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Enhanced Expression of Heme Oxygenase-1 and Carbon Monoxide Excitatory Effects in Oxytocin and Vasopressin Neurones During Water Deprivation

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Abstract

A growing body of evidence supports carbon monoxide (CO) as a gas neurotransmitter within the central nervous system. While CO has been shown to affect neurohypophyseal hormone release in response to osmotic stimuli, the precise sources, targets and mechanisms underlying CO actions within the magnocellular neurosecretory system remain largely unknown. In this study, we combined immunohistochemistry and patch-clamp electrophysiology to study the cellular distribution of the CO-synthase enzyme heme oxygenase type 1 (HO-1), as well as CO actions on oxytocin (OT) and vasopressin (VP) magnocellular neurosecretory cells (MNCs) in euhydrated (EU) and 48h water-deprived rats (48WD). Our results show expression of HO-1 immunoreactivity both in OT and VP neurones, as well as in a small proportion of astrocytes, both in the supraoptic (SON) and paraventricular (PVN) nuclei. HO-1 expression, and its colocalization with OT and VP neurones within SON and PVN were significantly enhanced in 48WD rats. Inhibition of HO activity (CrMP 20 μ M) resulted in a slight membrane hyperpolarization in SON neurones from EU rats, without significantly affecting their firing activity. In 48WD rats, on the other hand, CrMP resulted in a more robust membrane hyperpolarization, significantly decreasing neuronal firing discharge. Taken together, our results indicate that magnocellular SON and PVN neurones express HO-1, and that CO acts as an excitatory gas neurotransmitter in this system. Moreover, we found the expression and actions of CO to be enhanced in water-deprived rats, suggesting that the state-dependent up-regulation of the HO-1/CO signalling pathway contributes to enhance MNCs firing activity during an osmotic challenge.

Keywords

supraoptic; paraventricular; hypothalamus; dehydration; neuroendocrine

Introduction

Carbon monoxide (CO) is a biologically active gas (1, 2). While CO has been the focus of numerous studies in cerebral vascular biology (3), a growing body of evidence also supports CO as a gas neurotransmitter within the central nervous system (CNS) (1, 2, 4). CO is generated endogenously from the catabolism of heme oxygenase (HO), an enzyme that cleaves the heme derived from heme-proteins (Fe-protoporphyrin) such as hemoglobin, myoglobin and cytochromes, resulting in the formation of equimolar amounts of CO, free

iron and biliverdin (1, 2). Endogenous CO exerts its biological effects by activating sGC and thereby increasing intracellular cGMP levels (1, 2).

Three HO isoforms have been described so far: (a) the inducible HO-1, which can be upregulated by a variety of stimuli including oxidative stress and inflammation (2, 5); (b), the constitutive isoform HO-2, widely expressed in several tissues, and shown to be regulated by a few selected stimuli, including glucocorticoids (6); and (c) HO-3, whose function is yet unknown (7). While HO-2 is the most commonly found constitutive active isoform under basal conditions in the CNS (1, 8), HO-1 has also been found in specific groups of CNS neurones, particularly within hypothalamic nuclei, including the supraoptic (SON), paraventricular (PVN), ventromedial and preoptic nuclei (8, 9). Moreover, HO-1 was also found in glial cells where its gene expression can be induced by oxidative stress or high temperature (10, 11).

The presence of HO-1 within the SON and PVN indicates that CO may play an important role in the central regulation of fluid/electrolyte homeostasis via modulation of magnocellular neurosecretory cells (MNCs). This is supported by several studies showing a modulatory role of CO on oxytocin (OT) and vasopressin (VP) release induced by osmotic stimulation (12), endotoxemia (13) or direct K^+ stimulation (14). Despite this evidence, the precise cellular sources, targets and mechanisms by which CO regulates neurohypophysial hormone release, particularly during osmotic challenges, remain unknown. To address this important gap in our knowledge, we performed here a detailed characterization of HO-1 expression in immunoidentified SON and PVN OT and VP neurones and astroglial cells, both in euhydrated (EU) and 48h water-deprived (48WD) rats. Moreover, given that the release of OT and VP from neurohypophysial terminals is directly dependent on the degree and pattern of electrical activity of MNCs (15), we performed *in vitro* patch-clamp recordings to investigate whether endogenous CO regulates SON firing activity, and whether this effect is dependent on the hydrated status of the animal.

Materials and Methods

Animals

Male Wistar rats (200 - 300g) were purchased from Harlan Laboratories (Indianapolis, IN) and housed in a 12-12 hour light-dark cycle and given free access to food and water. All the procedures used in this study were carried out in agreement with Georgia Health Sciences University Institutional Animal Care and Use Committee guidelines. Animals were then divided in two groups, one with water access *ad libitum* (euhydrated, EU), and another one submitted to water deprivation for 48 hours (48WD). Plasma osmolarity was measured by an osmometer (Precision Systems, Natick, MA), and mean values obtained were 286 ± 3 and 316 ± 4 mOsm/Kg H_2O , in EU and 48WD groups, respectively.

Immunohistochemistry

EU and 48WD rats ($n = 3/\text{group}$) were deeply anaesthetised with sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially with 0.01M phosphate-buffered saline (PBS, 150 ml at pH 7.4) followed by 4% paraformaldehyde (PFA, 350 ml). Brains were removed and postfixed in 4% PFA for 3 hours at 4°C. Fixed brains were cryoprotected at 4°C with 0.01M PBS containing 30% sucrose for 72 hours. Sequential coronal sections (25 μm) through the frozen hypothalamus, between bregma -0.92 and -2.12 (16), were cut with a Leica CM3050S cryostat (Nussloch, Germany) in three series and stored at 4°C until used, and then incubated in a solution of 0.01 MPBS with 0.1% Triton X-100, 0.04% NaN_3 , and 10% normal horse serum for 1 hour. Sections were then incubated for 24 hours in a cocktail of primary antibodies containing either a rabbit anti-heme oxygenase type 1 (HO-1) polyclonal

antibody (SPA-895, 1:1000; StressGen, Ann Arbor, MI), along with a guinea pig anti-oxytocin (OT) polyclonal antibody (T-5021, 1:50000; Peninsula Laboratories, San Carlos, CA) or a guinea pig anti-vasopressin (VP) polyclonal antibody (T-5048, 1:20000; Peninsula Laboratories, San Carlos, CA). According to the manufacturer, the immunogen for the HO-1 antibody is a recombinant rat HO-1 (Hsp32) lacking the membrane spanning region, detecting a band of ~32kDa by Western blot. Reactions with primary antibodies were followed by 4 hours incubation in the presence of fluorescently labelled secondary antibody donkey anti-rabbit FITC-labelled (1:250) and donkey anti-guinea pig Cy5-labelled (1:50) from Jackson ImmunoResearch Laboratories (West Grove, PA). Another series of sections were incubated for 24 hours in a cocktail of primary antibodies containing both a rabbit anti-heme oxygenase type 1 (HO-1) polyclonal antibody (SPA-895, 1:1000) and a mouse anti-S-100 β -subunit (S100 β) monoclonal antibody (S 2532, 1:1000; Sigma-Aldrich, Saint Louis, MO). Reactions with these primary antibodies were followed by 4 hours incubation in the presence of the secondary antibodies donkey anti-rabbit FITC-labelled (1:250) and donkey anti-mouse Cy5-labelled (1:50). Preabsorption controls were run in order to test the specificity of the anti-HO1 primary antibody, using the proper HO-1 recombinant antigen protein (ADI-SPP-730, 1:200; Enzo Life Sciences INC, Farmingdale, NY), using procedures previously described (17). The antigen was used at a concentration 5 $\times$ that of the primary antibody. The mixtures were incubated in PBS containing 0.1% Triton X-100 and Na₂S₂O₃ 0.04% for 2 hours at room temperature, centrifuged for 5 minutes at 10,000g, and the supernatants applied to the tissue, following the same procedure described above. Another control experiment consisted of omitting the primary antibody from the immunohistochemical reaction. Finally, in a separate set of experiments we also perform an immunohistochemical reaction using an anti-heme oxygenase type 2 (HO-2) polyclonal antibody (OSA-200, 1:1000; StressGen, Ann Arbor, MI), using similar procedures as described above. According to the manufacturer, the immunogen for this antibody is the native rat HO-2 protein isolated from rat testes, and in Western Blot analysis it detects a band of ~36kDa.

Confocal imaging acquisition and quantitative analysis

Stained sections were examined with a Zeiss LSM 510 Confocal Microscope System (Carl Zeiss, Oberkochen, Germany). A helium-neon laser was used to excite fluorescein isothiocyanate (FITC) at 488nm and an argon-krypton laser was used to excite Cy5 fluorochrome at 633nm. Fluorescent signal cross-talk among the channels was avoided by setting image acquisition parameters with individually labelled sections. Ten consecutive optical focal planes (2 μ m interval) were acquired from three sections/rat of different rostro-caudal levels of the SON and PVN nuclei. All images from the different experimental groups were digitised with identical acquisition settings. To determine the degree of colocalization among signals, as well as the mean signal intensity, a densitometry analysis based on a threshold paradigm using Image ProPlus Software 5.1 (Media Cybernetics, Silver Spring, MD), was used as previously described (18). Briefly, OT and VP immunoreactive MNCs from the SON and the lateral and anterior magnocellular PVN subnuclei, as well as S100 β immunoreactive astrocytes, were traced using a freehand tool within Image ProPlus. The channel was then switched to the one containing the HO-1 immunoreactivity, and the mean HO-1 immunofluorescence intensity within the traced cell was calculated. Background intensity was calculated from adjacent areas and was subtracted from all images. Cells were then arbitrarily considered to be HO-1 positive if the mean intensity was above 1.5 times the background threshold immunofluorescence. A mean value of all sampled sections/animal was calculated (18). All quantitative analyses were carried out blindly.

Electrophysiology

Conventional whole-cell patch-clamp recordings were obtained, as previously described (19). Briefly, rats (n= 8 EU and 7 48WD) were briefly anaesthetised with sodium pentobarbital and perfused through the heart with cold artificial cerebrospinal fluid (aCSF; NaCl 120mM, KCl 2.5mM, NaH₂PO₄ 1.25mM, MgSO₄ 1mM, CaCl₂ 2mM, NaHCO₃ 26mM, glucose 20mM and ascorbic acid 0.4mM in pH 7.4) in which NaCl was replaced by an equimolar amount of sucrose. Coronal hypothalamic slices containing the SON were cut (200 µm) using a Leica VT1000S vibroslicer (Nussloch, Germany), and placed in a holding chamber containing aCSF and stored at room temperature for at least 1 hour until used. For electrophysiological recordings, a slice was transferred to a submersion-type recording chamber, continuously perfused (~2ml/min⁻¹ at 30-32°C) with a standard aCSF bubbled with a gas mixture of 95% O₂ and 5% CO₂. To be consistent with previous electrophysiological studies in MNCs from dehydrated rats (20-22), recordings in both groups were obtained at the same aCSF osmolarity (290-300 mOsmol/l). Patch pipettes (4-8MΩ) were pulled from thin-walled (outer diameter, 1.5mm; inner diameter, 1.7mm) borosilicate glass (GC150T-7.5; Clark, Reading, United Kingdom) on a horizontal electrode puller (P-97; Sutter Instruments, Novato, CA). The pipette internal solution contained (mM): D-gluconic acid 130, KCl 20, Hepes 10, EGTA 0.5, Mg-ATP 0.9 and Na-GTP 0.3. The pH was slowly titrated to 7.25-7.30. Electrical recordings were obtained using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier. The series resistance was monitored throughout the experiment, and data were discarded if the series resistance during recordings increased by two-fold. The voltage output was digitised at 16-bit resolution in conjunction with pClamp 8 software (Digidata 1320; Axon Instruments, Union City, CA). Data were digitised at 10kHz. Firing activity was recorded in the current-clamp mode, and the effects of the relative specific HO inhibitor chromium mesoporphyrin IX chloride (CrMP, 20µM; Porphyrin Products, Logan, UT) (23) was tested. Due to the large variability in basal firing activity, and the fact that in some instances recorded neurones were silent, neurones in both groups were subjected to DC current injection through the pipette in order to attempt to obtain a relative homogenous basal activity of ~0-8-1.2 Hz in both groups. The amount of DC current injection between the two groups was similar (range: 7-10 pA). Control firing rate (defined as the number of spikes s⁻¹) was measured over a 2 minutes period before drug application. Due to cell-to-cell variability in the latency of drug-induced responses (range: 133-218s), the peak of the drug-induced response was determined, and the mean firing frequency for the drug effect was calculated during a period comprising 1 minute before and 1 minute after peak effect.

Statistical analysis

Results are expressed as mean ± SEM. Immunohistochemical data was analysed using a One-Way ANOVA, non-parametric Kruskal-Wallis test, followed by Dunn's post hoc tests. Results from electrophysiological studies were analysed using a Two-Way ANOVA repeated measures analysis, followed by Bonferroni's post hoc tests. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at $P<0.05$.

Results

HO-1 is densely expressed in the supraoptic (SON) and paraventricular (PVN) nuclei

To determine whether the inducible heme-oxygenase isoform HO-1 is expressed in the SON and PVN, hypothalamic sections from euhydrated rats were immunostained with a specific antibody raised against HO-1. Results from these studies showed a dense HO-1 immunoreactivity in both nuclei, which appeared to be localised mostly in large principal neurones (Fig.1). In the SON, MNCs were strongly immunoreactive for HO-1 (Fig.1-A1).

Within the PVN, HO-1 staining also appeared particularly strong in MNCs-containing regions, including the anterior (Fig.1-A2) and the lateral magnocellular subnuclei (Fig. 1-A3). HO-1 staining was also visualised in smaller cell bodies (putative astrocytes, *see more below*) located in the SON ventral glial laminae, as well as in the periventricular region of PVN (arrowheads in Fig.1- A1,A2, respectively). Preabsorption of the antibody with the corresponding antigen (Fig.1- B1,B2), as well as omission of primary antibody (Fig.1-B3), resulted in a lack of immunostaining. In a separate set of experiments, we immunostained for the HO-2 subunit. As shown in Fig.1-C1,C2, while HO-2 immunoreactivity was present in the SON and PVN, staining was clearly weaker than that of HO-1. Moreover, HO-2 immunoreactivity appeared more punctate, and was absent in glia-enriched areas (i.e., SON ventral glial laminae and PVN periventricular region, opened arrowheads). The remaining of this work was focused specifically on the HO-1 isoform.

HO-1 is expressed both in oxytocin (OT) and vasopressin (VP) SON and PVN neurones

To assess the neurochemical phenotypes of SON and PVN MNCs that express HO-1, we performed dual immunofluorescence protocols using specific antibodies raised against HO-1 and OT or VP. Representative examples obtained in control (euhydrated, EU) rats are shown Figs.2- A,C and Fig.3- A,C (SON and PVN, respectively). A summary from a quantitative analysis evaluating the number of immunoreactive neurones, immunoreactivity intensities, and proportions of double-labelled neurones, is shown in Fig.4. As depicted in the representative images and summarised in Fig.4, we found HO-1 to be expressed both in OT and VP neurones of the SON and PVN. In the SON, we found HO-1 in ~30 % of OT neurones and ~19 % of VP neurones, being the incidence of HO-1 staining in OT neurones significantly higher than in VP neurones ($P<0.05$, Dunn's post-hoc test, Fig.4-C). Similar results were observed in the PVN where the degree HO-1 expression in OT neurones (~40 %), was significantly higher (Fig.4-C, $P<0.05$ Dunn's post-hoc test) than in VP neurones (~18 %).

Enhanced HO-1 expression in SON and PVN neurones during water deprivation

Based on previous results from our laboratories showing that CO modulates OT secretion from hypothalamic explants exposed to hypertonic stimulation (12), we aimed here to determine whether HO-1 expression within the SON and PVN changed in response to an osmotic stimulation. To this end, we compared HO-1 immunoreactivity between EU and 48 hours water-deprived (48WD) rats. Representative examples of HO-1, OT and VP immunostaining in the SON and PVN of 48WD rats are shown in Fig.2- B,D and Fig.3- B,D, respectively. As summarised in Fig.4, we found a significant increase in the number of HO-1 positive MNCs in 48WD rats when compared to EU rats, both in the SON and PVN (Fig.4-A, $P<0.001$ in both cases, Dunn's post-hoc test). Moreover, the HO-1 immunoreactivity intensity was also significantly enhanced in 48WD rats when compared to EU rats in both nuclei (Fig.4-B, $P<0.001$ Dunn's post-hoc test). Finally, we found the degree of OT and VP neurones expressing HO-1 in the SON of 48WD rats (~94 % and ~41 %, respectively) to be significantly higher than that observed in the SON of EU rats (Fig.4-C **left**, $P<0.01$ in both cases, Dunn's post-hoc test). A similar increase in the degree of OT/HO-1 and VP/HO-1 colocalization (~87 % and ~53 %, respectively) was observed in the PVN (Fig.4-C **right**, $P<0.01$ in both cases, Dunn's post-hoc test). Interestingly, both in EU and 48WD, and in the SON and PVN rats, the degree of OT neurones expressing HO-1 was significantly higher than that observed in VP neurones (Fig.4-C, $P<0.05$, Dunn's post-hoc test).

HO-1 is expressed in a subpopulation of SON and PVN astrocytes

Previous studies reported the presence of HO-1 containing astrocytes in discrete regions of the rat brain (9, 11). To determine whether this was also the case in the SON and PVN, we

assessed for HO-1 immunoreactivity in S100 β -stained astrocytes, both in EU and 48WD rats. Representative examples are shown in Fig.5- A,B for the SON and Fig.5- C,D for the PVN. As summarised in Fig.5-E, we found a small proportion of SON and PVN astrocytes (~10 %), mostly located in the ventral glial laminae and in periventricular regions, respectively, to express HO-1. Differently from what we observed in SON and PVN MNCs, neither the incidence nor the intensity of astrocytic HO-1 immunoreactivity was modified in 48WD rats (Fig.5- E,F).

Endogenous CO stimulates SON firing activity, an effect enhanced in water deprived rats

To determine whether the expression of HO-1 in the magnocellular system is functionally relevant, we evaluated whether endogenous CO production influenced MNCs firing activity, and whether this effect was dependent on the hydrated status of the rats. To this end, we obtained patch-clamp recordings from MNCs in SON slices, and monitored the effect of an HO inhibitor (CrMP, 20 μ M) on MNCs firing activity in EU and 48WD rats. Fig.6-A shows representative examples of the effect of CrMP on the firing activity of SON neurones from EU and a 48WD rats, and the summary data is shown in Fig.6- B,C. Bath application of CrMP (5-7 minutes) in SON neurones from EU rats (n=8) induced a slight membrane hyperpolarization and decrease in firing rate, which were however not statistically different ($P > 0.05$ in both cases). CrMP effects were more pronounced in SON neurones from 48WD rats (n=7), leading to a larger and significant membrane hyperpolarization (5.7 ± 1.5 mV, $P < 0.05$, Bonferroni's post hoc test), as well as a significant inhibition of firing activity (75.8 ± 11.2 %, Bonferroni's post hoc test, Fig.6-C). No correlation between the control basal firing activity of the recorded neurones and the magnitude of the CrMP effect was observed (Pearsons $r = 0.465$ $P > 0.05$).

Discussion

Gas neurotransmitters, particularly nitric oxide (NO), have been shown to play important roles in neuroendocrine regulation (24-26). In this work, we aimed to determine whether carbon monoxide (CO), another functionally relevant gas molecule in the brain (1, 2), plays a role in the modulation of magnocellular neurosecretory activity. Combining immunohistochemistry and electrophysiology in brain slices, we aimed to determine the cellular distribution and actions of HO-1 and CO, respectively, within the hypothalamic SON and PVN. Results from this work can be summarised as follows: **1.** HO-1 immunostaining was localised primarily both in oxytocin (OT) and vasopressin (VP) neurones, and it was also observed in a small proportion of astrocytes; **2.** In control euhydrated (EU) rats, inhibition of HO activity resulted in a slight membrane hyperpolarization of recorded SON neurones, without significantly affecting their firing activity; **3.** In 48h water-deprived (48WD) rats, we found an increased HO-1 immunoreactivity, which corresponded also with a more robust membrane hyperpolarization and decreased firing rate evoked by HO-1 inhibition. Taken together, results from our studies support the presence of HO-1 in OT and VP neurones, expression of which is upregulated in water-deprived rats. In addition, our results show that CO acts as an excitatory endogenous gas molecule, contributing to enhanced firing activity of MNCs during osmotic challenges.

Cell-type distribution of heme-oxygenase 1 (HO-1) in the SON and PVN

A main finding of our study is that the CO-synthesizing enzyme HO-1 is present in MNCs of the SON and PVN nuclei. Despite the fact that HO-2 has been reported to be the most abundant isoform found in the normal adult rat brain (2, 9), our results are in agreement with previous studies showing relatively high basal expression of HO-1 in discrete hypothalamic neuronal populations (8, 9). Results from double immunofluorescence studies show that

HO-1 is present in OT and VP neurones, suggesting that both neuronal types have the capability of acting as sources of CO within the SON and PVN. Whether the higher incidence of HO-1 expression observed in OT neurones is indicative of a more predominant contribution to CO levels by these neurones remains to be determined.

In addition to MNCs, our results indicate that a small proportion of SON and PVN astrocytes, mostly located in ventral glial laminae and periventricular regions, respectively, expressed HO-1 as well. This finding is consistent with recent reports supporting the presence of HO isoforms in brain astrocytes (27). It is well-established that astrocytes play critical roles in influencing magnocellular neurosecretory activity via a plethora of mechanisms and signals, including modulation of synaptic efficacy and density (28), ambient levels of neurotransmitters (22, 29), and release of neuroactive substances, including ATP (30) and nitric oxide (31). Thus, the expression of HO-1 in SON/PVN astrocytes suggest that they could also potentially act as CO sources within these hypothalamic regions.

Functional significance of CO in the magnocellular neurosecretory system

Results from our electrophysiological studies support CO as a biologically relevant gas in the control of MNC activity. We found that pharmacological inhibition of HO activity in control rats induced a slight membrane hyperpolarization, which was insufficient however, to significantly affect their firing activity. These effects, on the other hand, were enhanced in water-deprived rats, a challenge known to result in the robust activation of the magnocellular system (32). Thus, compared to euhydrated controls, the HO-1 inhibitor CrMP induced a larger and significant membrane hyperpolarization, accompanied by a diminished firing activity of SON neurones in 48WD rats. Several important conclusions can be drawn from these results. Firstly, our data supports endogenous CO as an excitatory gas molecule, which can efficiently affect MNCs membrane potential and firing activity. Secondly, despite a relatively dense expression of the CO-synthesizing enzyme in control rats, its activity, and thus constitutive CO bioavailability, does not appear to be sufficiently high, at least in the slice preparation, to affect SON firing activity. Finally, the HO/CO signalling mechanism in the SON and PVN is regulated in a state-dependent manner. This is supported both by the increased HO-1 expression in OT and VP MNCs, as well as the enhanced CO-mediated effects on MNCs firing activity in 48WD rats. Whether the increased HO-1 immunoreactivity during water deprivation reflects a transcriptional upregulation, or alternatively, a diminished protein degradation, is at present unknown. Moreover, given the well-established diminished enwrappment of neuronal somata by astrocyte processes during dehydration (33), the possibility of enhanced antibody penetration in this condition needs to be considered. Future studies are then warranted to determine the precise mechanisms contributing to enhanced HO-1 immunoreactivity in 48WD rats.

Hormonal secretion from the posterior pituitary is dependent on the degree and pattern of MNC firing activity (15). Thus, in conditions such as dehydration, MNCs increase their firing activity in order to cope with the increased hormonal demand (34). Thus, taken together, our immunohistochemical and electrophysiological data support an enhanced HO-1 expression and activity within individual MNCs, resulting in an enhanced bioavailability of CO in 48WD rats. It is reasonable to speculate then that the up-regulation of the HO/CO system during water deprivation could act as a mechanism to stimulate MNCs activity, contributing in turn to neuronal homeostatic responses during dehydration.

Our results are in general agreement with recent reports addressing the effects of CO on OT and VP hormonal release. Thus, Gomes and colleagues (12) showed that HO inhibition decreased osmotically-stimulated OT release, supporting an excitatory role for endogenous

CO during osmotic challenges. Moreover, CO was shown to induce prostaglandin formation, a well-established stimulator of OT and VP neuronal activity and hormone release (35). Conversely, others reported opposite effects of CO during endotoxemia or interleukin stimulation (13, 14). Thus, while these studies support a regulatory role for CO on OT and VP neuronal activity and hormone release, they also suggest that these effects may be dependent on the type of challenge. Alternatively, differences in experimental conditions, including concentration and type of drugs used, among others, may account for the reported discrepancies.

It is also worth considering that HO-1 is a member of the heat shock protein HSP32 family (36), and that its expression is augmented following stressful stimuli (2, 5). In fact, numerous studies support HO-1 as an ubiquitous cellular protective mechanisms against a variety of stressors, including osmotic stress (37, 38), hypertension (39), and endotoxemia (40), actions that could result from the HO-1 activity byproduct biliverdin, a cellular antioxidant (41). Thus, an alternative functional outcome of the increased HO-1 expression during dehydration could be increased MNCs protection in face of a sustained osmotic stress. Clearly, more studies are needed to verify a potential neuroprotective role for HO-1 in MNCs.

Some important caveats in our studies need to be taken into consideration. Firstly, even though the neurochemical phenotype of neurones in the electrophysiological studies was not determined, similar responses were obtained in all MNCs, suggesting that both OT and VP neurones were likely affected by CO. This is also in line with the fact that both neurones expressed relatively high HO-1 levels, and that being a gas, CO can act in a rather diffuse manner beyond its cellular source. Secondly, the lack of available HO isoform-selective inhibitors limited our ability to differentiate the contribution of HO-1 vs. HO-2 to the CO-mediated effects on SON neuronal activity reported here. In fact, our studies also demonstrate the presence of the HO-2 isoform in the SON and PVN. While HO-2 immunoreactivity (at least under basal conditions) was weaker than that of HO-1, particularly in the PVN, we cannot discard at present its contribution to basal CO levels within the SON. Future studies are warranted to determine whether the HO-2 isoform is also regulated in an activity-dependent manner in the magnocellular system. Finally, and to be consistent with previous electrophysiological studies in MNCs from dehydrated rats (20-22), electrophysiological recordings in both experimental groups were obtained at the same “normal” aCSF osmolarity. Please note that before recordings, slices were maintained in a holding chamber containing normal aCSF for at least 1 hour, minimizing the possibility that the differences observed in dehydrated rats were the consequence of rapid reversal from hyperosmolarity, rather than chronic hyperosmolarity itself.

Perspectives

Given the previously well-established role of nitric oxide in the regulation of the magnocellular system (24-26) it seems relevant to draw comparisons between these two gas neurotransmitters. In this sense, numerous studies demonstrated high expression levels of the neuronal isoform of nitric oxide synthase (nNOS) in both OT and VP neurons (31, 42) which similar to our findings with HO-1, is upregulated in an activity-dependent manner during osmotic challenges (43, 44). Differently from the HO/CO system however, the general consensus is that NO is constitutively produced within the SON and PVN, acting as a strong inhibitor of neuronal activity (31, 45, 46), and hormone release from the posterior pituitary (24, 25, 47, 48). Taken together, these studies support the notion that NO and CO are two functionally relevant gas molecules with opposing actions in the magnocellular system (i.e., NO inhibitory and CO excitatory). Moreover, it is reasonable to speculate that a dynamic balance between their bioavailability and efficacy constitutes an important determinant of neurosecretory activity in these nuclei. Within this frame, it will be important

in future studies to elucidate signaling mechanisms that either selectively or commonly regulate nNOS and/or HO activity. Moreover, given that the HO/CO and NOS/NO systems have been shown to directly interact with each other (49), including CO-mediated NOS inactivation (50), it will also be important to determine whether the CO-mediated excitatory effects described in this study involve a diminished NO bioavailability. These important questions will be the focus of future studies.

In conclusion, our results indicate that magnocellular SON and PVN neurones express HO-1, and in contrast to nitric oxide, CO acts as a functionally relevant excitatory gas neurotransmitter in this system. Moreover, we found the expression and actions of CO to be enhanced in water-deprived rats, indicating that the state-dependent regulation of HO-1/CO in the magnocellular system could constitute a homeostatic mechanism contributing to the needed enhanced neuronal activity during osmotic challenges.

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Fig. 1. Confocal photomicrographs obtained from euhydrated rats, showing HO-1 (**A,B**) and HO-2 (**C**) immunoreactivities in the supraoptic (**A1, B1-3 and C1** bregma level $\sim -1.40\text{mm}$) and paraventricular anterior (**A2**, bregma level $\sim -1.60\text{mm}$) and medial, at the level of the lateral magnocellular subnucleus (**A3 and C2**, bregma level $\sim -1.80\text{mm}$). A preabsorption control for HO-1 immunoreactivity showing complete lack of staining is shown in **B1**. In **B2** the confocal acquisition settings were enhanced to show the background tissue. A representative control example of omission of primary antibodies is shown in **B3**. Arrows and filled arrowheads point to labeled neurones and putative astrocytes, respectively. Opened arrowheads mark the SON ventral glial laminae or periventricular PVN region. OX, optic chiasm. 3V, third ventricle. Scale bars: $50\mu\text{m}$.

Fig. 2. Confocal photomicrographs displaying HO-1 (*A1-D1*, green), OT (*A2* and *B2*, red) and VP (*C2* and *D2*, red) immunoreactivities in the supraoptic nucleus of euhydrated and 48h water-deprived rats. *A3-D3*, show the respective images superimposed. Arrows point to examples of double-labelled neurones. OX, optic chiasm. 3V, third ventricle. Scale bars: 50µm.

Fig. 3. Confocal photomicrographs displaying HO-1 (**A1-D1**, green), OT (**A2** and **B2**, red) and VP (**C2** and **D2**, red) immunoreactivities in the lateral magnocellular subnucleus of the PVN of euhydrated and 48h water-deprived rats. **A3-D3**, show the respective images superimposed. Arrows point to examples of double-labelled neurones. OX, optic chiasm. 3V, third ventricle. Scale bars: 50µm.

Fig. 4.

Bar graphs showing the mean number of immunoreactive neurones (**A**) and mean immunoreactivity intensity (**B**) of HO-1, OT or VP neurones in the SON and in the lateral magnocellular subnucleus of the PVN in euhydrated (EU) and 48h water-deprived (48WD) rats. **C**, Bar graph showing the mean proportion of OT and VP neurones showing HO-1 immunoreactivity (double-labelled neurones) in the SON and PVN of EU and 48WD rats. Data are reported as means $\pm$ SEM. One-Way ANOVA followed by Dunn's post-hoc tests was used to determine differences among groups within the SON and PVN, respectively. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to EU group. + $P < 0.05$ compared to respective group.

Fig. 5. Confocal photomicrographs showing HO-1 (A1-D1, green) and S100β (A2-D2, red) immunoreactivities in the SON ventral glial laminae (A and B) and the periventricular PVN region (C and D) of euhydrated (EU) and 48h water-deprived (48WD) rats. A3, B3, C3, and D3, show the respective images superimposed. Arrows point to examples of double-labelled astrocytes. OX, optic chiasm. 3V, third ventricle. Scale bars: 50μm. Bar graphs showing the proportion of double-labelled S100β/HO-1 astrocytes, as well as HO-1 immunoreactivity intensity within the SON ventral glial lamina and the periventricular PVN region of EU or 48WD rats are shown in E and F, respectively.

Fig. 6.

Representative examples showing the effects of HO inhibition (CrMP 20 μ M) on the membrane potential and firing activity of SON neurones recorded from a euhydrated (EU, upper trace) and a 48h water-deprived (48WD, lower trace) rat (**A**). Bar graphs summarizing the effects HO inhibition on the mean resting V_m (**B**) and firing activity (**C**) of SON neurones in EU and 48WD rats. * $P < 0.05$ compared to EU rats.