Carbon monoxide and nitric oxide modulate hyperosmolality-induced oxytocin secretion by the hypothalamus in vitro

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Synopsis
OT (oxytocin) is secreted from the posterior pituitary gland, and its secretion has been shown to be modulated by NO (nitric oxide). In rats, OT secretion is also stimulated by hyperosmolarity of the extracellular fluid. Furthermore, NOS (nitric oxide synthase) is located in hypothalamic areas involved in fluid balance control. In the present study, we evaluated the role of the NOS/NO and HO (haem oxygenase)/CO (carbon monoxide) systems in the osmotic regulation of OT release from rat hypothalamus in vitro. We conducted experiments on hypothalamic fragments to determine the following: (i) whether NO donors and NOS inhibitors modulate OT release and (ii) whether the changes in OT response occur concurrently with changes in NOS or HO activity in the hypothalamus. Hyperosmotic stimulation induced a significant increase in OT release that was associated with a reduction in nitrite production. Osmotic stimulation of OT release was inhibited by NO donors. NOS inhibitors did not affect either basal or osmotically stimulated OT release. Blockade of HO inhibited both basal and osmotically stimulated OT release, and induced a marked increase in NOS activity. These results indicate the involvement of CO in the regulation of NOS activity. The present data demonstrate that hypothalamic OT release induced by osmotic stimuli is modulated, at least in part, by interactions between NO and CO.

Key words: carbon monoxide, haem oxygenase, hypothalamus, nitric oxide, osmotic regulation, oxytocin (OT)

INTRODUCTION
Mammals control the volume and osmolality of their body fluids via stimuli that arise from both intracellular and extracellular fluid compartments. These stimuli are sensed by two kinds of receptors: osmoreceptor-Na+ receptors and volume or pressure receptors. This information is conveyed to specific areas of the CNS (central nervous system) and generates an integrative response. This response requires the integrity of the AV3V (anteroventral third ventricle) region [e.g. OVLT (organum vasculosum of the lamina terminalis), MnPO (median preoptic nucleus) and SFO (subfornical organ)], the neurohypophyseal system and brainstem structures [1].

The hypothalamo-neurohypophyseal system plays an important role in the maintenance of body fluid homeostasis through AVP (vasopressin) and OT (oxytocin) release in response to osmotic and non-osmotic stimuli. Central administration of OT inhibits water intake in rats subjected to water deprivation, hypertonic saline administration and angiotensin II injection [2]. In addition, numerous studies have demonstrated that central OT inhibits salt appetite [3]. Indeed, OT-deficient mice have enhanced salt intake [4]. Sodium chloride intake is more prominent when pituitary OT release is low or at basal levels, and it is inhibited when OT release is stimulated [3]. These results support the idea that, in addition to its activity in the mammary gland and during parturition, the oxytocinergic system plays an important role in fluid balance regulation.

NO (nitric oxide) and CO (carbon monoxide) are neuromodulators that influence physiological and pathological processes in the peripheral nervous system and CNS [5]. NOS (nitric oxide synthase), which generates NO, has been identified in several

Abbreviations used: ANP, atrial natriuretic peptide; AVP, vasopressin; AV3V, anteroventral third ventricle; CNS, central nervous system; HO, haem oxygenase; KRBG, Krebs–Ringer bicarbonate buffer; LDH, lactate dehydrogenase; l-NAME, N^6-nitro-l-arginine methyl ester; l-NMMA, N^6-monomethyl-l-arginine; MnPO, median preoptic nucleus; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one; OT, oxytocin; OVL, organum vasculosum of the lamina terminalis; PVN, paraventricular nucleus; SFO, subfornical organ; sGC, soluble guanylate cyclase; SIN-1, 3-morpholinosydnonimine hydrochloride; SNP, sodium nitroprusside; SON, supraoptic nucleus; ZnPPIX, protoporphyrin IX zinc(II).

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structures within the neural circuit that regulate body fluid homeostasis, including SFO, MnPO, OVLT and the hypothalamo-neurohypophyseal system [5,6]. Neurons from these structures have direct and indirect anatomical connections with magnocellular cells, suggesting a potential function of NO in the modulation of AVP and OT release [1]. These actions are at least partly mediated by the modulation of magnocellular neuron activity in the hypothalamic SON (supraoptic nucleus) and PVN (paraventricular nucleus). For example, NO regulates (usually through inhibition) the electrical activity of vasopressinergic and oxytocinergic neurons [7,8]. In addition, in vitro and in vivo studies have shown that NO inhibits the release of OT [9–12] and ANP (atrial natriuretic peptide) [13].

Carbon monoxide is generated by HO (haem oxygenase), which is also found in several areas in the hypothalamus [14]. HO is expressed in the PVN and the SON [14]. Increased CO generation was clearly associated with K+–stimulated OT release [15] and inhibition of AVP [16] release from rat hypothalamic explants. It also modulates the release of CRH (corticotropin-releasing hormone) [17], GnRH (gonadotropin-releasing hormone) [18] and ANP [13], demonstrating that CO plays an important role in neuroendocrine regulation.

Physiologically, NO and CO display notable similarities. For example, both NO and CO can bind the iron atom of the haem moiety associated with sGC (soluble guanylate cyclase), thereby activating the enzyme and increasing intracellular cGMP production [19]. However, other reports have demonstrated that CO and NO have opposite effects [20]. Furthermore, these two gases do not always work independently, but can modulate each other’s actions [21].

We hypothesized that CO may be involved in the control of body fluid homeostasis. The present study aimed to determine the relationship between NOS/NO and HO/CO systems in the control of in vitro OT secretion by hypothalamic fragments under basal and hyperosmotic conditions.

### MATERIALS AND METHODS

#### Animals and experimental preparation

Adult male Wistar rats weighing 180–200 g were housed in individual cages in a temperature-controlled environment (23 ± 2°C) with a 12 h light/12 h dark cycle (lights were switched on at 06:00 h), with free access to rat chow and tap water. All experiments were performed between 09:00 h and 10:00 h. All the experimental procedures involving animals were performed in accordance with the ‘Guide for the care and use of laboratory animals’ (Brazilian College of Animal Experimentation) and approved by the local ethics committee (School of Medicine of Ribeirao Preto of the University of Sao Paulo – Ethical Commission on Animal Research).

The method of hypothalamic incubation used in the present study has been previously described and validated [13]. After decapitation, the brain was quickly removed and the hypothalamic was immediately dissected. The hypothalamic fragment included an area 1.0 mm lateral to the midline beginning at the anterior border of the optic chiasma and ending at the anterior border of the mammillary bodies with a depth of approx. 2.5 mm. The hypothalamic fragment included OVLT, MnPO, SON, PVN, supra-chiasmatic and arcuate nuclei. The total dissection time was less than 1 min after decapitation. Each basal hypothalamus was transferred to a well containing 0.5 ml of cold KRBB (Krebs—Ringer bicarbonate buffer; 118.46 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.18 mM NaH2PO4, 1.18 mM MgSO4 and 24.88 mM NaHCO3, pH 7.4) with 1% glucose and pre-incubated in a Dubnoff shaker (50 cycles per min) at 37°C for 60 min in an atmosphere of 95% O2 and 5% CO2. After the pre-incubation, the medium was gently aspirated and replaced with fresh medium containing KRBB with 280 or 340 mOsm/kg of water with NaCl and/or KRBB containing the substances to be tested. The fragments were then incubated for a further 30 min period. At the end of the incubation, all fragments were exposed to a 30 min pulse of 56 mM KCl to assess their ability to respond to a depolarizing stimulus. Medium obtained from each incubation period was kept frozen at −20°C until OT or nitrite determinations were performed.

#### Chemicals

SNP (sodium nitroprusside; 300 μM), l-NAME (N^6^-nitro-L-arginine methyl ester; 300 μM), l-NMMA (N^6^-monomethyl-L-arginine; 300 μM), 8-bromo-cGMP (10 and 100 μM) and ZnPPIX [protoporphyrin IX zinc(II); 10^−6 and 10^−7 M] were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). SNAP (5-nitroso-N-acetyl-L-penicillamine; 300 μM), SIN-1 (3-morpholinosydnonimine hydrochloride; 300 μM) and ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; 100 μM) were obtained from Tocris (Ellisville, MO, U.S.A.). ZnPPIX and ODQ were dissolved in DMSO, which was added to the control vehicle. All other drugs were dissolved in KRBB on the day of the experiment. All the doses used in the present study were based on previous reports in the literature [13,22–25].

#### NOS activity

For the determination of NOS activity, we used the method described by Bredd and Snyder [26], which measures the conversion of [14C]arginine into [14C]citrulline. After incubation (30 min), the hypothalamic fragments were homogenized with 0.2 ml of 20 mM Hepes (pH 7.4) containing 1.25 mM CaCl2, 1 mM DTT (dithiothreitol) and 100 mM tetrahydrobiopterin (BH4). After homogenization, 1 mM NADPH and 200 000 cpm of [14C]arginine (270 μCi/mmol) were added and the homogenates were incubated for 15 min at 37°C. The homogenates were then centrifuged at 10000 g for 10 min at 4°C. The resulting supernatants were applied to 2 ml columns of Dowex AG WX-8 (Na+ form) and eluted with 3 ml of double-distilled water. [14C]Citrulline levels were then determined using a beta counter. This method is based on the equimolar production of citrulline and NO from arginine, which is mediated by NOS. The results are expressed as citrulline production per mg of tissue.
CO and NO modulate oxytocin secretion

Viability of hypothalamic fragments
To validate the viability of the hypothalamic fragments, we used an automated method to measure the amount of LDH (lactate dehydrogenase) released into the incubation medium (Roche Diagnostic System).

RIA and protein determination
OT measurements were performed by RIA as previously described [27]. All samples from the same experiment were measured in a single assay in duplicate. The assay sensitivity and intra- and inter-assay coefficients of variation were 0.9 pg/ml, 12.6% and 7% respectively. Hormone release is expressed as pg/mg of protein. Protein content of the hypothalamic fragment was determined as previously described by Bradford [28].

Nitrite
Nitrite concentration (NO metabolite) in 10 μl of the incubation medium from each hypothalamic fragment was determined using a Sievers nitric oxide analyser (Sievers Instruments) [29]. Results are expressed as μM of nitrite production. This reflects the nitrite accumulation in the medium from each hypothalamic fragment.

Data analysis
The results are reported as means ± S.E.M. and were subjected to one-way ANOVA, followed by the Newman–Keuls post hoc test. The level of significance was set at P < 0.05.

RESULTS

Viability of hypothalamic fragments
As can be observed in Figure 1, there was no difference in the concentration of LDH in the medium when comparing the effects of incubation in isotonic or hypertonic solutions treated with NO donors. This result indicates that there is comparable tissue viability between the two conditions.

Effect of osmotic stimulation on OT release and nitrite production
To evaluate the influence of osmolality on OT release, hypothalamic fragments were incubated in iso-osmotic (280 mOsm/kg of water) or hyperosmotic medium (340 mOsm/kg of water, with sodium chloride). Exposing basal hypothalamus to the hyperosmotic medium resulted in a significant increase in OT release (107.2 ± 5.4 versus 177.5 ± 5.40 pg of OT/mg of protein, P < 0.001), which was associated with a significant decrease in nitrite production (0.58 ± 0.12 versus 0.25 ± 0.05 μM, P < 0.01). These results suggest that hyperosmolality increases OT secretion, concomitant with a reduction in NO production.

Effect of NO donors and NOS inhibitors on OT release
To address the direct role of NO on OT release, NO donors (SNP, SNAP or SIN-1; 300 μM) were used to increase NO levels in the hypothalamic fragment. As shown in Figure 2(A), addition of NO donors resulted in a significant decrease in osmotically stimulated OT secretion. In contrast, basal OT release was unaffected by incubation of the hypothalamus with NO donors. Non-specific NOS inhibitors (L-NAME and L-NMMA; 300 μM) did not alter either basal or osmotically stimulated OT release (Figure 2B).

Effect of HO inhibitor on OT release and NOS activity
To evaluate the participation of the HO/CO system in OT release, we used a well-established pharmacological approach to induce
Figure 3 Effects of ZnPPIX, an HO inhibitor, on OT release (A) and NOS activity (B) in hypothalamic fragments

Values are means ± S.E.M. *P < 0.05 (compared with vehicle at 280 mOsm/kg of water); ++P < 0.001 (ZnPPIX at 10⁻⁵ and 10⁻⁴ M compared with vehicle at 340 mOsm/kg of water). The number of hypothalamic fragments per group is given in parentheses.

changes in HO activity. HO inhibition using ZnPPIX (10⁻⁵ and 10⁻⁴ M) induced a decrease in OT secretion from hypothalamic fragments under both isotonic and hypertonic conditions (Figure 3A). To investigate the interactions between the HO/CO and NOS/NO systems, NOS activity was evaluated in the presence of ZnPPIX. We observed an increase in NOS activity, suggesting that CO may inhibit NO synthesis (Figure 3B).

Effect of ODQ on ZnPPIX- and SNP-induced inhibition of OT release

To further evaluate cross-talk between the HO/CO and NOS/NO systems and to investigate the mechanism by which ZnPPIX inhibits OT release, we used ODQ (100 μM), a selective inhibitor of sGC. We observed that in the presence of ODQ, the effect of ZnPPIX on both basal and hyperosmotically stimulated OT release was completely reversed (Figure 4A), indicating that inhibition of OT release by ZnPPIX involves the NO–sGC–cGMP pathway. To confirm that the NO donor (SNP) also activates the sGC–cGMP pathway, hypothalamic fragments were incubated with both SNP and ODQ. As shown in Figure 4(B), the inhibitory effect of SNP on OT release was also reversed by ODQ co-incubation under hyperosmotic stimulation.

Effect of 8-bromo-cGMP on OT release

We also tested the effects of a membrane-permeable cGMP derivative, 8-bromo-cGMP (10 and 100 μM), on OT release. Application of 8-bromo-cGMP under hyperosmotic conditions caused a significant decrease in OT release (Figure 4C), mimicking the effect of NO donors (see Figure 2A). This result suggests that the inhibition by NO is at least partly mediated by local activation of the sGC–cGMP system.

DISCUSSION

The present results show that in vitro incubation of hypothalamic fragments in hypertonic medium (from 280 to 340 mOsm/kg of water with sodium chloride) results in an increase in OT release. Similar to our results, previous in vivo and in vitro studies have also shown that hyperosmotic stimulation causes an increase in OT release [30,31]. It is well known that OT inhibits salt intake,
stimulates ANP release by the heart and also increases renal sodium excretion [1]. Therefore OT release contributes to the normalization of extracellular volume and plasma osmolality [1,32]. This osmotically induced OT release was associated with a decrease in nitrite production, which reflects reduced NO production. To further study the modulatory actions of NO on OT release, we used NO donors to increase NO levels. SNP, SNAP and SIN-1 had no effect on basal release (iso-osmotic condition), but effectually suppressed osmotically stimulated OT release, suggesting that NO selectively inhibits the OT release induced by osmotic stimuli.

The SON and PVN receive inputs from the forebrain osmosensitive network in the lamina terminalis (e.g. AV3V) to regulate OT and AVP secretion. A number of studies have reported NOS expression in the AV3V [5,6] and in magnocellular neurons of the hypothalamic neurosecretory system, including the SON and PVN [33]. In addition to OT, the hypothalamic magnocellular neurons co-express NOS [34], suggesting a functional role of NO in the regulation of OT secretion. A previous electrophysiological study has demonstrated that NO modulates the activity of the magnocellular system. In the present study, SNP (a spontaneous releaser of NO) inhibited the firing of OT neurons in the SON in vitro. This effect is prevented by Hb, an NO scavenger. These results indicate that NO reduces OT secretion [7]. Furthermore, Srisawat et al. [8] demonstrated in vivo that SNP applied locally to the SON inhibits the electrical activity of both OT and AVP cells. In addition, central administration of SNP inhibits the hyposmotic stimulation of OT secretion. Other studies directly measured the level of OT in the plasma or hypothalamus by RIA, and showed that NO inhibits OT secretion in rats [35,36]. It has also been demonstrated that intracerebroventricular administration of L-NAME during dehydration produces a selective increase in plasma OT levels, but not vasopressin levels [37].

However, in our study, the NOS inhibitors L-NMMA and L-NAME had no effect on basal or osmotically stimulated OT release. Using the same experimental design, we have previously shown that NOS activity decreases in response to hyposmotic stimulation. We speculate that addition of NOS inhibitors would not change OT release during osmotic stimulation because NOS activity is already decreased. Srisawat et al. [8] reported that NOS inhibitors had no effect on basal OT release, but did potentiate the already elevated plasma OT concentration evoked by hypertonic saline injected intraperitoneally. A possible explanation is that decreased NOS activity may contribute to increased OT release, which is presumably needed to promote natriuresis under hypertonic conditions in vivo [38].

We also investigated the possible involvement of the HO/CO system in OT release and NOS activity. To test this hypothesis, we used a specific HO inhibitor, ZnPPIX. HO inhibition decreased both basal and osmotically stimulated OT release. These results suggest that CO may be a positive modulator of both basal and osmotically stimulated OT release. However, another study reported inhibition of OT release from rat hypothalamic explants by CO. Kostoglou-Athanassiou et al. [15] reported that haemin (a substrate for HO) inhibited KCl-stimulated OT release, but ZnPPIX had no effect on basal or stimulated OT release. Differences in experimental models, time exposures and drug concentrations may account for these discrepancies.

The potential interplay between CO and NO occurs at different levels and may be synergistic or antagonistic, providing an integrated mechanism for the fine-tuning of cellular function. CO can directly bind to and inactivate NO, which decreases enzymatic activity in some preparations [39]. To address whether CO modulates NOS activity and NO production, hypothalamic fragments were incubated with ZnPPIX to inhibit HO activity. We observed that HO inhibition by ZnPPIX induced a marked increase in NOS activity, indicating that constitutive CO release may tonically inhibit NOS activity in the hypothalamus. These results are in accordance with the reports of McMillan et al. [40], Fan et al. [41] and Thorup et al. [42], which show that CO suppresses neuronal, inducible and endothelial NOS activities. Abu-Soud et al. [43] also demonstrated that CO inhibits NOS activity and prevents NO synthesis. On the other hand, Ischiropoulos et al. [44] showed that CO poisoning increases NO production in the rat brain, as detected by EPR spectroscopy. We speculate that under hyposmotic stimulation, CO inactivates NOS and subsequently decreases NO production. Therefore CO may act as an inhibitory modulator of NO signalling. Recent results from our laboratory have demonstrated that inhibition of HO using ZnDPBG (zinc deuteroporphyrin 2,4-bis glycol) produced a clear dose-related increase in NOS activity that was associated with inhibition of ANP release [15] from hypothalamic fragments. These results provide evidence that CO modulates NOS activity and therefore OT release in vitro.

As described above, ZnPPIX induced a dramatic increase in NOS activity, with a significant decrease in OT release. The inhibitory effect of ZnPPIX treatment on OT release could be due to stimulation of NO release and activation of the sGC/cGMP pathway. This assumption was confirmed by the prevention of OT decrease induced by ZnPPIX and SNP treatment in the presence of ODQ, a drug that specifically inhibits NO-sensitive GC. Additionally, we showed that the addition of 8-bromo-cGMP inhibits the OT release induced by hyposmolality, mimicking the effect of an NO donor. sGC is physiologically activated by the gaseous neurotransmitters NO and CO [45]. It is known that in the cerebellum [46], NO diffuses into nearby neurons and glia, where it binds to sGC and activates the enzyme, leading to elevated intracellular levels of cGMP [47]. A similar process may also occur in the hypothalamus. NO appears to release several hormones via the sGC–cGMP system, such as growth hormone [48], insulin [49] and vasopressin [50]. Thus our findings indicate that the inhibitory effect of NO on OT release from hypothalamic fragments involves, at least in part, activation of the sGC–cGMP system.

In conclusion, in the present study, we demonstrate that NO exerts an inhibitory effect on osmotically stimulated OT secretion from hypothalamic fragments, which is mediated by cGMP signalling. We also show that the HO–CO system modulates NOS activity and OT release. The molecular mechanism by which CO suppresses hypothalamic NOS activity remains to be elucidated.
REFERENCES


CO and NO modulate oxytocin secretion


