Elevated mitochondrial superoxide contributes to enhanced chemoreflex in heart failure rabbits

Yanfeng Ding,1 Yu-Long Li,2 Matthew C. Zimmerman,1 and Harold D. Schultz1

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

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Ding Y, Li Y, Zimmerman MC, Schultz HD. Elevated mitochondrial superoxide contributes to enhanced chemoreflex in heart failure rabbits. Am J Physiol Regul Integr Comp Physiol 298: R303–R311, 2010. First published November 18, 2009; doi:10.1152/ajpregu.00629.2009.—Peripheral chemoreflex sensitivity is enhanced in both clinical and experimental chronic heart failure (CHF). Here we investigated the role of manganese superoxide dismutase (MnSOD), the SOD isoform specially targeted to mitochondria, and mitochondrial superoxide levels in the enhanced chemoreceptor activity and function of the carotid body (CB) in CHF rabbits. CHF suppressed MnSOD protein expression and elevated mitochondrial superoxide levels in CB compared with that in sham CB. Adenovirus (Ad) MnSOD (1 × 108 plaque-forming units/ml) gene transfer selectively to the CBs normalized mitochondrial superoxide levels in glomus cells from CHF CB. In addition, Ad MnSOD reduced the elevation of superoxide level in CB tissue from CHF rabbits. Ad MnSOD significantly increased MnSOD expression in CHF CBs and normalized the baseline renal sympathetic nerve activity and the response of renal sympathetic nerve activity to hypoxia in CHF rabbits. Ad MnSOD decreased baseline single-fiber discharge from CB chemoreceptors compared with Ad Empty (6.3 ± 1.5 vs. 12.7 ± 1.4 imp/s at ~100-Torr Po2, P < 0.05) and in response to hypoxia (20.5 ± 1.8 vs. 32.6 ± 1.4 imp/s at ~40-Torr Po2, P < 0.05) in CHF rabbits. Compared with Ad Empty, Ad MnSOD reversed the blunted K+ currents in glomus cells from CHF rabbits (385 ± 11 vs. 551 ± 20 pA/pF at +70 mV, P < 0.05). The results suggest that decreased MnSOD in the CB and elevated mitochondrial superoxide levels contribute to the enhanced CB chemoreceptor activity and peripheral chemoreflex function in CHF rabbits.

Mitochondria produce significant amounts of reactive oxygen species (ROS) that may contribute to intracellular oxidative stress or activation of intracellular signaling pathways (1, 33, 37). Importantly, a substantial body of evidence has recognized that the mitochondria may act as a key O2 sensor in CB, pulmonary artery, and chromaffin cells (1, 3, 21, 33, 36, 38–41). Thus mitochondrial superoxide levels may play an important role in superoxide-mediated CB chemoreceptor hypersensitivity in CHF.

In the present study, we investigated the role of mitochondrial superoxide levels and the effect of adenoviral (Ad) MnSOD gene transfer to the CB on CB chemoreceptor hypersensitivity in CHF rabbits.

MATERIALS AND METHODS

Experimental animals and induction of CHF. Male New Zealand White rabbits, weighing 2.5–3.5 kg, were randomly assigned to sham and CHF groups. Rabbits were housed in individual cages under controlled temperature and humidity and a 12:12-h dark-light cycle and fed standard rabbit chow with water available ad libitum. The experimental protocols were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee, and the investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). Rabbits were anesthetized with a cocktail consisting of 1.2 mg acepromazine, 5.9 mg xylazine, and 58.8 mg ketamine, given as an intramuscular injection. Using sterile technique, a left thoracotomy was performed. The pericardium was opened, and wire loop electrodes were attached to the left ventricle for pacing. All leads exited the chest between the third and fourth ribs. The chest was closed in layers and evacuated. Rabbits were placed on an antibiotic regimen consisting of 5 mg/kg im Baytril for 5 days. After the rabbits recovered from the thoracotomy (−2 wk), the CHF was induced by pacing, as previously described (6, 7, 16, 17, 19, 20, 35). The progression of CHF was monitored by weekly echocardiograms (Acuson Sequoia 512C with a 4-MHz probe), with the pacemaker turned off for at least 30 min before the recordings were started. Sham-operated animals underwent a similar period of echocardiographic measurements. CHF was characterized by a >40% reduction in ejection fraction and fraction of shortening.

Gene transfer to the CB. Gene transfer to CB was performed as described in our laboratory’s studies (7, 16), using a replication incompetent Ad vector encoding human MnSOD cDNA (43–45).
Briefly, the left and right sinus region was temporarily vascularly isolated (including the common carotid artery, internal carotid artery, and external carotid artery), and the tip of a PE-10 catheter was positioned at the level of the CB via the external maxillary artery using sterile surgical technique. Once isolated, 200 μl of Ad Empty (as control Ad) or Ad MnSOD [1 × 10^8 plaque-forming units/ml, dissolved in 0.9% sodium chloride] was slowly injected via the catheter into the CB bilaterally in animals used for chemoreflex analysis. For CB analysis (CB afferent nerve recording, patch clamp of glomus cells, CB molecular measurements), Ad MnSOD was applied to one CB and Ad Empty to the contralateral CB in the same rabbit by the same technique. The catheter and snares around the vessels were removed, incision closed, and animal allowed to recover. Experiments were performed 3–4 days later, concurrent with optimal transgene expression.

**Immunofluorescence detection and Western blot analysis of MnSOD in the CB**. We performed immunofluorescence detection and Western blot analysis, as described in our laboratory’s previous studies (6, 7). For immunofluorescence staining, CB sections, 10 μm-thick, were mounted on precoated glass slides for MnSOD, cytochrome oxidase IV (COX IV), and tyrosine hydroxylase (TH) detection. CB sections were blocked with 10% normal donkey serum for 1 h and incubated with primary anti-MnSOD (Santa Cruz Biotechnology, Santa Cruz, CA), anti-COX IV (Abcam, Cambridge, MA), and anti-TH antibodies (Sigma-Aldrich, St. Louis, MO) overnight at 4°C, followed by incubation with appropriate secondary antibody (Molecular Probe in Invitrogen, Carlsbad, CA) for 1 h at room temperature. Slides were observed under a Leica fluorescent microscope with appropriate excitation/emission filters, and pictures were captured by a digital camera system. No staining was observed with the procedure described above using PBS instead of the primary antibody.

For protein measurement by Western analysis, CBs were rapidly removed and immediately frozen in dry ice and stored at −80°C until analyzed. The concentration of total protein extracted from CBs was measured using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Protein (10 μg) was fractionated in a 12% polyacrylamide gel, along with molecular weight standards, and transferred onto the polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was probed with primary antibody (1:1,000 dilutions of anti-MnSOD) and secondary antibody (1:5,000 dilutions) IgG-horseradish peroxidase (Pierce Chemical), respectively, and then treated with enhanced chemiluminescence substrate (Pierce Chemical). The bands in the membrane were visualized and analyzed using UVP Bio Imaging Systems. Protein loading was controlled by probing all Western blots with rabbit anti-COX IV antibody (1:1,000 dilution, Abcam), and MnSOD protein intensity was normalized to COX IV intensity.

**Isolation of CB glomus cells and detection of mitochondrial superoxide level**. CB glomus cells were isolated by a two-step enzymatic digestion protocol, cultured at 37°C in a humidified atmosphere of 95% air-5% CO2, and studied within 24 h of dissociation (7, 17, 19). For detection of mitochondrial superoxide levels in live cells (23, 29, 44), glomus cells were loaded with the fluorogenic probe MitoSOX Red (2 μmol/l, Molecular Probes) for 15 min. Cell were also incubated with MitoTracker Green (50 nmol/l, Molecular Probes) to confirm the localization of MitoSOX Red to mitochondria. After being washed with Hanks’ balanced salt solution to remove MitoSOX Red and MitoTracker Green, the cells were suspended in the Hanks’ solution. We used a Zeiss confocal LSM 510 Meta laser-scanning microscope with excitation at 488- and 405-nm wavelength for MitoTracker Green and MitoSOX Red, respectively, to capture fluorescent images. Notably, as previously characterized and described by Robinson and colleagues (29, 30), using the 405-nm excitation wavelength for MitoSOX Red allowed us to detect the superoxide-specific 2-hydroxyethidium product of oxidized MitoSOX Red. MitoTracker Green and MitoSOX Red fluorescence per cell was quantified using Image J analysis software.

**Chemoreflex evaluation with renal sympathetic nerve activity.** Renal sympathetic nerve activity (RSNA) recording electrodes were implanted, and changes in RSNA in response to stimulation of peripheral chemoreceptors were recorded, in sham and CHF rabbits in the conscious state as in our laboratory’s previous studies (6, 7, 15, 16, 20, 35). At the time of the renal nerve electrode surgery, arterial/venous catheters were inserted into the jugular vein and femoral artery to record arterial blood pressure (BP) and heart rate (HR). The experiments were carried out 3–4 days after surgery. RSNA was expressed as percent maximum, and maximal RSNA was determined in each rabbit by an intravenous bolus injection of sodium nitroprusside (100 μg/kg). Changes in RSNA, BP, and HR in response to stimulation of peripheral chemoreceptors were measured in sham and CHF rabbits in the conscious resting state. Peripheral chemoreceptors were stimulated preferentially by allowing the rabbits to breathe graded mixtures of hypoxic gas under isocapnic conditions. Different concentrations of O2 with balance of N2 were delivered into the chamber in the following sequence: 21% O2 (normoxia), 10% O2 (mild hypoxia), and 10% O2 (severe hypoxia). Each stimulation was held until a steady response was achieved for 3 min. Then the RSNA, BP, and HR were measured, and an arterial blood sample was taken from the arterial catheter for the measurement of arterial PO2, arterial PCO2, and pH. Because hypoxic stimulation of ventilation induces hyperventilatory hypocapnia, 2–4% CO2 were added to the hypoxic gases to maintain relatively constant arterial PCO2 during hyperventilation. While the rabbits were breathing control air (21% O2), sufficient recovery time was allowed between stimuli to ensure that all variables returned to baseline levels.

**Recording of afferent discharge of CB chemoreceptor.** Single-unit action potentials were recorded from CB chemoreceptor fibers in the carotid sinus nerve (CSN) in anesthetized rabbits, as in previous studies (7, 15, 16, 20, 34). Briefly, the left or right carotid sinus region was vascularly isolated and perfused with Krebs-Henseleit solution (in mM: 120 NaCl, 4.8 KCl, 2.0 CaCl2, 2.5 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 5.5 glucose; 10 ml/min, temperature 37°C). Perfusion was bubbled with O2/CO2/N2 gas mixture to maintain PO2 at 100–110 Torr, PCO2 at 30–35 Torr, and pH 7.4 as the normoxic condition. Flow through the isolated sinus was set at 10 ml/min at a perfusion pressure of 80 Torr and PO2 of the perfusate was altered by bubbling with gas mixtures containing the different O2 concentrations, a constant fraction of CO2, and a balance of N2 to achieve a PO2 of 55–65 and 35–45 Torr, respectively. All chemicals were obtained from Sigma-Aldrich Chemical, St. Louis, MO.

The CSN was exposed and transected near the petrosal ganglion to interrupt neural efferents to the CB. The CSN was covered with mineral oil, and fine slips of nerve filaments were placed on a silver electrode. Impulses were amplified with a bandwidth of 100 Hz-3 kHz (Grass P511, Grass Instrument, Quincy, MA), displayed on an oscilloscope (2120 Oscilloscope, BK Precision), and fed into a rate meter (FHC, Brunswick, ME), whose window discriminators were set to accept potentials of the particular amplitude. Bundles that had one, or at most two, easily distinguishable active fibers were used. Chemoreceptor afferents were identified by their sparse and irregular discharge at normoxia and by their response to hypoxia and NaCN.

**Recording of outward K+ currents in the CB glomus cells.** The procedures for isolation, identification of glomus cells, and recording of its K+ currents were as described previously (7, 17, 19). CB glomus cells were isolated by a two-step enzymatic digestion protocol and cultured at 37°C in a humidified atmosphere of 95% air-5% CO2, and studied within 24 h of dissociation. At the beginning of each experiment, cells were superfused with the normal extracellular solution containing the following composition (mM) 140 NaCl, 5.4 KCl, 2.5 CaCl2, 0.5 MgCl2, 5.5 HEPES, 11 glucose, 10 sucrose, pH 7.4, to identify glomus cells, which exhibited Na+ current. Once the presence of Na+ current was confirmed, the extracellular solution was changed...
RESULTS

Characteristics of the CHF state. After 3–4 wk of rapid ventricular pacing, CHF was induced and characterized by an enlarged heart and impaired contractile function as evaluated by the changes of heart weight, left ventricle chamber diameter, and the shortening and ejection fractions (Table 1), which is consistent with our laboratory’s previous studies (6, 7, 15, 16, 20). There was no difference in these parameters among CHF rabbits with/without Ad Empty or Ad MnSOD.

MnSOD protein expression in CB from sham and CHF rabbits. In CB tissue from sham rabbits, triple immunofluorescence staining showed MnSOD colocalized with TH (a marker of glomus cell cluster) and COX IV (a marker of the inner mitochondrial membrane)-positive cells, indicating MnSOD expression in the mitochondria of the glomus cell clusters (Fig. 1). Moreover, TH and MnSOD double immunofluorescence analysis showed that the level of immunofluorescence intensity for MnSOD was qualitatively lower in the CB from CHF rabbits than that from sham rabbits (Fig. 2A). Western blot analysis confirmed a decrease in MnSOD protein, but not COX IV, from CHF CBs compared with that from sham CBs (P < 0.05, Fig. 2, B and C).

Mitochondrial superoxide levels in glomus cells from sham and CHF rabbits. Due to the decreased MnSOD protein expression in the CBs from CHF rabbits, we hypothesized that mitochondrial-produced superoxide levels are elevated. To test this hypothesis, mitochondrial superoxide levels were measured using MitoSOX Red fluorescence in live glomus cells. We observed an increase of MitoSOX Red fluorescence in CB glomus cells from CHF rabbits compared with that from the sham group (P < 0.05, Fig. 3), thus indicating an increase in mitochondrial superoxide levels. Importantly, as shown in the representative confocal microscopy images (Fig. 3A), MitoSOX Red fluorescence colocalized with that of MitoTracker green (yellow fluorescence in merged image), thus confirming an increase in mitochondrial-generated superoxide levels in CB glomus cells from CHF rabbits. However, the intensity of MitoTracker green fluorescence was not different in glomus cells between sham and CHF rabbits (data not show), indicating that the number of MitoTracker green-labeled mitochondria in CB glomus cells was not affected by CHF.

MnSOD protein expression in CHF CB after Ad MnSOD gene transfer to CB. Gene transfer of Ad MnSOD to the CB of CHF rabbits markedly increased the level of immunolabeled MnSOD compared with that in the contralateral Ad Empty infected CB from CHF rabbits (Fig. 4A). Ad MnSOD also increased MnSOD protein expression blots in CHF CBs compared with CHF CBs infected by Ad Empty (P < 0.05, Fig. 4, B and C). Ad Empty did not affect MnSOD levels by immu-

Table 1. Body weight, ventricular weight, and cardiac function in sham and CHF rabbits

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Vehicle (both CBs)</th>
<th>Ad Empty (both CBs)</th>
<th>Ad MnSOD (both CBs)</th>
<th>Ad Empty/Ad MnSOD (contralateral CBs)</th>
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<td>21</td>
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<td>14</td>
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<tr>
<td>BW, kg</td>
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<td>3.8±0.2</td>
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<td>17.2±0.5*</td>
<td>17.6±0.7*</td>
<td>18.0±0.8*</td>
</tr>
<tr>
<td>LVESD, mm</td>
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<td>FS, %</td>
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<td>20.4±1.8*</td>
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<td>21.2±1.9*</td>
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<tr>
<td>EF, %</td>
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<td>44.2±0.9*</td>
<td>44.5±0.8*</td>
<td>43.8±1.0*</td>
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</tr>
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Values are means ± SE; n, no. of animals. CHF, chronic heart failure; CBs, carotid bodies; Ad, adenovirus; MnSOD, manganese superoxide dismutase; BW, body weight; LVW, left ventricular weight; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; EF, ejection fraction. *P < 0.05 vs. sham.
no fluorescent and Western detection in the CBs of CHF rabbits [Fig. 2 (CHF) vs. Fig. 4 (CHF + Ad Empty)]. We did not find enhanced MnSOD expression in the other tissues (heart, kidney, or brain) after local Ad MnSOD gene transfer to CB region in our experiments (data not show), which confirmed that gene transfer was successfully localized to CB tissue and is consistent with our laboratory’s previous studies by the same technique (7, 16).

Effect of MnSOD gene transfer on mitochondrial superoxide levels in glomus cell of CHF rabbits. To determine whether MnSOD gene transfer affected mitochondrial superoxide levels, confocal microscopy was used to capture MitoSOX Red fluorescence in glomus cells of CHF CBs infected with Ad Empty or Ad MnSOD. The representative images from confocal microscopy (Fig. 5A) and summary (Fig. 5B) data illustrate Ad MnSOD gene transfer to CB in CHF rabbits decreased MitoSOX Red fluorescence in glomus cells compared with that of Ad Empty infection ($P < 0.05$).

Effect of MnSOD gene transfer to CB on RSNA and hemodynamic parameters in CHF rabbits. CHF increased baseline RSNA (normoxia) and enhanced RSNA in response to hypoxia compared with that in sham rabbits (Fig. 6B), which is consistent with that in our laboratory’s previous studies (7). Bilateral CB infection by Ad MnSOD in CHF rabbits markedly reduced the baseline RSNA and the response of RSNA to hypoxia ($P < 0.05$, Fig. 6). Bilateral CB infection with Ad Empty did not alter the enhanced RSNA and ventilation at normoxic and hypoxic states in CHF rabbits (Fig. 6). Mean arterial BP was lower and HR was higher in CHF compared with sham rabbits, as typically observed in this CHF model (16, 35), and neither hemodynamic variable was influenced by Ad Empty or Ad MnSOD transfection in CHF animals (data not shown).

Effect of MnSOD gene transfer on CB chemoreceptor activity in CHF rabbits. The basal discharge of CB chemoreceptors during normoxia and the response to isocapnic hypoxia were elevated in CHF rabbits compared with that in sham rabbits (Fig. 7B), which is consistent with that in our laboratory’s previous studies (7). Ad MnSOD gene transfer to CB in CHF rabbits significantly blunted CB chemoreceptor activity during normoxia and hypoxia compared with that from the Ad Empty infection of contralateral CB ($P < 0.05$, Fig. 7) in the same CHF animals, and the activity reverted to that seen in sham rabbits. However, Ad Empty infection showed no effect on CB chemoreceptor activity (Fig. 7).
Effect of MnSOD gene transfer on $I_K$ of glomus cell in CHF rabbit. Figure 8A illustrates typical $I_K$ recordings and current-voltage curves obtained from glomus cells under conventional whole-cell patch technique. $I_K$ was attenuated in CB glomus cells from CHF rabbits compared with that in sham rabbits (Fig. 8), which confirms our laboratory’s previous finding (7). Ad MnSOD gene transfer to the CB in CHF rabbits significantly increased glomus cell $I_K$ compared with that in sham glucus cell. Wyatt and Buckler (41) have demonstrated that there is a close link between oxygen sensing and mitochondria, since three inhibitors of mitochondrial electron transport (rotenone, myxothiazol, and NaCN) all mimic the effects of hypoxia on intracellular calcium and background K$^+$ currents in neonatal rat glomus cell. Mitochondria are also a major cellular source of ROS involved in intracellular signaling (33, 37). Importantly, electron transport inhibitors shown to inhibit K$^+$ currents in rat glomus cells (41) are also known to enhance mitochondrial superoxide production (24). By utilizing the mitochondrial targeted superoxide-fluorogenic probe, MitoSOX Red (23, 29, 44), our present results clearly show that mitochondrial superoxide levels are increased in CB glomus cells in CHF rabbits compared with that in sham rabbits (Fig. 2). These data suggest the elevated mitochondrial superoxide, as well as the increased NADPH oxidase-derived superoxide, contributes to the enhanced CB chemoreceptor function in CHF.

**DISCUSSION**

The present study demonstrates that downregulation of MnSOD in the CBs contributes to enhanced CB chemoreceptor activity and reflex function in CHF rabbits. Restoring MnSOD expression by Ad MnSOD gene transfer to the CB reverses the chemoreflex hypersensitivity, the chemoreceptor hyperactivity, and the attenuated $I_K$ of glomus cells in CHF rabbits. Furthermore, Ad MnSOD gene transfer to CB effectively reduces the elevated mitochondrial superoxide levels in glomus cells in the CHF state. These results indicate that suppression of mitochondrial MnSOD and the elevated mitochondrial superoxide in CB glomus cell contribute to the enhanced CB chemoreceptor and reflex function in CHF.

ROS are metabolites of oxygen that play an important role in physiological and pathophysiological conditions, and superoxide is regarded as a particularly important radical, serving as the progenitor for other ROS in biological systems (13). Our previous studies have shown that NADPH oxidase-derived superoxide anion signaling pathway is involved in the enhanced CB chemoreceptor sensitivity to hypoxia in CHF rabbits. However, several lines of evidence suggest mitochondria play an important role in O$_2$ sensing, especially in CB glomus cell. Wyatt and Buckler (41) have demonstrated that there is a close link between oxygen sensing and mitochondria, since three inhibitors of mitochondrial electron transport (rotenone, myxothiazol, and NaCN) all mimic the effects of hypoxia on intracellular calcium and background K$^+$ currents in neonatal rat glomus cell. Mitochondria are also a major cellular source of ROS involved in intracellular signaling (33, 37). Importantly, electron transport inhibitors shown to inhibit K$^+$ currents in rat glomus cells (41) are also known to enhance mitochondrial superoxide production (24). By utilizing the mitochondrial targeted superoxide-fluorogenic probe, MitoSOX Red (23, 29, 44), our present results clearly show that mitochondrial superoxide levels are increased in CB glomus cells in CHF rabbits compared with that in sham rabbits (Fig. 2). These data suggest the elevated mitochondrial superoxide, as well as the increased NADPH oxidase-derived superoxide, contributes to the enhanced CB chemoreceptor function in CHF.
The present study addressed the role of the MnSOD isoform, which is specifically targeted to and expressed in mitochondria, on the CHF-enhanced CB chemoafferent hyperactivity in CHF rabbits by Ad-mediated MnSOD gene transfer technique. This technique has been used successfully to enhance gene expression selectively to the CBs in our laboratory’s prior studies (7, 16). We found the vector restored the expression of MnSOD in the CB and reversed the enhanced CB chemoreflex sensitivity and chemoafferent activity observed in CHF rabbits. A suppression of \( I_K \) in glomus cells contributes to a heightened excitability of these cells to drive elevated CB afferent activity and chemoreflex hypersensitivity in CHF (7, 12, 15, 17, 19, 28). Ad MnSOD localized to CB reduced the enhanced mitochondrial superoxide and the enhanced \( I_K \) activity of CB glomus cells induced by CHF.

Our laboratory’s previous studies have suggested that NADPH oxidase-derived superoxide plays an important role in both CHF- and ANG II-induced depression of \( I_K \) in CB glomus cells, as evidenced by the ability of NADPH oxidase inhibitors, superoxide scavenger tempol and Ad CuZnSOD, to markedly enhance \( I_K \) of glomus cells by reducing superoxide levels (7, 17, 19). Our laboratory also demonstrated that suppressed expression of CuZnSOD is involved in the elevated superoxide in CB tissue and contributes to the CB chemoafferent hyperactivity in CHF rabbits (7). The effects of MnSOD overexpression on CB chemoreceptor function in the CHF state shown here are similar to that of CuZnSOD overexpression (7). Similarly, in differentiated CATH.a neurons, gene transfer using either Ad CuZnSOD or Ad MnSOD significantly blunted the ANG II-inhibited \( I_K \) effect (42). Collectively, the evidence suggests that intracellular superoxide within both cytoplasmic

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**Fig. 6.** Effect of normoxic and hypoxic states on renal sympathetic nerve activity (RSNA) in sham, CHF, and CHF rabbits treated with either Ad MnSOD or Ad Empty bilaterally to CBs. **A:** representative recording of RSNA in CHF rabbits treated with either bilateral Ad MnSOD or Ad Empty to CBs. \( \text{PaO}_2 \), arterial \( \text{O}_2 \). **B:** relationship curves of RSNA with the different oxygen levels. Values are means ± SE. *P < 0.05 vs. sham group. #P < 0.05 vs. CHF + Ad MnSOD.

The present study addressed the role of the MnSOD isoform, which is specifically targeted to and expressed in mitochondria, on the CHF-enhanced CB chemoafferent hyperactivity in CHF rabbits by Ad-mediated MnSOD gene transfer technique. This technique has been used successfully to enhance gene expression selectively to the CBs in our laboratory’s prior studies (7, 16). We found the vector restored the expression of MnSOD in the CB and reversed the enhanced CB chemoreflex sensitivity and chemoafferent activity observed in CHF rabbits. A suppression of \( I_K \) in glomus cells contributes to a heightened excitability of these cells to drive elevated CB afferent activity and chemoreflex hypersensitivity in CHF (7, 12, 15, 17, 19, 28). Ad MnSOD localized to CB reduced the enhanced mitochondrial superoxide and the enhanced \( I_K \) activity of CB glomus cells induced by CHF.

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**Fig. 7.** Effect of normoxic and hypoxic states on CB chemoreceptor afferent discharge from sham, CHF, and CHF rabbits treated with contralateral Ad MnSOD vs. Ad Empty to CBs in the same animal. **A:** representative recording of CB chemoreceptor afferent discharge in CHF rabbits treated with Ad MnSOD vs. Ad Empty to contralateral CBs. **B:** relationship curves of CB chemoreceptor discharge with the different oxygen levels. DF, discharge frequency; AP, action potential. Values are means ± SE. *P < 0.05 vs. sham. #P < 0.05 vs. CHF + Ad MnSOD.

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and mitochondrial compartments contributes to CHF and ANG II-induced inhibition of voltage-activated $I_K$ in CB type I cells and neurons, respectively.

Further studies are required to explore, in more detail, the intracellular signaling of superoxide and how it influences $I_K$ channels in CB glomus cells and other neurons. An important question that arises from our studies is how downregulation of the two spatially distinct superoxide scavengers (MnSOD vs. CuZnSOD) mediates similar effects on CB function in CHF. One hypothesis is the superoxide generated in the cytosolic compartment increases mitochondrial-produced superoxide or vice versa (2), thus indicating a ROS-induced ROS mechanism. Recently, Doughan et al. (8) have shown that ANG II induces mitochondrial ROS production in aortic endothelial cells via activation of NADPH oxidase. A second hypothesis is that CuZnSOD overexpression via Ad increases this SOD isoform in mitochondria. Indeed CuZnSOD is known to be present in mitochondria (25), and Zimmerman et al. (44) have shown that CuZnSOD levels are increased in neuronal mitochondria following Ad CuZnSOD infection. Thus it is possible that overexpression of CuZnSOD may act via scavenging mitochondrial-produced superoxide due to its presence in mitochondria.

These changes in SOD function that occur in CHF are not unique to the CB. Gao at al. (11) reported that MnSOD and CuZnSOD expression is reduced in the rostral ventrolateral medulla of CHF rabbits. The mechanisms responsible for downregulation of SOD in the CB and rostral ventrolateral medulla in CHF are not known. Because both of these regions experience an enhancement of neuronal activity in CHF, the question arises as to whether the altered neuronal activity contributes to suppressed SOD expression. However, other changes also occur uniformly across these two autonomic sites in CHF that may play a role. These include enhanced ANG II-NADPH oxidase-mediated superoxide production (17, 20, 31, 46, 47) and downregulation of neuronal nitric oxide synthase with decreased bioavailable nitric oxide (16, 19, 31, 46), among other possible signaling processes. Presently, factors that influence both MnSOD and CuZnSOD transcriptional control are poorly understood. It is, however, an important question that deserves further analysis.

Although our present and previous studies have focused on the role of superoxide in enhanced CB chemoreceptor activity in CHF rabbits, additional studies are needed to determine the role of the other ROS, such as hydrogen peroxide, hydroxyl radical, lipid peroxy radical, and others. We should also consider its interaction with another relevant groups of molecules, such as the reactive nitrogen species, including nitric oxide, to produce radical intermediates that could also influence signaling pathways in the CB (13, 14).

**Perspectives and significance.** Profound activation of the sympathetic nervous system is characteristic of CHF and contributes to late-stage deterioration of cardiac function (9, 10, 22, 31, 48). The arterial chemoreflex is a contributory factor for the sympathetic hyperactivity in CHF patients and experimental animals (4–6, 20, 26, 27, 32, 35). The exacerbation of cardiac dysfunction should, in turn, amplify and perpetuate the peripheral chemoreceptor disturbance, thus resulting in a deleterious feed-forward feedback. Nevertheless, neither Ad CuZnSOD (7) nor MnSOD gene transfer to the CB improved cardiac function in our experiments over the course of several days. Additional studies using strategies over the long term are needed to address this issue.

In summary, CHF decreased MnSOD protein expression and increased superoxide levels in mitochondria of CB glomus cells. Ad MnSOD gene transfer to the CB in CHF rabbits normalized the enhanced CB chemoreflex, CB chemoafferent hypersensitivity, the suppressed $I_K$ of the glomus cell, and the
elevated superoxide levels in CB tissue and mitochondria of glomus cells. It is implicated that MnSOD deficiency of the CB and elevated mitochondrial superoxide levels in glomus cell contribute to CB dysfunction in CHF.

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DISCLOSURES
I am not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors’ academic institutions or employers.

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