Gene Transfer of Neuronal Nitric Oxide Synthase to Carotid Body Reverses Enhanced Chemoreceptor Function in Heart Failure Rabbits

Yu-Long Li, Yi-Fan Li, Dongmei Liu, Kurtis G. Cornish, Kaushik P. Patel, Irving H. Zucker, Keith M. Channon, Harold D. Schultz

Abstract—Our previous studies showed that decreased nitric oxide (NO) production enhanced carotid body (CB) chemoreceptor activity in chronic heart failure (CHF) rabbits. In the present study, we investigated the effects of neuronal NO synthase (nNOS) gene transfer on CB chemoreceptor activity in CHF rabbits. The nNOS protein expression and NO production were suppressed in CBs (P<0.05) of CHF rabbits, but were increased 3 days after application of an adenovirus expressing nNOS (Ad.nNOS) to the CB. As a control, nNOS and NO levels in CHF CBs were not affected by Ad.EGFP. Baseline single-fiber discharge during normoxia and the response to hypoxia were enhanced (P<0.05) from CB chemoreceptors in CHF versus sham rabbits. Ad.nNOS decreased the baseline discharge (4.5±0.3 versus 7.3±0.4 imp/s at 105±1.9 mm Hg) and the response to hypoxia (18.3±1.2 imp/s versus 35.6±1.1 at 40±2.1 mm Hg) from CB chemoreceptors in CHF rabbits (Ad.nNOS CB versus contralateral noninfected CB respectively, P<0.05). A specific nNOS inhibitor, S-Methyl-L-thiocitrulline (SMT), fully inhibited the effect of Ad.nNOS on the enhanced CB activity in CHF rabbits. In addition, nNOS gene transfer to the CBs also significantly blunted the baseline renal sympathetic nerve activity (RSNA) and the response of RSNA to hypoxia in CHF rabbits (P<0.05). These results indicate that decreased endogenous nNOS activity in the CB plays an important role in the enhanced activity of the CB chemoreceptors and peripheral chemoreflex function in CHF rabbits. (Circ Res. 2005;97:260-267.)

Key Words: nitric oxide • gene transfer • chemoreceptor • hypoxia • heart failure

The endogenous production of nitric oxide (NO) plays an important role in cardiovascular homeostasis through its action on the central and peripheral autonomic nervous systems.1 Although NO plays a significant excitatory role in the nucleus tractus solitarii (NTS),2–4 many studies have shown that NO produced within the carotid body (CB) is an inhibitory modulator of CB chemoreceptor activity.5–11 For example, the administration of the precursor L-arginine, NO donor molecules,5,6,9 or NO gas8 to the cat CB perfused in vitro reduces the chemoreceptor response to hypoxia. Conversely, inhibition of nitric oxide synthase (NOS) increases the frequency of CB chemoreceptor discharge in situ and in vitro.5,12,13

Profound activation of the sympathetic nervous system is characteristic of chronic heart failure (CHF).14–16 Peripheral chemoreceptor activation is an excitatory input that increases sympathetic outflow.17 Peripheral chemoreceptor sensitivity is enhanced in both clinical and experimental CHF18–20 and contributes to the tonic elevation in sympathetic function. Our recent studies have shown that a decreased NO production is involved in the enhanced CB chemoreceptor activity in CHF.13 We found that NOS inhibition enhanced the sensitivity of the CB chemoreceptors13 and decreased the CB glomus cell outward potassium currents (I_K)21 in sham rabbits but was without effect in CHF rabbits. Whereas, the NO donor (S-nitroso-N-acetyl-penicillamine [SNAP]) normalized the enhanced sensitivity of CB chemoreceptors and augmented glomus cell I_K in CHF rabbits.13,21

At least 3 isoforms of NOS have been isolated:22 neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). Histochmical studies in cat CB11 have demonstrated that intrinsic neurons innervating the intraglomic arterioles and glomus cells in addition to intraglomular vascular endothelial cells are positive for NOS (nNOS and eNOS). However, the contribution of nNOS and eNOS isoforms to the production of NO in the CB has not yet been adequately explored. Two studies concluded that a nonspecific NOS inhibitor, L-NAME, significantly enhanced the ventilatory response to NaCN in rat1

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and the CB chemoreceptor response to hypoxia in cats;\textsuperscript{23} whereas specific nNOS inhibitors were ineffective.\textsuperscript{3,23} Conversely, Kline et al.\textsuperscript{24,25} using mutant mice deficient in nNOS and eNOS isoforms, found that mice lacking nNOS showed greater ventilatory responses to hypoxia than wild-type controls; whereas responses to hypoxia were blunted in mutant mice lacking eNOS compared with the wild-type. Until now, even less is known about which isoform of NOS contributes to the enhanced peripheral chemoreceptor activity in CHF rabbits. Therefore, in the present study, we investigated the effect of Ad.nNOS gene transfer to the CB on the enhanced peripheral chemoreceptor activity in CHF rabbits.

### Materials and Methods

#### Pacemaker Implant and Production of CHF

All experiments were performed on male New Zealand White rabbits weighing 2.5 to 3.5 Kg. Experiments were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health and the American Physiological Society’s Guidelines 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 12:12-hour dark-light cycle and fed standard rabbit chow (Harlan Teklab) with water available ad libitum.

Rabbits underwent sterile thoracic instrumentation and were then paced to induce CHF, as previously described.\textsuperscript{19} Rabbits with >40\% reductions in $\Delta V_{\text{max}}$, and shortening fraction were considered in CHF (generally after 3 to 4 weeks). Sham-operated animals underwent a similar period of sonographic measurements with the pacemaker turned off. Any rabbit exhibiting abnormal arterial blood gases (PaO$_2$<85 mm Hg; 45 mm Hg < PaCO$_2$<30 mm Hg) was excluded from the study. See online supplement available at http://circres.ahajournals.org for details of instrumentation and cardiac function analysis.

#### Gene Transfer with Ad.EGFP or Ad.nNOS to the CB

The Ad.nNOS originally described by Channon et al.\textsuperscript{26} was used in these experiments. This Ad.nNOS, containing a rat nNOS cDNA, expresses functional nNOS protein when perfused in carotid arteries of rabbits.\textsuperscript{27} Three days before the experiment, using sterile surgical technique, the left and right carotid sinus regions were exposed via a small incision. The 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 carotid body via the external maxillary artery. After these arteries were occluded with snares, 200 $\mu$L of Ad.EGFP (as control adenoviruses) or Ad.nNOS (1×10$^7$ pfu/mL, dissolved in 0.9\% sodium chloride\textsuperscript{28}) was slowly injected into the carotid body via the catheter and the snares around the vessels were removed. A similar sham surgery, without adenoviral injection, was performed on the contralateral sinus region as a control in the same animal. In reflex experiments (see below), application of either Ad.EGFP or Ad.nNOS was performed on both right and left CBs in the same animal. The incision was closed, and the rabbits were placed on an antibiotic regimen consisting of 5 mg/kg Baytril i.m. daily. Ad.EGFP or Ad.nNOS showed no signs of damage (cell fragments) to the CB as observed from light microscopic evaluation of histological sections.

#### Examination of Infection Efficiency of the Adenoviruses and Immunofluorescence for nNOS Detection in the CB

Carotid bodies were obtained from sham (unpaced) and from CHF rabbits. Each rabbit was perfused transcardially with 500 mL heparinized saline followed by 1500 mL of freshly prepared 4\% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4).

For Ad.EGFP measurement (3 CHF rabbits), both CBs in each rabbit were rapidly removed. The CB was blocked in the coronal plane and sectioned at 30 $\mu$m thickness in a cryostat. The sections were mounted onto chrome-alum–coated slides. The slides were dried. EGFP was directly measured under a Leica microscope at 510 nm with single excitation peak and at 490 nm of green light\textsuperscript{29} to evaluate the infection efficiency of the adenovirus.\textsuperscript{30}

For nNOS immunofluorescence detection (5 sham and 5 CHF rabbits), both CBs in each rabbit were rapidly removed and postfixed in 4\% paraformaldehyde in 0.1 mol/L PBS for 12 hours at 4°C, followed by soaking the CBs in 30\% of sucrose for 12 hours at 4°C for cryostat protection. The CB was cut into 30 $\mu$m-thick sections. The CB sections were mounted onto precoated glass slides for immunofluorescence for nNOS detection (see online supplement for details).

#### Western Blot Analysis for nNOS in the CB

Carotid bodies from sham and CHF rabbits were rapidly removed and immediately frozen in dry ice and stored at −80°C until analyzed. The protein was extracted with the lysing buffer (10 mmol/L PBS, 1\% Nonidet P-40, 0.5\% sodium deoxycholate, 1\% SDS) plus protease inhibitor cocktail (Sigma, 100 $\mu$L/mL). After a centrifugation at 12 000g for 20 minutes at 4°C, the protein concentration in the supernatant was determined using a BCA protein assay kit (Pierce Chemical). The protein sample was used for Western blot analysis\textsuperscript{31} for nNOS (see online supplement for details).

#### NO Measurement in the CB

Homogenates were prepared from CB samples. Total protein concentration was determined using a BCA protein assay kit (Pierce Chemical). NO was measured using a gas-phase chemiluminescent method\textsuperscript{32} (NOA 280i, Sievers). See online supplement for details.

#### Recording of Afferent Discharge of CB Chemoreceptors

Single unit action potentials were recorded from CB chemoreceptor fibers in the carotid sinus nerve as we have described previously (8 sham and 16 CHF rabbits).\textsuperscript{33} Both sinus regions (adenoviral infected CB versus control CB) were vascularly isolated and perfused with Krebs-Henseleit solution (in mM: 120 NaCl, 4.8 KCl, 2.0 CaCl$_2$, 2.5 MgSO$_4$, 1.2 KH$_2$PO$_4$, 25 NaHCO$_3$, 0.1 l-arginine, and 5.5 glucose). Perfusate was bubbled with O$_2$/CO$_2$/N$_2$ gas mixture. CB nerve recordings were performed 3 days after exposure to adenovirus. See online supplement for details.

#### Peripherial Chemoreflex Control of Renal Sympathetic Nerve Activity and Ventilation

Renal sympathetic nerve recording electrodes were implanted as we have described previously.\textsuperscript{19} At that time, arterial/venous catheters were inserted into the right carotid artery and jugular vein, and either Ad.EGFP or Ad.nNOS was injected into both right and left CBs in CHF rabbits as described above. Experiments (6 sham and 18 CHF rabbits) were performed 3 days after surgical instrumentation/ adenoviral application.

Changes in renal sympathetic nerve activity (RSNA) and minute ventilation (VE) in response to stimulation of peripheral chemoreceptors were measured in sham and CHF rabbits in the conscious resting state as described in our previous study.\textsuperscript{19} Peripheral chemoreceptors were stimulated preferentially by allowing the rabbits to breathe graded mixtures of hypoxic gas under isocapnic conditions. See online supplement for details.

#### Statistical Analysis

All data are expressed as mean±SEM. Statistical significance was determined by a 2-way ANOVA, followed by a Bonferroni procedure for post-hoc analysis for multiple comparisons. Statistical significance was accepted when $P<0.05$. 

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Results

Induction of CHF
Rapid left ventricular pacing induced CHF by the third or fourth week of pacing. LV dD/dt max and LV shortening fraction were reduced after 3 or 4 weeks of pacing, compared with prepared baseline (P<0.05) (data available in online supplement). There was no significant change in the LV dD/dt max and LV shortening fraction from baseline during 4 weeks in sham rabbits.

Confirmation of Adenovirus Gene Transfer
The expression of EGFP was used to confirm the efficacy of adenovirus infection. EGFP was visible in the CB from CHF rabbits (n=3) infected with Ad.EGFP (Figure 1B). However, no EGFP was observed in the contralateral CB (without Ad.EGFP injection) from these same rabbits (Figure 1D). There was no expression of EGFP in the heart and brain of these animals (data not shown). These results confirm that our method for selective gene transfer to the CB is feasible.

Expression of nNOS and NO Production in the CBs from CHF Rabbits After the Transfer of Ad.nNOS
Using immunohistochemical analysis, we found that the expression of endogenous nNOS was localized in nerve fibers in the CB (Figure 2). In addition, the expression of nNOS was lower in the CB from CHF rabbits than that from sham rabbits (Figure 2). Three days after injection of Ad.nNOS (200 μL, 1×10⁸ pfu/mL) to the CB of CHF rabbits, the expression of nNOS in the CB (Figure 3B) was significantly increased, compared with that in the noninfected CB from the same animals (Figure 3A). However, Ad.EGFP did not affect the expression of nNOS in the CBs of CHF rabbits (Figure 3C and 3D).

We also used Western blot analysis to measure the protein expression of nNOS in each group. CHF markedly decreased the protein expression of endogenous nNOS in the CBs, compared with that in sham rabbits (Figure 4A and 4B). Ad.nNOS infection significantly enhanced the intensity of the bands of nNOS in the CBs from the CHF rabbits compared with that in the noninfected CBs from the CHF animals (Figure 4A and 4B). However, Ad.EGFP did not affect the protein expression of nNOS in the CBs of CHF rabbits (Figure 4A and 4B). The levels of nNOS protein expression in the CB measured by immunoblot (Figure 4A and 4B) are consistent with the degree of immunohistochemical staining of nNOS observed for each group (Figure 2 and 3).

The NO concentration in CBs from CHF rabbits was significantly less than that in sham rabbits (Figure 4C). Ad.nNOS gene transfer restored NO production in the CBs from CHF rabbits to normal levels. Ad.EGFP had no effect on NO production in the CBs of CHF rabbits (Figure 4C).

Effect of Ad.nNOS on CB Chemoreceptor Activity in CHF Rabbits
Previously, we showed that the baseline discharge of CB chemoreceptors during normoxia and the response to isocapnic hypoxia were enhanced in CHF rabbits compared with sham rabbits.20 In the present study, we observed similar results (Table). After Ad.nNOS infection of the CB of CHF rabbits, CB chemoreceptor activity during normoxia and hypoxia was significantly blunted (Table and Figure 5) as compared with that from the noninfected CB in the same animals. Ad.EGFP showed no effect on CB chemoreceptor activity (Table).
S-Methyl-l-thiocitrulline (SMTC, a specific nNOS inhibitor; Cayman Chemical Company) increased CB chemoreceptor activity during normoxia and hypoxia in CBs from sham rabbits and in CBs infected with Ad.nNOS from CHF rabbits (Figure 6). However, CB chemoreceptor activity during normoxia and hypoxia was not altered by SMTC in noninfected CBs from CHF rabbits or in CBs infected with Ad.EGFP.

Effect of Ad.nNOS on RSNA, VE, and MBP in CHF rabbits
We observed that RSNA at rest (normoxia) and the RSNA responses to hypoxia were elevated in CHF rabbits compared with that in sham rabbits (Figure 7A and 7B), which is consistent with that in our previous study. The ventilatory response to hypoxia was also enhanced in CHF rabbits (see online Table II). Ad.nNOS infection of both CBs in CHF rabbits markedly reduced resting RSNA and the RSNA and VE responses to hypoxia. However, these reflex responses to hypoxia were not reduced to the level seen in sham rabbits (Figure 7A, online Table II). Bilateral CB Ad.EGFP infection did not alter the enhanced RSNA at normoxic and hypoxic states in CHF rabbits (Figure 7A).

MBP was lower (Figure 7B) and HR higher (online supplement) in CHF compared with sham rabbits, and neither was influenced by Ad.nNOS or Ad.EGFP treatment.
Discussion

The present study showed that (1) the expression of nNOS and NO production was suppressed in the CB from CHF rabbits along with enhanced CB chemoreceptor activity; (2) Ad.nNOS gene transfer enhanced the expression of nNOS and NO production in the CB from CHF rabbits and reversed the enhanced CB chemoreceptor activity in CHF rabbits; (3) the specific nNOS inhibitor, SMTC, abolished the effect of Ad.nNOS on CB chemoreceptor activity; (4) localized Ad.nNOS gene transfer to the CBs lowered resting RSNA and reduced peripheral chemoreflex sensitivity in conscious CHF rabbits.

Adenovirus-mediated gene transfers have been reported previously.27,28,33 However, it can be difficult to transfect localized tissues and not to affect other tissues in in vivo experiments. Using Ad.EGFP to evaluate the adenovirus-mediated gene transfer, we found that gene transfer to the CB only induced the expression of Ad.EGFP in the CB region but not in the contralateral uninfected CB (Figure 1) and other tissues (heart and brain) from the same rabbits. The successful gene transfer to the CBs established the solid methodological foundation for investigating the role of nNOS expression in the CBs on peripheral chemoreflex function in CHF rabbits. The enhanced expression of nNOS in the CBs of CHF rabbits by gene transfer was confirmed by immunohistochemistry (Figure 3) and Western blot analysis (Figure 4). The dose and time period of the gene transfer technique we used in the present study was based on previous studies27,28 in which adenovirus-mediated gene expression was maximal without tissue injury when the dose of $2 \times 10^7$ pfu and the time course of 3 days were used.

The CBs are a pair of small arterial chemoreceptor organs, which sense blood PaO$_2$, Paco$_2$, and pH, and reflexly influ-

### Effects of Adenoviral Gene Transfer of nNOS and EGFP on CB Chemoreceptor Activity in CHF Rabbits

<table>
<thead>
<tr>
<th>P$_{O_2}$ (mm Hg)</th>
<th>Discharge Frequency (imp/s)</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
</tr>
<tr>
<td>105±1.9</td>
<td>2.2±0.6</td>
</tr>
<tr>
<td>63±2.4</td>
<td>7.5±1.3</td>
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<tr>
<td>40±2.1</td>
<td>17.3±1.0</td>
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Data are mean±SEM, n=8 in each group. *P<0.05 vs sham; #P<0.05 vs CHF.

Figure 4. nNOS protein and NO production in treated and untreated CBs. A, Representative gel of nNOS and β-tubulin proteins in sham (unpaced), CHF, CHF + Ad.nNOS, and CHF + Ad.EGFP-treated CBs. A positive nNOS protein control (brain paraventricular nucleus PVN) and negative (absence of primary antibody) control are shown on the same gel. B, Relative nNOS protein expression in sham, CHF, CHF + Ad.nNOS, and CHF + Ad.EGFP-treated CBs. n=6 in each group. C, NO concentration in sham, CHF, CHF + Ad.nNOS, and CHF + Ad.EGFP-treated CBs. n=4 in each group. Data are mean±SEM, *P<0.05 vs sham; #P<0.05 vs CHF rabbits.

Figure 5. Representative recordings of action potentials from CB chemoreceptors in a CHF rabbit. Left, Control (noninfected) CB. Right, Ad.nNOS-infected contralateral CB. DF indicates discharge frequency; AP, action potential.

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The present study showed that 1) the expression of nNOS and NO production was suppressed in the CB from CHF rabbits along with enhanced CB chemoreceptor activity; 2) Ad.nNOS gene transfer enhanced the expression of nNOS and NO production in the CB from CHF rabbits and reversed the enhanced CB chemoreceptor activity in CHF rabbits; 3) the specific nNOS inhibitor, SMTC, abolished the effect of Ad.nNOS on CB chemoreceptor activity; 4) localized Ad.nNOS gene transfer to the CBs lowered resting RSNA and reduced peripheral chemoreflex sensitivity in conscious CHF rabbits. Adenovirus-mediated gene transfers have been reported previously. However, it can be difficult to transfect localized tissues and not to affect other tissues in in vivo experiments. Using Ad.EGFP to evaluate the adenovirus-mediated gene transfer, we found that gene transfer to the CB only induced the expression of Ad.EGFP in the CB region but not in the contralateral uninfected CB (Figure 1) and other tissues (heart and brain) from the same rabbits. The successful gene transfer to the CBs established the solid methodological foundation for investigating the role of nNOS expression in the CBs on peripheral chemoreflex function in CHF rabbits. The enhanced expression of nNOS in the CBs of CHF rabbits by gene transfer was confirmed by immunohistochemistry (Figure 3) and Western blot analysis (Figure 4). The dose and time period of the gene transfer technique we used in the present study was based on previous studies in which adenovirus-mediated gene expression was maximal without tissue injury when the dose of $2 \times 10^7$ pfu and the time course of 3 days were used.

The CBs are a pair of small arterial chemoreceptor organs, which sense blood PaO$_2$, Paco$_2$, and pH, and reflexly influ-
ence cardiopulmonary function via primary afferent fibers of the carotid sinus nerve (CSN). Because rabbits lack functional aortic chemoreceptors, the peripheral chemoreflex is attributable mainly to the CBs in this species. Our previous studies have shown that CB chemoreflex sensitivity is enhanced in rabbits with CHF. This enhanced sensitivity of the CB chemoreflex contributes to the sympathetic activation in the CHF state because inhibition of CB chemoreceptor activity decreased resting RSNA in CHF but not in sham rabbits.19 Our studies have also demonstrated that a decrease in NO production in the CBs is involved in the enhanced CB chemoreceptor activity and peripheral chemoreflex function in CHF rabbits.19–21

The glomus cells in the CB are thought to be primary chemosensory transducers by releasing excitatory neurotransmitters that depolarize carotid sinus nerve afferents. We have previously demonstrated that I_K is blunted in CB glomus in CHF rabbits. This effect is mainly attributable to the suppression of KCa channels activity caused by decreased availability of NO. The KCa channel facilitation by NO in glomus cells is mediated by cGMP-dependent protein kinase G.21,39 NO also inhibits L-type Ca channels in glomus cells of the rabbit CB via a cGMP independent process.40 The ability of nNOS gene transfer to reduce CB chemoreceptor activity in CHF rabbits in the present study is consistent with these effects of NO on ion channel function in CB glomus cells.

Histochemical and immunological studies have demonstrated NOS enzymes in the CBs of mammals.11,22,41,42 The nNOS isoform is present in the intrinsic neurons innervating the intraglomic arterioles and glomus cells. Intraglomal vascular endothelial cells contain eNOS. Several studies have shown that a nonspecific NOS inhibitor, L-NAME, significantly enhances the ventilatory response to NaCN in rats and CB response to hypoxia in cats but specific nNOS inhibitors were ineffective on them. Conversely, using mutant mice deficient in nNOS and eNOS isoforms, Kline et al found that mice lacking nNOS showed greater ventilatory responses to hypoxia than wild-type controls; whereas responses to hypoxia were blunted in mutant mice lacking eNOS compared with the wild-type.

Our study confirms previous studies showing the presence of nNOS in nerve fibers in the CB. Furthermore, our results demonstrate that nNOS protein expression and NO production are markedly lower in the CB from CHF rabbits compared with that in sham rabbits. Gene transfer of nNOS to the CB enhanced protein expression and NO production in the CB and reversed the enhanced CB chemoreceptor activity of CHF rabbits. The specific nNOS inhibitor, SMTC, abolished the effect of Ad.nNOS on CB chemoreceptor activity. Equally important, SMTC alone enhanced CB chemoreceptor activity in sham rabbits, indicating that, in this species, nNOS provides a tonic inhibitory influence on CB chemoreceptor activity under normal conditions. By contrast, SMTC failed to increase CB chemoreceptor activity in CHF rabbits without nNOS gene transfer, indicating a loss of this tonic inhibitory influence in the CHF state. These results, taken together, demonstrate that a marked down regulation of endogenous NOS in the CB is involved in the enhanced CB chemoreceptor activity in CHF rabbits.

![Figure 6. Effect of SMTC (1 μmol/L) on the activity of CB chemoreceptors in sham, CHF, CHF/Ad.nNOS, and CHF/Ad.EGFP-treated CBs. Hypoxia: PaO_2=40±2.4 mm Hg. Data are mean±SEM, n=8 in each group. *P<0.05 vs normoxia; †P<0.05 vs control. ‡P<0.05 vs CHF Control or CHF/Ad.EGFP control.](http://circres.ahajournals.org/)

![Figure 7. Effect of bilateral CB gene transfer with either Ad.nNOS or Ad.EGFP (2×10^7 pfu) on RSNA (A) and MBP (B) under normoxic and hypoxic states in CHF rabbits, as compared with unpaced sham rabbits without gene transfer. Data are mean±SEM, n=6 for each group. *P<0.05 vs sham; †P<0.05 vs CHF.](http://circres.ahajournals.org/)
The adenoviral transfer of nNOS gene to the CB proved efficacious in elevating nNOS protein expression and NO production in treated CBs of CHF rabbits to levels found in sham rabbits. Yet, even though ad.nNOS treatment reduced CB chemoreceptor activity and chemoreflex function in CHF rabbits toward that seen in sham rabbits, the gene transfer did not completely normalize CB function. It is possible that the inability of this technique to target specific cell types within the CB influenced the efficacy of the gene transfer on chemoreceptor function. In addition, the role of endogenous eNOS on the CB chemoreceptor activity cannot be assessed from the present study.

Alternatively, other endogenous active substances besides NO (such as angiotensin II, Ang II) may also play a role in this pathophysiological process. In recent studies, we have found that Ang II enhanced the hypoxia-induced RSNA response in sham rabbits but not in CHF rabbits. Conversely, the AT1 receptor antagonist, l-158 809 attenuated hypoxia-induced increases in RSNA in CHF rabbits but not in sham rabbits.32 We also found that NADPH oxidase–derived superoxide anion mediated the Ang II-enhanced CB chemoreceptor activity in CHF rabbits.33 But the relationship among NO, Ang II, and superoxide anion on CB chemoreceptor function is not yet clear. Ang II may depress NOS gene expression34 and affect the bioavailability of NO via increasing endogenous superoxide anion production.35 Further study is needed to explore the mechanism of the enhanced CB chemoreceptor function in CHF rabbits that appears to be independent of, or interacts with, nNOS-derived NO.

In the present study, nNOS gene transfer to both CBs significantly blunted the enhanced RSNA at rest (normoxia) and during hypoxia in conscious CHF rabbits. These results demonstrate the important contribution of enhanced CB chemoreceptor input to elevated sympathetic outflow in CHF and the contribution of nNOS downregulation in the CB to this effect. The fact that enhanced gene expression of CB nNOS did not completely normalize peripheral chemoreflex function in CHF rabbits (Figure 7A) is expected given our observation that enhanced nNOS expression in the CB also did not completely normalize CB chemoreceptor activity in CHF rabbits (Table). In addition, it is well known that a number of other cardiovascular reflex and central neural alterations contribute to elevated sympathetic activity in CHF.36 Our results underscore the significance of a multiplicity of factors contributing to sympathetic hyperactivity in CHF.

In conclusion, we have described a model of nNOS gene transfer to the CBs for evaluating CB function. The present results demonstrate that the nNOS downregulation in the CB contributes to the enhanced CB chemoreceptor activity and the sympahto-excitation in the CHF state.

**Acknowledgments**

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Pacemaker implant and production of CHF

Rabbits were anesthetized with a cocktail consisting of 1.2 mg acepromazine, 5.9 mg xylazine and 58.8 mg ketamine, given as an i.m. 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. Two sonomicrometer crystals (Sonometrics Corp., London, ON, Canada) were attached to opposing walls of the lateral left ventricle for measuring external diameter. All leads exited the chest between the 3rd and 4th ribs. The chest were closed in layers and evacuated. Rabbits were placed on an antibiotic regimen consisting of 5 mg/kg Baytril i.m. for 5 days. After the rabbits recover from the thoracotomy (about 2 weeks), baseline left-ventricular end-systolic and end-diastolic external diameter (D), fractional shortening, and shortening velocity (dD/dt max) were measured by sonomicrometry (Triton Technology Inc., San Diego, CA, USA). An arterial blood gas sample (0.25 ml) was taken by needle puncture of an ear artery periodically to monitor arterial blood gases. The pacing was started at 320 bpm, which was held for 7 days, and then the rate was gradually increased to 380 bpm, with an increment of 20 bpm each week. Rabbits were paced with a pacemaker of own design, with it’s output usually being set at 4-5 V and 0.5 ms. Sonograms and blood gases (ABL5, Radiometer, Copenhagen) were acquired weekly, with the rabbits sitting quietly in a Plexiglas box and with the pacemaker turned off for at least 30 min before recordings were made.

Immunofluorescence detection for nNOS and neurofilament
After 30 minutes in PBS, CB sections on the glass slide were incubated with 10% of normal donkey serum for 1 hour followed by incubation with primary anti-nNOS and anti-neurofilament antibodies (Abcam) overnight at 4°C. Then the sections were washed with PBS and incubated with appropriate secondary antibody (Vector) for 30 minutes at room temperature. After 3 washes, sections were mounted by Fluormount (BDH Laboratory Supplies, London, England) and coverslipped. Slides were observed under a Leica microscope with appropriate excitation/emission filters. 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.

**Western blot analysis for nNOS and β-tubulin**

The protein sample was mixed with loading buffer containing β-mercaptoethanol and heated at 100°C for 5 min. Six µg of protein was loaded. Protein was fractionated in a 7.5% polyacrylamide gel along with molecular weight standards, transferred to an immobilon membrane, and subjected to a Western immunoblotting protocol. The membrane was probed with monoclonal anti-nNOS (Transduction Labs, Lexington, KY) and anti-β-tubulin (Santa Cruz) mouse antibodies, and peroxidase-conjugated goat anti-mouse IgG. The signal was detected using enhanced chemiluminescence substrate (Pierce Chemical, Rockford, IL) and Renaissance X-ray film (NEN-DuPont). The film was digitized using a Kodak digital camera and the net intensity was determined using Kodak 1D Image Analysis Software.

**Measurement of NO in the CB**

The proteins of CB homogenates were precipitated in equal volumes of 0.5 N NaOH and 10% ZnSO₄ for 15 minutes before being centrifuged at 14,000 x g for 5 minutes at 4°C.
Supernatant (10 µl) was injected into a reflux column containing 0.1 mol/L of VCl₃ in 1 mol/L of HCl at 80°C to reduce any nitrates and nitrites into NO. NO then combines with O₃ produced by the analyzer to form NO₂. The resulting emission from the excited NO₂ was detected by a photomultiplier tube and recorded digitally (mV). The values were then interpolated to a standard curve of NaNO₂ concentrations concurrently determined. Measurements were made in triplicate for each sample.

**Recording of afferent discharge of CB chemoreceptor**

3 days after Ad.nNOS was applied to the CB, rabbits were anesthetized with the anesthetic cocktail as above. Both carotid sinus regions were exposed gently. Each sinus region was vascularity 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) and L-arginine (0.1 mM), the endogenous substrate for NOS. Briefly, the internal carotid artery and all branches of the external carotid artery were ligated, and the common carotid and one branch of the external carotid arteries were cannulated to allow flow-through perfusion of the carotid sinus region with the buffer solution (10 ml/min, T 37°C). Perfusate was bubbled with O₂/CO₂/N₂ gas mixture to maintain PO₂ at 100-110 mm Hg, PCO₂ at 30-35 mm Hg, and pH at 7.4 as the normoxic condition. PO₂, PCO₂ and pH of the buffer solution perfusing the carotid sinuses were measured by gas- and ion-selective electrodes (Diamond General). The pressure in the carotid sinus was maintained at 80 mmHg.

Autonomic innervation of both carotid sinus regions was eliminated by stripping all visible neural connections among the carotid sinus, the superior cervical, and nodose ganglia. The
carotid sinus nerve was totally 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 placed on a silver electrode. Impulses were amplified with a bandwidth of 100 Hz-3 kHz (Grass P511), displayed on an oscilloscope (Gould 450), and fed into a rate meter (Frederick Haer) whose window discriminators were set to accept potentials of the particular amplitude. Impulses were counted by the rate meter in 1-second bins. The action potential and rate meter signals were fed into an A/D converter (PowerLab) attached to a Macintosh computer. 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 renal sympathetic nerve**

The left kidney was exposed retroperitoneally, and a branch of renal nerve was separated from the renal plexus and the surrounding connective tissues. A pair of stainless steel stranded Teflon coated recording electrodes was placed around the nerve branch. The nerve-electrode junction was insulated electrically from the surrounding tissues and covered with a fast setting silicone (Kwik-Sil, world Precision Instruments, Sarasota, FL). A ground wire was sutured to a nearby muscle before the incision was closed. The electrode wires were tunneled beneath the skin to exit on the upper back. Rabbits were allowed to recover for 3 days, depending on the quality of RSNA, before experiments commenced.

RSNA was recorded using a Grass P511 differential amplifier and a storage oscilloscope. The RSNA was filtered at a bandwidth of 100 Hz-3 KHz. The nerve signal was also fed to an audio amplifier and loudspeaker. The nerve signal was rectified, integrated, and both raw and
integrated signals were recorded. All analogue signals were captured and digitized by using a PowerLab (Model 8S, AD instruments Inc., Colorado Springs, CO) data Acquisition system.

RSNA was corrected for noise by subtracting the integrated noise level from the total integrated signal. The noise was determined by bolus administration of phenylephrine to increase MBP over 130 mm Hg in order to inhibit sympathetic outflow, at the end of the experiment.

**Recording of hemodynamics and ventilation**

At the time of the renal nerve electrode surgery, a microrenthane catheter was inserted into the right carotid artery and jugular vein for measurement of arterial blood pressure and injection of drugs, respectively. The catheters were tunneled beneath the skin and filled with heparin (1000 U/ml).

Arterial blood pressure (ABP) was recorded from the carotid catheter using a Hewlett-Packard transducer and Coulburn bridge amplifier. Mean blood pressure (MBP) and Heart rate (HR) were calculated from the pulsatile ABP.

Tidal volume (TV) and breathing rate (BR) were determined by unrestrained plethysmography described previously\(^1\). Minute ventilation (VE) was defined as TV X BR and was normalized to body weight. Rabbits were placed in a Plexiglas chamber (volume 11 liters) with exit ports for catheters and renal nerve electrodes. The chamber was sealed, except for an inlet and outlet port that allowed a continuous flow of air through the chamber. Different gas mixtures could be easily passed through the chamber to alter blood gases. TV was measured by temporarily (30 s) sealing the air ports and measuring the pressure changes in the sealed chamber by using a Validyne (MP-45) differential pressure transducer and amplifier connected
to the PowerLab analysis system. The chamber was sealed only for short intervals (30 s) to prevent appreciable changes in the composition of air in it. Different gas mixtures could be easily passed through the chamber to alter blood gases. All ventilatory gases were saturated with water vapor by passage through a warmed bubbling chamber.

Calibration of TV was performed dynamically; with the animal present in the chamber by varying the volume of the chamber with a calibrated plunger at 60 cycles/min. TV was calculated from the formula of Drorbaugh and Fenn².

**Measurement of arterial blood gases**

Arterial blood samples (0.2 ml) were drawn from the arterial catheter during the steady state of the response to various stimuli. PaO₂, PaCO₂ and pH of arterial blood were measured by a blood gas analyzer (ABL5, Radiometer, Copenhagen).

**Peripheral chemoreflex protocol**

Changes in RSNA, VE, HR, and MBP 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), 15% O₂ (mild hypoxia), and 10% O₂ (severe hypoxia). Each stimulation was held until a steady response was achieved (3-5 min). Then the RSNA, VE, MBP, and HR were measured during the 30-s plethysmograph maneuver (explained above), and an arterial blood sample was taken from the arterial catheter for the measurement of PaO₂, PaCO₂, and pH. Because hypoxic stimulation of ventilation induces hyperventilatory hypocapnia, 2-4% CO₂ was added to the
hypoxic gases to maintain relatively constant PaCO₂ 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.
Table 1s. Cardiac diameters and contractility in sham and CHF rabbits

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=20)</th>
<th>CHF (n=42)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>3-4 wk</td>
</tr>
<tr>
<td>ESD, % of control</td>
<td>100 ± 0.0</td>
<td>100.5 ± 0.9</td>
</tr>
<tr>
<td>EDD, % of control</td>
<td>100 ± 0.0</td>
<td>100.8 ± 0.7</td>
</tr>
<tr>
<td>$dD/dt_{\text{max}}$, mm/s</td>
<td>-10.8 ± 1.2</td>
<td>-10.7 ± 1.3</td>
</tr>
<tr>
<td>% shortening</td>
<td>10.5 ± 1.0</td>
<td>9.8 ± 1.1</td>
</tr>
</tbody>
</table>

Data are means ± SEM; ESD, left ventricular end-systolic diameter; EDD, left ventricular end-diastolic diameter; $dD/dt_{\text{max}}$, 1st derivative of change in diameter; %shortening=(EDD-ESD)/EDDx100%. *P<0.05 vs. baseline; #p<0.05 vs. sham rabbits at the same period.
Table 2s. Hemodynamic variables and ventilation during normoxia and hypoxia in conscious sham, CHF, CHF + Ad.nNOS, and CHF + Ad.EGFP rabbits

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Mild hypoxia</th>
<th>Severe Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MBP, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>82.4 ± 2.2</td>
<td>81.2 ± 2.7</td>
<td>81.7 ± 3.3</td>
</tr>
<tr>
<td>CHF</td>
<td>75.2 ± 2.7#</td>
<td>73.9 ± 3.0#</td>
<td>73.7 ± 2.4#</td>
</tr>
<tr>
<td>CHF + Ad.nNOS</td>
<td>76.3 ± 2.6#</td>
<td>76.1 ± 2.9#</td>
<td>75.8 ± 3.2#</td>
</tr>
<tr>
<td>CHF + Ad.EGFP</td>
<td>74.7 ± 2.1#</td>
<td>74.9 ± 2.8#</td>
<td>74.2 ± 2.3#</td>
</tr>
<tr>
<td><strong>HR, beats/min</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>244 ± 7</td>
<td>241 ± 12</td>
<td>246 ± 9</td>
</tr>
<tr>
<td>CHF</td>
<td>285 ± 6#</td>
<td>289 ± 10#</td>
<td>288 ± 13#</td>
</tr>
<tr>
<td>CHF + Ad.nNOS</td>
<td>288 ± 7#</td>
<td>292 ± 14#</td>
<td>294 ± 14#</td>
</tr>
<tr>
<td>CHF + Ad.EGFP</td>
<td>282 ± 10#</td>
<td>284 ± 11#</td>
<td>289 ± 13#</td>
</tr>
<tr>
<td><strong>VE, ml/min/kg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>253.8 ± 11.2</td>
<td>321.4 ± 13.6*</td>
<td>474.6 ± 21.8*</td>
</tr>
<tr>
<td>CHF</td>
<td>362.9 ± 24.2#</td>
<td>502.3 ± 26.3*#</td>
<td>634.7 ± 30.1*#</td>
</tr>
<tr>
<td>CHF + Ad.nNOS</td>
<td>277.1 ± 19.5</td>
<td>405.3 ± 21.4*#</td>
<td>547.5 ± 23.2*#</td>
</tr>
<tr>
<td>CHF + Ad.EGFP</td>
<td>366.2 ± 23.1*#</td>
<td>492.5 ± 25.8*#</td>
<td>642.3 ± 24.6*#</td>
</tr>
</tbody>
</table>

Data are means ± SEM, n=6 in each group. MBP, mean blood pressure; HR, heart rate; VE, minute ventilation. *P<0.05 vs. normoxia; #p<0.05 vs. sham.
Table 3s. Blood-gas variables during normoxia and hypoxia in conscious sham, CHF, CHF + Ad.nNOS, and CHF + Ad.EGFP rabbits

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Mild hypoxia</th>
<th>Severe Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PaO₂, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>91.2 ± 2.1</td>
<td>59.1 ± 2.8*</td>
<td>41.2 ± 2.4*</td>
</tr>
<tr>
<td>CHF</td>
<td>91.4 ± 2.7</td>
<td>60.8 ± 2.9*</td>
<td>42.0 ± 1.8*</td>
</tr>
<tr>
<td>CHF + Ad.nNOS</td>
<td>91.5 ± 2.3</td>
<td>61.4 ± 3.2*</td>
<td>42.3 ± 2.2*</td>
</tr>
<tr>
<td>CHF + Ad.EGFP</td>
<td>92.4 ± 2.5</td>
<td>60.9 ± 3.4*</td>
<td>42.0 ± 3.0*</td>
</tr>
<tr>
<td><strong>PaCO₂, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>35.1 ± 1.5</td>
<td>34.4 ± 1.2</td>
<td>33.7 ± 1.9</td>
</tr>
<tr>
<td>CHF</td>
<td>35.7 ± 1.6</td>
<td>35.1 ± 1.8</td>
<td>34.6 ± 1.7</td>
</tr>
<tr>
<td>CHF + Ad.nNOS</td>
<td>35.5 ± 1.4</td>
<td>35.2 ± 1.8</td>
<td>34.7 ± 2.2</td>
</tr>
<tr>
<td>CHF + Ad.EGFP</td>
<td>35.5 ± 1.8</td>
<td>34.8 ± 2.1</td>
<td>34.3 ± 2.3</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>7.433 ± 0.007</td>
<td>7.426 ± 0.008</td>
<td>7.414 ± 0.010</td>
</tr>
<tr>
<td>CHF</td>
<td>7.428 ± 0.012</td>
<td>7.422 ± 0.011</td>
<td>7.408 ± 0.014</td>
</tr>
<tr>
<td>CHF + Ad.nNOS</td>
<td>7.426 ± 0.011</td>
<td>7.428 ± 0.014</td>
<td>7.411 ± 0.010</td>
</tr>
<tr>
<td>CHF + Ad.EGFP</td>
<td>7.428 ± 0.008</td>
<td>7.425 ± 0.012</td>
<td>7.415 ± 0.013</td>
</tr>
</tbody>
</table>

Data are means ± SEM, n=6 in each group. *P<0.05 vs. normoxia.