Enhanced activity of carotid body chemoreceptors in rabbits with heart failure: role of nitric oxide

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Sun, Shu-Yu, W. Wang, I. H. Zucker, and H. D. Schultz. Enhanced activity of carotid body chemoreceptors in rabbits with heart failure: role of nitric oxide. J. Appl. Physiol. 86(4): 1273-1282, 1999.—An enhanced peripheral chemoreflex has been documented in patients with chronic heart failure (CHF). This study aimed to examine the characteristics of carotid body (CB) chemoreceptors in response to isocapnic hypoxia in a rabbit model of pacing-induced CHF and to evaluate the possible role that nitric oxide (NO) plays in the altered characteristics. The chemosensitive characteristics of the CB were evaluated by recording single-unit activity from the carotid sinus nerve in both an intact and a vascularly isolated preparation. It was found that the baseline discharge under normoxia (intact preparation: arterial Po₂ 90–95 Torr; isolated preparation: Po₂ 100-110 Torr) and the chemosensitivity in response to graded hypoxia (Po₂ 40-70 Torr) were enhanced in CHF vs. sham rabbits. These alterations were independent of the CB preparations (intact vs. isolated). NO synthase inhibition by N^{ω} -nitro-L-arginine increased the baseline discharge and the chemosensitivity in the intact preparation, whereas L-arginine (10^{-5} M) inhibited the baseline discharge and the chemosensitivity in the isolated preparation in sham but not in CHF rabbits. S-nitroso-N-acetylpenicillamine, an NO donor, inhibited the baseline discharge and the chemosensitivity in both CB preparations in CHF rabbits but only in the isolated preparation in sham rabbits. The amount of NO produced in vitro by the CB under normoxia was less in CHF rabbits than in sham rabbits (P < 0.05). NO synthase-positive varicosities of nerve fibers within the CB were less in CHF rabbits than in sham rabbits (P < 0.05). These data indicate that an enhanced input from CB occurs in the rabbit model of pacing-induced CHF and that an impairment of NO production may contribute to this alteration.

single-unit activity; carotid sinus nerve; *N*¹⁰-nitro-L-arginine; *S*-nitroso-*N*-acetyl-penicillamine; hypoxia

ACTIVATION OF SYMPATHETIC NERVOUS SYSTEM is a prominent part of the pathophysiology of chronic heart failure (CHF) (8, 10, 32). Because the magnitude of sympathohumoral activation in CHF is a good predictor of mortality (22), understanding the mechanism(s) responsible for the increased sympathetic activity is of major importance. A popular hypothesis explaining the generalized sympathetic activation in CHF has been the impairment of inhibitory arterial and cardiac baroreflexes (15, 32). However, other evidence suggests that excitatory reflexes may also play a role (31). Peripheral chemoreceptor activation is an excitatory input that results in increased sympathetic outflow and blood pressure. Recent studies have shown that chemoreflex sensitivity is enhanced in patients with CHF (4-7). Studies from our laboratory in a rabbit model of pacing-induced CHF have also shown an enhanced sensitivity of the peripheral chemoreflex (25, 26). This enhanced sensitivity of the peripheral chemoreflex contributes, at least in part, to the sympathetic activation in this rabbit model of CHF, because inhibition of peripheral chemoreceptor activity decreased renal sympathetic nerve activity (RSNA) in CHF, but not in sham, rabbits (26). It is not known whether this enhanced reflex sensitivity results from an alteration in the chemosensitive characteristics of the peripheral chemoreceptors or from changes in the central integration of the chemoreceptor input. Therefore, the first goal of this study was to determine whether the chemosensitive characteristics of the peripheral chemoreceptors are altered in the CHF state. For this purpose, the chemosensitive characteristics of the carotid body (CB) chemoreceptors in response to isocapnic hypoxia were examined in sham and CHF rabbits by recording the single-unit activity from the carotid sinus nerve (CSN). It was found that the sensitivity of CB chemoreceptors in response to hypoxia was enhanced in CHF rabbits.

On the basis of these observations, the second goal of this study was to evaluate the mechanism that is responsible for the enhanced sensitivity of the CB chemoreceptors in CHF rabbit. There is evidence indicating that administration of exogenous nitric oxide (NO) inhibits the activity of the CB chemoreceptors in normal animals (19). Furthermore, it has been demonstrated that NO synthesis is suppressed in CHF (24). Therefore, it is reasonable to speculate that an attenuated NO synthesis in the CB during the development of CHF may account for the enhanced sensitivity of the CB chemoreceptors in this condition. To test our hypothesis, we examined the effect of inhibition of endogenous NO synthesis and of administration of exogenous NO on the chemosensitive characteristics of the CB chemoreceptors in sham and CHF rabbits. We also measured the basal NO production in vitro from the CB and examined the density of nerve fibers positive for NO synthase (NOS) within the CB in sham and CHF rabbits. We found that the basal NO production and the NOS-positive nerve fibers were less in CHF rabbits than in sham rabbits. Blockade of NO synthesis increased the discharge of the CB chemoreceptors in sham rabbits while restoration of NO by administration of an NO donor inhibited the discharge of the CB chemoreceptors in CHF rabbits.

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MATERIALS AND METHODS

General Preparation

All experiments were carried out on male New Zealand White rabbits weighing 2.5–3.5 kg. 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 and the American Physiological Society's Guides for the Care and Use of Laboratory Animals. Rabbits were anesthetized with an anesthetic mixture consisting of 1.2 mg/kg acepromazine, 5.9 mg/kg xylazine, and 58.8 mg/kg ketamine, given as an intramuscular injection. Supplemental anesthesia was provided by pentobarbital sodium (1.7 mg/kg iv) as needed.

With the use of a sterile technique, a left thoracotomy was performed as previously described (18). Briefly, the pericardium was opened, and a pin electrode of our own design was implanted on the left ventricle for pacing. Two sonomicrometer crystals (Sonometrics) were attached to the epicardial surfaces at the base of the heart to measure external cardiac diameters. All leads exited the chest between the 3rd and 4th ribs. A ground lead was implanted on the left wall of the chest underneath the skin. The chest was then closed in layers and evacuated. Proper procedures were taken to ensure that atelectasis and pneumothorax were minimized. Rabbits were placed on an antibiotic regimen consisting of 2.3 mg/kg Baytril im for 5 days.

Production of Heart Failure

After the rabbits recovered from the thoracotomy (10-14 days), in the awake state, baseline cardiac end-systolic and end-diastolic external diameters, fractional shortening, and shortening velocity (dD/dt_{max}) were measured by sonomicrometry. An arterial blood sample (0.25 ml) was taken by needle puncture of an ear artery for measuring blood gas. After these prepacing measurements were completed, a pacing regimen was started. The pacing was started at a 320 beats/min rate, which was held for 7 days. Then the pacing rate was gradually increased to 380 beats/min, with an increment of 20 beats/min each week. The pacemaker was of our own design, with its output usually being set at 4-5 V and 0.5 ms. Sonograms were done and blood gases were measured 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. Rabbits with >40% reductions in dD/dt_{max} and shortening fraction were considered in CHF (generally after 3-4 wk). Sham-operated rabbits underwent a similar period of sonographic measurements. Rabbits exhibiting abnormal arterial blood gases [either arterial Po_2 (Pa_{0_2}) <80 Torr, arterial P_{CO_2} (Pa_{CO_2}) >45 Torr, or Pa_{CO_2} <30 Torr] were excluded from the study.

CB Preparations

On the day of the experiment, rabbits were anesthetized with urethan (0.8 g/kg iv) and α -chloralose (40 mg/kg iv). The trachea was intubated, and the lungs mechanically ventilated (250–300 ml·kg⁻¹·min⁻¹). Tidal volume was adjusted to maintain Pa_{CO₂} at 30–35 Torr. To prevent spontaneous breathing, the rabbit was paralyzed with pancuronium bromide (0.25 mg/kg iv). Supplemental doses were given as needed. Body temperature was maintained at 37–38°C by a heating pad. Polyethylene catheters were inserted into a femoral artery and a vein for the measurement of, respectively, arterial pressure, blood gases, and administration of drugs. Lactated Ringer solution was infused (3 ml·kg⁻¹·h⁻¹) through the venous line to avoid dehydration. Any metabolic

acidosis was corrected by and intravenous infusion of appropriate amounts (0.2 \times body wt \times base excess meq) of 1 M NaHCO_3.

Both carotid sinus regions were exposed gently. The vessels of one carotid sinus (randomly assigned) were left intact (intact preparation, perfused with animal's own arterial blood), and the CSN was exposed for recording chemoreceptor afferent activity (see Recording Techniques). An ultrasonic probe attached to a flowmeter (T106, Transonic Systems) was used to measure the blood flow of the common carotid artery, which supplied the carotid sinus and CB. After the protocol for afferent recordings was completed on the intact side, the opposite 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 dextrose). Briefly, the internal carotid artery and all branches of external carotid artery were ligated, and the common carotid and external maxillary arteries were cannulated to allow flow-through perfusion of the carotid sinus region with the buffer solution. Perfusate was bubbled with O₂-CO₂-N₂ gas mixture at proper proportion (see Construction of Stimulus-Response Curves) to maintain Po₂ at 100–110 Torr, Pco₂ at 30–35 Torr, and pH at 7.35–7.45 as the normoxic condition. The temperature of the perfusate was maintained at 36-38°C by using a water bath. Pressure in the carotid sinus was maintained at 80 mmHg by adjusting the height of the perfusion reservoir, and the flow was kept constant (10 ml/min) by adjusting the outlet of the effluent cannula.

Autonomic innervation of both carotid sinus regions was eliminated by stripping all visible neural connections between the carotid sinus and the superior cervical and nodose ganglia. The CSN was totally transected near the petrosal ganglion, not only to facilitate dissection of afferent fibers but also to interrupt neural efferents to the CB.

Recording Techniques

The CSN was covered with mineral oil and fine slips of nerve filaments placed on a silver electrode. Impulses was amplified with a band width of 100 Hz-3 kHz (Grass P511), displayed on an oscilloscope (Gould 450) and fed into a rate meter (Frederick Haer), window discriminators of which were set to accept potentials of a particular amplitude. Impulses were counted by the ratemeter in 1-s bins. The action potentials and ratemeter signals were fed into an analog-todigital converter (MacLab) 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.

In Vitro NO Assay

NOS activity in the CB was estimated by measuring the rate of NO release from the CB with a chemiluminescence NO analyzer (Sievers 208). At the end of afferent recording experiments, the blood-perfused sinus was removed, and the CB was dissected within ice-cold Krebs-Henseleit solution bubbled with 100% O_2 . The CB was then allowed to equilibrate at room temperature for 30 min in 30 ml of Krebs-Henseleit solution containing 1 mM of L-arginine and bubbled with 100% O_2 . After this incubation, the CB was removed and placed into a small incubation vial containing 0.5 ml of Krebs-Henseleit solution with 1 mM of L-arginine at 90–95 Torr $PO_2/30-40$ Torr PCO_2 (normoxia) and allowed to incubate at 37–38°C for 30 min. At the end of the incubation period, the CB was removed, and the incubation vial was sealed immediately and stored in a freezer (-20° C) for later measurement of

nitrite (NO₂⁻) and nitrate (NO₃⁻) concentration by the chemiluminescence analyzer. The analyzer was calibrated by using standard solutions of NaNO₂. This system regenerates all of the NO oxidized to NO₂⁻ or NO₃⁻. To control for background NO₂⁻ and NO₃⁻ signals, aliquots from incubation vials with Krebs-Henseleit solution without CB were assayed in parallel with those from vials containing CB, and the background level was subtracted from assay measurements. The CB was weighed at the end of the experiment, and NO release was expressed as picamoles per milligram wet weight per 30 min. NO release from the common carotid artery (CA) and the superior cervical ganglion (SCG) were also assayed for comparison.

Histochemical Studies

The localization of NOS in the CB of sham and CHF rabbits was examined by using NADPH-diaphorase histochemistry, as described previously (27). At the end of the afferent-recording experiments, the blood-perfused carotid sinus was vascularly isolated and perfused with Krebs-Henseleit solution until free of blood. Then the carotid sinus was perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 20 min at room temperature. The sinus was then infiltrated with 30% sucrose in 0.1 M phosphate buffer and stored frozen at -70° C until sectioned.

Cryostat sections (15 μm) were cut and incubated in 0.1 M phosphate buffer, pH 7.4, containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium, and 1.0 mg/ml \beta-NADPH at 37°C for 60 min. After the reaction, the sections were rinsed in phosphate buffer, pH 7.4, and mounted onto chrome-alumcoated slides. The slides were air-dried, then rinsed in distilled water, and dried again. Coverslips were then mounted directly with Entellan. As a control, some sections were incubated with the oxidized form of NADP along with nitroblue tetrazolium. Sections were examined under a microscope, and the density of the varicosities of nerve fibers positive for NADPH-diaphorase was counted within unit area (0.5 mm²). Five separate areas within each section were counted, and the values were averaged for the section. Three to five sections were counted from each CB. Values from sections of the same CB were averaged again as the value of the density of nerve fibers within this CB.

Protocols

Discharge characteristics of the carotid body chemoreceptors in sham and CHF rabbits. The stimulus-response curves of single-unit activity of CB chemoreceptors to alterations in Po2 were examined in eight sham and eight CHF rabbits. In the intact preparation, Pa_{O_2} was altered by ventilating the rabbits with 20% $O_2\text{--}80\%$ $N_2,\,15\%$ $O_2\text{--}85\%$ $N_2,\,10\%$ $O_2\text{--}90\%$ $N_2,$ and 100% O_2 sequentially. Each stimulus lasted until a steady response was reached (40-60 s). An arterial blood sample was obtained at the end of each stimulus for blood-gas measurement. Stimuli were separated by at least 10 min to allow complete recovery of the fiber. Pa_{CO}, was controlled by adjusting the tidal volume, and pH was adjusted by intravenous NaHCO₃. In the vascularly isolated preparation, Po₂ was altered by bubbling the perfusate with gas mixtures of different O_2 concentrations (50, 10, 5, 0%) to achieve a PO_2 of 300-400, 100-110, 60-70, and 40-50 Torr, respectively. The gas mixtures contained a constant fraction of 5% CO₂ and a balance of N₂. pH was adjusted by adding NaHCO₃ or HCl into the perfusate. The afferent responses of the CB chemoreceptors from the intact preparation and from the isolated preparation were compared between sham and CHF rabbits. The comparison allowed us to deduce whether changes in afferent sensitivity between sham and CHF rabbits were the consequence of either intrinsic differences within the CB (isolated sinus) or differences as a result of alterations in blood-borne substances that influenced chemoreceptor activity.

Contribution of NO to the alteration of chemosensitive characteristics of carotid body chemoreceptors in CHF rabbits. GROUP A: EFFECT OF NOS INHIBITION IN THE INTACT PREPARA-TION. The response of CB chemoreceptors to alterations in Pa_{O_2} was examined before and after treatment of the CB with a competetive NOS antagonist No-nitro-L-arginine (L-NNA) in both sham (n = 7) and CHF (n = 7) rabbits. After the control response was obtained, a bolus injection of L-NNA was given intravenously at a dose of 30 mg/kg. Twenty minutes later, the response to the same level of hypoxia was reexamined. Then a large dose (60 mg \cdot kg⁻¹ \cdot min⁻¹) of L-arginine, the endogenous substrate for NOS, was infused intravenously for 10 min. The response to hypoxia was examined for the third time with the infusion of L-arginine to determine whether the effect of L-NNA was reversible by its competitive agonist. Only the response to severe hypoxia (Pa $_{\rm O_2}$ at 40–50 Torr) was examined, since we had shown the graded characteristics of the CB chemoreceptors from the previous protocol.

GROUP B: EFFECT OF L-ARGININE IN THE ISOLATED PREPARA-TION. We initially conducted a protocol in the isolated preparation, similar to that explained above in the intact preparation (group A), but found no effect of L-NNA ($10^{-5}-10^{-4}$ M) on the response of CB chemoreceptors to hypoxia either in sham or in CHF rabbits. Because the doses of L-NNA we used were high enough to effectively block the NOS activity (17, 23), we speculated that the ineffectiveness of L-NNA in the isolated preparation may be due to absence of the substrate (L-arginine) for NOS within the buffer solution. Therefore, we switched the sequence of administration of L-NNA and L-arginine in the isolated preparation to examine the effect of L-arginine on the response of CB chemoreceptors in sham (n = 7) and CHF (n = 7) rabbits. After the control response was obtained, L-arginine (10^{-5} M) was added to the perfusate, and the response to hypoxia (Po2 at 40-50 Torr) was then examined as explained above. We then added L-NNA (10^{-3} M) to the L-arginine-supplemented perfusate to determine whether the effect of L-arginine on the response of the CB chemoreceptors could be blocked by its competitive antagonist.

GROUP C: EFFECT OF N^{ω} -NITRO-D-ARGININE (D-NNA) AND D-ARGININE IN SHAM RABBITS. As a negative control, the effect of biologically inactive isomers of L-NNA and L-arginine, i.e., D-NNA and D-arginine, on the response of the CB chemoreceptors to isocapnic hypoxia (Po₂ at 40–50 Torr) was examined in six sham rabbits. This was not done in CHF rabbits, because neither L-NNA nor L-arginine was found effective in CHF rabbits (see RESULTS). The effect of D-NNA was examined only in the intact preparation, whereas the effect of D-arginine was examined only in the isolated preparation.

GROUP D: EFFECT OF EXOGENOUS NO ON THE RESPONSE OF CB CHEMORECEPTORS. To assess whether the effects of endogenous NO on the sensitivity of the CB chemoreceptors were maximally expressed in each group of animals, the effect of an NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP), on the response of the CB chemoreceptors was examined in five sham and six CHF rabbits. Unlike L-arginine, which requires NOS to produce NO, SNAP spontaneously releases NO under aqueous conditions via homolytic cleavage of the *S*-*N* bond. In the intact preparation, SNAP was infused intravenously at a dose of 60 nmol·kg⁻¹·min⁻¹ throughout the experiment. In the isolated preparation, SNAP was added into the perfusate at a concentration of 10^{-4} M. The response of the CB chemoreceptors to isocapnic hypoxia (Po₂ at 40–50 Torr) was obtained before and during administration of SNAP.

Data Analysis

Baseline discharge of the CB chemoreceptors and the discharge at different levels of Po₂ were compared within group and between groups by using a two-way ANOVA for repeated measures. Post hoc comparison was done by using the Bonferroni procedure. To quantify the stimulus-response characteristics of the CB chemoreceptors, experimental data from each animal were also fitted to a hyperbolic function described previously (13) by using the following equation: y =a + b/(x - c), where y is the discharge frequency of the CB afferents and x is Po_2 . The values a, b, and c are constant, a being the horizontal asymptote, *b* a "shaping term," and *c* the vertical asymptote. The values of r^2 were at 0.95–0.99 (P <0.01) for all of the curve fittings. The parameters derived from curve fittings and those from NO measurement and histological study were compared between sham and CHF rabbits by unpaired Student's t-test. Statistical analyses were performed by using commercial statistical software (GB-STAT, Dynamic Microsystems). All data are expressed as means \pm SE. Statistical significance was accepted when P < 0.05.

RESULTS

As described in our accompanying paper (26), paced rabbits exhibited overt evidence of heart failure, including an enlarged heart, an attenuated contractility, and an elevated central venous pressure. Out of a total of 61 rabbits (33 sham, 28 CHF) used in the present study, 46 rabbits (26 sham and 20 CHF) were also used in the reflex study reported in our accompanying paper. Because the alterations of hemodynamics and cardiac dimensions due to cardiac pacing in the present study were not significantly different from those described in the reflex study, the reader is referred to Tables 1 and 2 in our accompanying paper (26) for these data.

Discharge Characteristics of the CB Chemoreceptors in Sham and CHF Rabbits

Isocapnic hypoxia increased the discharge frequency of the CB chemoreceptors in both sham and CHF rabbits in a level-dependent manner (Fig. 1). The Pco₂ and pH were maintained within the normal range during the hypoxic stimulation (Table 1). However, the baseline discharge in the normoxic state and the magnitude of the response to corresponding levels of hypoxia were greater in CHF than in sham rabbits (Fig. 2), suggesting an enhanced sensitivity of the CB chemoreceptors in CHF rabbits. These alterations were observed both in the intact and in the isolated CB preparations (Fig. 2). The discharge frequency of the CB chemoreceptors in the hyperoxic state (300-400 mmHg) was not different between sham and CHF rabbits in both preparations (Fig. 2). Comparisons of the parameters derived from the hyperbolic curve fitting showed that the shaping coefficient b was greater, whereas the vertical asymptote *c* was smaller in CHF than in sham rabbits (Table 2), again suggesting a left-upward shift of the Po₂-discharge relationship or a sensitization of the CB chemoreceptors in CHF rabbits.

We found that blood flow decreased in the carotid artery during severe hypoxia (Pa_{O_2} at 40–50 Torr),

Fig. 1. Representative recordings of afferent discharge of carotid body (CB) chemoreceptors during normoxia and 2 levels of isocapnic hypoxia from a sham (*left*) and a chronic heart failure (CHF) rabbit (*right*). CHF rabbit exhibited higher levels of discharge both in the normoxic state and at the equivalent levels of hypoxia, compared with sham rabbit. DF, discharge frequency [impulses (imp)/s]; AP, action potential; Pa_{O_2} , arterial PO_2 .



Table 1. Blood-gas data during hyperoxia, normoxia, and mild and severe hypoxia in sham (n = 8)and CHF (n = 8) rabbits

Hyperoxia	Normoxia	Mild Hypoxia	Severe Hypoxia			
Intact preparation						
$403.0 \pm 3.6^*$	97.0 ± 1.4	$62.8 \pm 0.7^*$	$44.2 \pm 1.1^*$			
$397.6 \pm 1.7^*$	93.2 ± 3.1	$62.0 \pm 0.6^*$	$41.1 \pm 0.9^*$			
31.5 ± 0.1	30.1 ± 0.2	33.2 ± 0.1	33.8 ± 0.1			
33.8 ± 0.1	32.8 ± 0.1	32.9 ± 0.1	33.0 ± 0.1			
$7.431 \!\pm\! 0.002$	7.433 ± 0.002	7.437 ± 0.003	7.426 ± 0.002			
7.421 ± 0.002	7.432 ± 0.002	7.436 ± 0.002	$7.425 \!\pm\! 0.001$			
Isolated preparation						
$354.3 \pm 5.3^*$	107.0 ± 2.8	$66.2 \pm 1.8^{*}$	$43.0 \pm 1.4^{*}$			
$355.1 \pm 5.0^*$	108.0 ± 1.7	$69.4 \pm 2.1^*$	$43.1 \pm 2.2^*$			
32.0 ± 0.1	32.6 ± 0.1	31.2 ± 0.1	31.3 ± 0.1			
31.4 ± 0.1	32.1 ± 0.1	31.2 ± 0.1	30.2 ± 0.1			
$7.408 \!\pm\! 0.004$	7.407 ± 0.005	7.412 ± 0.006	$7.416 \!\pm\! 0.007$			
7.411 ± 0.004	7.401 ± 0.005	7.416 ± 0.006	7.422 ± 0.004			
	Hyperoxia $403.0 \pm 3.6^*$ $397.6 \pm 1.7^*$ 31.5 ± 0.1 33.8 ± 0.1 7.431 ± 0.002 7.421 ± 0.002 I_{4} $354.3 \pm 5.3^*$ $355.1 \pm 5.0^*$ 32.0 ± 0.1 31.4 ± 0.1 7.408 ± 0.004 7.411 ± 0.004	Hyperoxia Normoxia Intact preparate $403.0 \pm 3.6^*$ 97.0 ± 1.4 $397.6 \pm 1.7^*$ 93.2 ± 3.1 31.5 ± 0.1 30.1 ± 0.2 33.8 ± 0.1 32.8 ± 0.1 7.431 ± 0.002 7.433 ± 0.002 7.421 ± 0.002 7.432 ± 0.002 $Isolated preparate 354.3 \pm 5.3^* 107.0 \pm 2.8 32.0 \pm 0.1 32.6 \pm 0.1 31.4 \pm 0.1 32.1 \pm 0.1 7.408 \pm 0.004 7.407 \pm 0.005 7.411 \pm 0.004 7.401 \pm 0.005 $	$\begin{array}{c c c c c c c c c c c c c c c c c c c $			

Values are means \pm SE. Pa_{O2}, arterial PO2; Pa_{CO2}, arterial PCO2; CHF, chronic heart failure. *P < 0.01, significantly different compared with normoxia.

when the afferent recording was performed in the intact preparation [sham: from 93 \pm 19 ml/min (normoxia) to 75 \pm 15 ml/min (hypoxia); CHF: from 91 \pm 18 ml/min (normoxia) to 74 \pm 17 ml/min (hypoxia)]. The magnitude of decrease in the carotid blood flow was not



Fig. 2. Po_2 -discharge relationships of CB chemoreceptors in intact (*A*) and isolated (*B*) preparations in sham and CHF rabbits, showing an elevated baseline discharge in normoxic state and an enhanced response to hypoxia in both preparations from CHF rabbits (**P* < 0.05; ***P* < 0.01, CHF vs. sham).

Table 2. Logistic parameters derived from hyperbolic curve fitting for the DF-P0₂ relationships in sham and CHF rabbits

	Intact Preparation		Isolated Preparation	
	Sham (<i>n</i> = 8)	CHF (<i>n</i> =8)	Sham (<i>n</i> =8)	CHF (<i>n</i> =8)
<i>a</i> , impulses/s <i>b</i> , impulses ·	0.4 ± 0.2	0.3 ± 0.2	0.7 ± 0.3	0.4 ± 0.1
s ⁻¹ ·Torr c, Torr	$\begin{array}{c} 309.6\pm80.1 \\ 29.6\pm3.2 \end{array}$	$\begin{array}{c} 972.8\pm89.2\dagger\\ 18.7\pm2.4^* \end{array}$	$\begin{array}{c} 326.7 \pm 62.6 \\ 25.8 \pm 3.1 \end{array}$	$\begin{array}{c} 855.7\pm 66.5 \\ 17.2\pm 2.7 * \end{array}$

Values are means \pm SE; *n* = no. of rabbits. DF, discharge frequency; *a*, *b*, *c*, logistic parameters (see text for more information). * *P* < 0.05, † *P* < 0.01, CHF vs. sham rabbits.

different between sham and CHF rabbits. Mean arterial blood pressure did not change significantly [sham: $75 \pm 2 \text{ mmHg}$ (normoxia) vs. $76 \pm 2 \text{ mmHg}$ (hypoxia), P > 0.05; CHF: $69 \pm 2 \text{ mmHg}$ (normoxia) vs. $70 \pm 4 \text{ mmHg}$ (hypoxia), P > 0.05] during the same interval. Neither carotid artery blood flow nor blood pressure changed in either group during mild hypoxia (Pa₀₂ at 60–70 Torr).

Contribution of NO to the Alteration of Chemosensitive Characteristics of the CB Chemoreceptors in CHF Rabbits

Group A. In the intact preparation, administration of the NOS inhibitor L-NNA (30 mg/kg) increased the baseline discharge in the normoxic state and the response to hypoxia in sham, but not in CHF, rabbits (Fig. 3). The effect of L-NNA was reversed by the NOS substrate L-arginine (60 mg·kg⁻¹·min⁻¹) (Fig. 3).

The administration of L-NNA elevated arterial blood pressure in both groups. The elevation was 27 ± 2 mmHg (from 77 ± 5 to 104 ± 7 mmHg) in sham rabbits and $2\overline{1} \pm 1$ mmHg (from 70 \pm 5 to $9\overline{2} \pm 6$ mmHg) in CHF rabbits. To assess the possibility that the elevation of the blood pressure contributed to the effect of L-NNA on the CB chemoreceptors, we increased blood pressure to an equivalent level by mechanical occlusion of the descending aorta in two sham and two CHF rabbits. One fiber was studied in each rabbit. It was found that the equivalent elevation of blood pressure induced mechanically did not increase the baseline discharge nor the response to hypoxia in these rabbits. Therefore, it seems unlikely that the difference in the effect of L-NNA on the chemoreceptor activity between sham and CHF rabbits was due to the changes in the arterial blood pressure.

Group B. In the isolated preparation, addition of L-NNA (10^{-5} and 10^{-4} M) to the buffer solution did not affect the baseline discharge or the response to hypoxia in either sham or CHF rabbits (data not shown). However, when L-arginine (10^{-4} M) alone was added into the perfusate, the baseline discharge and the responses to hypoxia were inhibited in sham but not in CHF rabbits (Fig. 4). The inhibitory effect of L-arginine on the activity of the CB chemoreceptors was reversed by the subsequent administration of L-NNA (10 mM) (Fig. 4).



Fig. 3. Effect of N° -nitro-L-arginine (L-NNA; 30 mg/kg iv) on activity of CB chemoreceptors under normoxic and hypoxic conditions (Pa₀₂ at 40–45 Torr) in intact preparations in sham (*A*; *n* = 7) and CHF (*B*; *n* = 7) rabbits. L-NNA increased baseline discharge and also the response to hypoxia in sham but not in CHF rabbits. Effect of L-NNA could be reversed by L-arginine (L-arg; 60 mg·kg⁻¹·min⁻¹). **P* < 0.05, L-NNA vs. control; †*P* < 0.05, hypoxia vs. normoxia.

Group C. D-NNA and D-arginine, when given in the same doses as the L-isomers, did not affect the activity of the CB chemoreceptors either in the intact or in the isolated preparation in sham rabbits (Fig 5).

Group D. In the intact preparation, administration of the NO donor SNAP (60 nmol \cdot kg⁻¹ \cdot min⁻¹ iv) inhibited the baseline discharge and the response to hypoxia in CHF but not in sham rabbits (Fig. 6). In the isolated preparation, SNAP (10⁻⁴ M) inhibited the baseline discharge and the response to hypoxia in both sham and CHF rabbits (Fig. 7). Administration of SNAP in the intact preparation decreased arterial blood pressure from 78 \pm 3 to 68 \pm 4 mmHg (P < 0.01) in sham rabbits and from 71 \pm 3 to 58 \pm 2 mmHg in CHF rabbits (P < 0.01). To assess the possibility that the decrease in blood pressure might contribute to the effect of SNAP on the CB chemoreceptors, experiments were conducted in one fiber from a sham and one fiber from a CHF rabbit, with the blood pressure maintained at the equivalent level induced by SNAP by partial occlusion of the inferior vena cava. It was found that an equivalent decrease in blood pressure caused by the vena cava occlusion did not alter the characteristics of either chemoreceptor in response to hypoxia.

In Vitro NO Production from the Carotid Bodies of Sham and CHF Rabbits

Basal NO production from the CB, CA, and SCG in the normoxic state was measured in vitro in six sham and eight CHF rabbits. It was found that the CBs from CHF rabbits produced markedly less NO than those from sham rabbits (Table 3). The NO production from the CA was also less in CHF than in sham rabbits (Table 3). Minimal NO was produced from the SCG, and no difference was found between CHF and sham rabbits (Table 3).

Histochemical Staining of NADPH-diaphorase in the CB from Sham and CHF Rabbits

NADPH-diaphorase histochemistry was carried out in the CB from seven sham and nine CHF rabbits. The histochemistry showed that the density of NADPHdiaphorase-positive sites within the CB from CHF rabbits was significantly less than that from sham rabbits (21 ± 5 vs. 34 ± 5 sites/0.5 mm², P < 0.05). Figure 8 illustrates the representative results from a CB of a sham rabbit and a CB of a CHF rabbit. No positive staining was found when the section was incubated with the oxidative form of NADP.



Fig. 4. Effect of L-arginine (10^{-5} M) on activity of CB chemoreceptors under normoxic and hypoxic conditions (Po₂ at 40–45 Torr) in isolated preparations in sham (*A*; *n* = 7) and CHF (*B*; *n* = 7) rabbits. L-arginine decreased baseline discharge and response to hypoxia in sham but not in CHF rabbits. **P* < 0.05, L-arginine vs. control; †*P* < 0.05, hypoxia vs. normoxia.



Fig. 5. Effect of D-NNA (30 mg/kg iv) in intact preparation (A; n = 6) and of D-arginine (10^{-5} M) in isolated preparation (B; n = 6) on activity of CB hemoreceptors in sham rabbits. These biologically inactive isomers did not affect baseline discharge or response to hypoxia (Po₂ at 40–45 Torr) of CB chemoreceptors in sham rabbits. $\dagger P < 0.05$, hypoxia vs. normoxia.

DISCUSSION

The major findings of the present study were as follows. 1) The baseline discharge in the normoxic state and the response to isocapnic hypoxia of the CB chemoreceptors were enhanced in CHF rabbits compared with sham rabbits. These alterations were independent of the CB preparations (intact vs. isolated). 2) NOS inhibition enhanced the sensitivity of CB chemoreceptors in sham but not in CHF rabbits. 3) Administration of NOS substrate, L-arginine, decreased the sensitivity of CB chemoreceptors in sham but not in CHF rabbits. 4) Increase in NO production independently of NOS activity by administration of SNAP inhibited the sensitivity of CB chemoreceptors in CHF rabbits. 5) The in vitro basal NO production from the CB and the varicosities of nerve fibers positive for NADPH-diaphorase within the CB were less in CHF rabbits than in sham rabbits. From these results, we conclude that input from CB chemoreceptors is enhanced in the rabbit model of pacing-induced CHF and that an attenuated NOS activity in the CB contributes to the enhanced activity of the CB chemoreceptors in this condition.

Previous studies have documented that an enhanced peripheral chemoreflex is a common finding in patients with increasing severity of heart failure (4-6). In our accompanying paper (26), we have reported similar results documenting that peripheral chemoreflex control of RSNA and ventilation is enhanced in conscious rabbits with pacing-induced CHF. The important question that arises from these studies is defining the mechanism(s) responsible for this altered chemoreflex function. Although we cannot tell from the present study whether the central gain of the peripheral chemoreflex is altered, our findings provide direct evidence for an enhanced afferent input from the CB chemoreceptors in the CHF state, which would provide a primary contribution to the augmentation of reflex function. Furthermore, the fact that the enhanced afferent sensitivity of the CB chemoreceptors is independent of the preparations (intact vs. isolated) suggests that an intrinsic alteration occurs within the CB in the CHF state.

Although there are numerous factors that may affect peripheral chemoreceptor function (1, 9), we were interested in the possible role that NO plays in this alteration. NO is of importance for several reasons. 1) NOS activity has been detected in the CB of rats (29), cats (11), and rabbits (19). 2) NO inhibits the activity of CB chemoreceptors in normal animals (19, 28). 3) NOS activity or NO production is decreased in the CHF state



Fig. 6. Effect of *S*-nitroso-*N*-acetyl-penicillamine (SNAP; 60 nmol·kg⁻¹·min⁻¹ iv) on activity of CB chemoreceptors in normoxic and hypoxic states (Pa₀₂ at 40–45 Torr) in intact preparation. SNAP inhibited activity of CB chemoreceptors in CHF rabbits (*B*; *n* = 6) but not in sham rabbits (*A*; *n* = 5). **P* < 0.05, SNAP vs. control; †*P* < 0.05, hypoxia vs. normoxia.



Fig. 7. Effect of SNAP (10^{-4} M) on activity of CB chemoreceptors in normoxic and hypoxic (Po₂ at 40–45 Torr) states in isolated preparation. SNAP inhibited activity of CB chemoreceptors both in sham (*A*; *n* = 5) and in CHF rabbits (*B*; *n* = 6). **P* < 0.05, SNAP vs. control; †*P* < 0.05, hypoxia vs. normoxia.

(16, 24, 30). These data imply that a reduction of NO release in the CHF state may lead to a disinhibition of the CB chemoreceptors, which could account for the enhanced afferent sensitivity of the CB chemoreceptors in the CHF condition.

The results of the present study provide direct evidence for the above hypothesis. We found that basal NO production from the CB and the density of NADPHdiaphorase-positive cells (a maker of NOS) (2, 27) within the CB were less in CHF rabbits than in sham rabbits. These results suggest an attenuated NOS activity and a lower content of NOS within the CB in CHF rabbits. Moreover, we found in the intact preparation that inhibition of NOS activity by administration of L-NNA enhanced the activity of the CB chemorecep-

Table 3. Basal NO production from CB, CA,and SCG in sham and CHF rabbits

	nmol	NO Production, nmol·mg wet tissue ⁻¹ ·30 min ⁻¹			
	СВ	CA	SCG		
Sham (<i>n</i> =6) CHF (<i>n</i> =8)	$\begin{array}{c} 8.40 \pm 1.71 \\ 1.83 \pm 0.19^* \end{array}$	$\begin{array}{c} 0.27 \pm 0.01 \\ 0.06 \pm 0.01 * \end{array}$	$\begin{array}{c} 0.07 \pm 0.01 \\ 0.08 \pm 0.01 \end{array}$		

Values are means \pm SE; n = no. of rabbits. NO, nitric oxide; CB, carotid body; CA, carotid artery; SCG, superior cervical ganglion. * P < 0.05, CHF vs. sham rabbits.



20 µm

Fig. 8. NADPH-diaphorase histochemistry in CB from a sham (A) and a CHF rabbit (B). Arrows indicate dense staining in neuronal structures.

tors in sham but not in CHF rabbits and that the administration of the NO donor SNAP inhibited the chemoreceptor activity in CHF but not in sham rabbits. These results indicate that a tonic inhibitory effect of NO on the activity of the CB chemoreceptors is fully expressed in the CB in sham rabbits but virtually absent in the CB in CHF rabbits. A possible nonspecific effect produced by L-NNA is unlikely, since the fact that the effect of L-NNA could be reversed by L-arginine and D-NNA was without affect. These data strongly support the idea that an attenuated NOS activity in the CB of CHF rabbits, rather than an inability to respond to NO, contributes to the enhanced activity of the CB chemoreceptors in this condition.

An augmented activity of the CB chemoreceptors was also identified in the isolated preparation in CHF rabbits. Unlike in the intact preparation, NOS inhibition produced by L-NNA (alone) did not affect the activity of the CB chemoreceptors in either sham or CHF rabbits. However, administration of the NOS substrate L-arginine (alone) to the isolated preparation attenuated the afferent activity of the CB chemoreceptors in sham but not in CHF rabbits. This result suggests that NO production in the isolated CB under our conditions was substrate limited in the sham group. By contrast, we found that SNAP (which produces NO independently of NOS activity) produced a similar inhibitory effect in the isolated preparation of both groups. This result further suggests that the enhanced sensitivity of the CB chemoreceptors in the isolated preparation from CHF animals could be linked to an attenuated NOS activity. These data support our hypothesis that the downregulation of NOS activity or a decrease in NO release in the carotid sinus region plays an important role in the alteration of the characteristics of the CB chemoreceptors in CHF rabbits.

Three isomers of NOS have been found, i.e., neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (3). From the data presented here it is not possible to determine which isomer is altered in the CB of rabbits with CHF. However, because NADPH-diaphorase histochemistry is relatively specific for the neuronal isomer (2), we believe that a downregulation of nNOS in the CB cells contributes to the reduced capacity to synthesize NO in the CHF state. The results from previous studies (19, 28, 29) indicate that NADPH-diaphorase-containing elements within the CB are mainly composed of nerve fibers and scattered neurons. We found that the density of NADPH-diaphorase-positive fibers is decreased in the CB in CHF rabbits.

However, a participation of eNOS in the alteration of the characteristics of the CB chemoreceptors in CHF rabbits cannot be excluded. A recent study supports that eNOS is more important than nNOS in the regulation of respiration in response to the stimulation of peripheral chemoreceptors in the normal condition (12). Because it is known that eNOS exists in the endothelium of the CB vasculature (19) and that eNOS activity is decreased in the CHF state (16, 24), it is reasonable to speculate that downregulation of NOS activity during CHF may result in a vasoconstriction within the CB and, therefore, lead to a decrease in the blood supply to the CB. The decreased blood supply may, in turn, cause the activation of the CB chemoreceptors.

It is not clear how NO alters O_2 sensitivity in the CB. The activation of chemoreceptors by hypoxia has been postulated to be due to decreased availability of an inhibitory chemical messenger (19, 20). It has been documented that *1*) many nerve plexuses innervating the chemoreceptor tissue were positive for NOS (19); *2*) inhibition of NOS activity augments CB chemoreceptor activity, indicating a tonic inhibitory effect of endogenous NO on the chemoreceptor activity (19, 28); and *3*) the NOS activity is less at low Po₂ compared with normoxic controls (21). Therefore, it is possible that low sensory activity during normoxia is due to a constant inhibitory action of NO, whereas increased sensory activity under hypoxia is due to disinhibition resulting from reduced production of NO. Because NOS activity or NO synthesis is attenuated in the CHF state (16, 24, 30, present study), less inhibition produced by NO and, therefore, an enhanced activity of the chemoreceptors should be expected in this condition.

Although one would expect little NO released from the CB in the isolated preparation from both sham and CHF rabbits because of the substrate limitation of the perfusate, an enhanced activity of the CB chemoreceptors (compared with shams) was still observed in the isolated preparation in CHF rabbits. This implies that, although NO is important in the alteration of the characteristics of the CB chemoreceptors in the CHF state, other factors besides NO may also play a role in this pathophysiological process. An alternative explanation is that the enhanced activity of the CB chemoreceptors results from an intrinsic alteration in the CB due to chronic downregulation of NO synthesis during the development of CHF, which cannot be mimicked by the acute depletion of NO as occurred during the preparation for the isolated carotid sinus.

We found that there was a reduction of blood flow in the carotid artery during severe hypoxia (Pa_{O_2} at 40–45 Torr) in both sham and CHF rabbits, when the afferent recording was performed in the intact preparation. Because the blood pressure did not change significantly during the same interval, the reduction of carotid blood flow was probably due to a vasoconstriction, which may have resulted from a sympathoadrenal activation in response to hypoxia and/or an attenuated cardiac function because of hypoxia. It is open to question whether the reduction of blood flow affected the sensitivity of the CB chemoreceptors to hypoxia in this instance. However, it seems unlikely that the reduction of blood flow to the carotid sinus area contributed to the difference in the sensitivity of the CB chemoreceptors between sham and CHF rabbits because the degree of reduction of blood flow was equivalent in both groups. Moreover, the enhanced sensitivity of the CB chemoreceptors to hypoxia in CHF was also observed in the isolated preparation where the flow was held constant.

Limitations

First, the present study was carried out in the anesthetized state. The anesthesia and the surgical procedure could directly or indirectly affect the characteristics of the CB chemoreceptor in response to hypoxia. Therefore, care should be taken when extrapolating the data obtained here to the clinical phenomena observed in patients. Second, we did not measure the conduction velocity of the afferent fibers of the CB. Therefore, it is not known what type of afferents from the CB was recruited in the present study. Third, a nonspecific NOS inhibitor L-NNA was employed in the present study. From the data presented here, it is impossible to determine which isomer is responsible for the alteration of the characteristics of the CB chemoreceptors in the CHF state. Further study is needed to evaluate the relative role of eNOS and nNOS.

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To our knowledge, this is the first time that the characteristics of the CB chemoreceptors have been examined in an experimental model of CHF by directly recording the single-unit activity from the CB afferents. The data shown in the present study provide direct evidence for an enhanced afferent input from the CB chemoreceptors in CHF rabbits, which suggests a possible mechanism for the augmentation of the peripheral chemoreflex function in the CHF state. Furthermore, our findings indicate that an attenuated NOS activity in the CB contributes to the enhanced activity of the CB chemoreceptors in this condition.

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