BRAIN RESEARCH 1069 (2006) 154–158

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Short Communication

Endothelins in the cat petrosal ganglion and carotid body: Effects and immunolocalization

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ARTICLE INFO

Article history:
Accepted 16 November 2005
Available online 20 December 2005

Keywords:
Chemoreceptor
Petrosal ganglion

ABSTRACT

In response to hypoxia, chemoreceptor cells of the carotid body (CB) release transmitters, which acting on the petrosal ganglion (PG) neuron terminals, increase the chemoafferent discharge. Additionally, vasoactive molecules produced within the CB may modulate hypoxic chemoreception by controlling blood flow and tissue PO_2. Endothelin-1 (ET-1) increases basal CB chemosensory discharges in situ, probably due to its vasoconstrictor action. However, the actions of ET-1 on PG neurons or its expression in the PG are not known. Using immunohistochemistry, we found that endothelin-like peptides are expressed in the cat PG and CB under normoxic conditions. Exogenous applications of ET-1 increased the chemosensory activity in the vascularly perfused CB but were ineffective on either the CB or PG superfused preparations, both of which are devoid of vascular control. Thus, our data indicate that the excitatory effect of ET-1 in the carotid chemoreceptor system appears to be mainly due to a vasoconstrictor effect in the CB blood vessels.

Stimulation of the carotid body (CB) by hypoxia, hypercapnia and acidosis induces the release of transmitters from glomus cells, which in turn increases the discharge of the nerve terminals of petrosal ganglion (PG) neurons (Iturriaga and Alcayaga, 2004). The CB is a highly vascularized organ, with the highest blood flow reported to date (Barnett et al., 1988; De Burgh Daly et al., 1954; McCloskey and Torrance, 1971; O'Regan, 1981). Accordingly, vasoactive molecules produced within the CB may modulate the chemosensory process by regulating the blood flow and tissue PO_2. Endothelin-1 (ET-1), one of the most potent known vasoconstrictors, is involved in central and peripheral cardiorespiratory control (Kuwaki et al., 1996). Autoradiographic studies have shown the presence of [^{125}I] ET-1 binding sites in the CB, nodose ganglion and brain stem of cats and rats (McQueen et al., 1995; Spyer et al., 1991). More recently, immunohistochemical studies showed the presence of endothelins (ETs) in the rat CB (Chen et al., 2002a,b). Intravenous injections of ET-1 increase ventilation in rats in a dose-dependent manner (McQueen et al., 1995; McQueen et al., 1994), effect that has been attributed to CB stimulation, because it is abolished by bilateral carotid sinus neurotomy (McQueen et al., 1994). Recordings of the neural discharge in the carotid sinus nerve (CSN) show that exogenously applied ET-1 increases chemosensory discharges in situ (McQueen et al., 1994; Rey and Iturriaga, 2004) but not in the superfused CB in vitro (Chen et al., 2000a; Rey and Iturriaga, 2004), a preparation devoid of vascular control. These results suggest that the excitatory effect of ET-1 on CB chemoreception is probably mediated by its vasoconstrictor properties. However, ET-1 may have a direct excitatory effect

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doi:10.1016/j.brainres.2005.11.051

on PG neurons that innervate the CB chemoreceptor (glomus) cells. Indeed, some studies have suggested an excitatory role of ET-1 in primary sensory neurons (Houck et al., 2004; Pomonis et al., 2001). In situ hybridization for ET-1 mRNA and immunohistochemistry studies indicate that ETs are synthesized in the spinal cord and dorsal root ganglia (Giaid et al., 1989; Yoshizawa et al., 1989). Moreover, ET-1 activates Na^+ currents and increases intracellular Ca^{2+} in sensory neurons (Zhou et al., 2001; Zhou et al., 2002). However, it is not known whether ETs are expressed in the PG and if ET-1 stimulates PG neurons. Thus, we studied the ET-like immunoreactivity (ET-ir) in the cat PG and CB. In addition, we compared the effect of the application of ET-1 to the isolated PG preparation (Alcayaga et al., 1998) and to the perfused (Iturriaga et al., 1991) and superfused (Alcayaga et al., 1988) preparations of the CB.

Experiments were performed in 9 male cats (2.0–4.5 kg) anesthetized with sodium pentobarbitone (40 mg/kg, i.p.). The experimental protocol was approved by the Ethical Committees of the Facultad de Ciencias Biológicas of the P. Universidad Católica de Chile and the Facultad de Ciencias of the Universidad de Chile. For immunohistochemistry, the CBs and the PGs obtained from 3 anesthetized cats were fixed with neutral-buffered formalin, dehydrated in ethanol, included in paraffin, cut in 5-μm sections and mounted on silanized slides. Deparaffinized samples were incubated for 18 h at 4 °C with a rabbit anti-ET-1 antibody (1:600) directed against the complete ET-1 peptide (T-4051, Bachem, CA, USA) and stained with a biotin–streptavidin peroxidase kit (DakoCytomation, CA, USA), using 0.1% 3-3′-diaminobenzidine as a chromophore. Slides were counterstained with Harris' hematoxylin and covered. Myocardial tissue was used as a positive control and sections of CB tissue without the primary antibody as a negative control. The cross-reactivity of the ET-1 antibody was Big Endothelin <1%; ET-2, 91% and ET-3, 0.05%. Photomicrographs were taken at 400×, digitized and analyzed with the ImageJ software (NIH, MD, USA). ET-ir area was measured with a color deconvolution algorithm (Ruifrok and Johnston, 2001) and averaged from three fields in each sampled slide. On each sampled field, the total tissue area was measured to calculate the percentage of positive ET-ir area.

To assess the effect of ET-1 on PG neurons, the PG with the CSN was removed from cats and placed in a chamber as previously described (Alcayaga et al., 1998). The isolated PG was superfused with Hank's balanced solution containing 5 mM HEPES at 38.0 ± 0.5 °C, pH 7.42, and equilibrated with 21% O_2. The effect of ET-1 on CB chemosensory activity was assessed using in vitro preparations that allow the separation of vascular effects. The superfused CB preparation has been extensively used to study chemoreceptor function without the interference of vascular control (Alcayaga et al., 1988; Eyzaguirre and Koyano, 1965). The CB with the CSN was excised from cats and superfused in vitro with modified Tyrode solution, pH 7.40 at 37.5 ± 0.5 °C, and equilibrated with 20% O_2 + 5% CO_2 (Alcayaga et al., 1988). To study the participation of the blood vessels in the CB chemosensory process, we used the arterially perfused CB preparation, which conserve its functional vascularization (Belmonte and Eyzaguirre, 1974; Iturriaga et al., 1991). The carotid bifurcation including the CB, with the CSN attached to it, was cannulated through the common carotid artery, excised from the cat and perfused in vitro by gravity at a constant pressure of 80 Torr with modified Tyrode solution equilibrated with 20% O_2 + 5% CO_2 at 37.5 ± 0.5 °C, pH 7.40 (Iturriaga et al., 1991). To record chemosensory discharges in the PG and CB preparations, the CSN was placed on paired Pt electrodes and lifted into mineral oil that covered the preparation. Neural signals were preamplified and amplified, filtered and fed to an electronic spike amplitude discriminator, which allowed the selection of action potentials of given amplitude above the noise. The selected impulses were counted with a frequency meter to assess the frequency of CSN discharges (f_{CSN}), expressed in Hertz. The signal was digitized with an analog-digital board. The ET-1 was exogenously applied to the superfused PG and CB in 20 μl boluses with a microdispenser, whose tip was placed about 1 mm from the surface of the organs. In the perfused CB, ET-1 was applied in 200 μl boluses into the arterial line. The maximal responsiveness of the PG and CB preparations was tested using acetylcholine (ACh) and hypoxic stimulation (PO_2 ~25 Torr), respectively. The relation between f_{CSN} and the ET-1 doses was assessed using the best fit of the standardized responses to a logistic expression: $(f_{CSN}/\max f_{CSN}) = 1/(1 + \{ED_{50}/ET\text{-}1\}^S)$ (Alcayaga et al., 1998).

Immunohistochemistry showed that ET-ir was present in both the PG and the CB. In both organs, low magnification images indicated that positive staining was mostly located in the cell cytoplasm (Figs. 1A, E). In the PG, ET-ir was usually distributed longitudinally in the middle region of the ganglion (Fig. 1A). At high magnification, staining was observed in neuron cell bodies (Fig. 1B), associated with axonal processes and occasionally in the endothelium of capillaries (Fig. 1C). In the CB, ET-ir was faint and occupied a lower percentage of the total area (1.9 ± 1.2% in the CB vs. 11.2 ± 2.6% in the PG, $P < 0.05$ t test). In fact, positive ET-ir staining was found in glomus cell clusters (Figs. 1E, F) and in the endothelium of interlobular vessels (Figs. 1F, G). Positive ET-ir staining in control myocardial tissue was confined to endothelial cells (Fig. 1H). Negative controls, omitting the primary antibody, were consistently devoid of staining (Fig. 1F, inset).

The exogenous application of ET-1 (0.1 to 2.0 μg) to the isolated PG was ineffective in modifying f_{CSN}, while ACh (0.1 to 500 μg) increased f_{CSN} as previously reported (Alcayaga et al., 1998). Fig. 2A shows that in the isolated PG, a bolus injection of 2.0 μg of ET-1 had no effect on f_{CSN}, while the application of 200 μg of ACh increased f_{CSN} up to 280 Hz. Similarly, in the superfused CB, the application of large doses of ET-1, up to 10.0 μg, did not modify the basal f_{CSN}. Fig. 2B illustrates that in the superfused cat CB, the application of 1.0 μg of ET-1 did not increase f_{CSN}, while the superfusion with hypoxic modified Tyrode increased f_{CSN} up to 250 Hz in the same preparation. On the contrary, in the perfused CB preparation, small doses of ET-1 (0.1–0.5 μg) produced a delayed, large and long-lasting increase of f_{CSN} (Fig. 2C). It is noteworthy that the increases in CB chemosensory discharges induced by 0.5–1.0 μg of ET-1 lasted for about 60 min before returning to baseline values. Fig. 3 summarizes and compares the effects of ET-1 (0.01 to 2.0 μg) on f_{CSN} when applied to the isolated PG and to the perfused and superfused CB preparations. In most experiments, ET-1 doses of 0.5–1.0 μg were enough to elicit maximal chemosensory responses in the perfused CBs, similar to those attained

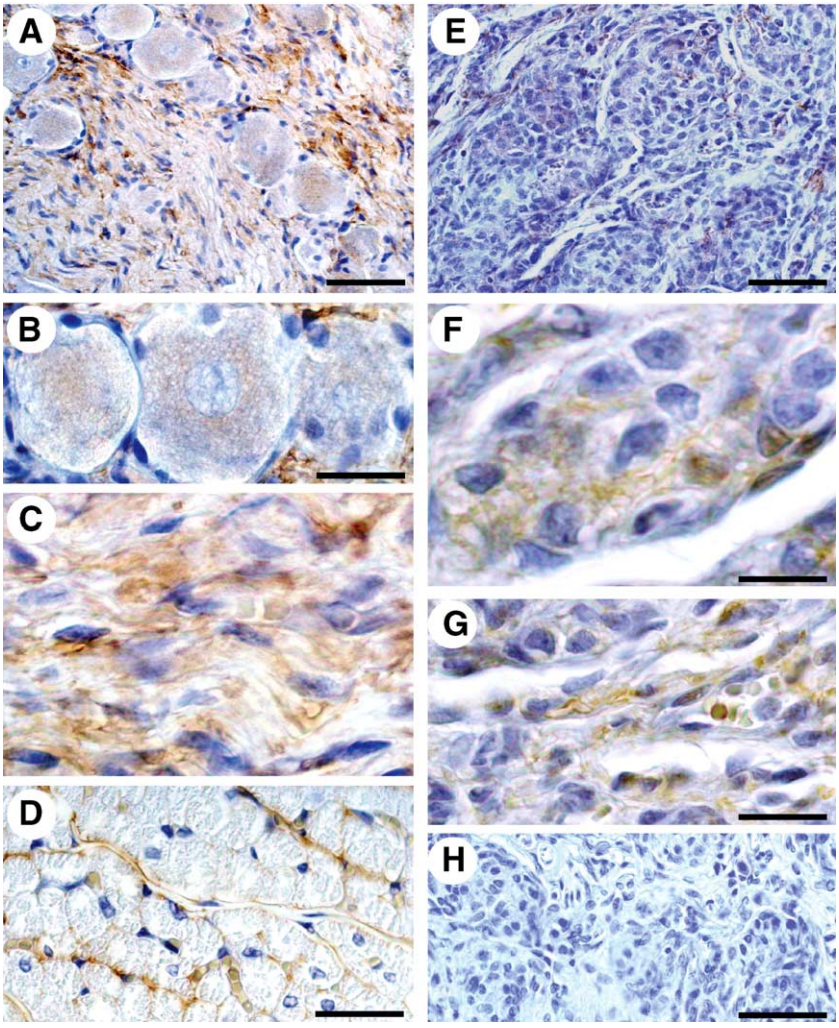

Fig. 1 – Immunolocalization of ETs in the cat PG and CB. ET-ir is observed in 400× photographs of the PG (A) and CB (E). Left column: positive staining is present in PG neuronal bodies (B) and along the axonal processes (C). Right column: in the CB, ET-ir is present in the glomus cell cytoplasm (F) and in the endothelium of interlobular blood vessels (G). Myocardium, positive control (D); negative control is devoid of staining (H). Scale bars: 50 μm (A, E, H); 25 μm (B, C, D, F, G).

with hypoxic perfusion. The ED_{50} for the ET-1 excitatory effect on the perfused CB chemosensory discharges was 0.10 ± 0.02 μg (n = 5 CBs).

To our knowledge, this is the first study that reports the localization of ETs in the PG. Indeed, we found positive staining in the neuron cell bodies and along the axonal processes of PG neurons. However, the resolution of the immunohistochemical technique preclude us from identifying if ET-ir is present in PG neurons and/or Schwann cells in the nerve bundles. Indeed, expressions of ET-like peptides and ET-1 binding sites have been found in sensory neurons but also in satellite and Schwann cells (Franco-Cereceda et al., 1991; Giaid et al., 1989; Milner et al., 2000; Pomonis et al., 2001; Yoshizawa et al., 1989). Our recordings in the isolated PG indicate that ET-1 has not a direct excitatory effect on these sensory neurons, suggesting that ET-1 is not directly involved in the generation of the increases in chemosensory afferent discharges. However, it has been proposed that ET-1 is a neuromodulator in other primary sensory systems. Dymshitz and Vasko (1994) found that in isolated sensory neurons, the release of substance P and calcitonin gene-related peptide was enhanced by pretreatment with ET-1, but ET-1 per-se did not evoke the release of these peptides. In the nucleus tractus solitarius, ET-1 increases the neuronal activity through the endothelin receptor type-A (ET_A-R) and enhances the response evoked by the putative transmitter glutamate (Shihara et al., 1998). In addition, pharmacological studies suggest that ET receptor activation may play a role in neuropathic and chronic inflammatory pain (Griswold et al., 1999; Jarvis et al., 2000). Pomonis et al. (2001), using autoradiography and immunolocalization techniques, examined the distribution of ET_A-R and endothelin receptor type-B (ET_B-R) in the dorsal root ganglion and peripheral nerves of the rat, rabbit and monkey. They found that in dorsal root ganglia and peripheral nerves, ET_A-R was present in a subset of small-sized peptidergic and non-peptidergic sensory neurons and their axons, while satellite and Schwann cells were positive for ET_B-R (Pomonis et al., 2001).

Our results confirm and extend previous observations regarding the immunolocalization of ETs in the normoxic

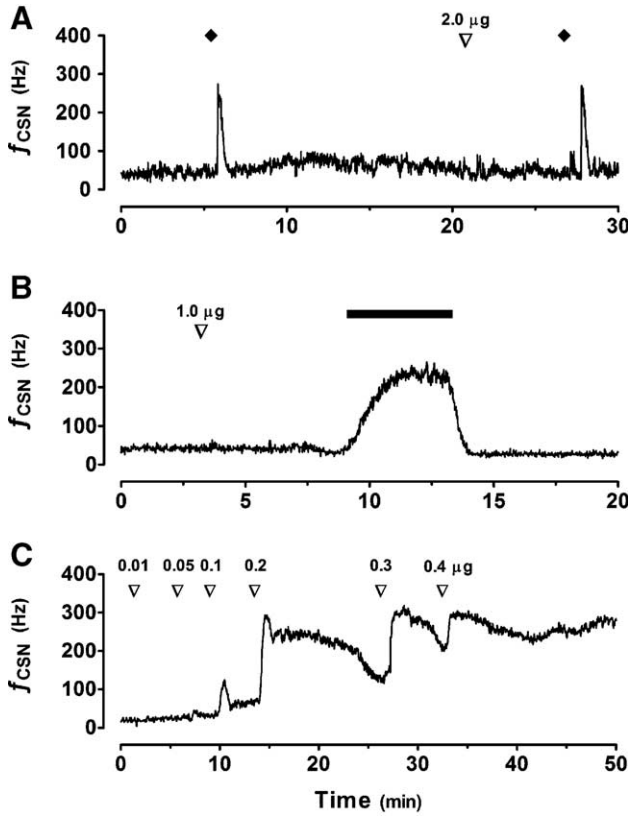

Fig. 2 – Effect of ET-1 on f_{CSN} in single preparations. (A) Isolated petrosal ganglion. (B) Superfused CB. (C) Perfused CB. f_{CSN}, frequency of chemosensory discharges expressed in Hertz. Empty arrowheads indicate ET-1 injections. Bar, hypoxic superfusion. ♦, 200 µg ACh injections.

CB. Present immunohistochemical data show a diffuse cytoplasmic staining of glomus cells and endothelium of interlobular blood vessels in the CB. It has been found that ETs in the rat CB are not confined to the CB blood vessels but are also found in glomus cells (Chen et al., 2002a,b). However, Ozaka et al. (1997) reported the presence of ET-1 immunoreactive gold particles only in endothelial cells within the rat CB but not in glomus cells. Studies performed in the rabbit CB superfused in vitro showed that ET-1 did not increase basal chemosensory discharges perse, but potentiated the chemosensory response induced by hypoxia (Chen et al., 2002a). This modulatory effect of ET-1 on hypoxic chemoreception was attributed to the activation of ET_A-R (Chen et al., 2002a,b). Indeed, these studies showed that ET-1, acting on Ca^{2+} channels, increases Ca^{2+} influx in rabbit glomus cells. Accordingly, they proposed that ET-1, acting through ET_A-R may modulate several components of the O_2-sensing mechanisms in glomus cells, including intracellular levels of cAMP and Ca^{2+} (Chen et al., 2000a,b). Even though ET-1 may enhance chemosensory responses to hypoxia in the superfused CB, a preparation devoid of vascular effects, the excitatory effect of ET-1 on CB chemoreception is most likely mediated by its vasoconstrictor effect. Our results showed that exogenously applied ET-1 produces chemosensory excitation in the perfused CB but not in the superfused CB and PG preparations. Accordingly, the excitatory effect of ET-1 on CB chemosensory activity seems to be mainly mediated by vasoconstriction. Thus, ETs would act as local regulators of CB blood flow, modulating the O_2 content in the CB tissue, and therefore modifying the chemosensory discharges.

Immunohistochemical, electrophysiological and pharmacological data indicate that ET-1 is involved in the increased CB chemosensory responses to acute hypoxia induced by chronic hypoxic exposure in rats (Chen et al., 2002a,b). In fact, Chen et al. (2002a) found that the ET_A-R antagonist BQ-123 reduced the chemosensory response to hypoxia by 11% in the superfused rat CB, indicating that ET-1 exerts some excitatory tone in the CB. However, they found that BQ-123 reduced the hypoxic chemosensory response to hypoxia by 50% in CBs from rats exposed to hypoxia for 9–16 days. In addition, Chen et al. (2002b) found that the increase in intracellular $[Ca^{2+}]$ induced by ET-1 in glomus cells was 49% greater in rats exposed to hypoxia for 4 weeks. Immunohistochemical studies have shown that chronic hypoxia increases ET-ir and ET_A-R expression in the rat CB (Chen et al., 2002a,b). Thus, the potentiation of the effect of ET-1 on the CB is consistent with an excitatory role of the ET-1 and ET_A-R in the CB during chronic hypoxia. It is plausible that during chronic hypoxia, an increased ET-1 level in the CB may excite the nerve terminals of the PG neurons. However, our data showed that large doses of ET-1, which evoked maximal increases in the perfused CB chemosensory discharges, fail to evoke neural responses in the PG isolated preparation.

In summary, present results show that under normoxic conditions ET-s are expressed in the cat CB and PG. The immunolocalization of ETs in the CB is predominantly associated with blood vessels, but we cannot exclude a modulatory effect of ETs on glomus cells. Expression of ET in the PG is located among the axonal processes of the neurons and in the neuron cell bodies. Application of exogenous ET-1 only elicits chemosensory excitation in the perfused CB, but not in the superfused CB and PG, indicating that its excitatory effect is mainly due to vasoconstriction.

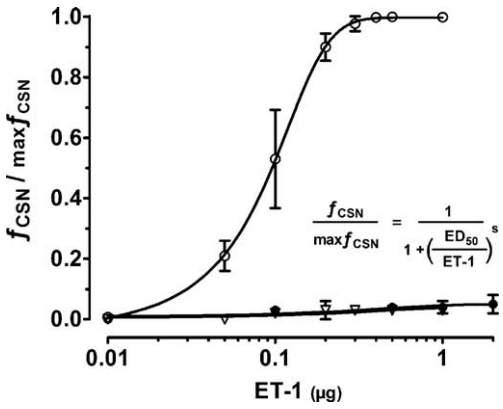

Fig. 3 – Dose response-curve for the effect of ET-1 on f_{CSN} in 5 perfused CBs (○), 2 superfused CBs (●) and 3 isolated PGs (▽). f_{CSN}, frequency of chemosensory discharges expressed as percentage of the maximal responses induced by ACh (PGs) or hypoxia (CBs).

Acknowledgments

This work was supported by grant 1030330 from the National Fund for Scientific and Technological Development of Chile (FONDECYT). We would like to thank Mrs. Jenny Corthorn and Cecilia Chacon for their assistance in the immunohistochemical techniques.

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