Expression of Neuronal Nitric Oxide Synthase in Rabbit Carotid Body Glomus Cells Regulates Large-Conductance Ca\(^{2+}\)-Activated Potassium Currents

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Li Y-L, Zheng H, Ding Y, Schultz HD. Expression of neuronal nitric oxide synthase in rabbit carotid body glomus cells regulates large-conductance Ca\(^{2+}\)-activated potassium currents. J Neurophysiol 103: 3027–3033, 2010. First published March 31, 2010; doi:10.1152/jn.01138.2009. Our previous studies show that a decrease in endogenous nitric oxide (NO) is involved in the blunted outward K\(^{+}\) currents in carotid body (CB) glomus cells from chronic heart failure (CHF) rabbits. In the present study, we measured the effects of the neuronal nitric oxide synthase (nNOS) transgene on the K\(^{+}\) currents in CB glomus cells from pacing-induced CHF rabbits. Using single-cell real-time RT-PCR and immunofluorescent techniques, we found that nNOS mRNA and protein are expressed in the rabbit CB glomus cells and CHF decreased the expression of nNOS mRNA and protein in CB glomus cells. After 3 days of an adenoviral nNOS (Ad.nNOS) gene transfection, the expression of nNOS protein was increased to the level found in sham CB glomus cells. In whole cell patch-clamp experiments, Ad.nNOS markedly reversed the attenuated K\(^{+}\) currents in CB glomus cells from CHF rabbits. The specific nNOS inhibitor (S-methyl-L-thio-citrulline [SMTC]) and large-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channel blocker (iberiotoxin) fully abolished the effect of Ad.nNOS on the K\(^{+}\) currents in the CB glomus cells from CHF rabbits. However, neither CHF nor Ad.nNOS altered the protein expression of BK channel \(\alpha\)-subunit. These results suggest that a decrease of NO induced by an attenuated nNOS activity lowers the activation of the BK channels but not the protein expression of the BK channel \(\alpha\)-subunit in the CB glomus cells during CHF.

INTRODUCTION

It is known that enhanced peripheral chemoreflex sensitivity occurs in pacing-induced chronic heart failure (CHF) rabbits (Sun et al. 1999a), which contributes to increased sympathetic outflow in the CHF state, given that inhibition of peripheral chemoreceptor activity is shown to reduce resting renal sympathetic nerve activity (RSNA) (Sun et al. 1999a). Our previous study has found that an augmented afferent signal from the carotid body (CB) chemoreceptors mediates the enhancement of peripheral chemoreflex sensitivity in CHF rabbits (Sun et al. 1999b).

As the primary sensor of the peripheral chemoreflex, the CB is composed of neurotransmitter-enriched glomus cells and glial-like sustentacular cells among other neuronal structures. Although many voltage-gated ion channels exist in the glomus cell membrane, the suppression of outward K\(^{+}\) channels is assumed to contribute to the initial depolarization of cells, release of neurotransmitter, and augmentation of afferent signal input in the carotid sinus nerve (Gonzalez et al. 1994; Prabhakar 1994). Nitric oxide (NO) is a gas molecule that functions as a modulator in both the peripheral and the central nervous systems and is synthesized by NO synthase (NOS) including neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) (Snyder 1992). Our previous study documented that a decreased availability of NO reduced K\(^{+}\) currents in CB glomus cells from CHF rabbits. Further, we ascertained that down-regulation of NO synthesis in the CB from CHF rabbits contributed to the CB chemoreceptor hypersensitivity (Sun et al. 1999b) and transgene overexpression of nNOS (adenoviral nNOS [Ad.nNOS]) significantly reversed enhanced chemoreceptor function in CHF rabbits (Li et al. 2005). Therefore our first purpose in the present study was to test the effect of Ad.nNOS on the K\(^{+}\) currents in sham and CHF CB glomus cells.

Although our studies have found that NO plays an important role in regulating K\(^{+}\) channels of glomus cells, chemoreceptor discharge, and chemoreflex sensitivity in sham and CHF rabbits (Ding et al. 2008; Li et al. 2004, 2005; Sun et al. 1999b), it is unclear whether NOS is present in the rabbit CB glomus cell. In rats and cats, NOS was found to exist in the extensive plexus of nerve fibers and vessels surrounding glomus cells, but not within glomus cells (Dvorakova and Kummer 2005; Wang et al. 1993, 1994). Therefore our second purpose was to establish whether CB glomus cells from rabbits express nNOS mRNA and protein by single-cell real-time RT-PCR and immunofluorescent techniques and whether nNOS expression in glomus cells is altered in CHF.

METHODS

Pacemaker implantation and production of CHF

All experiments were carried out on male New Zealand White rabbits weighing 2.5–3.5 kg (n = 51 rabbits). Experiments 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 (Publication No. 85-23, revised 1996) and the American Physiological Society’s Guide for the Care and Use of Laboratory Animals. Rabbits were assigned to sham-operated and CHF groups. They were housed in individual cages under controlled temperature and humidity and a 12-h/12-h dark–light cycle and fed standard rabbit chow with water available without restriction.

Rabbits were anesthetized with a cocktail consisting of 5.8 mg/kg xylazine, 35 mg/kg ketamine, and 0.01 mg/kg atropine given as an
intramuscular (im) injection, intubated, and connected to a small animal anesthesia respiration unit using 2.0–5.0% inhalation isoflu-rane with oxygen for the duration of the surgery. Using sterile technique, a left thoracotomy was performed as previously described (Sun et al. 1999a). Briefly, a pin electrode was attached to the left ventricle and a ground electrode was secured to the left atrium for pacing. All wires were tunneled beneath the skin and exited in the midscapular area. The chest was closed. Rabbits were placed on an antibiotic regimen consisting of 5 mg/kg Baytril, im, for 5 days. After 2 wk, the pacing was started at 340 beats/min (bpm), held for 7 days, after which the rate was gradually increased to 380 bpm, with an increment of 20 bpm each week. The progression of CHF was recorded and identified with the pacemaker turned off for ≥30 min before the recordings were started. Sham-operated animals underwent a similar protocol of echocardiographic measurements. CHF was characterized by a >40% reduction in ejection fraction (EF) and fraction of shortening (FS) and dilation of the left ventricle in both systole and diastole.

**Isolation and identification of CB glomus cells**

The isolation and identification of CB glomus cells were performed as described in our previous study (Li et al. 2004). Briefly, the carotid bifurcations on both sides were removed surgically from sham or CHF rabbits (Channon et al. 1998). After isolated CB cells were cultured in vitro, 2 mg/ml); that for the second step contained collagenase (4 mg/ml) and bovine serum albumin (BSA, 5 mg/ml). The isolated cells including glomus cells, other neural cells, and vascular cells were cocultured at 37°C in a humidified atmosphere of 95% air-5% CO2.

Culture cells were superfused with an extracellular solution containing Na+. Those cells that exhibited Na+ current were considered to be glomus cells (Overholt and Prabhakar 1997).

**Gene transfer with Ad.EGFP or Ad.nNOS to the CB glomus cells**

The Ad.nNOS originally described by Channon et al. (1996) was used in these experiments. This Ad.nNOS, containing a nNOS cDNA, expresses functional nNOS protein when perfused in carotid arteries of rabbits (Channon et al. 1998). After isolated CB cells were cultured about 6 h, Ad.EGFP (as control adenoviruses) or Ad.nNOS (1 × 107 pfu/ml, 10 μl) was slowly added into the culture dish containing isolated primary CB cells. All experiments were performed 3 days after gene transfer. Enhanced green fluorescent protein (EGFP) was directly measured under a Leica microscope at 510 nm with single excitation peak and at 490 nm of green light (Hajjar et al. 1998) to evaluate the infection efficiency of the adenovirus (Xia et al. 2002). Enhanced green fluorescent protein (EGFP) was used instead of the primary antibody. Expression of nNOS or BK channels was quantified using Adobe Photoshop CS3 (Photoshop Extended). The image (red color) of immunofluorescence for nNOS, BK-α channel and tyrosine hydroxylase

**Immunofluorescence for nNOS, BK-α channel and tyrosine hydroxylase**

Isolated CB cells plated onto coverslips were fixed with 50/50 mixture of ethanol and methanol for 20 min at −20°C, washed with PBS–Triton solution (phosphate-buffered saline + 0.1% Triton X-100), and blocked with 10% of normal goat serum for 1 h at room temperature. Primary anti-nNOS (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-BK-α (Alomone Labs, Jerusalem, Israel) and anti-tyrosine hydroxylase (TH) antibodies (a marker for CB glomus cells; Santa Cruz Biotechnology) were incubated with the CB cells overnight at 4°C. Then the CB cells were incubated with appropriate secondary antibodies (Santa Cruz Biotechnology) for 60 min at room temperature. After being washed in PBS, the coverslips were mounted on precleaned microscope slides. The CB cells were observed under a Leica fluorescent microscope with appropriate excitation/emission filters. Pictures were captured by a digital camera system. No staining was seen when the above-described procedure was used, but PBS was used instead of the primary antibody.

Expression of nNOS or BK channels was quantified using Adobe Photoshop CS3 (Photoshop Extended). The image (red color) of nNOS or BK channels in the cells colocalized with TH in each glomus cell cluster was selected using the lasso tool. After a measurement scale was set (1 pixel = 1 pixel), the integrated density (optical density [OD]) of the nNOS or BK channel image was automatically calculated. TABLE 1. Primer sequence

<table>
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<th>Gene Accession Number</th>
<th>Primer Name</th>
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<th>Sequence Start</th>
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<td></td>
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<tr>
<td>L18-internal</td>
<td>GGCAAGAGGCGGAGAGGCCGAG</td>
<td>31</td>
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</table>

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Values are means ± SE, where n represents number of animals. CHF, chronic heart failure; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; EF, ejection fraction.

measured by clicking record measurements in the analysis menu. Similarly, the total pixels (area) of all glomus cells (TH-labeled) were measured in the same cell cluster. The quantitative data were calculated by the integrated density of the nNOS or BK channel image/total measured in the same cell cluster. The quantitative data were calculated by the integrated density of the nNOS or BK channel image/total pixels of all glomus cells and presented as OD/pixel. Five clusters from each animal (n = 4 rabbits for each group) were scored.

Recording of whole cell currents

K⁺ currents were measured in the perforated whole cell configuration of the patch-clamp technique (Li et al. 2004) using a Warner PC-505B patch-clamp amplifier (Warner Instruments, Hamden, CT). Patch pipettes had resistances of 4–6 MΩ when filled with (in mM) 105 potassium aspartate, 20 KCl, 1 CaCl₂, 10 EGTA, 5 Mg-ATP, 10 Hepes, and 25 glucose (pH 7.2). In the perforated whole cell configuration, nystatin was added to the patch-pipette solution at a final concentration of 300 μg ml⁻¹ immediately before recording. The extracellular solution had the following composition (in mM): 140 NaCl, 5.4 KCl, 2.5 CaCl₂, 0.5 MgCl₂, 5.5 Hepes, 11 glucose, and 10 sucrose (pH 7.4). Na⁺ channels were blocked by tetrodotoxin (TTX, 0.5 μM). Current–voltage (I–V) relations were elicited by 400 ms test pulses from a holding potential of −80 to +70 mV applied in 10 mV increments (5 steps between steps).

Ca²⁺ currents were recorded in a similar perforated whole cell configuration. The intracellular solution contained (in mM) 115 CsCl, 20 tetraethylammonium-Cl (TEA), 10 phosphocreatine, 5 EGTA, 5 Mg-ATP, 0.2 TrisGTP, and 5 Hepes (pH 7.2). The extracellular solution had the following composition (in mM): 140 NaCl, 5.4 KCl, 2.5 CaCl₂, 0.5 MgCl₂, 5.5 Hepes, 11 glucose, and 10 sucrose (pH 7.4). Na⁺ channels were blocked by tetrodotoxin (TTX, 0.5 μM). The I–V relations were elicited by 25 ms test pulse steps (5 steps between steps) from a holding potential of −80 mV to test pulses (−50 to +50 mV, 10 mV increments).

Cell membrane capacitance (Cₘ) was determined by integrating the capacitive current evoked by a 5 mV voltage step and dividing the resulting charge by the voltage step. Mean series resistances, as determined from the decay of the capacitive transient, were 12.8 ± 0.4 MΩ in perforated whole cell mode and were compensated electronically by 80–90%. Current traces were sampled at 10 kHz and filtered at 5 kHz. Peak currents were measured for each test potential and were plotted against the corresponding test potential.

Statistical analysis

All data are expressed as means ± SE. Statistical significance was determined by Student’s unpaired t-test for hemodynamics, mRNA, and Ca²⁺ currents and by a two-way ANOVA, followed by a Bonferroni procedure for post hoc analysis for multiple comparisons in protein and K⁺ currents. Statistical significance was accepted when P < 0.05. A power analysis was conducted to assess whether the sample size was sufficient to ensure P < 0.05.

RESULTS

Cardiac function in sham and CHF animals

The data for each parameter were collected from all sham and CHF rabbits. Measurements were made with the pacer turned off for ≥30 min. CHF was induced by 4 wk of rapid left ventricular pacing. CHF was characterized by an enlarged left ventricular weight-to-body weight ratio, left ventricular end-diastolic diameter, and left ventricular end-systolic diameter, and reduced shortening and ejection fractions (Table 2).

nNOS expression in CB glomus cells

Using single-cell real-time RT-PCR, we found that rabbit CB glomus cells express the nNOS gene (Fig. 1). CHF induced a significant decrease in the nNOS mRNA, compared with sham (P < 0.05). Using immunofluorescent staining, we further confirmed that rabbit CB glomus cells express nNOS protein (Fig. 2). In addition, the immunofluorescent detection of nNOS was lower in the CB glomus cells from CHF rabbits than that from sham rabbits (Fig. 2).

Three days after incubation of CB glomus cells of sham and CHF rabbits with Ad.nNOS (10 μl, 1 × 10⁷ pfu/ml), the expression of nNOS protein was significantly increased in the CHF CB glomus cells but not sham cells (Fig. 2). The expression of EGFP was used to confirm the efficacy of adenovirus infection. EGFP was visible in nearly all isolated primary glomus cells (n = 3 CHF rabbits) incubated with Ad.EGFP (Fig. 3B). No EGFP was observed in the glomus cells without exposure to Ad.EGFP from these same rabbits (Fig. 3D). Ad.EGFP did not affect the expression of nNOS in the CB glomus cells from sham or CHF rabbits (data not shown).

Effect of Ad.nNOS on K⁺ currents in sham and CHF CB glomus cells

Outward K⁺ currents were recorded in the perforated whole cell patch-clamp mode. K⁺ currents were significantly blunted

![FIG. 1. Expression of neuronal nitric oxide synthase (nNOS) mRNA in carotid body (CB) glomus cells from sham and chronic heart failure (CHF) rabbits, measured by single cell real-time RT-PCR. Data are mean ± SE, n = 20 primary isolated glomus cells in each group. *P < 0.05 vs. sham.](http://jn.physiology.org/). by 10.20.33.2 on October 25, 2016
in the CB glomus cells from CHF rabbits compared with those from sham rabbits, which is consistent with our previous study (Li et al. 2004). Ad.nNOS infection increased K⁺ currents in the CHF CB glomus cells, but did not affect K⁺ currents in the sham CB glomus cells (Fig. 4). Ad.EGFP had no effect on K⁺ currents in the CB glomus cells from both sham and CHF rabbits (Fig. 4D). In addition, S-methyl-l-thiocitrulline (SMTC, 1 μM, a specific nNOS inhibitor) markedly reduced the K⁺ currents of glomus cells in sham, sham + Ad.nNOS, and CHF + Ad.nNOS groups but not the CHF group (Fig. 5).

Effects of iberiotoxin on K⁺ currents in sham and CHF CB glomus cells

Large-conductance Ca²⁺-activated K⁺ (BK) channel currents are present in rabbit CB glomus cells (Lopez-Lopez et al. 1993) and are regulated by NO (Li et al. 2004). Therefore we investigated whether Ad.nNOS could modulate the activation of BK channels. Iberiotoxin (100 nM, a selective BK channel blocker) decreased the K⁺ currents of CB glomus cells in sham, sham + Ad.nNOS, and CHF + Ad.nNOS groups but not the CHF group (Fig. 6). There was no significant difference in the expression of BK channel protein among the sham, CHF, and CHF + Ad.nNOS groups (Fig. 7). Because the BK channel is Ca²⁺ sensitive, we assessed whether Ca²⁺ currents may be suppressed in CHF CB glomus cells. Quite the opposite, we found voltage-sensitive Ca²⁺ currents were elevated in CB glomus cells from CHF rabbits as compared with that from the sham group (Fig. 8).

**DISCUSSION**

The findings reported here indicate that: 1) nNOS mRNA and protein are expressed in the rabbit CB glomus cells and CHF markedly decreases nNOS mRNA and protein in these cells; 2) Ad.nNOS transgene expression partially improves the attenuated K⁺ currents in CHF CB glomus cells; 3) a specific nNOS inhibitor (SMTC) and BK channel blocker (iberiotoxin) suppress K⁺ currents in the CB glomus cells and fully abolish the effect of Ad.nNOS on the K⁺ currents in CHF cells; and 4) BK currents in CHF CB glomus cells are attenuated despite enhanced Ca²⁺ channel activity. These results indicate that endogenous nNOS is involved in the regulation of glomus cell BK channels in rabbit CB glomus cells and, importantly, a reduced nNOS expression mediates the suppression of BK currents in glomus cells from CHF rabbits.

Although many studies have demonstrated that endogenous NO plays an important role in the CB chemoreceptor function as an inhibitory modulator (Chugh et al. 1994; Iturriaga et al. 2000; Li et al. 2004, 2005; Prabhakar et al. 1993; Sun et al.
1999b; Wang et al. 1994), the source(s) of the NO and the localization of NOS in the CB are still unclear. In the cat CB, NOS immunoreactivity and disphorase activities have been found in endothelial cells, carotid sinus nerve but not in glomus cells, type II cells, and vascular smooth muscle cells (Grimes et al. 1995; Wang et al. 1993, 1994). Immunohistochemical evidence has shown that only carotid sinus nerves but not glomus cells are nNOS-immunoreactive in the rats (Dvorakova and Kummer 2005). On the other hand, guinea pig CB glomus cells do exhibit nNOS-immunoreactivity (Dvorakova and Kummer 2005). These results suggest that there are species differences in the nNOS localization in the CB.

In our previous study, the NOS inhibitor (L-NNA) reduced the outward K^+ currents in isolated primary CB glomus cells (Li et al. 2004). Based on the above-cited evidence, we proposed two possible explanations for this phenomenon: first, NOS might be present in glomus cells at low levels that are
difficult to detect, but sufficient to influence K\(^+\) channel activation; and second, even though glomus cells are dispersed in cell culture, other neural and endothelial cells of the CB coexisted with glomus cells might produce sufficient NO to exert a paracrine effect on the neighboring glomus cells (Li et al. 2004). In the present study, we demonstrate, using single-cell real-time RT-PCR and immunofluorescent staining, that nNOS mRNA and protein are expressed in the rabbit CB glomus cells (Figs. 1 and 2). Further, by using gene transfer of nNOS to the glomus cells, we demonstrate that NOS functions within rabbit CB glomus cells to modulate BK channel activation.

Our previous studies have documented the suppression of the outward K\(^+\) currents in the CB glomus cells from CHF rabbits (Li et al. 2004). The present study indicates that nNOS mRNA and protein are substantially lower in the CB glomus cells from CHF rabbits compared with that from sham rabbits. Transgene expression of nNOS via Ad.nNOS infection increased the expression of nNOS protein and K\(^+\) currents in the CB glomus cells from CHF rabbits. A specific nNOS inhibitor (SMTG) totally abolished the effect of Ad.nNOS on the K\(^+\) current in the CB glomus cells from CHF rabbits and also reduced the K\(^+\) currents in the CB glomus cells from sham rabbits, regardless of gene transfer of Ad.nNOS. Our previous study has demonstrated that Ad.nNOS gene transfer to the CB reverses the enhanced CB chemoreceptor activity and peripheral chemoreflex sensitivity in CHF rabbits (Li et al. 2005). These data suggest that the nNOS down-regulation in CB glomus cells reduces the outward K\(^+\) currents and contributes to the enhanced chemoreceptor activity in CHF rabbits.

K\(^+\) channels are functionally classified into voltage-gated K\(^+\) (K\(_v\)) channels, BK channels, ATP-sensitive K\(^+\) channels, inward rectifier K\(^+\) channels, and voltage-insensitive background K\(^+\) channels (Jan and Jan 1997). Of these K\(^+\) channels, K\(_v\) channels, BK channels, and HERG-like K\(^+\) channels are expressed in the rabbit CB glomus cells (Lopez-Lopez et al. 1993; Overholt et al. 2000). Our previous study has shown that the attenuated K\(^+\) currents in these glomus cells are mainly due to the suppressed current density of BK channels and the decreased availability of NO is responsible for the suppression of BK currents in the CB glomus cells from CHF rabbits (Li et al. 2004). In the present study, we confirm that NO modulates the activation of BK channels since the selective BK inhibitor (iberiotoxin) fully abolished the effect of Ad.nNOS on the K\(^+\) currents in the CB glomus cells from CHF rabbits (Fig. 6). Furthermore, this suppression of K\(^+\) current density in CHF glomus cells and the reversal by Ad.nNOS are not brought about via an influence of NO on the expression of the BK channel \(\alpha\)-subunit (Fig. 7). The possibility remains, however, that NO modulates BK channel activity in CB glomus cells via influencing the functionality or expression of BK channel \(\beta\)-subunits. Wu et al. (2002) found that the excitatory effect of NO on the BK channels is likely mediated by the BK channel \(\beta\)-subunit.

In the present study, even though nNOS gene transfer to CHF glomus cells increased nNOS expression to normal levels (Fig. 2), K\(^+\) currents were not completely normalized (Fig. 4). These data, although paradoxical, are consistent with our previous study (Jan and Jan 1997) in which CB chemoreceptor activity and chemoreflex function were not completely normalized in CHF rabbits after nNOS gene transfer to the CB. The results would suggest either that NO signaling downstream from nNOS also is altered in these cells in the CHF state and/or that changes occur in the expression of BK \(\beta\)-subunits or functionality of the BK channel. Alternatively, it is possible...
that other endogenous factors, such as angiotensin II as implicated in our other study (Li and Schultz 2006), also modulate the activation of the BK channels. These issues warrant further study.

It is worthy of consideration whether NO raises cytosolic Ca\(^{2+}\) concentration since the BK channel is Ca\(^{2+}\) sensitive. However, this viewpoint is not supported by many studies. First, NO inhibits L-type Ca\(^{2+}\) channels via a cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent protein kinase in cell-attached patches of bovine chromaffin cells (Carabelli et al. 2002) or cGMP-independent mechanism in whole cell recording of rabbit CB glomus cells (Summers et al. 1999).

Second, NO decreases the cytosolic free Ca\(^{2+}\) concentration via increasing Ca\(^{2+}\) stores in sarcoplasmic reticulum and inhibiting store-operated Ca\(^{2+}\) influx in the smooth muscle cells from rabbit aorta (Cohen et al. 1999). Furthermore, NOS inhibition activates L- and T-type Ca\(^{2+}\) channels in renal afferent and efferent arteries (Feng and Navar 2006). Importantly, our data show that Ca\(^{2+}\) currents are enhanced in the CB glomus cells from CHF rabbits despite impaired NOS expression and K\(^{+}\) channel activity (Fig. 8). Therefore it is not likely that the modulatory effect of NO on BK channel activity by the CB glomus cell is secondary to changes in intracellular Ca\(^{2+}\).

In conclusion, our results indicate that the nNOS expressed in the rabbit CB glomus cells and the reduced endogenous nNOS expression decrease the activation of BK channels, but not involving altered expression of BK-\(\alpha\) in the CHF condition.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

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