Dual effects of nitric oxide on cat carotid body chemoreception

RODRIGO ITURRIAGA, SANDRA VILLANUEVA, AND MATIAS MOSQUEIRA

Laboratorio de Neurobiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago 1, Chile

Received 4 February 2000; accepted in final form 2 May 2000.

Iturriaga, Rodrigo, Sandra Villanueva, and Matias Mosqueira. Dual effects of nitric oxide on cat carotid body chemoreception. J Appl Physiol 89: 1005–1012, 2000.—We studied the effects of nitric oxide (NO) released by NO donors on cat carotid body (CB) chemosensory activity during normoxia and hypoxia. CBs excised from pentobarbital sodium-anaesthetized cats were perfused with Tyrode at 38°C and pH 7.40. The frequency of chemosensory discharges (fx) was recorded from the carotid sinus nerve, and changes of NO concentration were measured by a chronoamperometric technique, with NO-selective carbon-fiber microelectrodes inserted into the CB. During steady chemosensory excitation induced by hypoxia, bolus injections of NO (∆NO = 0.5–12 μM), released by S-nitroso-N-acetylpenicillamine (SNAP) and 6-(2-hydroxy-1-methyl-nitrosohydrazino)-N-methyl-1-hexanamine (NOC-9), transiently reduced fx in a dose-dependent manner. However, during normoxia, the same concentration of NO (∆NO = 0.5–13 μM) released by the NO donors increased fx in a dose-dependent manner. The present results show a dual effect of NO on CB chemoreception that is dependent on the PO2 levels. During hypoxia, NO is predominantly an inhibitor of chemoreception, whereas, in normoxia, NO increased fx. The mechanisms by which NO produces chemosensory excitation during normoxia remain to be determined.

dual effect; hypoxia; oxygen

IT HAS BEEN PROPOSED THAT nitric oxide (NO) gas produced within the carotid body (CB) is an inhibitory modulator of hypoxic chemoreception (8, 28, 31, 34). Indeed, in the cat CB perfused in vitro, the administration of L-arginine (34), NO donors such as sodium nitroprusside (8) and nitroglycerine (34), and 25 ppm NO gas (18) reduces the amplitude of the chemosensory response to hypoxia. On the other hand, the NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) enhances the hypoxic response in the cat CB in situ (17) and in vitro (34). Furthermore, the inhibition of NOS increases basal chemosensory discharges in the cat CB in situ (17) and in vitro (8, 20, 28, 34). Sodium nitroprusside (SNP) reverses this excitatory effect in the cat CB perfused in vitro (8) but not in situ (17).

Recently, our laboratory studied the effects of SNP (1–2 mg/kg iv) on the carotid chemosensory responses to sodium cyanide (NaCN), dopamine, and hyperoxia in paralyzed and artificially ventilated cats (17). Unexpectedly, we found that SNP increased basal chemosensory discharges, although reducing both the NaCN-induced chemosensory excitation over baseline and the transient chemosensory inhibition induced by dopamine. Contrary to what is seen in the in situ preparation, our laboratory (2) and others (8, 35) have found that SNP reduced or had no effect on basal frequency of chemosensory discharge (fx) in the cat CB preparations superfused or perfused in vitro with saline solutions. A major difference between in vitro and in situ CB preparations is the presence of large amounts of endothelium and vascular smooth muscle tissue in the whole cat, which is needed to activate SNP for releasing NO (22). Thus it is likely that the large amounts of NO released from SNP in situ may account for the increased basal chemosensory activity. Indeed, it is well known that NO may impair the electron transport chain and oxidative phosphorylation (4, 6), conditions that are expected to increase chemosensory discharge (5, 25, 26). Accordingly, we would expect that high concentrations of NO should increase chemosensory discharge. To test this hypothesis, we studied the effects of NO released by NO donors on CB chemosensory activity during normoxia and hypoxia. Because we are aware that SNP is a potential releaser of cyanide ions (15), two different spontaneous NO donors, 6-(2-hydroxy-1-methyl-nitrosohydrazino)-N-methyl-1-hexanamine (NOC-9) and S-nitroso-N-acetylpenicillamine (SNAP) were used. This study was performed using a perfused cat CB, preparation in vitro that allowed fast delivery of the stimuli through the CB vessels and simultaneous recording of CB chemosensory discharge and chronoamperometric measurements of NO with carbon microelectrodes.

METHODS

Experiments were performed on 10 CBs excised from seven adult cats. The cats were anesthetized with pentobarbital sodium (40 mg/kg ip), followed by additional doses (12 mg iv) to maintain a level of surgical anesthesia. The carotid...
bifurcation including the CB and the carotid sinus nerve (CSN) was perfused with Tyrode solution as previously described (19). Briefly, the carotid bifurcation was cannulated through the common carotid artery, excised from the cat, and placed in a chamber. Each CB was perfused by gravity with Tyrode at pH 7.40, equilibrated with 20% O2 and 5% CO2, and simultaneously superfused with Tyrode equilibrated with 95% N2 and 5% CO2. The composition of the Tyrode was, in mM, 154.0 Na, 4.7 K, 2.2 Ca, 1.1 Mg, 123.0 Cl, 21.0 glutamate, 21.3 HCO3, 5.5 D-glucose, and 5.0 HEPES. The temperature of the fluid in the chamber was maintained at 38.0 ± 0.5°C with a regulated heating system. Chemosensory discharges were recorded from the CSN, which was placed on a pair of platinum electrodes and lifted into mineral oil. The neural signals were preamplified and amplified, filtered (10 Hz-1 kHz, notch filter 50 Hz), and fed to an electronic amplitude discriminator that allowed the selection of action potentials of a given amplitude above the baseline noise. The selected chemosensory impulses were counted with a frequency meter to measure ƒx expressed in Hz. The ƒx signal was digitized with an analog-digital board (DIGITADA 1200A, Axon Instruments) for later analyses.

NO was measured through a computerized chronoamperometric system (IVEC 10, Medical System). NO-sensitive electrodes consisting of single carbon fibers (30-μm diameter) covered with porphyrin and Nafion (Quanteon) were gently inserted into the CB. A potential of 0.9 V, with respect to the reference Ag-AgCl electrode, was applied for 100 ms at a rate of 5 Hz. The resulting oxidation current was digitally integrated during the last 80 ms of each pulse, averaged for five cycles, displayed at a rate of 1 Hz, and stored in the computer. The electrodes were calibrated with SNAP (100–600 μM) in Tyrode solution at pH 7.40 (see Ref. 33 for a similar method). Only carbon microelectrodes showing high sensitivity and linear currents (r2 > 0.95) were used. Using an independent technique for measuring NO, based on a gas-phase chemiluminescent reaction between NO and ozone, with a NO analyzer (Sievers 280, Sievers Instruments), we found that SNAP produced NO in a molar ratio of 1 to 0.01 in Tyrode solution (100 μM SNAP produces 1 μM NO). The integrated oxidation current was expressed as changes of NO concentration over baseline levels (ΔNO).

SNAP (18–1,800 μg) and NOC-9 (20–900 μg) were dissolved in Tyrode and injected into the perfusate line in boluses of 0.2–0.5 ml during normoxic (PO2 = 125 Torr) or hypoxic (PO2 = 30 Torr) perfusion of the CBs. Results were expressed as means ± SE. Statistical differences for multiple-dependent samples and for multiple samples were assessed with Kruskal-Wallis test, followed by paired comparisons through Conover test (30).

RESULTS

CB levels of NO during hypoxia. Figure 1 shows the effects of hypoxic stimulation on the NO-electrochemical signal and chemosensory discharges in two CBs. Hypoxia promptly increased ƒx to a maximal level, which showed a slight adaptation to a steady level. In all CBs, hypoxic perfusion increased ƒx from a baseline of 56.2 ± 12 Hz to a steady value of 334.7 ± 32.5 Hz (P < 0.01). The most common NO-electrochemical response observed in 9 out of 10 CBs is shown in Fig. 1A. Hypoxia produced a transient reduction of the NO-electrochemical signal that was followed by a recovery to the previous baseline value. However, hypoxia induced a late increase of the electrochemical signal after 10 min of hypoxia in only one CB (Fig. 1B). The time course of this late increase in the electrochemical signal resembles the delayed efflux of dopamine previously measured in the cat CB (17). Although porphyrin carbon fiber microelectrodes are more selective to NO than to dopamine (1 > 100), a high concentration of dopamine could be detected. This interpretation is supported by the fact that the amplitude of this electrochemical signal was reduced by repeated hypoxic stimuli, as happens with dopamine (16).

Effects of NO on carotid chemosensory activity during normoxia. Figure 2 shows the excitatory effects of increasing concentrations of NO produced by injections of 20, 40, and 200 μg NOC-9 and 1,800 μg SNAP on carotid chemosensory discharges during normoxic perfusion (PO2 = 125 Torr). Note that the ƒx peak preceded the corresponding NO peak by ~15–20 s. The inflexion of the NO signal after the injection of NOC-9 was due to the lower temperature of the NOC-9 solutions (room temperature vs. 38.5°C). Although the amplitude of the maximal ƒx responses induced by 40 and 200 μg NOC-9 were similar, the chemosensory response induced by 200 μg NOC-9 showed a secondary response. In this case, the chemosensory response induced by SNAP also consisted of two phases. An initial and rapid increase in ƒx, followed by a slow secondary response. This biphasic response resembles the chemosensory response to large doses of NaCN (50–100 μg). When we repeated the injections of NOC-9 or SNAP at intervals
as short as 2 min, we observed some degree of desensitization to the excitatory effects of NO.

The effects of different doses of SNAP and NOC-9 on $f_x$ during normoxia in six CBs are summarized in Fig. 3. The response to 180 $\mu$g SNAP was not significantly different from the control baseline, because, in most preparations, the dose of 180 $\mu$g did not increase NO. To study the correlation between the maximal concentrations of NO produced by injections of the NO donors and the maximal change in chemosensory activity

Fig. 2. Excitatory effects of three injections of 6-(2-hydroxy-1-methyl-nitrosohydrazino)-N-methyl-1-hexanamine (NOC-9; 20, 40, and 200 $\mu$g in 0.2 ml) and one injection of S-nitroso-N-acetylpenicillamine (SNAP; 1.800 $\mu$g in 0.2 ml) into one CB during normoxia. Arrowheads indicate NO donor injections.

Fig. 3. Summary of the excitatory effects produced by several doses of SNAP (A) and NOC-9 (B) on chemosensory activity during normoxia in 6 cat CBs. Maximal $f_x$ was attained during hypoxia (solid bars) and basal $f_x$ (open bars). *$P < 0.01$, +$P < 0.05$, statistical difference compared with basal $f_x$.

Effects of NO on carotid chemosensory activity during hypoxia. Figure 6 compares the effects of three doses of NOC-9 on two CBs during steady chemosensory excitation induced by hypoxia ($P_{O_2} \approx 30$ Torr). Hypoxia increased $f_x$ to a steady level, whereas NO released by NOC-9 transiently reduced the increased $f_x$ in a dose-dependent manner. The doses of 40 and 200 $\mu$g NOC-9 that increased NO to ~1 and 6 $\mu$M, respectively, reduced, but did not entirely abolish, the

Fig. 4. Linear correlation between maximal changes in NO produced by NOC-9 and SNAP and changes in chemosensory activity ($\Delta f_x$) during normoxia in 5 CBs. At least 2–3 different doses of NOC-9 or SNAP were injected in each CB. $\Delta f_x$ = maximal $f_x$ – basal $f_x$; $r$, correlations coefficient.

Fig. 5. Effect of the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide (PTIO) on the chemosensory excitation induced by 900 $\mu$g of SNAP. Arrowheads indicate bolus injections of SNAP and PTIO.
chemosensory excitation induced by hypoxia (Fig. 6A). However, a large dose of NOC-9 (400 μg), which increased NO up to 11 μM, entirely abolished the hypoxia-induced chemosensory excitation (Fig. 6B). When low doses of NOC-9 were injected during hypoxia, chemosensory activity returned to the previous baseline values on withdrawal of the hypoxic perfusion (Fig. 6A). However, after the application of large doses of NOC-9 (400–800 μg) during hypoxia, the chemosensory activity presented large excitatory rebound, as is shown in Fig. 6B. This rebound was observed in all CBs, even when they were perfused with normoxic Tyrode solution. Note that the 200-μg dose of NOC-9 initially produced a brief chemosensory excitation, which was then followed by a more marked inhibition (Fig. 6A). This dual effect of NO on \( f_x \) during hypoxia was commonly observed, but its magnitude was variable. Figure 7 shows the clear dual effect of NO on \( f_x \) during hypoxia. Almost the same level of NO produced by 1,800 μg SNAP caused chemosensory excitation during normoxia. However, during hypoxia, NO produced a dual effect consisting of an initial but brief chemosensory excitation followed by a more marked inhibition of chemosensory activity.

Figure 8 illustrates the linear correlation between the maximal peak of NO and the change in \( f_x \) (\( \Delta f_x = \text{minimum } f_x - \text{steady } f_x \)) attained during steady chemosensory excitation induced by hypoxia in five CBs. In this case, the correlation \((r = 0.87, P < 0.05)\) was negative and significant, indicating that large NO amounts produced chemosensory inhibition in a dose-dependent manner during hypoxia.

When testing the responses to repeated injections of NOC-9 and SNAP during hypoxia, we observed some desensitization of the inhibitory effects of NO. Figure 9 shows the responses to three NOC-9 injections of 200 μg and one SNAP injection of 900 μg during steady chemosensory excitation induced by hypoxia. The first two injections of NOC-9 that increased NO \( \sim 4–5 \) μM produced a sharp reduction of chemosensory discharges. However, two successive injections of a large dose of NOC-9 and SNAP performed 3 min later produced a modest reduction of \( f_x \), although they increased NO up to 8 and 6 μM, respectively.

Effects of several doses of NO on CB oxygen sensing. In six preparations, we tested the effects of repeated large doses of NO donors. After several injections of
large doses of NO donors during normoxia, we observed a spontaneous increase in basal chemosensory discharges, up to maximal discharge, in three of the CB preparations. This spontaneous increase of basal discharges was reversed by hyperoxic perfusion. Figure 10 shows an example in which, after eight large doses of SNAP (900 and 1,800 μg) applied during normoxia, basal $f_x$ spontaneously increased up to 400 Hz. The increased $f_x$ returned to baseline only when the CB was perfused with Tyrode equilibrated with hyperoxia (95% O₂ and 5% CO₂). Switching back from hyperoxia to hypoxia or normoxia resulted in an increase of $f_x$ to maximal discharge. Note that during hyperoxic or normoxic perfusion, SNAP did not further increase $f_x$ (Fig. 10B). These results suggest that oxygen sensing was affected by large NO concentrations.

**DISCUSSION**

According to the current hypothesis of chemoreception, the glomus cells of the CB are the primary sites of transduction of the hypoxic stimulus. In response to hypoxia, glomus cells are expected to release one (or more) excitatory transmitter(s) that in turn increases the frequency of discharges in nerve terminals of chemosensory petrosal neurons (13). In addition to the excitatory transmitter(s), other molecules produced within the CB may act as chemical modulator(s) of the chemosensory process. It has been proposed that endogenous NO is a tonic inhibitor of carotid chemoreception to hypoxia (8, 20, 27, 35). This proposition is based on the immunocytochemical localization of NOS in autonomic and petrosal sensory fibers (14, 28, 34, 35) and on the use of pharmacological tools, i.e., NO donors and NOS inhibitors (8, 20, 28, 34). The present results agree and extend previous observations that administration of NO donors such as nitroglycerine and SNP (8, 34) to the cat CB perfused in vitro reduces the chemosensory response to hypoxia. In addition, we found, in the present study, that large doses of NO donors abolished the chemosensory excitation induced by hypoxia. This observation suggests a crucial role for NO on hypoxic chemoreception. However, we do not know if the complete inhibition of NO on $f_x$ is a physiological or pharmacological effect because the level of NO in the CB is unknown. Our results cannot rule out that the inhibition of chemosensory excitation during hypoxia induced by NO was, in part, due to a vasodilatation (23, 34, 35). Recently, Lahiri and Buerk (23), using a similar in vitro perfused preparation of the cat CB, found that SNP infusion increases CB tissue Po₂ and reduces basal discharges, supporting the idea that part of the inhibitory effect of NO on CB chemoreception is secondary to vascular changes. However, it is not clear how a vasodilatation may totally explain the inhibition of the increased $f_x$ during perfusion and superfusion of the CB with hypoxic media (Po₂=30 Torr). In addition to vascular-dependent mechanisms, the glomus cells and petrosal neurons are other sites for the inhibitory actions of NO. Indeed, Summers et al. (29) found that NO donors such as SNP and spermine inhibit L-type Ca²⁺ currents in rabbit glomus cell through a cGMP-independent mechanism, which is mediated by a direct modification of the thiol groups of the calcium channel proteins. On the other hand, we found that SNP and L-NAME modulate the acetylcholine-induced activity in isolated petrosal ganglion neurons that selectively project through the carotid sinus nerve (3). SNP reduced the sensitivity and amplitude of dose-dependent increases of carotid sinus nerve frequency of discharge induced by acetylcholine, whereas L-NAME slightly enhanced the response (1). In the isolated petrosal ganglion, the...
superfusion with a low concentration of SNP did not change the basal frequency of carotid sinus nerve discharge; however, we did not study the effect of other NO donors (1).

In addition to the chemosensory inhibition produced by NO during hypoxia, our results show that the same concentrations of NO produced chemosensory excitation after normoxic perfusion. Furthermore, after several large doses of SNAP or NOC-9, basal \( f_x \) increased spontaneously up to the maximal discharge in one-half of the CBs studied. Similar to what we found in situ, the increased basal \( f_x \) returned to normal baseline levels only when the CB was perfused with hyperoxia, suggesting that the oxygen sensing mechanisms of the chemoreceptor cells were impaired. The transient chemosensory excitation produced by bolus injections of NO donors seems to be mediated by NO, because the selective NO scavenger PTIO reduced the excitatory response elicited by SNAP. The mechanisms underlying the transient and prolonged excitatory effects of NO on chemosensory discharges during normoxia remain to be identified. However, the most possible targets for the action of NO are the soluble guanylyl cyclase and the cytochrome oxidase, because these enzymes are highly sensitive to NO in the nM and \( \mu \)M range (4, 9). As it was mentioned above, a vasodilator effect of NO produced by the activation of guanylyl cyclase in smooth muscle is expected to reduce chemosensory discharge (23, 35), but an impairment of cytochrome oxidase redox activity is expected to increase chemosensory discharges (5, 25, 26). In recent years, the effects of NO on the respiratory chain and oxidative phosphorylation have received great attention. It is well known that NO inhibits mitochondrial respiration at different levels, reducing oxygen consumption. Indeed, NO at low concentrations (50 nM-5 \( \mu \)M) specifically and reversibly inhibits cytochrome oxidase (complex IV) in competition with \( O_2 \) (4). Nevertheless, at higher concentrations (>5 \( \mu \)M) NO also inhibits other complexes of the respiratory chain in isolated rat heart mitochondria (6). Several mechanisms, such as nitrosylation, oxidation of protein thiols, and removal of iron from iron-sulphur centers have been proposed to explain the effects of NO on the respiratory chain (4). Thus it is likely that the concentrations of NO released by NO donors in our experiments (0.5–13 \( \mu \)M) may impair the electron transport chain and oxidative phosphorylation in the mitochondria of the glomus cells. However, at high levels, NO may also interact with other nonrespiratory molecules such as free radicals, oxygen, superoxide anion, and iron and thiol groups in protein. Some of these reactions result in the oxidation of NO to nitrite and nitrate, which terminates its effect, but the products of other potential reactions may modify protein structure and function. Consequently, we cannot rule out any effects of large amounts of NO on glomus cell intracellular calcium and neurotransmitter release.

Another possible mediator for the NO effects is its metabolite peroxinitrite. NO reacts with the superoxide anion to form peroxinitrite on a molar basis. Peroxinitrite is a potent oxidant and nitrating agent, which may react with the amino acid tyrosine in cellular proteins, converting it to nitrosotyrosine. Nitration may in turn affect protein function. Recent studies have shown that peroxinitrite formation mediates prolonged vasoconstrictor in cerebral (10) and cardiac vascular territories after hypoxia-reoxygenation (38). Because we used a perfused preparation of the CB, it is possible that peroxinitrite, acting as a vasoconstrictor, may have counteracted the vasodilator effect of NO and mediated the excitatory effects of NO donors on CB chemoreception in normoxia. However, the vasoconstrictor effect of peroxinitrite occurred at concentrations >25 \( \mu \)M, whereas vasodilatation prevailed at lower concentrations (10). This value exceeded the maximal NO level released by the donors in this study (NO = 13 \( \mu \)M). In addition, peroxinitrite acts as a potent vasodilator in other vascular territories (7, 32); however, it may impair vascular relaxation, which can be prevented by coinfusion with SNAP (32). High concentrations of peroxinitrite can also produce inhibition of mitochondrial complexes I, II, and IV, damage to the mitochondrial membrane, mitochondrial swelling, depolarization, and calcium release (4, 6).

It has been proposed that CB chemosensory excitation induced by hypoxia may be the result of a decreased availability of an inhibitory chemical messenger such as NO (27). Given the fact that inhibition of NOS activity increases basal chemosensory discharges and NOS activity is reduced at low \( P_{O_2} \) related to normoxic controls (28), it is likely that at low concentrations of endogenous NO may exert a tonic inhibitory effect on chemosensory discharges during normoxia. Thus it is possible that the increased chemosensory activity induced by hypoxia resulted from reduced production of NO. Our results did not support this hypothesis because they showed that NO production did change or was only initially reduced during hypoxia and then remained constant during 2–5 min of hypoxic stimulation. The method used in the present study did not allow for measuring of the actual NO levels in the CB, because the NO oxidation current was expressed as changes of NO concentration over baseline. The basal level of NO in the CB remains unknown. However, the physiological tissue concentration of NO ranged between 20 nM-1 \( \mu \)M (21). From the above discussion, it is clear that NO may influence oxygen sensing in the CB by several mechanisms. However, a novel possibility is that the ratio NO/\( O_2 \) may be crucial to regulating the respiratory rate, playing a physiological role in oxygen sensing. Moreover, the recent finding that the mitochondria produce significant amounts of NO to regulate their own respiration (11, 12) suggests that NO may be important for the physiological regulation of energy metabolism. It is possible that the NO inhibition of cytochrome oxidase may be involved in the physiological regulation of respiratory rate (11, 12); however, there is no definitive evidence showing that NO regulation of mitochondrial respiration occurs in situ, and the interpretation is complicated because NO may also affect tissue respiration by cGMP-depen-
dient mechanisms (4). The dual effects of NO on chemoreception observed herein resemble the effects of carbon monoxide (CO) on cat CB chemoreception to hypoxia (24). At low concentrations, CO inhibits hypoxic chemoreception, presumably by binding to a membrane CO-binding pigment, which is not the cytochrome oxidase a3. At high concentrations, CO produces chemosensory excitation, which is fully reversed by bright light (21). Wilson et al. (36) found that the photochemical action spectrum showed a 432:590 nm ratio of ~6, which is characteristic of the CO complex of mitochondrial cytochrome oxidase.

In summary, our results show that NO induced a dual effect on carotid chemosensory discharges depending on the PO2 level. At low PO2, NO is predominantly an inhibitor of the increased chemosensory discharges, whereas, at normoxia, NO increases chemosensory discharges. During hypoxic stimulation in some CBs, we also observed a dual effect consisting in a rapid excitation followed by a more prolonged inhibition. The inhibitory effect of NO is compatible with its known action on CB blood vessels (23, 34), glomus cells (29, 34), and petrosal neuron activity (1). The mechanism underlying the excitative effect on chemosensory discharges is unknown, but it seems to be related with the oxygen sensing mechanism of the chemoreceptor cells (5).

We thank Carolina Larraín for assistance in the preparation of the experiments and the manuscript.

This work was supported by National Fund for Scientific and Technological Development of Chile Grant 198–0965.

REFERENCES

33. Wada K, Kamisaki Y, Ohkura T, Kanda G, Nakamoto K, Kishimoto Y, Ashida K, and Itoh T. Direct measurement of


