ORIGINAL ARTICLE

The presence of heme-oxygenase and biliverdin reductase in human cranial ganglia indicates a role for carbon monoxide in neural transmission

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Abstract

OBJECTIVES: The purpose of this study was to localize in cranial ganglia of man the occurrence of the putative gaseous neural messenger carbon monoxide (CO) and the biliverdin degrading enzyme biliverdin reductase (BVR).
METHODS: Immunocytochemistry with antibodies against the CO-inducing enzymes HO-1, HO-2, BVR and calcitonin gene-related peptide was used.
RESULTS: In the trigeminal ganglion about 60% of the cell bodies exhibited HO-2- and about 40% BVR-immunoreactivity. HO-2- as well as BVR-immunoreactivity was predominantly (78%) expressed in medium-sized cells (30–60 μm). In the superior cervical ganglion about 40% of the cell bodies exhibited HO-2-immunoreactivity. In the sphenopalatine and otic ganglia only a few cell bodies were HO-2 immunoreactive. HO-1, the inducible isoform of heme oxygenase, gave only very weak immunoreactivity in all ganglia examined. Double immunostaining revealed that in the human trigeminal ganglion HO-2 and BVR co-localized with calcitonin gene-related peptide.
CONCLUSIONS: The finding suggests that CO might serve as a modulator of synaptic transmission in man.

Introduction

The main source for the endogenous production of carbon monoxide (CO) is through oxidative cleavage of heme by heme oxygenase (HO) [15,16]. In addition, free iron and biliverdin is generated. Biliverdin can subsequently be reduced to bilirubin by the enzyme biliverdin reductase (BVR) [17]. CO has been suggested to function as a messenger molecule in signal transduction in the central and peripheral nervous systems [18] whereas bilirubin used to be considered a waste product of heme metabolism. However, recent studies indicate that biliverdin posseses antioxidant effects and perhaps modulates inflammatory responses [25]. There are two major isoforms of HO: HO-1, which is up-regulated by a variety of different stimuli [22] and HO-2 which is constitutively expressed under normal physiologic conditions [14,24]. A third isoform, HO-3, has been described and might have a regulatory role in heme-dependent cellular processes [19].

The presence of HO-2 in many neurons combined with functional studies demonstrating multiple effects of CO on both neurons [6,27,28,29,31] and innervated tissue [4,21,26,30] has led to speculations that, like nitric oxide (NO), CO might serve as an inter- and intracellular neuronal messenger molecule [27]. CO mediates its effects in nerves and other tissues by binding to the heme moiety of the soluble isoform of guanylate cyclase [9,27]. Upon binding guanylate cyclase, CO induces formation of the second messenger cyclic guanosine monophosphate (cGMP), which has neuromodulatory effects in the central and peripheral nervous systems and mediates a variety of effects in non-neural tissues including relaxation of smooth muscle [2]. BVR has, like HO-2, been demonstrated in peripheral ganglia of laboratory animals. The aim of the current study was to examine whether HO-1, HO-2 and BVR also could be found in human cranial ganglia.

Material and methods

Materials. Trigeminal, superior cervical, sphenopalatine and otic ganglia were obtained from adult subjects in accordance with the Szeged University Medical School guidelines for ethics in human tissue experiments. At autopsy trigeminal ganglia were taken out from 6 subjects, superior cervical ganglia were obtained from 3 subjects and one sphenopalatine and one otic ganglion from one subject. The subjects had an age of 74.8 years (51–85 years). None of the patients suffered from any central nervous system disease. Cause of death was related to cardiac disease. Tissues were collected within 24 h of death.

Fixation. The ganglia were immersed overnight in a fixative consisting of 2% paraformaldehyde and 0.2% picric acid in

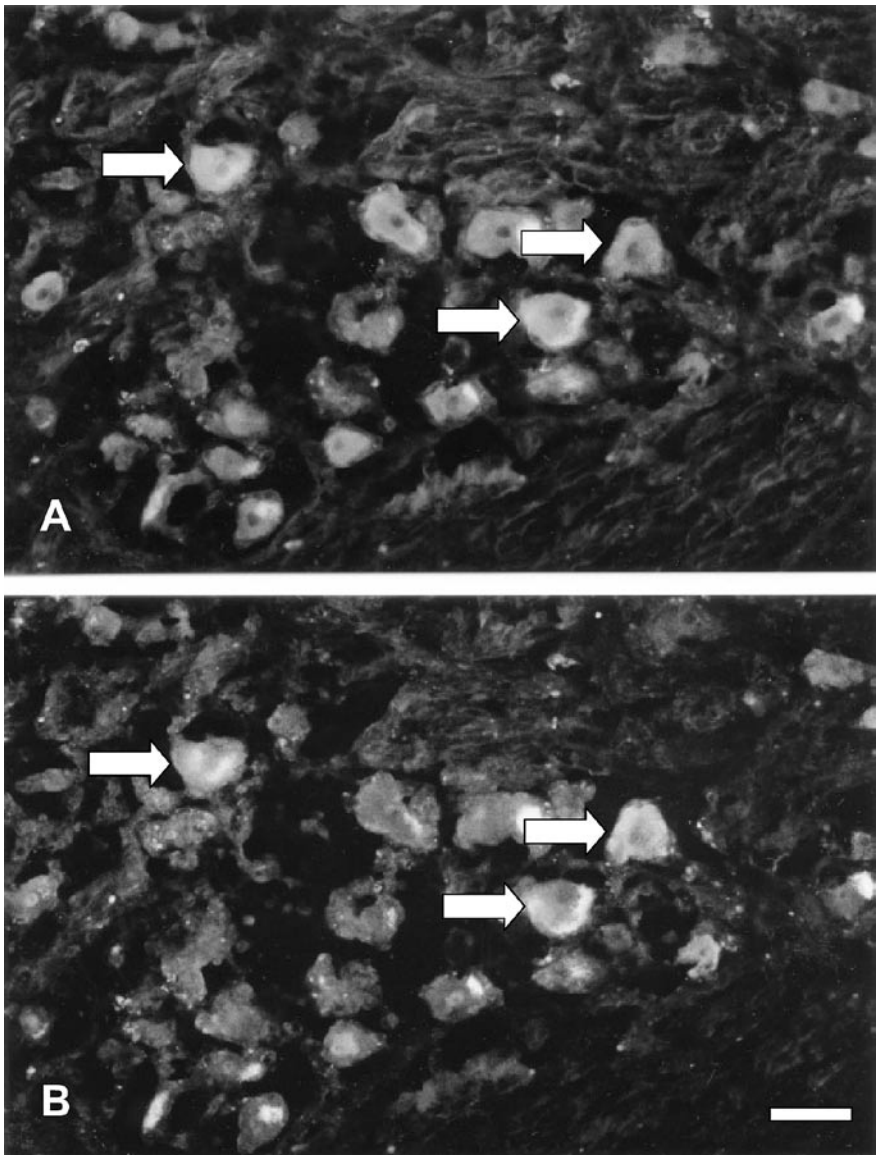

Figure 1: Double immunostaining in human trigeminal ganglion. Colocalization of HO-2 (A) with CGRP (B) (arrows). Calibration = 50 µm. (Publisher's note: Fig. 90% original size)

0.1mol/L phosphate buffer, pH 7.2. After fixation the specimens were rinsed repeatedly in sucrose-enriched (10%) Tyrode solution. The ganglia were frozen on dry ice and stored at –70° C. Sections were cut at 10 μm thickness in a cryostat and mounted on Superfrost Plus glass slides.

Immunocytochemistry. The sections were processed for the immunocytochemical demonstration of the CO-inducing enzymes, HO-1 and HO-2, BVR and for CGRP. The antiheme oxygenase-1 polyclonal antibody (OSA-100), the antiheme oxygenase-2 polyclonal antibody (OSA-200) and anti-biliverdin reductase (BVR) (Stressgen Biotech, Victoria, Alberta, Canada) were raised in rabbits. The antibodies were used in dilutions 1:1000, 1:500 and 1:100, respectively. The CGRP antibody (Eurodiagnostica, Malmö, Sweden) was raised in guinea-pig and used in a dilution of 1: 800. For the demonstration of the antigen-antibody reaction indirect immunofluorescence was used. Briefly, the cryostat sections were first washed with PBS and then rinsed in PBS for 15 min followed by incubation for 45 min with secondary antibodies raised against either rabbit or goat IgG and conjugated to either FITC or TRITC (1:80; swine anti-rabbit FITC, DAKO, Copenhagen, Denmark and 1:100; donkey anti-goat TRITC, Chemicon, Sweden). Slides were coverslipped in glycerol/PBS 2:1 (v/v) containing DAP I (1mg/μL) and observed under microscope with chromefluorescense filters.

To investigate the co-localization of HO-2 and BVR with CGRP double immunofluorescence was performed. The sections were incubated with the primary antibodies HO-2, BVR and CGRP (raised in guinea–pig). Secondary antibodies raised in rabbit or goat IgG and conjugated to either FITC or TRITC were used. By shifting the microscope filters (Leica filter settings no. K3), FITC- and TRITC-conjugated secondary antibodies could be determined in the same section. In control experiments, no specific immunoreactivity could be detected when the primary antibody was omitted. In tissues incubated with the primary antiserum (OSA-100) preadsorbed with an excess of recombinant HO-1 (SPP-730, 5ug/mL) the immunoreactivity was suppressed. Cross-reactivity with unidentified peptides containing amino acid sequences recognized by the different antisera cannot be excluded. It would therefore be appropriate to refer to the immunoreactive material as HO-2-like, CGRP-like etc. For brevity, the immunoreactive material is referred to as HO-2 and CGRP in the text.

Results

In the trigeminal ganglion about 60% (58 ± 18%) of the cell bodies exhibited HO-2 immunoreactivity. HO-2 immunoreactivity appeared in cell bodies of all sizes but was predominantly (78%) seen in medium-sized cells (30–60 μm). Double immunostaining

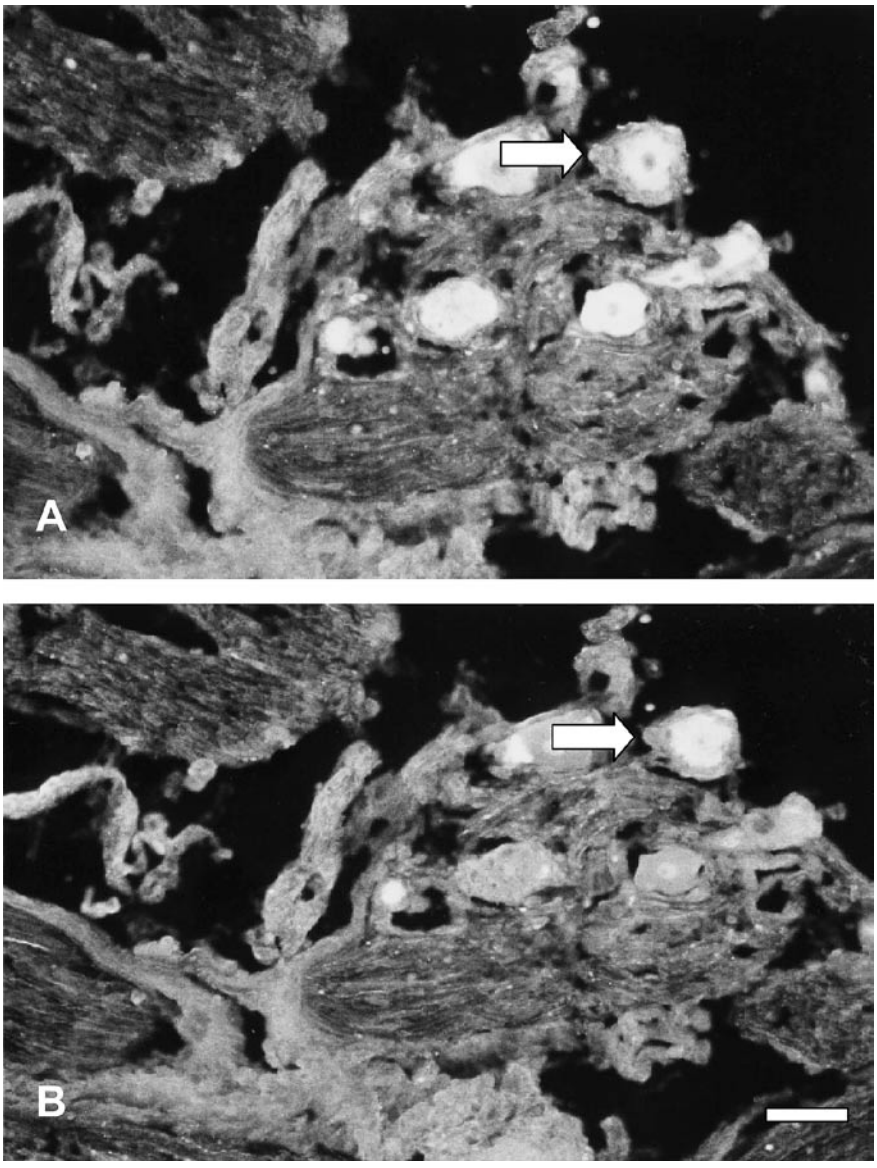

Figure 2: Human trigeminal ganglion. Double immunostaining. Colocalization of BVR (A) with CGRP (B) (arrow). Calibration = 50 µm.
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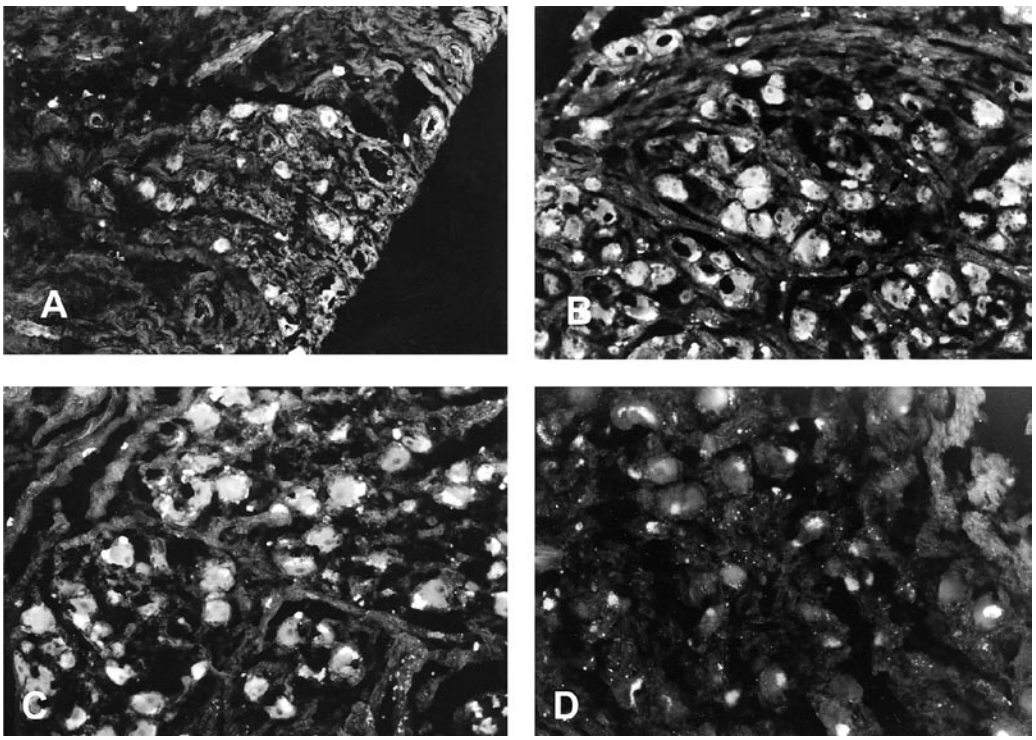

Figure 3: Immunofluorescence micrograph of HO-2 in the human superior cervical ganglion (A), otic ganglion (B) and sphenopalatine ganglion (C). A large number of HO-2 immunoreactive cell bodies can be seen (arrows). HO-1 immunoreactivity in the trigeminal ganglion (D). Only weak immunofluorescence is seen. Autofluorescent lipofuscins are seen in some cell bodies. (Publisher's note: Fig. 90% original size)

demonstrated that in the human trigeminal ganglion about 40% of HO-2-immunoreactive cell bodies co-localized with CGRP (Fig. 1). In the trigeminal ganglion about 40% of the cell bodies exhibited BVR immunoreactivity. BVR was predominantly (78%) expressed in medium-sized cells. In several cells a positive staining for BVR was also seen in the nuclei of the cell bodies. Double immunostaining revealed that about 30% of BVR-immunoreactive cell bodies co-localized with CGRP (Fig. 2). In the superior cervical ganglion about 40% of the cell bodies were HO-2 immunopositive. In the parasympathetic otic and sphenopalatine ganglia numerous cell bodies were HO-2 immunoreactive (Fig. 3). HO-1, the inducible isoform of heme oxygenase, displayed only very weak immunoreactivity in the ganglia examined (Fig. 3).

The immunoreactivity was abolished when the primary antibodies were omitted.

Discussion

The present study demonstrates HO-2- and BVR-immunoreactivity in human cranial ganglia. In the trigeminal ganglion the immunoreactive staining was predominantly seen in association with medium-sized cell bodies and double immunostaining revealed that some HO-2- and BVR-immunoreactive cell bodies also harbored CGRP. The HO-1 and HO-2 isoenzymes cleave heme b molecules converting it to CO, biliverdin and free iron. Biliverdin is then converted to bilirubin by BVR. CO is reported to modulate physiological actions such as smooth muscle relaxation, inhibition of platelet aggregation and to act as a neuronal messenger [27,31]. Of the three isoforms of HO, HO-2 is the most prevalent form in the brain [7,8]. In the periphery, HO-2 has been demonstrated in ganglia of sensory, sympathetic and parasympathetic origin in laboratory animals [13,16]. Our results differ to some extent to what is reported for laboratory animals. In laboratory animals almost all cell bodies show immunostaining for HO-2 whereas we found that about 60% of the cell bodies in the human trigeminal ganglion were immunoreactive. There may be several reasons for this difference. The human material was obtained from of elderly people and several cell bodies contained lipofuchsin as a sign of this. Neither can it be ruled out that the cause of death in this material may affect the histochemical outcome. The specimens were dissected within 24 h and not immediately taken out and transferred to the fixative as for laboratory animals.

BVR has previously been found in mucosal epithelial cells and in the endothelium of intramural vessels of human and porcine fundus. In addition, distinct BVR-immunoreactivity is seen in nerve cell bodies of both submucous and myenteric ganglia [5]. In contrast, only very weak BVR immunoreactivity was detected in peripheral ganglia of rats [13].

The finding that CGRP co-localized with HO-2 in neurons of the trigeminal ganglion is somewhat surprising. NO and CO display several similarities in their neuronal distribution and co-localization between NO and CGRP has previously not been reported [1,20]. In

the present study, about 30% of the HO-2- and BVR-immunoreactive cell bodies in the trigeminal ganglion displayed CGRP. The co-localization of CO and NO has been established in several peripheral tissues [12].

Only very weak immunoreactivity was seen with antibodies directed against HO-1. During normal conditions, low levels of HO-1 are seen in most tissues but the spleen. However, HO-1 activity can be enhanced by a variety of agents. During culture for 24–48 h a strong up-regulation of HO-1 immunoreactivity can be seen in satellite cells and in nerve cell bodies of cranial ganglia in rodents [13].

HO-2 might have a neuroprotective role since biliverdin formed from heme is rapidly reduced to bilirubin, preventing accumulation of the former [23]. In rats, only weak BVR immunoreactivity was seen in normal ganglia, following culture a distinct up-regulation was seen in some neural cell bodies [13]. In the gastrointestinal tract the distribution of BVR and HO-2 has been reported to be more or less symmetrical in both man and pig [5]. In contrast, we found somewhat more HO-2- than BVR-immunoreactive cell bodies in the trigeminal ganglion. The fact that HO-2 is present in nerve cell bodies but absent in fibers innervating peripheral tissues suggests that CO does not act as a regular neurotransmitter but perhaps as a modulator of synaptic transmission [3].

In the periphery, HO-2-like immunoreactivity has been reported in epithelial cell populations, endothelial cells and vascular and non-vascular smooth muscle cells [10]. It has been suggested that the finding of HO-2 and BVR in the endothelium of small blood vessels reflects a protective role for bilirubin with regard to vascular inflammation [11].

In conclusion, HO-2 and BVR are expressed in nerve cell bodies of human trigeminal, otic, sphenopalatine and superior cervical ganglia. In the trigeminal ganglion, HO-2 and BVR are most abundant in medium-sized cell bodies and seem to co-localize with neurotransmitters such as CGRP indicating a role for CO in synaptic transmission. However, the full importance of the HO/CO pathways in cell to cell communication remains to be explored [10].

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