Hypothalamic release of nitric oxide and interaction with amino acid neurotransmitters in chronically uraemic rats

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Abstract

Background. The activity of the hypothalamic gonadotrophin-releasing hormone (GnRH) pulse generator is diminished in uraemia. Since GnRH release is influenced by nitric oxide (NO) neurotransmission, we examined the integrity of hypothalamic NO neurotransmission in the chronically uraemic rat model.

Methods. Adult male castrated rats were rendered uraemic by two-stage 5/6 nephrectomy. Basal, N-methyl-D-aspartate (NMDA)-stimulated and DL-2-amino-5-phosphonovaleric acid (AP-5)-inhibited NO outflow was measured in uraemic and sham-nephrectomized control animals via a microdialysis probe in the medial preoptic area (MPOA). The influence of the noradrenergic system was evaluated by blocking noradrenergic neurons with N-(2-chloroethyl)-N-ethyl 2-bromobenzylamine (DSP-4). The activity of different NO synthase (NOS) isoforms was investigated by administration of the isoform-specific NOS inhibitors S-methyl-L-thiocitrulline (SMLT) and L-N6-(1-iminoethyl)-lysine (L-NIL). Moreover, hypothalamic mRNA expression of the individual NOS isoforms was quantitated by real-time reverse transcriptase–polymerase chain reaction. Effects of NO on amino acid outflow were assessed by addition of the NO donor S-nitroso-N-acetyl-penicillamine (SNAP).

Results. The expression of different NOS species and basal NO outflow did not differ between uraemic and control animals. Administration of the NO donor SNAP increased local NO production and amino acid outflow similarly in both groups. SMLT but not L-NIL, an inhibitor of the inducible NOS isoform, reduced NO outflow in both groups. AP-5 equally decreased, and noradrenergic blockade increased NMDA-stimulated NO outflow in both groups.

Conclusions. NO is produced locally and may interfere with amino acid neurotransmission in the rat MPOA. Uraemia did not interfere with NO neurotransmission in our study.

Keywords: amino acids; hypothalamus; microdialysis; nitric oxide; uraemia

Introduction

Chronic renal failure (CRF) is commonly associated with alterations of the reproductive system, manifested as delayed or arrested puberty in childhood [1], and hypogonadism or infertility in adult life [2]. In addition to alterations at the gonadal and pituitary level, a neuroendocrine defect has been identified in vitro and in vivo which is characterized by reduced activity of the hypothalamic gonadotrophin-releasing hormone (GnRH) pulse generator (see [1] for a review). This neuroendocrine pacemaker system consists of a group of neurons located in the medial preoptic area (MPOA) which episodically release GnRH into pituitary portal vessels, eventually eliciting pulsatile gonadotrophin secretion from the pituitary into the systemic circulation. We and others have previously described that the rate of luteinizing hormone release from the pituitary is diminished in human and experimental uraemia [3], and the amount of GnRH peptide per secretory burst arriving in the pituitary is markedly reduced [4]. This finding prompted us to evaluate further the neurotransmitter milieu to which the neurons of the GnRH pulse generator are exposed. While the generation of GnRH pulses by synchronized release of the peptide is apparently an intrinsic property of the neurosecretory cells [5], their activity is modulated by a complex network of neuronal afferences involving, among others, noradrenergic, glutaminergic, GABAergic, and...
Nitric oxide (NO) is produced by NO synthases (NOSs). Three isoforms have been identified so far: in contrast to the inducible nitric oxide synthase isoform (iNOS), the endothelial (eNOS) and neuronal (nNOS) isoforms are stimulated via a calcium- and calmodulin-dependent pathway which can be activated by neurotransmitters such as noradrenaline and glutamate [8].

Recent research has confirmed the existence of NOS-containing neurons in the MPOA. Large NO fibres are scattered throughout the MPOA [9], and local NO neurons expressing glutaminergic N-methyl-D-aspartate (NMDA) receptors have been observed as well [10]. NOS stimulation leads to increased, and inhibition to decreased GnRH release [11,12]. The stimulation of GnRH release by glutamate and noadrenaline as well as the inhibitory effect of γ-aminobutyric acid (GABA) can be modulated by NO [11,12]. We recently demonstrated alterations of the amino acid neurotransmitter milieu in the MPOA in experimental CRF [13].

Intracerebral microdialysis via stereotactically implanted membranes permits investigation of the local neurotransmitter milieu with minimal mechanical and no osmotic tissue trauma, and the conditions of neurotransmission can be selectively influenced by adding compounds to the dialysis fluid which interfere with neurotransmitter synthesis, release or degradation. In this study, we performed intracerebral microdialysis in the MPOA of unanaesthetized, freely moving, chronically uraemic and control rats to evaluate in vivo the contribution of NO to altered GnRH secretion in CRF.

Methods

Animals and experimental groups

Eight-week-old male Sprague–Dawley rats (Ivanovas Co., Kisslegg, Germany) were housed under controlled temperature (21–22°C) with lights on from 7 a.m. to 9 p.m. After an adaptation period of at least 7 days, the animals were randomly allocated to two experimental groups: uraemic animals and sham-operated controls (n = 4–6). Regular chow food (Altromin, Lage, Germany) was offered to all animals.

Abdominal surgery

All surgical interventions were performed as described previously [13]. Briefly, experimental uraemia was induced by removal of the upper and the lower pole of the left kidney, followed by complete removal of the right kidney 7 days later. In the control animals, sham procedures involving two-stage kidney decapsulation were performed. All animals were orchidectomized simultaneously with the initial partial nephrectomy/sham surgery.

Microdialysis experiments

Six days after the second abdominal surgical intervention, an intracerebral guide cannula was implanted adjacent dorsally to the right hypothalamic MPOA as described previously [13]. Two days after insertion of the guide cannula, a microdialysis probe (concentric double-lumen microdialysis probes with polycarbonate hollow fibre dialysis membranes (length 2 mm, outer diameter 500 μm, cut-off 20 kDa), CMA/12, CMA/Microdialysis AB, Stockholm, Sweden) was inserted with the tip extending 2.5 mm into the MPOA and connected to the collection assembly under light ether anaesthesia. The inlet tubing of the probe was connected to a 1 ml Hamilton gas-tight syringe (Zimmer, Mannheim, Germany) in a perfusion pump precidor (INFORS AG, Bottmingen, Switzerland) and continuously perfused with artificial cerebrospinal fluid (CSF) (concentrations in mM: 144 NaCl, 2.5 KCl, 1.3 CaCl2, buffered to pH 7.4 by 2 mM sodium phosphate buffer) at a flow rate of 2 μl/min. The total dead space of the outlet part of the probe and the outlet tubing was 6.5 μl. After an equilibration period of at least 1.5 h, samples were collected in 10 min fractions.

The six experimental protocols performed are illustrated in Table 1. Four to six experiments per treatment (uraemic and control) with confirmed probe location were performed with each protocol.

In order to assess the influence of glutamate and noadrenaline on local NO production, the glutamate agonist NMDA (1 mM) was added to the perfusion media alone (protocols A and C) or in combination with the NMDA antagonist D-2-amino-5-phosphonovaleric acid (AP-5, 1 mM; protocol B). To assess possible influences of the noradrenergic system on local NO production, the irreversible neurotoxin N-(2-chloroethyl)-N-ethyl 2-bromobenzylamine (DSP-4, 50 mg/kg intraperitoneally) was applied 1 week prior to the microdialysis experiment in protocol C. DSP-4 leads to an irreversible subtotal damage of ~75% of the noradrenergic system. Protocols D–F were performed to evaluate the activities of several NOS isoforms in the MPOA and the effect of local NO synthesis on amino acid neurotransmitter release. After a 40 min baseline period, the NO donor S-nitroso-N-acetylpenicillamine (SNAP, 1 mM) was added to the perfusion medium (protocol D). To assess the influence of selective inhibitors of NOS isoforms L-N^6-(1-iminoethyl)-lysine (L-NIL, 100 μM), a selective inhibitor of iNOS, and S-methyl-L-thiocitrulline (SMLT, 1 mM), a selective inhibitor of nNOS, were added in protocols E and F (see Table 1).

In protocol D, the perfusion rate of artificial CSF was 4 μl/min, and the fractions were divided for amino acid and NO measurements, respectively.

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<th>Table 1. Microdialysate collection protocols after 90 min of equilibration time</th>
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*aDSP-4 7 days earlier.
A gas-tight liquid switch (CMA/110, CMA/Microdialysis AB, Stockholm, Sweden) was used to change between the different perfusion media. The samples were snap-frozen immediately after collection and stored at −70°C. Immediately after the end of the study, the animals were sacrificed by aortic puncture under chloral hydrate anaesthesia. In order to verify the probe location, eosin was injected through a used, membraneless probe. The brains were conserved in 10% formaldehyde and embedded in paraffin. The probe track was located by inspection of sequential 50 µm thick transversal brain slices counter-stained with haematoxylin. Animals with incorrect probe placement (n = 12; 20% of all animals) were excluded from further protocols. The microdialysis probes were used up to three times. After each use, the probes were flushed with water and stored for 12 h in an 0.5 mM HCl solution to remove any matrix layers from the membranes. The mean in vitro recovery rates, determined for each probe prior to each experiment by perfusion at 2 µl/min in a fluid containing a known amount of NO or of the amino acids, were 19% for nitrite, 16% for nitrate, 30% for glutamate, 18% for aspartate and 16% for GABA. The recovery rates decreased by an average of 3% per use. Since the relationship between in vitro and in vivo solute recovery is complex, the measured dialysate concentrations were not corrected for in vitro recovery.

**Neurotransmitter and NO analysis**

Microdialysate samples were analysed as described elsewhere [14]. Briefly, nitrite and nitrate were measured using an isocratic high-performance liquid chromatography (HPLC) system with fluorometric detection after pre-column derivatization. The system consisted of a degasser (CMA/252), an isocratic pump (CMA/250), a refrigerated autoinjector (CMA/200), a C18 column (150 × 4.6 mm Zorbax Eclipse XDB-C18 column, particle size 5 µm) and a fluorescence detector (CMA/280). The mobile phase consisted of 10 mM borate buffer (pH 9.0) and 25% acetonitrile (Sigma, St Louis, MO). The lower limit of detection was 100 fmol/20 µl. The dose–signal relationship was linear between 100 fmol/20 µl and 500 pmol/20 µl for nitrite and nitrate. The coefficients of variation at concentrations of 0.2 and 10 pmol/20 µl were 6.2 and 3%, respectively. Amino acids were analysed as described previously [4] by the HPLC system described above using a C18 column (100 × 3.1 mm Zorbax, 3 µm). The mobile phase consisted of 0.1 M acetate (pH 5.4) with 17.5% acetonitrile and 2.5% borate buffer (pH 9.0) and 25% acetonitrile (Sigma, St Louis, MO). The mean in vitro recovery rates, determined for each probe prior to each experiment by perfusion at 2 µl/min in a fluid containing a known amount of NO or of the amino acids, were 19% for nitrite, 16% for nitrate, 30% for glutamate, 18% for aspartate and 16% for GABA. The recovery rates decreased by an average of 3% per use. Since the relationship between in vitro and in vivo solute recovery is complex, the measured dialysate concentrations were not corrected for in vitro recovery.

**Real-time RT–PCR assay**

A separate set of animals was used to analyse specific NOS mRNA expression in the MPOA by real-time reverse transcription-polymerase chain reaction (RT–PCR). One week after 5/6 nephrectomy, the animals were sacrificed. The brains were removed immediately and frozen in iso-pentane at −60°C. The MPOA was dissected and cut into 6 µm sections at −20°C in a cryomicrotome. Sections were collected in an Eppendorf vial and homogenized with Ultra Turrax in TRI reagent (Sigma, St Louis, MO). Total RNA was isolated, checked for integrity on an agarose gel and quantitated photometrically. A 1 µg aliquot of total RNA was reverse transcribed using oligo(dT)/random hexamer primers (1/10). Real-time RT–PCR was performed using specific primers for iNOS (forward, AGTGGTGGAGCCGATTGTGTC; reverse, GCTGAGCCAGTTCAAGTACG; amplicon length 205 bp), eNOS (forward, GTCAGTTACCAGCTGCGCAAGT; reverse, CTGGGCTTCTGC TCAATTTCC; amplicon length 93 bp), nNOS (forward, CAAGGAGCCTGTGATGTT; reverse, CCATACAC GTGAGCGGGACC; amplicon length 119 bp) and iNOS (forward, CATACCTTCAGTATGCCTTGGGTTT; reverse, GGTGAGAGCATTTCTGGTCGATG; amplicon length 94 bp).

mRNA levels were normalized to corresponding 18S quantities determined within the same run. (RT–) RNA controls in which the cDNA synthesis step was omitted and controls without template did not show a detectable amplification product. Each sample was analysed in duplicate in individual assays.

**Statistics**

Data are given as means ± SEM. After evaluation of the mode of distribution using the Shapiro–Wilk statistic, those variables that did not exhibit a Gaussian distribution were log-transformed. Subsequently, data were analysed using parametric techniques, i.e. two-way analysis of variance (ANOVA) with treatment (uraemic and control) as covariates, followed by Duncan’s test for multiple comparisons, for evaluation of between-group differences, and two-tailed paired t-tests for assessment of longitudinal (within-group) differences. P-values < 0.05 were considered as statistically significant.

**Results**

The uraemic rats consumed on average 18% less food than the control animals (food intake 301 ± 5.71 g in uraemic and 367 ± 7.04 g in control animals). The two-stage 5/6 nephrectomy resulted in a 55% reduction in renal mass (right kidney, 0.93 ± 0.02 g in uraemic and 1.02 ± 0.03 g in control rats; left kidney, 0 g in uraemic and 1.04 ± 0.02 g in control rats), and a 3-fold increase of serum creatinine and urea levels (urea, 87.67 ± 6.76 mg/dl in uraemic and 36.7 ± 3.77 mg/dl in control rats; creatinine, 1.6 ± 0.69 in uraemic and 0.55 ± 0.05 mg/dl in control rats).

**NO outflow**

The baseline periods of all experiments (protocols A–F) were combined to evaluate the effect of uremia on basal NO outflow. Basal NO levels did not differ between uraemic and control animals (1.44 ± 1.02 and 1.50 ± 1.20 µM, respectively).

In protocol A, stimulation with NMDA (1 mM) resulted in restlessness in all animals, necessitating
interrupted the experiments. Thus, only three animals per group were treated with this protocol. A 284% increased NO outflow was observed in nephrectomized animals, whereas there was no increase in control animals (NS).

Addition of the competitive NMDA antagonist AP-5 partially reversed the NMDA-induced increase of NO outflow; when used in combination with AP-5, NMDA stimulated NO production to 133 ± 51% of the respective basal secretion, without any significant differences between uraemic or control animals (uraemic, 135 ± 39% vs controls, 130 ± 62%; protocol B). After partial blockade of the noradrenergic system by DSP-4 1 week prior to the microdialysis experiment, basal NO levels remained unchanged (uraemic, 1.08 ± 0.63 μM vs controls, 1.16 ± 0.83 μM; protocol C). The NMDA-induced stimulation of NO outflow was increased without significant differences between uraemic and control animals (uraemic, 190 ± 61% vs controls, 164 ± 52%, NS, see Figure 1).

In protocol C, addition of the NO donor SNAP resulted in an immediate and reversible 40-fold increase of NO outflow without significant differences between uraemic and control animals (uraemic, basal 2.51 ± 2.43 μM, stimulated 84.5 ± 34.4 μM; control, basal 1.67 ± 1.19 μM, stimulated 61.5 ± 18.1 μM, NS; see Figure 2).

The nNOS inhibitor SMLT but not l-NIL, an inhibitor of the iNOS isoform, reduced NO outflow by 76% without significant differences between uraemic and control animals (protocols E and F). This inhibitory effect lasted for the 30 min post-stimulation period (see Figure 3).

Amino acid outflow

The NO donor SNAP significantly increased amino acid outflow without differences between the treatment groups (glutamate, 150%; aspartate, 130%; GABA, 3260%; see Figure 2).

NOS mRNA expression

Real-time PCR assays revealed no difference in mRNA expression of nNOS, eNOS or iNOS in uraemic or control animals (see Figure 4).

Discussion

This is the first study providing evidence for local NO production and influence of NO on other neurotransmitter systems in the MPOA of freely moving, awake rats. All experiments were performed in uraemic and non-uraemic animals, respectively, in order to assess a possible involvement of the NO system in the pathogenesis of uraemic hypogonadism.

For technical reasons, it was not possible to measure directly the influence of these manipulations upon GnRH release: first, this would have required the implantation of a second microdialysis probe in
of the direct vicinity of the MPOA, causing mechanical problems. Secondly, when blended with the manipulated GnRH release from the ipsilateral side, the uninfluenced GnRH release from the contralateral MPOA might have diminished any effects of agonists or inhibitors applied via the microdialysis probe implanted into one MPOA only. Mean basal NO concentrations in MPOA microdialysates were \( \sim 1.45 \text{ mmol/l} \). Taking into account a recovery rate of the microdialysis probe of 17\%, the extracellular concentration of NO would be estimated at \( \sim 8.5 \text{ mmol/l} \). This concentration is 2- to 3-fold lower than NO concentrations previously reported from other brain regions [15]. Puo et al. [16] had reported significantly decreased levels of cGMP, the only known second messenger of the NO system, after castration in the MPOA. In our study, all animals were orchidectomized to exclude any influences of decreased sex steroid levels on the NO system in uraemic hypogonadism. Thus, the relatively low basal NO concentrations may be partially explained by the castration procedure. However, further studies are necessary to clarify these differences.

We observed an increase of NO generation after local stimulation with the glutamate agonist NMDA associated with restless movements which prompted us to perform these experiments in only three animals/group. No discomfort was observed in animals simultaneously treated with the NMDA antagonist AP-5, or after previous partial destruction of noradrenergic input. The NMDA-induced NO generation was blunted after application of AP-5 but increased after partial

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**Fig. 2.** Mean±SD dialysate concentrations of NO, glutamate, aspartate and GABA measured simultaneously during the 40min baseline period followed by 40min of perfusion with 1 mM of the NO donor SNAP.
destruction of noradrenergic neurons. These findings provide a strong argument for the presence of local NO production in the MPOA which might be regulated by glutamergic and noradrenergic neuronal systems.

Furthermore, in order to assess possible effects of NO on amino acid neurotransmitter release in the MPOA, we measured amino acid outflow after perfusion with SNAP, an NO donor, and with inhibitors of different NOS isoforms. We observed an immediate increase in glutamate and GABA release, and a delayed increase of aspartate release after application of SNAP.

Application of SMLT, an inhibitor of nNOS, resulted in a decrease of glutamate, aspartate and GABA release by ∼30–40%. Our findings provide evidence for a local cross-talk of NO with amino acid neurotransmitter systems of the MPOA predominantly involving nNOS, but not iNOS.

In the uraemic animals, basal NO outflow was not significantly different from controls. Aiello et al. [17] had reported increased NO serum levels under uraemic conditions. Since NO crosses the blood–brain barrier, one would expect increased NO microdialysate levels if blood-derived NO metabolites accounted for the major part of NO in the extracellular space of the MPOA. The unaffected basal NO levels we found in uraemic animals suggest either that extracellular NO in the MPOA is derived predominantly from local production or that local NO production is concomitantly decreased in uraemia, as reported from other tissues [17]. Decreased NO synthesis could result from NOS inhibitors accumulating in uraemia [18]. On the other hand, Noris et al. [19] had reported increased cytokine concentrations in uraemia. Cytokines are known to induce an upregulation of the iNOS isoform [20]. Thus, we studied the effects of selective inhibition of the three NOS isoforms. SMLT but not l-NIL, an inhibitor of the iNOS isoform, reduced NO outflow without significant differences between uraemic and control animals. Moreover, we observed no alterations of basal gene expression of any of the different NOS isoforms. Together with the unaffected basal NO synthesis and the unchanged effects of NO stimulation (SNAP and NMDA), these results argue against different NOS activities in the MPOA of uraemic animals. Thus, it seems unlikely that local NO production in the MPOA of rats is altered by uraemia.
Uraemia may interfere not only with NO synthesis and release, but also with its receptor binding or post-receptor signal transduction cascades. To this end, we studied local NO action by measuring amino acid neurotransmitter outflow after stimulation and inhibition of NO release in uraemic and control animals. We did not observe any effects of stimulating NO release or inhibiting NOS activity on amino acid outflow between uraemic and control animals.

In summary, we found evidence that NO is produced locally and may interfere with amino acid neurotransmission in the rat MPOA. We did not, however, find evidence for altered local NO production or local action of NOS inhibitors in the MPOA of uraemic rats.

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Conflict of interest statement. None declared.

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