheng et al. 2006; Zhu et al. 2004). The degrees of left ventricular dysfunction and heart failure were determined using both hemodynamic and anatomic criteria. On the day of the terminal experiment, left ventricular end-diastolic pressure (LVEDP) was measured using a pressure transducer (SPR-524; size...
3.5-Fr; Millar Instruments, Houston, TX). To measure infarct size, the heart was dissected, and the atria and right ventricle were removed. A digital image of the left ventricle was captured using a digital camera (Canon, Japan). The percentage of infarct size to total left ventricle was quantified using Adobe Photoshop CS3 Extended (Adobe Systems, San Jose, CA). Rats with both LVEDP > 15 mmHg and infarct size > 30% of total left ventricle were considered as CHF.

Blue native gel electrophoresis for protein of mitochondrial complex enzymes. Nodose ganglia (NGs) from four rats were pooled to obtain sufficient tissue for the analysis. NGs were homogenized and suspended in blue native sample buffer (50 mM NaCl, 5 mM 6-aminohexanoic acid, 50 mM imidazole, pH 7.0) with 5% dodecylmalto-side. Then, the samples were centrifuged at 14,000 rpm for 20 min to remove cellular debris. Coomassie blue (5%) was added into the supernatant of samples, according to the ratio (1:6 w/w) of Coomassie blue/dodecylmaltoside. The blue native polyacrylamide gel was prepared as described by Schagger (1996) with slight modification. Equal amounts of the protein samples were loaded and then separated on a 4.5–13% gradient polyacrylamide gel at 4°C. After one-third of the run, deep blue cathode buffer was replaced by colorless cathode buffer, and then the electrophoresis was run continuously until the front of the blue dye reached the end of the gel, which was incubated in the staining solution (Colloidal Blue Staining Kit, Invitrogen, Carlsbad, CA) overnight at room temperature. After 7 h incubation with distilled water (water was changed three to four times during incubation), the image was captured and analyzed using the UVP Bioimaging and Analysis System (UVP, Upland, CA).

Spectrophotometric measurement of mitochondrial complex enzyme activities. Assays were performed in NG homogenates using a spectrophotometer (DU-640 Series; Beckman Instruments, Fullerton, CA). Complex I (NADH dehydrogenase) enzyme activity was measured as a function of the decrease in absorbance from NADH oxidation by decylubiquinone before and after rotenone addition (Birch-Machin et al. 1994). The specific activity of complex I reported in this study is rotenone-sensitive NADH dehydrogenase (ubiquinone) activity. Complex II (succinate dehydrogenase) activity was measured as a function of the decrease in absorbance from 2,6-dichloroindophenol reduction (Birch-Machin et al. 1994). Complex III [ubiquinol cytochrome c oxidoreductase subunit III (COX III)] activity was determined as a function of the increase in absorbance from cytochrome c reduction (Krahenbuhl et al. 1994). The nonenzymatic reduction in cytochrome c was measured under the same conditions after the addition of antimycin A and was subtracted from the total activity of complex III to calculate the activity specifically due to complex III. Complex IV (COX IV) activity was determined as a function of the decrease in absorbance from COX (Birch-Machin et al. 1994). The specificity of activities of the respiratory complexes was determined by monitoring changes in absorbance in the presence of the specific inhibitors: complex I (rotenone, 5 μM), complex II (malonate, 10 mM), complex III (antimycin A, 5 μM), and complex IV (potassium cyanide, 2 mM). The nmol/min/mg protein was used to express the activities of complex I, II, and III, whereas km/min/mg protein was used to present the activity of complex IV, where k is the first-order velocity constant (k = [2.3 log A (time 1)/A (time 0) + 1] nmol/min).

Western blot measurement of MnSOD protein. NGs were removed rapidly, frozen immediately in liquid nitrogen, and stored at −80°C until analyzed. Briefly, total protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of the protein samples were loaded, and Western blotting was performed as described previously (Tran et al. 2011). MnSOD protein was normalized by β-actin (a housekeeping protein).

MnSOD activity measurement. MnSOD activity was measured by a modification of the nitrite method (Beauchamp and Fridovich 1971; Elstner and Heupel 1976; Oyamagui 1984). Briefly, a SOD activity reference set (0, 6.25, 12.5, 25, 50, 100, 200, and 400 ng MnSOD/20 μl) was prepared with standard MnSOD (Sigma-Aldrich, St. Louis, MO). The unknowns were prepared in duplicates by adding NG homogenates at a dilution of 1/20 to the activity reference sets. The signal reduction of the reference and sample sets was plotted as a function of the concentration of MnSOD in the activity reference set. The shift of the X-intercept at the 50% end point of the sample curve relative to the 50% end point of the reference curve was a linear function (R² = 0.997) of the concentration of MnSOD added to the reference set. The standard SOD activity unit in each sample was computed as nanogram equivalents of standard MnSOD and normalized by total protein.

Labeling of aortic baroreceptor neurons and isolation of NG neurons. Aortic baroreceptor neurons in the NGs were selectively retrograde labeled by a transported fluorescent dye, 1,1′-dioctadecyl 3,3′,3′-tetramethylinodocarbocyanine perchlorate (DiI; red color; Molecular Probes, Eugene, OR), as described previously (Tu et al. 2010). Briefly, under sterile condition, rats were anesthetized with isoflurane at 2% for a thoracotomy at the third intercostal space, and DiI (2 μl) was injected into the adventitia of the aortic arch with a fine-tipped glass pipette. After the application of the dye, the surgical incision was closed. After a 1-wk recovery period to allow the dye to be transported to the aortic baroreceptor neurons in the NGs, the NG neurons were isolated by a two-step enzymatic digestion protocol (Tu et al. 2010). The isolated cells were resuspended in culture medium and plated onto culture wells. The culture medium consisted of a 50/50 mixture of DMEM and Ham’s F12 medium, supplemented with antibiotics and 10% FBS. The NG cells were cultured at 37°C in a humidified atmosphere of 95% air–5% CO₂ until cells were used for the experiments.

Gene transfer to the isolated NG cells. Replication-deficient recombinant human Ad.MnSOD and control adenovirus containing only the cytomegalovirus promoter (Ad.empty), constructed as described previously (Zwacka et al. 1998a, 1998b), were obtained from Gene Transfer Vector Core (University of Iowa, Iowa City, IA). Adenoviral titers were 4 × 10¹⁰ plaque-forming units (pfu/ml) for Ad.MnSOD and 9 × 10¹⁰ pfu/ml for Ad.empty. Our pilot study showed that 50 multiplicity of infection (MOI) of adenovirus was enough to induce gene expression without inducing neuronal toxicity (Yin et al. 2010); therefore, the isolated NG cells were infected with 50 MOI of adenovirus for 6 h and then cultured for 3 days before the experiments.

Immunofluorescent staining for MnSOD and Nav channel proteins. Isolated NG cells plated onto coverslips were fixed with 4% paraformaldehyde in 0.1 M PBS for 10 min at 4°C, washed with PBS–Triton solution (PBS + 0.25% Triton X-100), and blocked with 10% of normal goat serum for 1 h at room temperature. Primary antibodies against MnSOD (eBioscience, San Diego, CA) and COX IV (a mitochondria marker; Abcam, Cambridge, MA) or Na channel subunits (Alomone Labs, Jerusalem, Israel) and RT97 (an A-type neuron marker; Abcam) were incubated with the NG cells overnight at 4°C. Then, the NG cells were incubated with 4′,6′-

| Table 1. Hemodynamic and morphological characteristics of sham and CHF rats |
|---------------------------------------------|------------------|
| **Sham (n = 66)**                          | **CHF (n = 70)**  |
| Body weight (g)                            | 393 ± 7          | 408 ± 14         |
| Heart weight (g)                           | 1.37 ± 0.09      | 2.21 ± 0.12*     |
| Lung weight (g)                            | 2.05 ± 0.07      | 2.92 ± 0.09      |
| Heart weight/body weight (mg/g)            | 3.72 ± 0.06      | 5.51 ± 0.07*     |
| Lung weight/body weight (mg/g)             | 5.38 ± 0.07      | 7.46 ± 0.12*     |
| Infarct size (% of left ventricle)         | 0                | 36.2 ± 2.6*      |
| LVEDP (mmHg)                               | 1.8 ± 0.4        | 18.6 ± 1.3*      |
| LV dP/dt_max (mmHg/s)                      | 8.547 ± 219      | 5.731 ± 315*     |

Data are means ± SEM. CHF, chronic heart failure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_max, left ventricular maximal first derivative. *P < 0.05 vs. sham.
diamidino-2-phenylidole (DAPI; a nucleus marker; Santa Cruz Biotechnology, Santa Cruz, CA) and appropriate secondary antibodies (Invitrogen) or Alexa Fluor488-conjugated isolectin-B4 (a C-type neuron marker; Invitrogen) for 1 h at room temperature. The NG cells were observed under a Leica fluorescent microscope with appropriate excitation/emission filters (Leica Microsystems, Buffalo Grove, IL). Pictures were captured by a digital camera system. No staining was seen when the procedure described above was used, but PBS was used instead of the primary antibody. Expression of MnSOD or Nav channel subunits was quantified using Adobe Photoshop CS3 Extended (Adobe Systems).

**Measurement of mitochondria-derived superoxide.** The level of mitochondria-derived superoxide was measured using the mitochondria-targeted, superoxide-sensitive fluorescent probe MitoSOX Red (Invitrogen), as described previously (Yin et al. 2010). Briefly, fresh NG cells were loaded with MitoSOX Red (1 μM), MitoTracker Green (50 nM; a mitochondria marker; Invitrogen), and DAPI for 40 min. The images were captured using a Leica fluorescent microscope and quantified using Adobe Photoshop CS3 Extended (Adobe Systems).

**Recording of Na+ currents and action potential.** Only DiI-labeled NG neurons (aortic baroreceptor neurons) were selected for the recording of Na+, currents and action potential. Na+ currents and action potential were recorded by the whole-cell patch-clamp technique using Axopatch 200B patch-clamp amplifier (Axon Instruments, Sunnyvale, CA).

In the voltage-clamp experiments, resistance of the patch pipette was 1–3 MΩ when filled with the following solution (in mM): 105 CsCl, 25 triethylamine (TEA), 1 CaCl2, 10 EGTA, 5 MgATP, and 25 glucose (pH 7.3; 320 mOsM/L). The extracellular solution consisted of (in mM): 70 NaCl, 10 CsCl, 60 choline-Cl, 0.1 CdCl2, 10 TEA, 4 MgCl2, 10 HEPS, and 10 glucose (pH 7.4; 330 mOsM/L). In extracellular solution, 70 mM Na+ was used, because normal, extracellular Na+ (140 mM) is sufficiently large to saturate the patch-clamp amplifier (Ikeda et al. 1986). Series resistance of 5–13 MΩ was electronically compensated 30–80%. Junction potential was calculated to be +9.9 mV using pCLAMP 10.2 software (Axon Instruments), and all values of membrane potential given throughout were corrected using this value. Current traces were sampled at 10 kHz and filtered at 5 kHz. The holding potential was −100 mV, and current–voltage (I–V) relationships were elicited by 10 mV step increments to potentials between −90 and 40 mV for 40 ms. Peak currents were measured for each test potential, and current density was calculated by dividing peak current by cell membrane capacitance (Cm). Na+ current decay was fitted with a biexponential function 

\[ y = y_0 + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \]

where \( \tau_1 \) and \( \tau_2 \) are the amplitudes of the fast and the slow processes.
inactivating components, and $\tau_1$ and $\tau_2$ are their respective decay time constants.

In the current-clamp experiments, action potential was elicited by a ramp current injection of 0–250 pA, and the current threshold-inducing action potential was measured at the beginning of the first action potential. The number of action potentials was measured in a 1-s current-clamp. The patch pipette solution was composed of (in mM): 105 K-aspartate, 1 CaCl$_2$, 5 MgATP, 10 HEPES, 10 EGTA, and 25 glucose (pH 7.2; 320 mOsm/L). The bath solution was composed of (in mM): 140 NaCl, 5.4 KCl, 0.5 MgCl$_2$, 2.5 CaCl$_2$, 5.5 HEPES, 11 glucose, and 10 sucrose (pH 7.4; 330 mOsm/L). The pCLAMP 10.2 program (Axon Instruments) was used for data acquisition and analysis. All experiments were done at room temperature.

A-type and C-type aortic baroreceptor neurons were identified as in a previous study (Tu et al. 2010). Briefly, after data collection, neurons were depolarized at 0 mV from holding potential to –80 mV to record the fast transient sodium currents before and after 1 μM TTX treatment in the extracellular bath. The inhibited sodium current was defined as TTX sensitive and the remaining sodium current as TTX resistant. C-type neurons express both TTX sensitive and TTX resistant, but A-type neurons express only TTX sensitive (Doan and Kunze 1999).

Data analysis. All data are presented as means ± SE. SigmaStat 3.5 was used for data analysis. Statistical significance was determined by Student’s unpaired t-test for hemodynamic and morphological characteristics and expressions and activities of mitochondrial complex enzymes and MnSOD. Therefore, we compared the protein expression and activity of mitochondrial complex enzymes and MnSOD in the NG tissue between sham and CHF rats. Data from blue native gel electrophoresis, Western blots, and spectrophotometry showed that CHF significantly decreased the protein expression and activity of mitochondrial complex I, II, and III enzymes and also reduced MnSOD protein expression and activity in the NG tissue, compared with those in sham rats (Figs. 1 and 2). The changes of these enzymes

Fig. 3. Adenovirus gene transfection-induced overexpression of MnSOD in mitochondria. A: representative images showing cytochrome c oxidase subunit IV (CoX IV; mitochondrial marker, green fluorescence), MnSOD (red fluorescence), 4',6'-diamidino-2-phenylidole (DAPI; cell nucleus marker, blue fluorescence), and colocalization of MnSOD and Cox IV in mitochondria (yellow fluorescence) from sham and CHF nodose neurons, with or without adenoviral MnSOD (Ad.MnSOD) gene transfection [50 multiplicity of infection (MOI)]. B: quantitative data for protein expression of MnSOD in mitochondria from non-transfected control, control adenovirus containing only the cytomegalovirus promoter (Ad.empty)-transfected, and Ad.MnSOD-transfected nodose neurons in sham and CHF rats. Data are mean ± SE; n = 20 cells in each group. *P < 0.05 vs. sham control; #P < 0.05 vs. CHF control.

RESULTS

Hemodynamic and morphological characteristics of sham and CHF rats. Table 1 summarizes the hemodynamic and morphological characteristics of sham and CHF rats. In the CHF rats, a gross examination displayed a dense scar in the anterior ventricular wall and an average myocardial infarct size over 30% of the left ventricular area. Heart weight- and lung weight-to-body weight ratios were significantly higher in CHF rats compared with sham rats. These changes indicate cardiac hypertrophy and retention of water in CHF rats. In addition, LVEDP was increased, and left ventricular maximal first derivative was decreased in CHF rats. These data suggest the development of CHF with failed cardiac contractile function.

Mitochondria-derived superoxide production. Mitochondria have been thought to be a main source of the endogenous superoxide production. The level of mitochondria-derived superoxide is determined by the activities of mitochondrial complex enzymes and MnSOD. Therefore, we compared the protein expression and activity of mitochondrial complex enzymes and MnSOD in the NG tissue between sham and CHF rats. Data from blue native gel electrophoresis, Western blots, and spectrophotometry showed that CHF significantly decreased the protein expression and activity of mitochondrial complex I, II, and III enzymes and also reduced MnSOD protein expression and activity in the NG tissue, compared with those in sham rats (Figs. 1 and 2). The changes of these enzymes

J Neurophysiol • doi:10.1152/jn.00754.2011 • www.jn.org
provide the possibility that endogenous mitochondria-derived superoxide production is elevated in the NG tissue from CHF rats.

With the use of the immunofluorescence staining method, we found that the protein expression of endogenous MnSOD was localized in the NG neuron mitochondria, because Mn-SOD red fluorescence colocalized with COX IV (a mitochondria marker) green fluorescence, merging into yellow fluorescence (Fig. 3A). In addition, the protein expression of MnSOD was lower in the NG neuron mitochondria from CHF rats than that from sham rats (Fig. 3). Three days after Ad.MnSOD gene transfection (50 MOI) to the NG neurons, the protein expression of MnSOD was increased significantly in the sham and CHF NG neuron mitochondria, compared with those in the nontransfected (control) sham and CHF NG neuron mitochondria (Fig. 3). However, Ad.empty did not affect the protein expression of MnSOD in the NG neuron mitochondria from sham and CHF rats (Fig. 3).

Superoxide-sensitive probe MitoSOX Red is a useful tool for measurement of mitochondria-derived superoxide. With the use of this method, we observed that CHF significantly increased superoxide levels in the NG neuron mitochondria, because the enhancement of MitoSOX Red fluorescence induced by CHF indicates an increase in mitochondrial-derived superoxide (Fig. 4), which is corroborated by colocalizing MitoSOX Red fluorescence with MitoTracker Green (a mitochondria marker) to merge a yellow fluorescence (Fig. 4). In addition, Ad.MnSOD gene transfection markedly decreased mitochondria-derived superoxide in the NG neurons from CHF rats but not sham rats (Fig. 4). Ad.empty showed no effect on mitochondria-derived superoxide in the NG neurons from sham and CHF rats (Fig. 4). These results show that CHF enhances the mitochondria-derived superoxide in NG neurons via decreasing the protein expressions and activities of mitochondrial complex enzymes and MnSOD and that overexpression of MnSOD partially reduces the elevated superoxide level induced by CHF.

**Effect of Ad.MnSOD on Na\(_v\) currents in aortic baroreceptor neurons from sham and CHF rats.** Our previous study has shown that Na\(_v\) channel dysfunction is involved in the attenuation of aortic baroreceptor neuron excitability and subsequently contributes to the impairment of baroreflex in CHF rats (Tu et al. 2010). To determine the possible mechanisms responsible for the Na\(_v\) channel dysfunction in the CHF state, we measured the changes of the Na\(_v\) currents induced by overexpressing MnSOD in aortic baroreceptor neurons from sham and CHF rats. In the present study, Na\(_v\) currents were recorded in the aortic baroreceptor neurons, selectively labeled by DiI (see METHODS). The aortic baroreceptor neurons were separated into A-type and C-type neurons by their sensitivity to TTX (Schild and Kunze 1997; Tu et al. 2010). Although we found that the whole-cell \(C_m\) in C-type aortic baroreceptor neurons is smaller than that in A-type aortic baroreceptor neurons, there is no significant

![Fig. 4. Effect of Ad.MnSOD gene transfection on CHF-increased mitochondrial superoxide level. A: representative images showing MitoTracker (mitochondrial marker, green fluorescence), MitoSOX (mitochondrial superoxide marker, red fluorescence), DAPI (cell nucleus marker, blue fluorescence), and colocalization of MitoSOX and MitoTracker in mitochondria (yellow fluorescence) from sham and CHF nodose neurons, with or without Ad.MnSOD gene transfection (50 MOI). B: quantitative data for mitochondria-derived superoxide in mitochondria from nontransfected control, Ad.empty-transfected, and Ad.MnSOD-transfected nodose neurons in sham and CHF rats. Data are mean ± SE; \(n = 18\) cells in each group. *\(P < 0.05\) vs. sham control; \#\(P < 0.05\) vs. CHF control.](http://jn.physiology.org/doi/10.1152/jn.00754.2011/fig4)
Ad.MnSOD gene transfection significantly reduced the current threshold in A- and C-type aortic baroreceptor neurons from CHF rats, whereas Ad.empty did not change the current threshold in CHF aortic baroreceptor neurons (Fig. 6D).

The number of action potentials was also measured under 1-s current-clamp (50 pA for A-type neurons and 250 pA for C-type neurons). The number of action potentials was decreased significantly in A- and C-type aortic baroreceptor neurons from CHF rats, compared with that from sham rats (Fig. 6, A–C). Ad.MnSOD but not Ad.empty enhanced the number of action potentials in A- and C-type aortic baroreceptor neurons from CHF rats (Fig. 6, A–C).

Additionally, CHF markedly decreased input resistance and slowed down the maximum rate of depolarization of action potential (Table 3). Ad.MnSOD improved CHF-induced alterations of input resistance and the maximum rate of depolarization of action potential (Table 3). However, there was no difference in resting membrane potential, action potential duration at 90% repolarization, and overshoot among all groups (Table 3). The above data show that mitochondrial-derived superoxide is also involved in the attenuated aortic baroreceptor neuron excitability.

Determining how mitochondria-derived superoxide modulated the Na\textsubscript{\text{c}} channel function in aortic baroreceptor neurons from CHF rats. Modulating ion channel function includes acutely influencing the ion currents and chronically altering the expression of ion channels. Although the above data showed that Ad.MnSOD increased the Na\textsubscript{\text{c}} current density in CHF aortic baroreceptor neurons (Fig. 5), it is unclear how endogenous mitochondria-derived superoxide modulated the Na\textsubscript{\text{c}} channels in CHF aortic baroreceptor neurons. We investigated the effect of Ad.MnSOD gene transfection on protein expression of Na\textsubscript{\text{c}} channels in the NG neurons from sham and CHF rats. Previous study has confirmed that a TTX-sensitive Na\textsubscript{\text{c}} channel (Na\textsubscript{\text{c}},1.7) is expressed in A- and C-type NG neurons, but TTX-resistant Na\textsubscript{\text{c}} channels (Na\textsubscript{\text{c}},1.8 and Na\textsubscript{\text{c}},1.9) are located only in C-type NG neurons (Tu et al. 2010). As with our previous study (Tu et al. 2010), the protein expression levels of Na\textsubscript{\text{c}} channels (Na\textsubscript{\text{c}},1.7, Na\textsubscript{\text{c}},1.8, and Na\textsubscript{\text{c}},1.9) were lowered in the nodose neurons from CHF rats compared with sham rats (Fig. 7). In addition, Ad.MnSOD transfection significantly increased the protein expression of Na\textsubscript{\text{c}} channels in the nodose neurons from CHF rats (P < 0.05; Fig. 7).

Rotenone (mitochondrial complex I inhibitor) and antimycin A (mitochondrial complex III inhibitor) are reported to increase mitochondrial superoxide production (Cadenas et al. 1977; Murphy 2009; Tran et al. 2011). In sham rats, we found that rotenone or antimycin A acutely increased mitochondria-derived superoxide in the nodose neurons and reduced Na\textsubscript{\text{c}} current density in the aortic baroreceptor neurons (Fig. 7), which indirectly showed that elevated mitochondria-derived superoxide acutely reduced Na\textsubscript{\text{c}} currents. However, rotenone or antimycin A did not change mitochondrial superoxide production and Na\textsubscript{\text{c}} currents in CHF A- and C-type neurons (Fig. 8). It is possible that CHF already induced enough mitochondria-derived superoxide production to acutely reduce Na\textsubscript{\text{c}} currents.

The above results indicate that endogenous mitochondria-derived superoxide modulates the Na\textsubscript{\text{c}} channel function via acutely reducing Na\textsubscript{\text{c}} currents and chronically changing the protein expression of the Na\textsubscript{\text{c}} channels in the aortic baroreceptor neurons from CHF rats.
DISCUSSION

Our present study demonstrates that CHF induces the hyperactivation of the mitochondrial oxidative system (the decreases of mitochondrial complex enzyme expression and activity) and the inhibition of the mitochondrial antioxidative system (the reductions of the MnSOD expression and activity) in the NG tissue. This imbalance between the mitochondrial oxidative and antioxidative systems contributes to the elevation of superoxide production in CHF aortic baroreceptor neurons, with or without Ad.MnSOD gene transfection. Data are mean ± SE; n = 9 cells in each group. #P < 0.05 versus sham control; †P < 0.05 versus CHF control. Na+ current density in D measured in response to a test pulse at −10 mV from holding potential −100 mV.

Table 3. Electrophysiological changes on action potential in sham and CHF aortic baroreceptor neurons, with or without Ad.MnSOD gene transfection

<table>
<thead>
<tr>
<th></th>
<th>RMP (mV)</th>
<th>R_i (GΩ)</th>
<th>V_max (mV/ ms)</th>
<th>APD90 (ms)</th>
<th>Overshoot (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A-type neurons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>−60.2 ± 1.4</td>
<td>0.93 ± 0.06</td>
<td>82.6 ± 3.8</td>
<td>3.6 ± 0.2</td>
<td>40.1 ± 1.5</td>
</tr>
<tr>
<td>Sham + Ad.empty</td>
<td>−61.2 ± 1.6</td>
<td>0.90 ± 0.04</td>
<td>80.9 ± 5.1</td>
<td>3.5 ± 0.1</td>
<td>41.4 ± 0.8</td>
</tr>
<tr>
<td>Sham + Ad.MnSOD</td>
<td>−58.5 ± 2.2</td>
<td>0.94 ± 0.07</td>
<td>83.3 ± 4.2</td>
<td>3.5 ± 0.2</td>
<td>41.6 ± 1.2</td>
</tr>
<tr>
<td>CHF</td>
<td>−60.5 ± 4.2</td>
<td>0.46 ± 0.03*</td>
<td>66.6 ± 7.2*</td>
<td>3.9 ± 0.2</td>
<td>39.3 ± 1.3</td>
</tr>
<tr>
<td>CHF + Ad.empty</td>
<td>−60.2 ± 2.6</td>
<td>0.45 ± 0.03*</td>
<td>65.3 ± 5.3*</td>
<td>4.0 ± 0.2</td>
<td>39.8 ± 1.2</td>
</tr>
<tr>
<td>CHF + Ad.MnSOD</td>
<td>−58.9 ± 4.3</td>
<td>0.82 ± 0.05†</td>
<td>77.5 ± 5.4†</td>
<td>3.6 ± 0.2</td>
<td>40.0 ± 1.6</td>
</tr>
<tr>
<td><strong>C-type neurons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>−61.0 ± 3.6</td>
<td>0.31 ± 0.04</td>
<td>31.0 ± 3.3</td>
<td>7.2 ± 0.3</td>
<td>40.4 ± 2.1</td>
</tr>
<tr>
<td>Sham + Ad.empty</td>
<td>−59.7 ± 3.2</td>
<td>0.30 ± 0.03</td>
<td>29.8 ± 2.0</td>
<td>7.3 ± 0.3</td>
<td>40.3 ± 1.6</td>
</tr>
<tr>
<td>Sham + Ad.MnSOD</td>
<td>−60.1 ± 3.1</td>
<td>0.31 ± 0.02</td>
<td>32.6 ± 2.5</td>
<td>7.2 ± 0.3</td>
<td>41.9 ± 2.8</td>
</tr>
<tr>
<td>CHF</td>
<td>−60.3 ± 3.8</td>
<td>0.17 ± 0.01*</td>
<td>21.4 ± 1.5*</td>
<td>7.5 ± 0.3</td>
<td>39.3 ± 1.7</td>
</tr>
<tr>
<td>CHF + Ad.empty</td>
<td>−61.3 ± 2.5</td>
<td>0.16 ± 0.01*</td>
<td>19.8 ± 1.6*</td>
<td>7.6 ± 0.4</td>
<td>38.8 ± 1.0</td>
</tr>
<tr>
<td>CHF + Ad.MnSOD</td>
<td>−59.7 ± 3.6</td>
<td>0.26 ± 0.01†</td>
<td>28.2 ± 1.1†</td>
<td>7.3 ± 0.2</td>
<td>40.9 ± 1.1</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 9 cells in each group. RMP, resting membrane potential; R_i, input resistance; V_max, maximum rate of depolarization of action potentials; APD90, action potential duration at 90% repolarization. *P < 0.05 versus sham; †P < 0.05 versus CHF.
of mitochondria-derived superoxide level, and the latter is involved in the reduced Na\textsubscript{a} channel activity in CHF aortic baroreceptor neurons through acutely decreasing Na\textsubscript{a} currents and chronically reducing the Na\textsubscript{a} channel expression. Additionally, restoring MnSOD expression by Ad.MnSOD gene transfection to the nodose neurons significantly enhances the Na\textsubscript{a} current density and improves the cell excitability of the aortic baroreceptor neurons in CHF rats via decreasing the elevated mitochondrial superoxide level. These results suggest that the elevation of endogenous mitochondria-derived superoxide mediates the reduced Na\textsubscript{a} channel activity and attenuated cell excitability of the aortic baroreceptor neurons in CHF rats.

Under a physiological condition, the mitochondrial electron transport chain (mitochondrial complex enzymes) consumes molecular oxygen to transport the electron for ATP production, and there is a very low level of the molecular oxygen (1–2%) to produce superoxide (Cadenas and Davies 2000). In pathophysiological conditions, mitochondria are thought to be an important source of superoxide in most cells (Adam-Vizi and Chinopoulos 2006; Balaban et al. 2005; Cadenas and Davies 2000; Murphy 2009; Turrens 2003). There are oxidative (mitochondrial complex enzymes) and antioxidative (MnSOD) systems in the mitochondria. The mitochondria-derived superoxide level is elevated when the mitochondrial complex enzymes are inhibited (Cadenas et al. 1977; Murphy 2009), and the MnSOD activity is reduced (Robinson 1998; Wallace 2001). Some studies have reported that superoxide production is increased in inherited mitochondrial complex I deficiency,
which is primarily a consequence of the reduction in cellular mitochondrial complex I activity (Pitkanen and Robinson 1996; Verkaart et al. 2007). Our present study found that the protein expressions and activities of the mitochondrial complex enzymes (complex I, II, and III) and MnSOD were reduced in the NG from CHF rats (Figs. 1–3). Mitochondrial superoxide production measured by MitoSOX Red (a specific dye for mitochondrial superoxide) was enhanced in the NG neurons from CHF rats (Fig. 4). Furthermore, acute treatment with rotenone (mitochondrial complex I inhibitor) or antimycin A (mitochondrial complex III inhibitor) elevated mitochondria-derived superoxide production in the sham rat nodose neurons (Fig. 8). These results provide not only the direct evidence for the elevation of the mitochondria-derived superoxide level but also the molecular bases for the enhanced mitochondria-derived superoxide level in the NG neurons from CHF rats. However, until now, we don’t know why inhibiting mitochondrial complex enzymes induces the elevation of the mitochondria-derived superoxide level. One possible explanation is that CHF-reduced activity of each mitochondrial complex enzyme is uneven, and subsequently, these alterations cause the uncoupling of the mitochondrial electron transport chain and finally increase the electron leak and superoxide production. Further studies are needed to address this issue.

Our previous study has shown that CHF reduces the expression and activation of the Na\textsubscript{v} channels and attenuates the cell excitability in the aortic baroreceptor neurons, which contributes to the blunted arterial baroreflex in the CHF state (Tu et al.

Fig. 7. Effect of Ad.MnSOD gene transfection on CHF-reduced protein expression of Na\textsubscript{v} channel subunits in nodose neurons. A and B: representative images showing RT97 or isolecitin-B4 (IB4; A- or C-type neuron marker, respectively, green fluorescence), a TTX-sensitive Na\textsubscript{v} channel (Na\textsubscript{v}1.7; red fluorescence), DAPI (cell nucleus marker, blue fluorescence), and colocalization of Na\textsubscript{v}1.7 and RT97 or IB4 (yellow fluorescence) from sham, CHF, and Ad.MnSOD-plus-CHF nodose neurons. C: quantitative data for protein expression of Na\textsubscript{v} channel subunits in A- and C-type nodose neurons from sham, CHF, and Ad.MnSOD-plus-CHF groups. Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9, TTX-resistant Na\textsubscript{v} channels; OD, optical density. Data are mean ± SE; n = 16 cells in each group. *P < 0.05 vs. sham; #P < 0.05 vs. CHF.
Antimycin A markedly decreased the Nav current density, also found that mitochondrial enzyme inhibitors (rotenone and antimycin A) on mitochondrial superoxide (A) and Na⁺ current density (B) in sham and CHF neurons. Data are mean ± SE; n = 10 cells in each group. *p < 0.05 vs. respective control.

In the present study, CHF increased the mitochondria-derived superoxide level in the nodose neurons (Fig. 4). In addition, we used the Ad.MnSOD gene transfection to the nodose neurons to clarify whether the elevation of the mitochondria-derived superoxide is involved in the reduced Na⁺ channel activation and cell excitability in the CHF aortic baroreceptor neurons, because MnSOD is the SOD isoform known to exist in mitochondria. This technique has been used in our previous study selectively to increase protein expression of MnSOD in the mitochondria of the catecholaminergic neurons (Yin et al. 2010). Our present data confirmed that Ad.MnSOD gene transfection totally restored the protein expression of MnSOD and reduced the mitochondria-derived superoxide level in CHF nodose neurons. At the same time, the reduced expression and current density of the Na⁺ channels and the attenuated cell excitability in CHF aortic baroreceptor neurons were partially reversed by Ad.MnSOD gene transfection. We also found that mitochondrial enzyme inhibitors (rotenone and antimycin A) markedly decreased the Na⁺ current density, accompanied by elevating mitochondria-derived superoxide production in sham neurons (Fig. 8). These results strongly suggest that elevation of the endogenous mitochondria-derived superoxide level contributes to the reduced Na⁺ current density and the attenuated cell excitability in the CHF aortic baroreceptor neurons through chronically reducing protein expression of the Na⁺ channels and acutely decreasing the Na⁺ currents. However, our present study cannot exclude the involvement of other ion channels (such as calcium and potassium channels) during the Ad.MnSOD gene transfection, because any ion channel can be responsible for the alteration of cell excitability, and increasing evidence has shown that superoxide also regulates the function of other ion channels (Archer et al. 2008; Hool 2009; Hool and Corry 2007; Liu and Gutterman 2002; Wang 2006).

Ad.MnSOD gene transfection into the isolated NG neurons proved efficacious in increasing MnSOD protein expression in mitochondria of the CHF NG neurons, which is even higher than that in the sham NG neurons (Fig. 3). Yet, Ad.MnSOD gene transfection did not totally normalize the mitochondria-derived superoxide level (Fig. 4) and the protein expression and activation of Na⁺ channels in CHF nodose neurons (Figs. 5 and 7) to levels found in sham nodose neurons. There may be at least two possible explanations for this inconsistency. First, the mitochondria-derived superoxide level is dependent on both oxidative (mitochondrial complex enzymes) and antioxidative (MnSOD) systems in the mitochondria. Normalizing the ability of scavenging mitochondrial superoxide (Ad.MnSOD-induced overexpression of MnSOD) may not be enough to scavenge oxidative system-induced superoxide overproduction, because function of the mitochondrial complex enzymes did not improve in the present study. Second, it is possible that cytosolic superoxide and other endogenous factors also mediate the reduced Na⁺ channel activity of the aortic baroreceptor neurons induced by CHF.

Although we used Ad.MnSOD gene transfection to enhance MnSOD expression in neuron mitochondria, it should be considered that the Ad.MnSOD gene-expressed MnSOD protein may also locate other cell organelles besides mitochondria. Some studies have found that MnSOD protein is activated by loading with manganese only when the protein is unfolded and transported into mitochondria (Culotta et al. 2006; Luk et al. 2005), which indicates that endogenous or Ad.MnSOD gene-expressed MnSOD in other cell organelles (such as cytoplasm) is inactive. In addition, our present data showed that an enhanced mitochondria-derived superoxide level in CHF NG neurons was significantly lowered by Ad.MnSOD gene transfection. Therefore, this potential possibility cannot influence us to clarify the relationship between mitochondria-derived superoxide and Na⁺ channels in the CHF state.

Although we found that Ad.MnSOD gene transfection significantly increased the protein expression of MnSOD and decreased mitochondria-derived superoxide in CHF nodose neurons, we cannot clearly confirm the effect of Ad.MnSOD gene transfection in the CHF aortic baroreceptor neurons due to the limitation of the method (DiI labeling was lost from cells during the immunofluorescent-staining procedure and could not be used as a marker of the aortic baroreceptor neurons with immunofluorescent staining). However, it is reasonable to assume that Ad.MnSOD gene transfection alters the protein expression of MnSOD and mitochondria-derived superoxide production in the CHF aortic baroreceptor neurons, because the aortic baroreceptor neurons are a component of the NG neurons, and electrophysiological data indicate that Ad.MnSOD increases the Na⁺ currents and improves the cell excitability in CHF aortic baroreceptor neurons. Of course, we will use other tracers (such as biocytin and fluoro-gold) to clarify this point in future study.

There are very few studies focusing on the mechanisms by which superoxide modulates the expression and electrophysiological properties of the ion channels, especially no report
about the mitochondria-derived superoxide. Recent studies have shown that ROS down-regulates the expression of cardiac Na\textsubscript{v},1.5 channels (TTX-resistant) through NF-κB (a transcription factor) (Shang et al. 2008). Although an inside-out or outside-out single-channel recording, but not a whole-cell patch-clamp recording, can measure whether the superoxide directly modulates the single-channel open probability, it is impossible to measure the direct effect of the mitochondria-derived superoxide on the Na\textsubscript{v} channels due to loss of the mitochondria in a single-channel recording. Therefore, further studies are needed to explore the intracellular signaling of the mitochondria-derived superoxide and how the Na\textsubscript{v} channels are modulated by the mitochondria-derived superoxide.

In the present study, measurements of MnSOD, mitochondria-derived superoxide, Na\textsubscript{v} currents, and cell excitability were performed 3 days after the neurons were isolated and cultured, because 3 days are required for Ad.MnSOD gene transfection to obtain the sufficient protein expression of the MnSOD (Yin et al. 2010). It is possible that culture conditions alter electrophysiological characteristics of Na\textsubscript{v} channels in the aortic baroreceptor neurons. However, we do not think this is likely, because our pilot data showed that there was no significant difference on the Na\textsubscript{v} current density and cell excitability at the 1st, 2nd, and 3rd days in sham and CHF aortic baroreceptor neurons (data not shown). Additionally, a recent study has shown that the Na\textsubscript{v} currents recorded in the acutely dissociated nodose neurons are the same as that obtained in the nodose neurons cultured at 24 h (Kwong et al. 2008).

In summary, CHF decreased the protein expression and activity of the mitochondrial complex enzymes and MnSOD and enhanced the mitochondria-derived superoxide level in the mitochondria of the nodose neurons. Ad.MnSOD gene transfection to the CHF NG neurons partially reversed the reduced Na\textsubscript{v} currents and cell excitability via scavenging the elevated mitochondrial superoxide. These results suggest that mitochondrial dysfunction, including MnSOD deficiency and elevation of mitochondrial superoxide, is at least partially responsible for the reduced Na\textsubscript{v} currents and cell excitability in the CHF aortic baroreceptor neurons. Our previous study has shown that the reduced Na\textsubscript{v} channel activation in the aortic baroreceptor neurons is implicated in the arterial baroreflex impairment in CHF (Tu et al. 2010). From these data, therefore, we can consider that overexpression of the MnSOD in the aortic baroreceptor neurons may be a therapeutic target for improving arterial baroreflex function in the CHF state.

ACKNOWLEDGMENTS

The authors thank Dr. Kurtis Cornish for surgical assistance.

GRANTS

Support for this study was provided by a grant from the National Heart, Lung, and Blood Institute (HL-098503) to Y.-L. Li.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


Hool LC. The L-type Ca\textsubscript{2+} channel as a potential mediator of pathol-ogy during alterations in cellular redox state. Heart Lung Circ 18: 3–10, 2009.


Kang YM, Zhang ZH, Xue B, Weiss RM, Felder RB. Inhibition of brain proinflammatory cytokine synthesis reduces hypothalamic excitation in rats


