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Nitric oxide modulation of ET<sub>B</sub> receptor-induced vasopressin release by rat and mouse hypothalamo-neurohypophyseal explants

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<sup>1</sup>Departments of Medicine and Physiology, Wayne State University School of Medicine and John D. Dingell VA Medical Center; and <sup>2</sup>Department of Physiology, Wayne State University School of Medicine and Henry Ford Hospital System, Detroit, Michigan

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Rossi, Noreen F., and William H. Beierwaltes. Nitric oxide modulation of ET<sub>B</sub> receptor-induced vasopressin release by rat and mouse hypothalamo-neurohypophyseal explants. Am J Physiol Regul Integr Comp Physiol 290: R1208–R1215, 2006. First published December 15, 2005; doi:10.1152/ajpregu.00701.2005.—Endothelin (ET) peptides stimulate vasopressin (AVP) secretion via ET<sub>B</sub> receptors at hypothalamic loci. Nitric oxide modulates the actions of ET in the cardiovascular system and also influences neurotransmission and specifically suppresses firing of magnocellular neurons. The purpose of these studies was to ascertain whether nitric oxide, generated in response to ET<sub>B</sub> receptor stimulation, buffers the stimulatory effect of ET and suppresses AVP release. Studies were performed using a pharmacological approach in hypothalamo-neurohypophyseal explants from rats, and an alternative strategy using explants from mice with an inactivating mutation of neuronal NOS (nNOS<sup>−/−</sup>) and their wild-type parent strain. Whole explants in standard culture or only the hypothalamus of compartmentalized explants was exposed to the ET<sub>B</sub> selective agonist, IRL 1620 (10<sup>−11</sup> to 10<sup>−8</sup> M). Rat and wild-type mouse explants displayed similar responses, although absolute basal release rates were higher from murine explants. Maximal AVP release at 0.1 nM IRL 1620 was 311 ± 63 (rat) and 422 ± 112% basal-<sup>−</sup>1·h<sup>−1</sup> (mouse). Sodium nitroprusside (SNP; 0.1 mM) suppressed maximal AVP release to basal values, N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), 0.1 μM, which did not itself stimulate AVP secretion, more than doubled the response to 1 μM IRL 1620, from 136 ± 28 to 295 ± 49% basal-<sup>−</sup>1·h<sup>−1</sup> (P < 0.05) by rat explants. Explants from wild-type mice responded similarly. Explants from nNOS<sup>−/−</sup> mice had higher basal AVP secretory rate in response to 1 μM IRL 1620: 271 ± 48 compared with 150 ± 24% basal-<sup>−</sup>1·h<sup>−1</sup> (P < 0.05) from wild-type murine explants. In the nNOS<sup>−/−</sup>, SNP suppressed stimulated release, and L-NAME exerted no additional stimulatory effect: 243 ± 38% basal-<sup>−</sup>1·h<sup>−1</sup>. Thus nitric oxide inhibits the AVP secretory response induced by ET<sub>B</sub> receptor activation within the hypothalamo-neurohypophyseal system and is generated primarily by the nNOS isoform. The modulation of AVP secretion by ET and also nitric oxide can take place independently from their effects on cerebral blood flow, systemic hemodynamics, or the arterial baroreflex.

endothelin; nitric oxide synthase; neuronal nitric oxide synthase; posterior pituitary

THE ENDOTHELINS (ET) ARE PEPTIDES THAT EXHIBIT POTENT VASOACTIVE ACTIONS. ET PEPTIDES ALSO PARTICIPATE IN NEUROTHERMATION AND NEUROMODULATION WITHIN THE CENTRAL NERVOUS SYSTEM (12, 16).

All components of the ET system, including both ET-1 and ET-3 isoforms, their respective mRNAs, ET-converting enzyme activity and both ETA and ETB receptor subtypes, exist within nonvascular brain tissues, and specifically in loci involved in vasopressin (AVP) secretion (13, 15, 17, 38, 45). Moreover, several studies have implicated ET peptides in the regulation of AVP secretion in vivo (24, 27, 29, 30) and in vitro (23–26, 28, 31, 34).

The actions of ET on the vasopressinergic system are complex and depend on the specific ET isoform, as well as the site of action and the receptor subtype involved (26, 41). ETA receptors are located on magnocellular neurons within the supraoptic and paraventricular nucleus and also vasopressinergic neurons within the hypothalamus (13, 43). ETB receptors are distributed in the hypothalamus, most prominently in the organum vasculosum of the lamina terminalis and median eminence (43). At the hypothalamic level, selective activation of ETA receptors inhibits AVP release from the neurohypophysis. In contrast, stimulation of ETB receptors increases both somatodendritic and neurohypophyseal AVP release (26).

The interactions of nitric oxide and ET in the cardiovascular system are well recognized (10, 40). Recent studies indicate that nitric oxide generated by neural-astroglial sources modifies ET signaling within the central nervous system (19, 27, 42). Immunohistochemical and in situ hybridization studies have demonstrated nitric oxide synthase (NOS), citrulline, and NADPH-diaphorase activity within the hypothalamo-neurohypophyseal system (HNS; 11, 39, 42). Osmotic or hypovolemic stimuli that increase activity of magnocellular neurons are associated with enhanced NADPH-diaphorase activity in this region (21, 32). Application of a nitric oxide donor reduces activity of phasically firing, vasopressinergic neurons in vitro (14, 37). In contrast, the firing rates of the AVP neurons increase after injection of a NOS inhibitor (18), consistent with a tonic inhibitory nitricergic influence on AVP release. Results from in vivo studies are more variable depending upon the use and type of anesthetic, the hydration state of the animal, or whether the nitric oxide donor or NOS inhibitor was injected...

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intravenously or into the cerebral ventricles (11). Notably, pharmacological inhibition of NOS is frequently associated with a concurrent increase in arterial pressure that may suppress AVP release via baroreflex mechanisms, thereby masking an effect by nitric oxide (27, 29). Although the mechanism of interaction of ET, AVP, and nitric oxide has been studied in several other tissues (10, 19, 44), the direct effect of nitric oxide on ETB receptor-induced AVP secretion has not been studied.

In light of the pressor response that accompanies either central ET injection, as well as inhibition of nitric oxide formation, the present studies were carried out on hypothalamo-neurohypophyseal explants. We hypothesize that nitric oxide generated in response to ETB receptor stimulation will suppress AVP release by the explants. We used a two-pronged approach: a classical pharmacological method in explants from rats, as well as an alternative strategy using explants from mice with an inactivating mutation of neuronal NOS (nNOS).

METHODS

Male Long-Evans rats (8 wk old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The nNOS knockout mice (nNOS−/−; 10 wk old) were obtained from Jackson ImmunoResearch Laboratories (Bar Harbor, Maine). The strain used was B6129S4-Nos1tm1Plh/J and were bred on a C57BL/6J×129/SV background to be homozygous for the Nos1tm1Plh targeted mutation, originally developed by Paul Huang (9, 33). C57BL/6J×129/SV mice served as the wild-type control. All rodents were housed at constant temperature with an 12:12-h light-dark cycle and given free access to water and standard chow. All procedures were reviewed and approved by the institutional Animal Investigation Committee and were in compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Dissection and Culture of Explants

Explants of the hypothalamo-neurohypophyseal complex were dissected as previously described (36). The explants encompassed both supraoptic nuclei with intact axonal projections to the neural lobe with the intermediate lobe attached. The organum vasculosum of the lamina terminalis and the arcuate, suprachiasmatic, preoptic and paraventricular nuclei were also present, but the subfornical organ and paraventricular nuclei were not contained within the slice.

Explants in standard culture. Each explant cultured by standard methods was placed ventral side down onto a Teflon mesh. The hypothalamic and posterior pituitary (PP) components of the explant existed as a single block in the same culture well. The culture medium was composed of Ham F-12 nutrient mixture supplemented with 5.5 mM dextrose, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY), fortified with 20% FBS (HyClone, Logan, UT), with a final osmolality of 297 ± 1 mosmol/kg H2O. The medium was changed every 24 h or at the end of an experimental protocol.

Explants in compartmentalized cultures. After dissection, compartmentalized explants were immediately placed into custom-fabricated sterile incubation chambers that have a two-piece barrier separating the hypothalamus from the PP (7, 8, 25, 26). The explants were positioned onto Teflon mesh with the intact infundibular stalk lying in a 0.33 mm wide × 0.2 mm deep notch in the lower half of the barrier. The upper half of the barrier was then slid into place. The barrier interfaces and gaps were sealed with sterile silicone grease to prevent leaking from one chamber to the other. Each compartment of the chamber was filled with medium. The medium in each compartment was changed every 24 h. When properly positioned and sealed, the only communication between the two compartments was via the pituitary stalk. Diffusion via the infundibular recess could not be entirely eliminated; however, the extent of leaking between compartments was assessed at the end of the experiment for each explant by adding 3H-labeled water (as a 150 mM NaCl solution) to one compartment and measuring its radioactivity in the opposite compartment. Explants displaying greater than 0.05%/h leak were discarded from analysis. At the end of the experiments, the upper barrier was removed, and each explant was scrutinized under a stereomicroscope (magnification ×25) for trauma to the infundibular stalk. Explants were classified as damaged if there were visual evidence of attenuation or lengthening of the stalk (commonly seen with excessive traction); nicks or cuts in the fibers of the stalk or any part of the explant (usually incurred while positioning the explant within the chamber); or complete separation of the hypothalamus and pituitary. Tissues exhibiting damage to the stalk were discarded prospectively from further analysis. All explants were kept at 37°C under an humidified atmosphere of 95% O2–5% CO2 at pH 7.4.

Sampling Procedures

The sampling techniques followed procedures reported earlier (8, 36). Unless otherwise stated, all protocols were performed 48 h after dissection. To prevent peptide degradation, 0.05 mg/ml bacitracin (Sigma, St. Louis, MO) was added to the medium at the beginning of the protocols (8, 36). Each culture well or compartment was treated identically in all respects. During the basal period, AVP release rate was ascertained after 1 h exposure to standard medium administered to both sides of the barrier. A 1-h test period followed immediately, and the test agent(s) dissolved directly in medium was delivered only to the specified compartment. Unless otherwise stated, control medium was administered to the other compartment. Samples were obtained for determination of AVP degradation during basal and test periods from both chambers, described in detail previously (36). For example, two samples of media were removed at the beginning of the basal period. One sample was immediately frozen at −70°C. The second sample was maintained at 37°C in the incubator for the 1-h period, and then frozen. The difference in the frozen and incubated samples was taken as an estimate of the degradation rate during the basal period. The same was done for the test period. Explants in standard cultures were treated as a single compartment. Samples for AVP radioimmunoassay were frozen and stored at −70°C. Osmolality was determined on the remaining medium.

Compartmentalized Protocols

Dose response to ETB agonist. At the beginning of the test period, IRL 1620 (N-succinyl-[Glu9,Ala11,15]-endothelin-1 fragment 8-21; Sigma RBI), a selective ETB receptor agonist, was added to the hypothalamic side of the barrier. Concentration of IRL 1620 ranged from 0.1 μM to 10 μM. Standard medium was added to the PP side. We have previously found that the selective ETB agonist does not induce AVP release when applied solely to the PP (26). Thus experiments were not performed with IRL 1620 added to the neurohypophyseal compartment. At the end of the test hour, samples for release obtained from both hypothalamic and pituitary compartments regardless of the site of application of agonist. Each explant was used only once and exposed to only one concentration of IRL 1620.

Standard Explant Protocols

In all protocols, each explant was used for only one basal and test period unless stated otherwise. We found that the dose-response curves for pituitary release of AVP after addition of IRL 1620 to either the hypothalamic or pituitary compartments were similar, so subsequent experiments were performed in explants maintained in standard cultures rather than in compartments. The dose-response curve to IRL 1620 (0.1 μM to 10 nM) was repeated in the standard

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culture paradigm for explants from both Long-Evans rats and wild-type mice.

Effects of NOS antagonist or nitric oxide donor on ET<sub>B</sub> receptor activation. N<sup>-</sup>ω-nitro-L-arginine methyl ester (L-NAME, Sigma RBI), a nonselective NOS inhibitor, was added to the medium at the beginning of the test period to bring the final concentration to either 1, 10, or 100 μM.

On the basis of results from the foregoing experiments, submaximally stimulatory doses of both the ET<sub>B</sub> agonist and L-NAME were added to the medium bathing explants from either Long-Evans rats or wild-type mice. Final concentrations were 1 pM IRL 1620 and 1 μM L-NAME. In separate explants, AVP release was assessed in the presence of a maximally stimulating dose of IRL 1620, 100 pM, plus 0.1 mM of the nitric oxide donor, sodium nitroprusside (SNP, Sigma RBI) or with SNP alone.

Experiments in nNOS<sup>−/−</sup> mice. The reproducibility of the AVP secretory response up to 5 days after dissection has been demonstrated by Sladek and Knigge (36) and verified by our laboratory (31). Because of the limited availability and cost of nNOS<sup>−/−</sup> mice, explants from the these mice were tested for AVP release during exposure to the submaximally stimulatory dose, 1 pM IRL 1620. The medium was changed, and the next day, the AVP secretory response to 1 pM IRL 1620 with 0.1 mM SNP was assessed. Finally, on the third day, these explants were tested for AVP release after exposure to 1 pM IRL 1620 with 1 μM L-NAME.

Response to osmotic stimulation. As a positive control for AVP stimulation, explants from wild-type and nNOS<sup>−/−</sup> mice were tested for their response to an increase of 20 mosmol/kgH<sub>2</sub>O in medium osmolality delivered as hypertonic NaCl.

Analytical Methods

AVP content of the medium was measured by radioimmunoassay with methods previously reported from our laboratory (20–25). The lower limit of detection was 0.1 pg/assay tube and 50% displacement was at 5.1 pg/tube. Intra-assay variability was 7.6% in the middle and high range; variability in the low range (<1.0 pg/tube) was 12.3%. Interassay variability of the four assays in this series of experiments was 11%. Samples of medium were diluted 1:50 or 1:100 so as to achieve an assay concentration within the midpoint of the curve (2.5 to 15 pg/tube) and directly assayed in duplicate. Media from a given set of experiments were assayed together to avoid interassay variability. Standards were prepared with purified AVP with a designated activity of 400 U/mg (Ferring, Malmö, Sweden). [125I]iodotyrosyl-AVP was used as the tracer (Amersham Pharmacia Biotech, Piscataway, NJ). Anti-AVP serum no. 2849 (prepared by J. Durr, Veterans Affairs Medical Center, Tampa, FL) was used at a final dilution of 1:3.6 × 10<sup>6</sup>. Medium osmolality was measured by freezing point depression (Precision Systems, Sudbury, MA).

Statistical Analysis

Release rates for basal and test periods were calculated in picograms per explant per hour of incubation, and corrected for changes in the volume of medium and for degradation, as reported previously (36). All comparisons of AVP release between basal period and test period release rates were performed on the absolute release rates after correction. Each explant acted as its own control. Since basal secretory rates varied from explant to explant, the release of AVP during the test hour was normalized as the percentage of AVP released during the preceding basal hour for the same compartment (or whole explant), and reported as %basal·explant<sup>−1</sup>·h<sup>−1</sup>. Comparisons among test periods from different groups of explants were then performed on the normalized release rates.

Differences between basal and test period release of AVP in the same explants were compared using a paired t-test. Comparisons among test periods from separate groups of explants were assessed by ANOVA and the Tukey-Kramer post hoc test for multiple comparisons. All data are expressed as means ± SE. A P value of less than 0.05 was considered significant.

RESULTS

The first experiments were run to ascertain an optimal dose at which IRL 1620 would stimulate AVP in both rats and mice. Figure 1 shows AVP release when the ET<sub>B</sub> receptor agonist was added to the hypothalamic (HT) compartment. Figure 1A depicts release of AVP by the hypothalamus, and Fig. 1B shows secretion by the PP during the test period. Overall, basal AVP release averaged 20 ± 3 pg·HT<sup>−1</sup>·h<sup>−1</sup> by the hypothalamus and 45 ± 6 pg·PP<sup>−1</sup>·h<sup>−1</sup> by the pituitary (n = 36). Maximal stimulation was observed at a concentration of 0.1 nM IRL 1620 for both hypothalamic release, 364 ± 82% basal·HT<sup>−1</sup>·h<sup>−1</sup> (P < 0.025 vs. basal, n = 6) and neurohypophyseal secretion, 344 ± 60% basal·PP<sup>−1</sup>·h<sup>−1</sup> (P < 0.05 vs. basal, n = 6). In time control experiments with basal medium added to each side of the barrier during both basal and test periods, AVP secretory rates remained stable from hypo-
thalamus, 100 ± 20 vs. 70 ± 27% basal-HT−1·h−1 (n = 6) and from PP, 100 ± 27 vs. 74 ± 19% basal-PP−1·h−1 (n = 6).

Because the dose-response curves for both hypothalamic and PP release were similar, the response to IRL 1620 was ascertained from explants in standard culture (Fig. 2). Maximal stimulation occurred with 0.1 nM IRL 1620 with explants from both Long-Evans rats and wild-type mice. Normalized AVP secretion from the murine explants did not significantly differ at any concentration from that of the rat explants. However, absolute basal release rate, corrected for degradation, was approximately fivefold higher from wild-type murine explants, 528 ± 59 pg·explant−1·h−1 (n = 60) compared with rat explants, 103 ± 8 pg·explant−1·h−1 (n = 88, P < 0.0001). Degradation averaged 3.8 ± 0.9% and did not exceed 6.3%. Time control experiments with control medium added during both basal and test periods showed no change in the AVP secretory rate for explants from either rats: 100 ± 30 (basal) vs. 93 ± 25% basal·explant−1·h−1 (test; n = 6) or wild-type mice 100 ± 24 (basal) vs. 88 ± 34% basal·explant−1·h−1 (test, n = 5). Like rat explants, absolute basal release rate at 96 h (253 ± 76% basal·explant−1·h−1, n = 12) is roughly half that observed at 48 h from wild-type murine explants (528 ± 59 pg·explant−1·h−1, n = 60; P < 0.05). The effect of an ~20 mosmol/kgH2O increase in medium osmolality from 299 ± 1 to 322 ± 2 mosmol/kgH2O on AVP secretion from wild-type mouse explants after 96 h in culture is displayed in Table 1 and is comparable to normalized values reported from rat explants at 48, 72, and 96 h postdissection from our own (31) and other laboratories (36).

Nonselective blockade of nitric oxide formation by L-NAME resulted in a concentration-dependent increase in AVP secretion (Table 2). Figure 3 shows that 1 μM L-NAME significantly potentiated AVP secretion by a submaximally stimulating dose of IRL 1620 by explants from both rat and wild-type mice to a similar extent. Conversely, when the maximally stimulating dose of IRL 1620, 0.1 nM, was added in the presence of SNP, AVP release was suppressed (Fig. 4). Although attenuation of the AVP secretory response was significant with 0.1 mM SNP in both rats and wild-type mice, AVP secretion decreased to basal levels only with the rat explants. SNP alone did not alter basal release rates.

Table 1. AVP release from wild-type mouse explants after osmotic stimulation with NaCl 96 h after dissection

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<th>Basal Release</th>
<th>Osmotic Stimulus</th>
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<td></td>
<td>253 ± 76</td>
<td>1109 ± 370*</td>
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Values are expressed as means ± SE; *P < 0.05 vs. basal release rate. The osmotic stimulus is given as 20 mosmol/kgH2O.

Absolute basal release rate by nNOS−/− mouse explants was 1,673 pg·explant−1·h−1 at 48 h, which was significantly higher than that from wild-type mouse explants at the same time period (see above, P < 0.0001). Similar to explants from rats and wild-type mice, absolute release rates decreased with time: 1,396 ± 386 pg·explant−1·h−1 at 72 h and 839 ± 63 pg·explant−1·h−1 at 96 h. Interestingly, absolute basal release rate at 96 h was roughly 50% of the rate at 48 h for both wild-type and nNOS−/− mice.

Normalized AVP release by explants from nNOS−/− mice in response to 1 pM IRL 1620 was 271 ± 52% basal·explant−1·h−1 (n = 5). This was significantly higher than basal release (Fig. 5 and Table 3). Moreover, AVP secretion by the nNOS−/− explants was significantly higher than that from wild-type murine explants after administration of 1 pM IRL 1620 150 ± 24% basal·explant−1·h−1 (P < 0.05). This release rate was comparable to that from wild-type mouse explants in the presence of 1 pM IRL 1620 with 1 μM L-NAME, 250 ± 18% basal·explant−1·h−1 (n = 6, P > 0.05). Furthermore, adding L-NAME to IRL 1620 did not increase AVP release beyond the value already seen with 1 pM IRL 1620 alone in the nNOS−/− mouse explants (Table 3 and Figs. 3 and 5). On the next day, when a source of nitric oxide was provided in the form of SNP, 1 pM IRL 1620 did not elicit an increase in AVP release by the nNOS−/− mouse explants. This was comparable to the suppressive effect of SNP on the 0.1 nM maximally stimulating dose of IRL 1620 on explants from wild-type mice (Table 3, Fig. 4 and 5).

Except for experiments with hypertonic sodium chloride, medium osmolality at the end of the experiments averaged 302 ± 1 mosmol/kgH2O.

DISCUSSION

The present studies indicate that inhibition of nitric oxide formation by hypothalamo-neurohypophyseal explants from both rats and mice potentiated ETb receptor-induced AVP secretion. Conversely, administration of an exogenous source of nitric oxide suppressed AVP release in response to ETb receptor activation. Importantly, the potentiation of AVP release seen with nonselective pharmacological blockade of NOS was similar to that with targeted inactivation of the nNOS gene. Thus our results suggest that ET can stimulate both AVP and nitric oxide production and that nitric oxide buffers the secretion of AVP. Moreover, the observation that L-NAME did not further enhance ETb receptor-induced AVP secretion by
explants from nNOS−/− mice suggests that the nitric oxide is largely, if not entirely, generated by the nNOS isoform. Because these studies were performed in vitro using explants, the responses are independent of the dynamic effects exerted by the ETB agonist or by nitric oxide on cerebral blood flow, systemic hemodynamics, or the arterial baroreflex.

Several studies have shown that ET peptides influence AVP release, both in vivo and in vitro in the rat (23–30, 34). More specifically, the present data demonstrate that administration of low concentrations of IRL 1620 to the hypothalamus of explants dose-dependently increases AVP release from both somatodendritic and neurohypophyseal sites. Pharmacological studies from our laboratory have shown that concentrations of IRL 1620 less than or equal to 0.1 nM are selective for the hypothalamic ETB receptor. At higher concentrations, IRL 1620 is no longer ETB selective and begins to activate inhibitory hypothalamic ETA receptors, resulting in the observed biphasic response (25). It is also important to note that ETB receptor activation plays neither a stimulatory nor an inhibitory role at the level of the posterior pituitary (25). Consistent with our previous report, the current results show that the curves for the release rates for AVP by rat explants in compartmentalized and standard culture are similar. This provided justification for performing subsequent testing with explants in standard cultures.

Although basal absolute AVP secretion by explants from wild-type mice was approximately fivefold higher than from

<table>
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<tr>
<th>l-NAME, µM</th>
<th>n</th>
<th>Basal Release, pg/explant−1·h−1</th>
<th>Test Release, pg/explant−1·h−1</th>
<th>Normalized Basal Release, %basal/explant−1·h−1</th>
<th>Normalized Test Release %basal/explant−1·h−1</th>
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<td>277±49*</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>54±23</td>
<td>242±81*</td>
<td>100±42</td>
<td>448±150*</td>
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Values are expressed as means ± SE; *P < 0.05 vs. basal release rate. l-NAME, N\(^{\text{N}}\)-nitro-l-arginine methyl ester.

Fig. 3. Effect of nonselective nitric oxide synthase inhibition by 0.1 µM N\(^{\text{N}}\)-nitro-l-arginine methyl ester (l-NAME) on AVP release by 1 µM IRL 1620 from explants cultured by standard methods. A: explants from Long-Evans rats, basal period (open bars) and test period (solid bars). B: explants from wild-type mice, basal period (hatched bars) and test period (gray bars). Values are expressed as means ± SE; n = 6 for each observation. *P < 0.05 vs. basal, †P < 0.01 vs. IRL + l-NAME.

Fig. 4. Effect of the nitric oxide donor, 0.1 mM sodium nitroprusside (SNP) on AVP release by 0.1 nM IRL 1620 from explants cultured by standard methods. A: explants from Long-Evans rats, basal period (open bars) and test period (closed bars). B: explants from wild-type mice, basal period (hatched bars) and test period (gray bars). Values are expressed as means ± SE; n = 6 for each observation. *P < 0.05 vs. basal, †P < 0.05 vs. IRL alone.
rats, the murine explants displayed the same pattern of AVP secretion to ET<sub>B</sub> receptor stimulation or a standard osmotic stimulus (7, 31, 36). The rate of decline of basal absolute AVP release by nNOS<sup>−/−</sup> explants (~50% from 48 to 96 h) is comparable to that for wild-type mice and to values reported for rat explants from our own (31) and other laboratories (36). Verification of the response by compartmentalized murine explants was not technically feasible at this time. Nonetheless, these findings, as well as the parallel responses to L-NAME and SNP, suggest that murine explants respond in an identical manner to those from rats, at least with respect to osmolality, ET<sub>B</sub> agonism, and the nitric oxide system.

In light of the dose-dependent increase in AVP release with L-NAME alone, the high baseline absolute AVP release rate exhibited by explants from nNOS<sup>−/−</sup> mice likely reflects the absence of a tonic inhibitory influence of endogenously generated nitric oxide. The lack of a significant independent effect of SNP on basal release rate may be less straightforward. In explants from normal rats and mice, basal AVP release rate decreases some 21%. In rat explants, the nitric oxide donor inhibited maximal IRL 1620-induced AVP release to basal values. The same dose of SNP significantly inhibited the AVP secretory response by explants from the wild-type mice, although not completely to basal levels. Overall, the responses of rats and mice were remarkably similar.

The augmentation of AVP release in response to a submaximally stimulating dose of the ET<sub>B</sub> agonist by nonselective NOS inhibition and suppression of the maximal AVP secretory response by the nitric oxide donor is congruent with electrophysiological studies showing inhibition of phasically firing, putative AVP neurons (14, 20, 37). This effect may be direct, as nitric oxide activates large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels in the PP (1). These channels are involved in repolarization and fast afterhyperpolarization of the action potential. Activation of these channels by nitric oxide could decrease the excitability of the terminals and attenuate AVP release. However, an indirect mechanism may also be involved. In neurons, nNOS has been shown to be compartmentalized with N-methyl-d-aspartate (NMDA) receptors (5, 35). Activation of NMDA receptors results in postsynaptic excitation in magnocellular neurons (2). NMDA receptor activation, in turn, results in increased inhibitory transmission by GABA. This GABAergic inhibition can be blocked by L-NAME, as well as bicuculline. This suggests that enhanced nitric oxide production associated with NMDA receptor stimulation mitigates the excitatory stimuli and can thereby reduce AVP secretion (2, 4).

Importantly, ET<sub>B</sub> receptor-induced AVP release is mediated by an NMDA mechanism and inhibited by muscimol, a GABA agonist in explants (28). The potentiation of AVP release by nonselective NOS inhibition and the robust AVP release with only 1 μM IRL 1620 by nNOS<sup>−/−</sup> explants are compatible with either the direct or indirect mechanism, or both.

In addition to corroborating the response to L-NAME, the experiments with nNOS<sup>−/−</sup> explants provide unique evidence not possible by a purely pharmacological approach. Besides inhibiting NOS, L-NAME exhibits anti-muscarinic effects (3). This action of L-NAME is not as well appreciated and may independently enhance AVP secretion via muscarinic receptor modulation of supraoptic nucleus neurons (6) and AVP secretion (7). Because AVP release by the nNOS<sup>−/−</sup> explants was augmented, the data implicating nitric oxide are more convincing. Moreover, L-NAME inhibits endothelial NOS and inducible NOS isoforms as well as nNOS. Notably, L-NAME did not further augment AVP secretion when added to IRL 1620 in nNOS<sup>−/−</sup> explants, thereby supporting the conclusion that the nNOS isoform is the primary source of nitric oxide that modulates AVP secretion in response to ET<sub>B</sub> receptor stimulation.

Table 3. Effect of L-NAME and sodium nitroprusside on IRL 1620-induced AVP release by explants from wild-type and nNOS<sup>−/−</sup> mice

<table>
<thead>
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<th>Wild-Type</th>
<th>nNOS&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td>Test Period Release, %basal-explant&lt;sup&gt;−1&lt;/sup&gt;·h&lt;sup&gt;−1&lt;/sup&gt;</td>
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<td>1 pM IRL 1620</td>
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<tr>
<td>1 pM IRL 1620 + 1 μM L-NAME</td>
<td>6</td>
</tr>
<tr>
<td>1 pM IRL 1620 + 0.1 mM SNP</td>
<td>5</td>
</tr>
<tr>
<td>0.1 nM IRL 1620</td>
<td>6</td>
</tr>
<tr>
<td>0.1 nM IRL 1620 + 0.1 mM SNP</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE. SNP, sodium nitroprusside. *P < 0.05 vs. basal release rate (see RESULTS); †P < 0.05 vs 1 pM IRL 1620; ‡P < 0.05 vs 0.1 mM IRL 1620.
In summary, the present findings indicate that nitric oxide inhibits the AVP secretory response induced by ET<sub>B</sub> receptor activation within the HNS. The aggregate data from pharmacological studies and targeted gene deletion indicate that the nitric oxide is generated primarily by the nNOS isoform. Moreover, the nitrergic modulation of ET<sub>B</sub>-induced AVP secretion occurred in murine and rat explants and is, therefore, independent of the effects of ET or nitric oxide on cerebral blood flow, systemic hemodynamics, or the arterial baroreflex.

Perspectives

Nitric oxide has been recognized as a modulator of ET signaling in both the cardiovascular system and the brain. In particular, several lines of evidence using nonselective pharmacological inhibitors have implicated locally produced nitric oxide in the response of AVP magnocellular neurons. By using a combined pharmacological and gene knockout approach, the present studies clearly identify nNOS as the isoform responsible for locally produced nitric oxide that dampens ET<sub>B</sub> receptor activated AVP release. These data also provide strong evidence that the inhibitory nitrergic influence on AVP secretion occurs independent of changes in cerebral blood flow, systemic hemodynamics, or the arterial baroreflex. Under pathophysiological conditions, such as traumatic brain injury, ET-1 and inducible NOS are both increased within brain microvasculature. However, our data from explants suggest that nitric oxide totally derived from nNOS is sufficient to modulate ET<sub>B</sub> receptor-induced AVP secretion. Moreover, it is very likely that nitrergic modulation of AVP release is not exclusive to ET signaling but is a more general buffering mechanism that occurs in response to other stimulatory neurotransmitter and/or osmotic stimuli.

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GRANTS

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REFERENCES


