Involvement of substance P in neutral endopeptidase modulation of carotid body sensory responses to hypoxia

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Kumar, Ganesh K., Yu Ru Kou, Jeffrey L. Overholt, and Nanduri R. Prabhakar. Involvement of substance P in neutral endopeptidase modulation of carotid body sensory responses to hypoxia. J. Appl. Physiol. 88: 195–202, 2000.—Previously, we showed that carotid bodies express neutral endopeptidase (NEP)-like enzyme activity and that phosphoramidon, a potent inhibitor of NEP, potentiates the chemosensory response of the carotid body to hypoxia in vivo. NEP has been shown to hydrolyze methionine enkephalin (Met-Enk) and substance P (SP) in neuronal tissues. The purpose of the present study is to determine whether NEP hydrolyzes Met-Enk and SP in the carotid body and if so whether these peptides contribute to phosphoramidon-induced potentiation of the sensory response to hypoxia. Experiments were performed on carotid bodies excised from anesthetized adult cats (n = 72 carotid bodies). The hydrolysis of Met-Enk and SP was analyzed by HPLC. The results showed that both SP and Met-Enk were hydrolyzed by the carotid body, but the rate of Met-Enk hydrolysis was approximately fourfold higher than that of SP. Phosphoramidon (400 µM) markedly inhibited SP hydrolysis (90%) but had only a marginal effect on Met-Enk hydrolysis (15% inhibition). Hypoxia (Prpo2, 68-8750-7587/00 $5.00 Copyright © 2000 the American Physiological Society 195http://www.jap.org
response to hypoxia as well as to SP, and these effects could be abolished or attenuated by SP-receptor antagonists.

**MATERIALS AND METHODS**

**General Preparation of the Animals**

Experiments were performed in adult cats of either sex anesthetized with pentobarbital sodium (35–45 mg/kg ip). After tracheal intubation, the trachea and the esophagus were ligated above the site of tracheal cannulation, sectioned, and retracted rostrally to expose the carotid bifurcation. Heparin (1,000 U/kg) was administered intravenously before carotid body removal. Both the common and external carotid arteries were ligated, and the carotid bodies along with the sinus nerve were excised and placed in ice-cold Krebs-Ringer solution preoxygenated with 100% O₂.

**Measurement of Peptide Hydrolysis in Carotid Bodies**

For in vitro degradation of neuropeptides, freshly isolated carotid bodies were used. Thin slices of carotid bodies were prepared to facilitate diffusion and were incubated at 37°C in 0.1 M Tris·HCl buffer, pH 7.4 (total volume, 750 µl), containing either SP (40 nmol) or Met-Enk (60 nmol) with and without phosphoramidon (400 µM) or EDTA disodium salt (1 mM). Fifty-microliter aliquots of the reaction medium were removed every 15 min and added to 50 µl of 0.2% (vol/vol) trifluoroacetic acid to terminate the reaction. The concentrations of peptides in the samples were determined by reverse-phase HPLC analysis by using a Shimadzu HPLC system with a gradient generator and a Chromatopak integrator (Hitachi). Peptides were resolved on a reverse-phase column (Vydac C18) using 0.1% (vol/vol) trifluoroacetic acid-water (solvent A) and 0.1% (vol/vol) trifluoroacetic acid-acetonitrile (solvent B) gradient solvent systems. Peptides were eluted using a linear gradient of 0–60% solvent B for 30 min, and the elution was monitored at 215 nm. The concentrations of peptides were determined from standard curves constructed with known concentrations of Met-Enk and SP. The peptide fragments resulting from the hydrolysis were identified from coelution studies with a variety of synthetic SP and Met-Enk peptide fragments obtained commercially. At the end of the experiment, tissue slices were homogenized in 0.1 M Tris·HCl buffer, pH 7.4, containing 0.1% (vol/vol) Triton X-100, and the protein concentration was determined by a colloidal-gold procedure using BSA as the standard (34).

**Recording of Sensory Discharge from Carotid Bodies in Vitro**

Sensory discharge from carotid bodies in vitro was recorded by using the procedures as described previously (21). Briefly, the carotid body along with the sinus nerve was placed in a lucite chamber (volume of 3.5 ml) and superfused with medium having the following composition (mM): 110 NaCl, 5 KCl, 0.5 MgCl₂, 2.2 CaCl₂, 54 sucrose, 5.5 glucose, 5 HEPEs; pH 7.4 (21). The temperature of the superfusing medium was maintained between 3 and 4°C/min. The chamber containing the carotid body was sealed with a lid to minimize exposure to atmospheric air. Gas-impermeable tubing was used to connect the chamber containing the carotid body to the reservoirs containing the superfusion solution. The superfusion medium was equilibrated with either 100% O₂ (hyperoxia) or with 10% O₂-balance N₂ (hypoxia). Aliquots of the superfusion medium were withdrawn from the chamber containing the carotid body for measurement of PO₂ by a blood-gas analyzer. In some experiments, PO₂ of the medium in the reservoirs was also measured and found to be comparable to the PO₂ values in the bath. Average PO₂ values under hyperoxia and hypoxia were found to be 376 ± 14 and 68 ± 6 Torr, respectively. The carotid sinus nerve was pulled through a small opening into another chamber. The nerve was blocked with paraformaldehyde to prevent it from drying. The electrical activity from thin filaments, dissected from the sinus nerve, was recorded (1–3 active units) with a platinum-iridium electrode and an A–C amplifier (model Pr 12/1. Grass Instruments). Discharge frequency of the action potentials was counted by using a meter (Winston Rad II). Chemoreceptor units were identified by their 1) spontaneous sporadic discharge, 2) increase in activity when switched from hyperoxic to hypoxic medium, and 3) decrease in activity when switched back to superfusion solution equilibrated with 100% O₂.

**Pharmacological Agents Used in the Study**

The following drugs from Sigma Chemical (St. Louis, MO) were used: SP (acetate salt), SP(1–7), SP(1–8), Met-Enk, Met-Enk(2–5), Met-Enk(3–5) and phosphoramidon (sodium salt), and d-Arg₁-o-Pro₁-o-Trp₁⁴-Leu₁⁴-SP (Spatide; SP-receptor antagonist). The nonpeptidyl SP-receptor antagonist CP-96345 was a gift from Pfizer. Stock solutions of neuropeptides (1 mM), Spatide (0.7 mM), CP-96345 (0.5 mM), and CP-96344 (0.5 mM) were prepared in 0.01 M acetic acid, whereas phosphoramidon (8.5 mM) was prepared in 50% (vol/vol) methanol. During the experimental, desired doses of the drugs were prepared by suitable dilution of the stock solution with the superfusion medium. Unless otherwise mentioned, drugs were added to the reservoirs.

**Experimental Protocols**

Series 1. The effects of phosphoramidon on the hydrolysis of SP and Met-Enk in the carotid bodies were examined (n = 24 carotid bodies). In a given experiment, two carotid bodies were harvested, basal hydrolysis of either SP or Met-Enk was monitored in one carotid body, and the effect of phosphoramidon (400 µM) was tested in the other. The effect of EDTA (1 mM) on Met-Enk hydrolysis was tested in six additional carotid bodies.

Series 2. The effects of phosphoramidon on sensory response to hypoxia were tested in 12 carotid bodies. Basal sensory discharge was monitored for 5 min while superfusion medium was equilibrated with 100% O₂ (control medium; hyperoxia). Then, carotid bodies were challenged with medium equilibrated with 10% O₂-balance N₂ (hypoxia; P O₂, 68 ± 6 Torr). Hypoxic challenge was maintained for 5 min (excluding the lag time of 6 min as determined by the addition of cressyl violet dye to the medium in the reservoir) followed by return to the control medium. The protocols were repeated while carotid bodies were superfused with phosphoramidon (400 µM).

Series 3. The effects of SP on carotid body activity before and after phosphoramidon were studied in 12 carotid bodies. The protocols were the same as described in series 2, except that the carotid bodies were challenged with SP instead of hypoxia. SP, at doses of 1, 5, 10, and 20 nmol, was administered close to the carotid body in 0.2 ml medium over a period of 20 s. Results obtained with the same volume of vehicle (the superfusion medium) served as controls. In between the doses, 10 min were allowed for the recovery.

Series 4. In this series of experiments, first we analyzed the effects of phosphoramidon on the carotid body responses to hypoxia (P O₂, 68 ± 6 Torr) and SP (10 nmol). Thereafter, the protocols were repeated after administration of Spatide (20
µM; n = 8) or CP-96345 (600 nM; n = 5), peptidyl and nonpeptidyl SP-receptor antagonists, respectively. Parallel experiments were performed with CP-96344 (600 nM), an inactive (2R,3R)-enantiomer of CP-96345, as controls (n = 5).

**Data analysis**

The rates of SP and Met-Enk hydrolysis were expressed in picomoles peptide hydrolyzed per hour per milligram protein. Chemoreceptor activity was averaged over 1 min during controls (preceding hypoxic challenge or SP administration) and during the peak activity after hypoxic or SP challenge. The changes in chemoreceptor activity were expressed as delta impulses per second (i.e., test – control). Average results are expressed as means ± SE. Statistical significance was evaluated by a paired t-test or by repeated-measures ANOVA. If ANOVA indicated a significant effect, the results were further compared by using Tukey’s test. *P* values <0.05 were considered significant.

**RESULTS**

**Effects of Phosphoramidon on the Hydrolysis of SP and Met-Enk in the Carotid Body**

Examples of HPLC profiles of SP and Met-Enk hydrolysis are shown in Fig. 1. Under our experimental condition, SP eluted at 23.8 min (Fig. 1A) and Met-Enk eluted at 17.4 min (Fig. 1D). Incubation with thin slices of carotid body for 1 h resulted in the hydrolysis of SP as evidenced by the appearance of SP fragments 1–7 and 1–8 (Fig. 1B, peak 3) and other shorter SP fragments (Fig. 1B, peaks 1 and 2). Met-Enk was also hydrolyzed by the carotid body to Met-Enk(2–5) and Met-Enk(3–5) (Fig. 1E, peak 4). The average results are summarized in Fig. 2. The hydrolysis of SP and Met-Enk could readily be detected as early as 15 min, and the hydrolysis increased linearly with the time of incubation. The rate of SP hydrolysis was 68 ± 3 nmol·h⁻¹·mg pro-

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Fig. 1. HPLC analysis of hydrolysis of substance P (SP; top) and methionine enkephalin (Met-Enk; bottom) by thin slices of carotid body (CB). A: SP alone; B: SP + CB; C: SP + CB preincubated with phosphoramidon (Phos; 400 µM) for 15 min; D: Met-Enk alone; E: Met-Enk + CB; and F: Met-Enk + CB preincubated with phosphoramidon (400 µM) for 15 min. In all experiments, incubation was for 1 h. Peaks 1 and 2, shorter fragments of SP; peak 3, SP(1–7) and (1–8), and peak 4, Met-Enk(2–5) and (3–5). Additional details are described in MATERIALS AND METHODS.

Fig. 2. Quantitative analysis of the hydrolysis of SP (A) and Met-Enk (B) by carotid body slices. Amount of peptide hydrolyzed is plotted as a function of time. Average data for hydrolysis of SP and Met-Enk in absence (Control) and in presence of either 400 µM Phos or 1 mM EDTA are shown. Details of peptide hydrolysis and HPLC quantitation of peptides are described in MATERIALS AND METHODS.
tein⁻¹, whereas that of Met-Enk was 280 ± 12 nmol·h⁻¹·mg protein⁻¹. Thus, under identical experimental conditions, the rate of Met-Enk hydrolysis was approximately fourfold higher than SP hydrolysis (P < 0.01; n = 6). In the presence of 400 µM phosphoramidon, the height of the SP peak remained nearly the same as in control, without the formation of SP fragments 1–7 and 1–8 (Fig. 1C). On average, the hydrolysis of SP was reduced by ~90% (Fig. 2A). By contrast, 400 µM phosphoramidon had only minimal effects on Met-Enk hydrolysis (~15%; P > 0.05; Figs. 1F and 2B). Higher concentration of phosphoramidon (800 µM) showed no further inhibition of Met-Enk hydrolysis in three additional carotid bodies. To determine whether other peptidases contribute to Met-Enk hydrolysis, the effect of EDTA, a broad-spectrum inhibitor of metallopeptidases, was tested. As shown in Fig. 2B, EDTA (1 mM) completely inhibited Met-Enk hydrolysis, suggesting the involvement of other metallopeptidases in the inactivation of Met-Enk in the carotid body. These observations clearly demonstrate that SP hydrolysis, but not Met-Enk hydrolysis, is significantly inhibited by phosphoramidon.

Effects of Phosphoramidon on the Sensory Response of the Carotid Body to Hypoxia in Vitro

For these experiments, we used an in vitro carotid body preparation. This preparation is advantageous in that it avoids potential complications resulting from effects of peptides and/or peptidase inhibitor on the cardiovascular system that may be themselves alter the chemosensory discharge. Figure 3A shows the effect of hypoxia (before and during application of 400 µM phosphoramidon) on chemosensory activity recorded from the carotid sinus nerve. It can be seen that sensory discharge frequency increased in response to hypoxia (Po₂, 68 ± 6 Torr). Average data from 12 carotid bodies are summarized in Fig. 3B. Phosphoramidon caused a transient increase (lasting for 1 min) in the basal discharge during hyperoxia (Po₂, 376 ± 14 Torr) in 4 of the 12 experiments. However, on average there was no consistent effect of phosphoramidon on the basal activity of the carotid body [control, 7 ± 1 vs. after phosphoramidon, 10 ± 3 impulses per second (imp/s); P > 0.05; n = 12]. More importantly, the effect of hypoxia was potentiated by phosphoramidon [Fig. 3, A (bottom) and B]. In the presence of phosphoramidon, the response to hypoxia increased by 80% (control, 46 ± 8 vs. phosphoramidon, 82 ± 12 imp/s; P < 0.01, n = 12). Phosphoramidon also shortened the duration of onset of the hypoxic response. In the presence of phosphoramidon, the latency of the sensory response to hypoxia was 174 ± 29 compared with 273 ± 17 s during control (P < 0.01; n = 12). These results demonstrate that phosphoramidon augments the sensory response of the carotid body to hypoxia in vitro, similar to the augmentation seen in in vivo carotid bodies as reported previously (14).

Effects of Phosphoramidon on Carotid Body Sensory Response to Exogenous Administration of SP

The biochemical studies described above show that phosphoramidon inhibits the hydrolysis of SP in the carotid body. Therefore, we tested whether SP contributes to the potentiation of the sensory response to hypoxia by phosphoramidon. As a first step, we recorded the sensory response of the carotid body to different doses of SP before and after phosphoramidon. In control experiments (without phosphoramidon), 10 and 20 nmol of SP significantly stimulated the sensory discharge, whereas doses below 5 nmol had no significant effect. An example illustrating the effect of phosphoramidon on the carotid body sensory response to 10 nmol of SP is shown in Fig. 4A. As can be seen, SP stimulated carotid body activity, and this response was enhanced by phosphoramidon. Average data for four different doses of SP before and after phosphoramidon are summarized in Fig. 4B. In the presence of phosphoramidon, the sensory responses to 10 and 20 nmol of SP were significantly greater than in prephosphoramidon controls (P < 0.01; n = 12). Although there was a tendency for potentiation at lower doses of SP (1 and 5 nmol), changes in sensory discharge were not significant (P > 0.05; n = 12). Phosphoramidon also shortened the onset of sensory response to SP. The latency for the SP response averaged 193 ± 16 s before
phosphoramidon, whereas it was reduced to 120 ± 15 s in the presence of phosphoramidon (P < 0.01; n = 12). None of the four doses of SP tested had any inhibitory effect on the carotid body activity. These results demonstrate that the stimulatory effects of SP on carotid body activity were potentiated by phosphoramidon in a manner similar to that seen with hypoxia.

Effects of SP-Receptor Antagonists on Phosphoramidon-Induced Augmentation of the Sensory Responses to Hypoxia and SP

If SP is involved in phosphoramidon-induced potentiation of the hypoxic response, then SP-receptor antagonists should block the effects of phosphoramidon. To test this possibility, we examined the effects of Spantide, a peptidyl SP-receptor antagonist (3), on chemoreceptor responses to different doses of substance P in the absence (Control; A) and presence (●) of Phos are shown. *Significance between sensory responses of carotid body before and after Phos; P < 0.01.

phosphoramidon, whereas it was increased by 89 ± 14 imp/s, whereas in the presence of Spantide the increase was only 17 ± 5 imp/s (81% reduction; P < 0.01; n = 8). As expected, Spantide also inhibited the stimulatory effects of SP on the carotid body. In the presence of phosphoramidon, SP (10 nmol) increased the sensory discharge by 18 ± 4 imp/s, whereas in the presence of Spantide the increase was only 2 ± 0.4 imp/s (P < 0.01; n = 8). In five additional carotid bodies, we also tested the effects of CP-96345, a nonpeptidyl SP-receptor [neurokinin-1 (NK1)] antagonist. As shown in Fig. 5, C and D, CP-96345 significantly attenuated the sensory responses to hypoxia as well as to SP in the presence of phosphoramidon. As a control, we also tested the effect of CP-96344, an inactive (2R,3R)-enantiomer of CP-96345, in five additional carotid bodies. Figure 5, C and D, shows that CP-96344 had no effect on the sensory responses to either SP or hypoxia in the presence of phosphoramidon. These results further support the idea that SP is involved in phosphoramidon-induced potentiation of the carotid body response to hypoxia.

DISCUSSION

We have previously shown that phosphoramidon enhances the carotid body sensory response to hypoxia in anesthetized animals (14). The main objective of the present study was to extend and elaborate on these findings by identifying the neuropeptide(s) that is involved in phosphoramidon augmentation of the hypoxic sensitivity of the carotid body. We found that phosphoramidon markedly inhibited the hydrolysis of SP but only marginally affected Met-Enk hydrolysis in the carotid body. Furthermore, we found that phosphoramidon potentiated the sensory response of the carotid body in vitro to both hypoxia and SP, and these effects were markedly attenuated or abolished by SP-receptor antagonists. These results clearly demonstrate the involvement of SP in the phosphoramidon augmentation of the carotid body sensory response to hypoxia.

Several lines of evidence suggest that phosphoramidon specifically inhibits NEP-like enzymes in many tissues (7, 28, 35), including the carotid body (12). It is well known that both SP and Met-Enk are the preferred substrates for NEP (7, 28, 35). In fact, NEP is often referred to as “enkephalinase” because of its ability to hydrolyze Met-Enk (7, 35). Although both SP and Met-Enk are hydrolyzed by the carotid body, much to our surprise, phosphoramidon markedly inhibited the hydrolysis of SP (~90%), whereas Met-Enk hydrolysis was only marginally affected (~15%). This lack of effect of phosphoramidon on Met-Enk was not due to a submaximal concentration, because doubling the concentration had no further effect. Rather, other metallopeptidases are likely to be involved in the hydrolysis of Met-Enk in the chemoreceptor tissue. Such a notion is supported by the finding that EDTA, a broad-spectrum inhibitor of metallopeptidases, completely inhibited Met-Enk hydrolysis in the carotid body. In the central nervous system, Met-Enks are hydrolyzed by EDTA-sensitive aminopeptidases that belong to the metallopeptidase family (29, 36). Enzyme activity resembling aminopeptidase has been found in the cat carotid body (12). It follows that aminopeptidase may be responsible for the hydrolysis of Met-Enk in the carotid body. It is also interesting to note that the rate of hydrolysis of Met-Enk is significantly greater than that of SP. The abundance of Met-Enk in glomus cells (8) and the
potential involvement of multiple metallopeptidases in the hydrolysis of Met-Enk may account for the higher rate of hydrolysis of Met-Enk observed in the carotid body. These observations suggest that, in the carotid body, NEP is involved in the degradation of SP but plays only a minor role in the degradation of Met-Enk.

The findings of the present study agree with those of our previous study in intact animals (14) that phosphoramidon augments the sensory response of the carotid body to hypoxia. The in vitro carotid body preparation avoids possible problems with effects of phosphoramidon on the cardiovascular system. Therefore, the fact that phosphoramidon inhibits SP hydrolysis and augments the effect of SP in the in vitro carotid body provides indirect evidence that SP is the neuropeptide involved in phosphoramidon augmentation of the chemosensory response to hypoxia.

The findings that phosphoramidon inhibits SP hydrolysis and augments the effect of SP in the in vitro carotid body provide indirect evidence that SP is the neuropeptide involved in phosphoramidon augmentation of the chemosensory response to hypoxia. In the nervous system, SP mediates its action via neurokinin receptors (27). We have previously showed that NK₁
receptors mediate excitatory actions of SP in the cat carotid body (23). To provide more direct evidence for the involvement of SP and neurokinin receptors in phosphoramidon-induced potentiation of the hypoxic response, we tested the effects of Spantide and CP-96345, peptidyl and nonpeptidyl (NK₁) SP-receptor antagonists, respectively. Spantide and CP-96345 both attenuated or abolished the phosphoramidon-induced augmentation of the responses to both hypoxia and SP. It could be argued that these inhibitory effects arise from a nonspecific action on the carotid body. However, such a possibility seems unlikely because both of these compounds had similar effects. Furthermore, inhibitory effects were not seen with CP-96344, an inactive enantiomer of CP-96345. Dashwood et al. (5) have analyzed NK₁ receptors in the cat carotid body by autoradiography. Their results showed that NK₁ receptors are present in the carotid body and that chronic unilateral sectioning of a carotid sinus nerve on 5-HT receptors and Chemoreceptor Reflexes

In summary, these findings support the notion that NEP modulates carotid body activity by maintaining low endogenous levels of excitatory peptides, especially SP. Furthermore, the finding that NEP can modulate the hypoxic response of the peripheral chemoreceptors suggests that strategies for NEP inhibitor-based drug development should take into consideration potential effects of NEP inhibitors on carotid body function. Stimulation of the carotid body by NEP inhibitors could have profound effects on both the cardiovascular and respiratory systems.

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