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Carbon monoxide: A role in carotid body chemoreception

(heme oxygenase 2/zinc protoporphyrin IX)

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ABSTRACT **Carbon monoxide (CO), produced endogenously by heme oxygenase, has been implicated as a neuronal messenger. Carotid bodies are sensory organs that regulate ventilation by responding to alterations of blood oxygen, CO_2, and pH. Changes in blood gases are sensed by glomus cells in the carotid body that synapse on afferent terminals of the carotid sinus nerve that projects to respiratory-related neurons in the brainstem. Using immunocytochemistry, we demonstrate that heme oxygenase 2 is localized to glomus cells in the cat and rat carotid bodies. Physiological studies show that zinc protoporphyrin IX, a potent heme oxygenase inhibitor, markedly increases carotid body sensory activity, while copper protoporphyrin IX, which does not inhibit the enzyme, is inactive. Exogenous CO reverses the stimulatory effects of zinc protoporphyrin IX. These results suggest that glomus cells are capable of synthesizing CO and endogenous CO appears to be a physiologic regulator of carotid body sensory activity.**

Carotid bodies are sensory organs that regulate respiratory responses to alterations in partial pressures of arterial blood oxygen (P_{O_2}). Hypoxia increases discharge frequency of the carotid sinus nerve, which conveys the sensory information from the carotid body to respiratory neurons in the brainstem (1). Several lines of evidence suggest that glomus cells (also called type I cells) are the primary chemoreceptor cells that detect changes in arterial P_{O_2}. While the mechanisms of transduction are debated, it seems certain that neurotransmitters released from glomus cells play important roles in carotid body chemoreception.

Recent studies suggest that carbon monoxide (CO) is formed in the nervous system and may function as a chemical messenger (2). CO is formed by heme oxygenase (HO), which oxidatively cleaves the heme ring, releasing CO and biliverdin. Two forms of HO have been isolated. One form, HO-1, is enriched in spleen and liver and is induced by heme and numerous oxidative stressors. By contrast, HO-2 is not inducible and is most abundant in brain (2), where HO-2 mRNA and protein are localized to discrete neuronal populations (2, 3). CO may influence some forms of synaptic plasticity, as hippocampal long-term potentiation is inhibited by zinc protoporphyrin IX (ZnPP-IX), an inhibitor of HO-2, and enhanced by exogenous administration of CO (4, 5). These studies suggest that CO may be a neurotransmitter in the nervous system. In the present study we demonstrate that the carotid body contains HO-2, the enzyme that synthesizes CO, and assess the effects of inhibition of this enzyme on carotid body sensory activity.

MATERIALS AND METHODS

Adult cats (2–3.4 kg) and rats (Sprague–Dawley, 100–150 g) of either sex were anesthetized with pentobarbital sodium (40 mg/kg; i.p.), the trachea was intubated, and a femoral artery and vein were cannulated. The arterial catheter was used for monitoring arterial blood pressure. Systemic administration of fluids was accomplished through the venous catheter. To expose the carotid bifurcation, the trachea and esophagus were ligated above the site of tracheal cannulation, sectioned, and retracted rostrally. The exposed tissues were covered with cotton gauze soaked in saline. Body temperature was maintained at 38 ± 1°C by a heating blanket governed by a rectal thermistor probe.

Antiserum to HO-2 was developed as follows. A peptide based on amino acids 247–258 of HO-2 (6) was synthesized, conjugated to bovine serum albumin, and injected into rabbits as described (7). Antiserum was affinity purified using a column consisting of a thyroglobulin–HO-2 conjugate immobilized on CNBr-activated Sepharose (7). For Western blot analysis, rat brain microsomes were prepared in 0.32 M sucrose, and protein (100 μg per lane) was separated on a 10% SDS/polyacrylamide gel, transferred to Immobilon-P membranes (Millipore), and probed with affinity-purified antibody (1 μg/ml) overnight. Blots were washed and developed by enhanced chemiluminescence (Amersham) and exposed to Kodak X-AR film. For preadsorption experiments, antibody was preincubated with a 20-fold excess of HO-2 peptide at 4°C for 24 hr.

For immunohistochemistry, anesthetized animals were perfused with heparinized saline (100 units/kg) followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3) at room temperature for 30 min. Carotid bodies were removed, postfixed in 4% paraformaldehyde in PB for 1 hr, rinsed in PB, and infiltrated in 30% sucrose in PB. Tissues were then placed in embedding medium (Tissue-Tek, Miles). Cryostat sections, 8 μm thick, were mounted on gelatin-coated slides. Slides were exposed to Tris-buffered saline/0.3% H_2O_2 for 15 min and permeabilized in 0.4% Triton X-100 for 30 min before being blocked for 1 hr in 4% normal goat serum. Slides were then incubated in primary antibody (8 μg/ml). For preadsorption experiments, primary antibody was incubated in a 20-fold excess of HO-2 synthetic peptide for 24 hr at 4°C. Immunostaining was developed with avidin-biotin using diaminobenzidine as a chromogen (Vectastatin ABC kit, Vector Laboratories).

Chemoreceptor activity was recorded from isolated carotid body as described (8). Briefly, the carotid bifurcation together with sinus nerve was dissected from anesthetized cats. Connective tissue was removed and the carotid bifurcation was placed in a Perspex chamber having two compartments, A and B. The carotid sinus nerve was pulled through the small opening in the partition separating the two compartments, placed on a platform in chamber B, and covered with warm mineral oil. The common carotid artery in chamber A was cannulated and superfused with Krebs solution at a rate of

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Abbreviations: ANOVA, one-way analysis of variance; CuPP-IX, copper protoporphyrin IX; HO-1, heme oxygenase 1; HO-2, heme oxygenase 2; ZnPP-IX, zinc protoporphyrin IX.

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2–4 ml/min and at a temperature of 36 ± 1°C. Chamber A was closed with a lid to prevent exposure of the carotid body to atmospheric air. The bathing medium (pH 7.38) contained (in mM) NaCl, 110; sucrose, 54; KCl, 5; glucose, 5.5; $MgCl_2$, 0.5; Hepes, 5; and $CaCl_2$, 2.2. Reservoirs with superfusion medium were bubbled with 21% O_2. Action potentials were recorded from thin filaments dissected from the sinus nerve using conventional electrophysiological equipment. Porphyrin analogues were added to the reservoirs containing the bathing medium.

Porphyrins—i.e., ZnPP-IX and copper protoporphyrin IX (CuPP-IX)—were obtained from Porphyrin Products (Logan, UT). Stock solutions were prepared by dissolving porphyrins in 9 ml of distilled H_2O adjusted to pH 11 with 1 M NaOH brought to 10 ml with 100% ethanol and diluted with the medium used for irrigating the carotid body. Maximum alcohol content did not exceed 0.1% and pH was maintained at 7.4. All results are expressed as mean ± SEM. Statistical analysis employed a paired *t* test or one-way analysis of variance (ANOVA) combined with Tukey's test.

RESULTS

HO-2 Is Localized to Glomus Cells. We developed antisera to HO-2 utilizing a synthetic peptide from the carboxyl-terminal region of rat HO-2. Western blot analysis indicates a single discrete band at 36 kDa in brain tissue, corresponding to authentic HO-2 (Fig. 1*A*). Immunocytochemical analysis of the rat (Fig. 1*B*) and cat (Fig. 1*C*) carotid body reveals intense staining of cells. Staining is cytoplasmic, with no staining of the nucleus. The HO-2-positive cells also stain for tyrosine hydroxylase, suggesting they are glomus cells (data not shown). Sustentacular or type II supporting cells and nerve fibers fail to stain for HO-2.

HO-2 Inhibition Augments Chemoreceptor Activity. To assess the functional relevance of HO, we monitored the effects of ZnPP-IX, a potent HO inhibitor, on sensory discharge of carotid bodies isolated from anesthetized cats. The *in vitro* carotid body preparation avoids potential systemic effects of HO inhibitors (e.g., changes in blood pressure) that may by themselves influence chemosensory activity. Lowering the pressure in the carotid sinus nerve by interrupting the flow for 10–15 s has no effect on the sensory activity, suggesting that the action potentials are not of baroreceptor origin. By contrast, hypoxia (perfusate P_{O_2} = 38 ± 11.4 mmHg; P_{CO_2} = 3 ± 1 mmHg; pH 7.35–7.42) increases sensory activity from 12.6 ± 2.8 to 30.5 ± 5.4 impulses per s ($P < 0.01$, $n = 8$), indicating that the sensory activity is of carotid body origin.

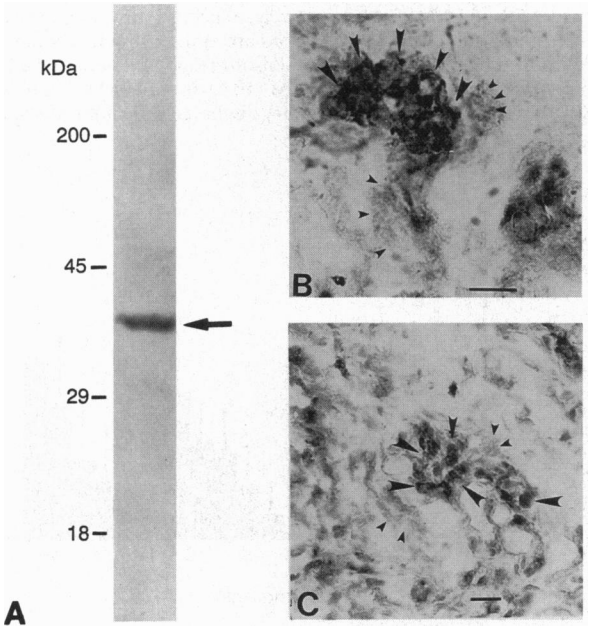

FIG. 1. (*A*) Immunoblot characterization of anti-HO-2 antibodies against rat brain microsomes. The immunoaffinity-purified antiserum to an HO-2-selective synthetic peptide recognizes a single band of 36 kDa (arrow), corresponding to authentic HO-2 (6). (*B* and *C*) HO-2-immunoreactive glomus cells in rat (*B*) and cat (*C*) carotid bodies. Glomeruli composed of individual glomus cells (large arrowheads) stain intensely for HO-2, whereas supporting elements (sustentacular or type II cells, small arrowheads) fail to demonstrate HO-2 immunoreactivity. (Bars = 25 μm.)

As little as 0.3 μM ZnPP-IX significantly enhances the sensory activity with maximal augmentation at 3 μM (Fig. 2). These potencies resemble those of ZnPP-IX in inhibiting HO (10). Effects of ZnPP-IX on carotid body activity are first discernable at 5 min and plateau by 10 min. Within 10 min after terminating the perfusion with ZnPP-IX, sensory activity returns to control values, indicating that the effects of ZnPP-IX are reversible. To examine the effect of vehicle on carotid body activity a volume equal to that required to dissolve the maximum dose of ZnPP-IX (10 μM) was placed in the bathing medium. Baseline activity in these six experiments, 14.7 ± 4.6 impulses per s, is unaltered by 15 min of perfusion with vehicle (12 ± 4.4, $P = >0.05$).

Four carotid body preparations with clear action potentials failed to show changes in discharge frequency in response to ZnPP-IX or hypoxia (data not shown), indicating that effects of ZnPP-IX depend on the viability of the preparation.

Substitution of other metalloporphyrins for the iron protoporphyrin normally bound to guanylyl cyclase results in inhibition of cyclase activation by NO (11). To assess whether guanylyl cyclase inhibition by ZnPP-IX accounts for the observed effects, we examined CuPP-IX, which inhibits guanylyl cyclase as potently as ZnPP-IX, but which does not inhibit HO (10). If the stimulatory effects of ZnPP-IX on the carotid body are due to its actions on guanylyl cyclase, then CuPP-IX at doses comparable to ZnPP-IX should augment the carotid body activity. CuPP-IX had either no effect or a very small stimulatory effect on carotid body activity (Fig. 3*A*). Average activity of the carotid body is not significantly affected at four doses of CuPP-IX tested (Fig. 3*B*).

Chemoreceptor tissue was perfused with ZnPP-IX at 3 μM, a dose that maximally stimulates the carotid body. During peak activation, 0.3 ml of CO-equilibrated medium was administered as a bolus close to the carotid body without interrupting the perfusion with ZnPP-IX (Fig. 4). CO prevents the carotid body stimulation by ZnPP-IX, and the effects of CO are reversible within 5 min. The same volume of superfusion medium without CO either has no effect or diminishes the activity transiently for 10 s.

DISCUSSION

The major findings of the present study are (*i*) HO-2 protein is present in glomus cells of the cat and rat carotid bodies; (*ii*) ZnPP-IX, an HO-2 inhibitor, augments carotid body activity in a dose-dependent manner, whereas CuPP-9 has no effect; and (*iii*) effects of ZnPP-IX are reversed by exogenous of CO.

HO-2 is localized to the glomus cells and not in the nerve fibers or other cell types. Thus, glomus cells are the primary source of HO-2 in the carotid body. Not all glomus cells stain for HO-2. Whether this reflects functional heterogeneity among glomus cells is unclear.

The following observations indicate that ZnPP-IX-induced carotid body activation is due to blockade of HO-2. CuPP-9, which does not inhibit HO-2 (10), fails to influence the carotid sinus nerve activity (Fig. 2). Since CuPP-9 is as potent as ZnPP-IX in inhibiting guanylyl cyclase, the stimulatory effects of ZnPP-IX cannot be explained by its direct actions on

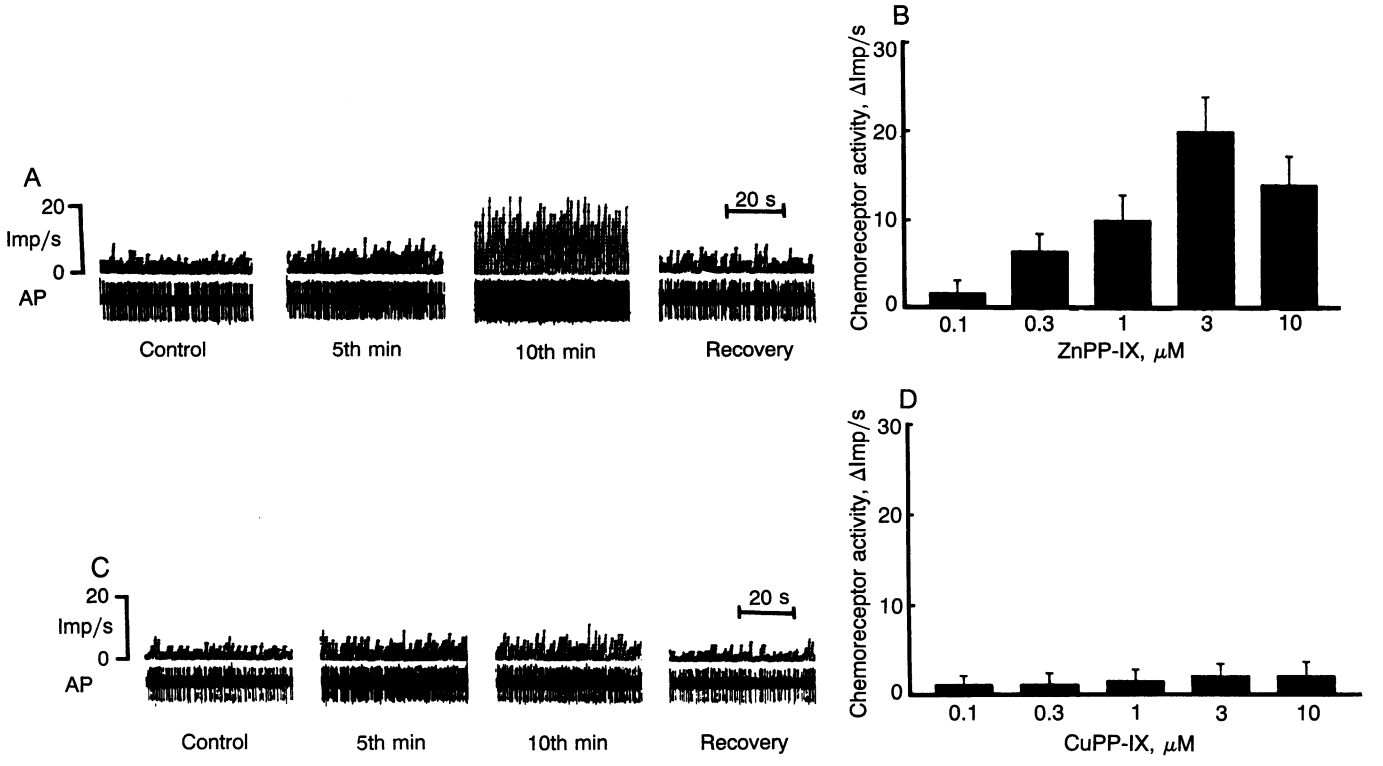

FIG. 2. ZnPP-IX but not CuPP-IX increases carotid sinus nerve firing. (*A*) ZnPP-IX (3 μM) enhances carotid body sensory activity *in vitro*. The carotid body was perfused and superfused with Krebs solution as described (8, 9). The P_{O_2} and P_{CO_2} of the bathing medium were 142 and 2 mmHg, respectively (pH 7.4; 36°C). Imp/s, integrated sensory discharge frequency impulses per s; AP, action potentials recorded from the carotid sinus nerve; Control, baseline sensory activity during normoxia; 5th and 10th min, sensory activity at the 5th and 10th min, respectively, of perfusion with ZnPP-IX; Recovery, sensory activity 15 min after terminating the perfusion with ZnPP-IX. Sensory activity increased progressively in the presence of ZnPP-IX. After terminating the infusion, activity returned to baseline, reflecting reversibility of the response. (*B*) Augmented carotid body sensory response to different doses of ZnPP-IX. Data are means ± SEM from 9 carotid bodies for all doses except for 10 μM (6 carotid bodies). ZnPP-IX augmented the carotid body activity in a dose-dependent manner. Compared to 3 μM, there was a tendency for a decrease in activity at 10 μM, but the difference was not significant ($P > 0.05$; ANOVA). (*C*) CuPP-IX (3 μM) fails to affect carotid body sensory activity *in vitro*. Control, baseline sensory activity during normoxia; 5th and 10th min, sensory activity at 5th and 10th min, respectively, of perfusion and superfusion with CuPP-IX; Recovery, sensory activity 15 min after terminating the perfusion with CuPP-IX. Other abbreviations are as in *A*. In some instances, CuPP-IX elicited a small increase in firing, but this effect was not consistently reproducible. (*D*) Carotid body sensory response is unaffected by different concentrations of CuPP-IX. Data are means ± SEM from 8 carotid bodies for all doses except for 3 μM (10 carotid bodies). There was a tendency for small increases in sensory activity with increasing doses of CuPP-IX. However, the increases in sensory discharge were not statistically significant compared to control baseline activity ($P > 0.05$; ANOVA).

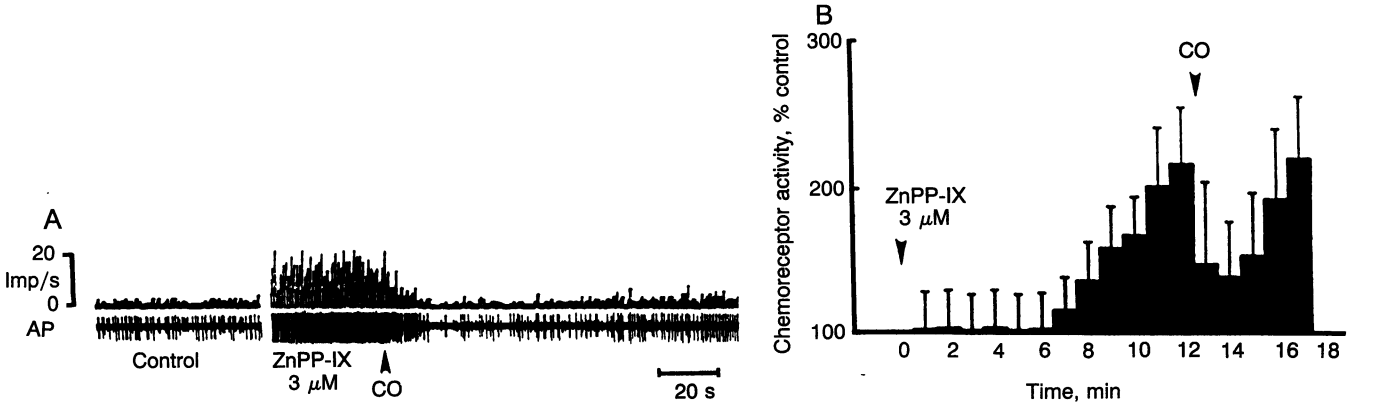

FIG. 3. (*A*) CO reverses ZnPP-IX-induced augmentation of carotid body activity. Control, baseline sensory discharge during normoxia; ZnPP-IX, sensory discharge during 10th min of perfusion with ZnPP-IX (3 μM). At the arrowhead, 0.3 ml of superfusing solution bubbled with 100% CO (for 15 min) was given close to the carotid body, without interrupting the perfusion to the carotid body. The volume of the perfusion chamber was 1.5 ml and the rate of perfusion of the carotid body was 4 ml/min. CO markedly attenuated ZnPP-IX-induced augmentation of the carotid body. Administration of the same volume of superfusion medium (control) either had no effect or diminished the activity transiently for 10 s (data not shown). (*B*) Time course for the reversal by CO of chemosensory response to ZnPP-IX (3 μM). Changes in chemoreceptor activity are presented as percent of baseline activity. Increase in sensory activity commenced from the 6th min of perfusion with ZnPP-IX (including the lag time of 1.5 min for the perfusion medium to reach from the reservoir to the carotid body). Sensory activity progressively increased in the ensuing 6 min. CO markedly attenuated the ZnPP-IX-induced augmentation of sensory activity, and the effects were reversible within 5 min. Data are means ± SEM from 5 carotid bodies.

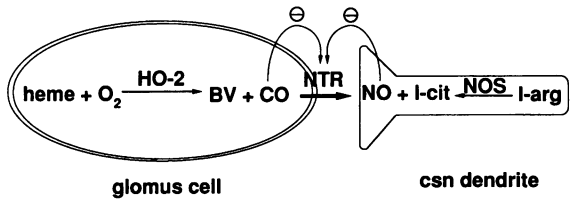

FIG. 4. Model of carotid body function. HO-2 cleaves heme to form CO and biliverdin (BV) in a reaction that is absolutely dependent upon molecular O_2. HO-2 is localized to glomus cells. Glomus cells release an excitatory transmitter (NTR) to augment firing of carotid sinus nerve. CO inhibits release of this transmitter by an autocrine or paracrine action on glomus cells. Nitric oxide (NO) synthase (NOS) converts arginine to NO and citrulline (cit). NOS is localized to carotid sinus neurons (csn), which receive synaptic input from glomus cells (8, 9). NOS inhibition accelerates carotid sinus nerve firing by removing a tonic inhibition on glomus cell activity physiologically mediated by NO. Thus, NO from carotid sinus neurons appears to regulate glomus cells as a retrograde messenger as has been suggested for NO influences on hippocampal long-term potentiation. Hypoxia augments glomus cell discharge and carotid sinus nerve firing (1). Hypoxia might directly inhibit HO-2 activity by decreasing O_2 availability, accelerating carotid sinus nerve firing as occurs with ZnPP-IX, a potent HO-2 inhibitor.

guanylyl cyclase. HO-2 converts heme to iron and biliverdin as well as CO. Inhibition of CO formation likely accounts for ZnPP-IX effects, because exogenous CO attenuates the carotid body response to ZnPP-IX.

Our findings implicate CO as a physiologic inhibitor of carotid body activity. In addition to our data with exogenous CO, Lahiri *et al.* (12) recently reported that low but not high doses of CO inhibit the sensory activity of the isolated carotid body. Since ZnPP-IX increases baseline activity, CO may maintain carotid body sensory activity at low levels in normoxia.

How might CO act in the carotid body? Hypoxia causes glomus cells to release a presumed excitatory transmitter that augments sinus nerve activity (1). The selective localization of HO-2 to glomus cells implicates CO in this process (Fig. 4). CO activates guanylyl cyclase by binding to iron in heme, which is at the active center of the enzyme, analogous to the actions of NO (13). cGMP immunoreactivity in the carotid body is selectively localized to glomus cells and to muscle layers of small blood vessels (14). Several lines of evidence suggest that cGMP is inhibitory to the carotid body activity. For instance, NO synthase is localized to carotid sinus nerve fibers that innervate glomus cells; NO elevates cGMP levels and inhibits chemosensory activity in the carotid body (8). Atrial natriuretic peptide (ANP) immunoreactivity occurs in glomus cells; ANP also enhances guanylyl cyclase activity and inhibits chemoreceptor activity (9). In addition, CO may directly modulate K^+ channel activity of the glomus cells (15). Conceivably, CO formed by glomus cells influences cGMP formation and/or K^+ channel activity in adjacent glomus cells, a paracrine effect, or may act back on the same cell, an autocrine effect. CO might directly affect the afferent terminals of the sinus nerve.

CO could influence carotid body function by altering blood flow, as CO can relax blood vessels by stimulating cGMP formation, analogous to the effects of NO (16, 17). Blood vessel relaxation would enhance oxygenation of the glomus cells and slow the discharge frequency of the carotid sinus nerve (18).

Molecular mechanisms whereby P_{O_2} regulates the glomus cell activity leading to an increase in sensory discharge have been obscure. It has long been postulated that heme-related proteins are important for oxygen sensing in the carotid body (1), which would fit elegantly with a role for HO and CO. HO has an absolute requirement for molecular oxygen and is sensitive to changes in oxygenation (19, 20). Conceivably, hypoxia directly lowers HO activity and CO formation. In our model (Fig. 4), hypoxia diminishes CO formation, leading to an increase in carotid sinus nerve activity. Thus, endogenous CO appears to function as an important physiologic regulator of the chemosensory activity of the carotid body.

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