Vasopressin Increases Intracellular NO Concentration via Ca\textsuperscript{2+} Signaling in Inner Medullary Collecting Duct

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Abstract—The present study was designed to determine whether arginine vasopressin (AVP) stimulates NO production in the epithelial collecting duct cells of the inner medulla (IMCDs) and if this is mediated through Ca\textsuperscript{2+} signaling. Thin tissue layers containing IMCDs were dissected from Sprague-Dawley rats. Intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) and NO production were measured in IMCDs by a fluorescence imaging system with the use of fura 2-AM and the cell-permeable form of the NO-sensitive dye 4,5-diaminofluorescein (DAF-2), respectively. AVP (100 nmol/L) produced a rapid peak increase in [Ca\textsuperscript{2+}], of 320±70 nmol/L within a few seconds and a sustained increase of 120±62 nmol/L. The peak increase in [Ca\textsuperscript{2+}], was followed by a significant increase of NO production (34±7 U). This was similar to that produced by 20 μmol/L of the NO donor DETA-NONOate (42±11 U). The NO scavenger carboxy-PTIO (100 μmol/L) or depletion of [Ca\textsuperscript{2+}], by preincubation with 5 μmol/L of the Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin in Ca\textsuperscript{2+}-free buffer abolished the NO response to AVP. We conclude that AVP mobilizes Ca\textsuperscript{2+} to produce NO in IMCDs.

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Key Words: vasopressins  ■ nitric oxide  ■ calcium  ■ kidney  ■ signal transduction

Several observations in our laboratory have suggested indirectly that arginine vasopressin (AVP) may stimulate the production of NO in the epithelial collecting duct cells of the inner medulla (IMCDs). Using an oxyhemoglobin-trapping microdialysis technique,\textsuperscript{1} we have found that renal medullary interstitial administration of AVP increases NO in the renal medulla. NO synthase (NOS), measured by L-arginine/L-citrulline conversion, was also observed to be highest in IMCDs, as determined from microdissected tubular and vascular segments.\textsuperscript{2} In addition, we have reported that AVP stimulates medullary NO production only through vasopressin V\textsubscript{2}-like receptors\textsuperscript{1} and that the mRNA for V\textsubscript{2} receptors was present only in tubules such as medullary thick ascending limbs and collecting ducts but not in the renal vasculature or in the vasa recta. On the basis of these observations, we have proposed that IMCDs may play the largest part in the renal medullary production of NO in response to AVP.

The well-recognized classical pathway transducing the effects of AVP on IMCDs is through V\textsubscript{2} receptor activation of adenylyl cyclase, resulting in increased cAMP\textsuperscript{4} and an increase in the hydraulic conductivity of IMCDs via the aquaporin shuttle system.\textsuperscript{5} AVP has also been shown to increase intracellular Ca\textsuperscript{2+} in IMCDs possibly via V\textsubscript{2} receptors.\textsuperscript{6} However, the effects of this release of Ca\textsuperscript{2+} have been largely unexplored, and the effect of AVP stimulation on NO production in IMCDs is unknown.

 Constitutive NOS is known to be modulated by intracellular Ca\textsuperscript{2+},\textsuperscript{7} and the aim of the present study was to determine whether AVP can stimulate NO production in IMCDs and to clarify whether this is a Ca\textsuperscript{2+}-dependent pathway. Kojima et al\textsuperscript{8} recently established the use of the NO-sensitive dye DAF-2, and the cell-permeable form of this dye (DAF-2DA) has allowed NO detection in individual cells. Therefore, an imaging technique applied to these renal medullary tissue slices that enables the application of the NO-sensitive dye DAF-2DA and the Ca\textsuperscript{2+}-sensitive dye fura 2-AM has been developed. The present study reports the temporal relationship of changes in intracellular Ca\textsuperscript{2+} and NO production that are due to AVP stimulation in IMCDs.

Methods

Experimental Animals

The present study used male Sprague-Dawley rats weighing 250 to 350 g (Harlan Inc, Madison, Wis) maintained ad libitum on water and a standard pellet diet (Purina Mills) in the Animal Resource Center of the Medical College of Wisconsin.

Preparation of Inner Medullary Collecting Duct

Rats were anesthetized with pentobarbital (50 mg/kg IP), and the aorta distal to the origin of the left renal artery was cannulated with PE-50 tubing and ligated above the left renal artery. The left kidney was perfused with 10 mL Hanks’ balanced salt solution (HBSS, Life Technologies) with 20 mmol/L HEPES buffer (Sigma Chemical Co) and 1 mg/mL BSA (Sigma) at 3 mL/min. The renal pedical was ligated, and the kidneys were excised and cut sagittally for the removal of the inner medulla. The inner medulla was then transferred into a Petri dish filled with ice-cold HBSS with HEPES buffer and 1 mg/mL BSA. The Petri dish was mounted on a cooling microscope.
stage and maintained at 4°C during dissection. Microdissection was performed under a Leica M3Z stereomicroscope. Single layers of collecting ducts were dissected from the inner medulla by stripping along the collecting ducts with the use of 30-gauge needles. Approximately 200–500-µm-sized tissue strips containing 2 or 3 collecting ducts were cut out with the tip of the 30-gauge fine needle. Care was taken not to collapse the lumen. These tissue strips contained segments of the inner medullary collecting duct, vasa recta, and thin limbs of Henle. The inner medullary collecting duct was readily distinguishable from the other segments by its significantly larger diameter and rough lumen appearance. Dissected tissues were placed on round glass coverslips coated with the tissue adhesive Cell-tak (BD Biosciences) for fluorescent imaging. Dissection was performed within 30 minutes after the removal of the kidney. The experimental buffer was HBSS with 20 mmol/L HEPES and 1 mmol/L L-arginine (Sigma), adjusted to pH 7.4.

Fluorescence Detection

Fluorescence measurements were made by using a Nikon Diaphot inverted microscope with a ×25 (numerical aperture 0.65) objective. The signal was detected by using a CCD video camera (Hamamatsu Co) coupled to a Gen II image intensifier (Hamamatsu Co), and excitation was provided by a Sutter DG-4 175-W xenon arc lamp (Inovision Co), which allowed high-speed excitation wavelength switching. For the experiments, coverslips were placed in an imaging chamber (maintained at 37°C) mounted on the stage of the inverted microscope, which allowed the superfusion of the experimental buffer and buffer containing the agonist. Regions, ~50 µm in length, were selected along the inner medulla collecting duct to quantify changes in fluorescence intensity by using MetaFluor imaging software (Universal Imaging Co).

Intracellular Ca²⁺ Measurement in IMCDs

The IMCDs isolated on coverslips were incubated in 5 µmol/L fura 2-AM (Molecular Probes Inc) for 30 minutes at room temperature and then washed to remove excess dye. Fluronic F127 (Molecular Probes Inc) was used to dissolve the fura 2-AM dye to prevent dye compartmentalization on loading. The coverslips were again incubated in the experimental buffer for 15 minutes before the experiments. Fura 2 signal fluorescence was stimulated by dual-wavelength excitation at 340 nm and 380 nm. A 510/40-nm bandpass emission filter was used to collect fura 2 signals at 0.25-second intervals. Ratios between the fluorescence intensity stimulated by 340-nm excitation at 340 nm and 380 nm. A 510/40-nm bandpass emission filter was used to prevent fura 2 fluorescence bleaching and to balance 340/380-nm excitation intensities.

Fluorescent signals were recorded in the experimental buffer and for 500 seconds after the bath was exchanged to the agonist vehicle or the vehicle containing 100 nmol/L AVP. During the 500-second period after the solution exchange, fluorescent signals were recorded in the presence of the agonists. Intracellular Ca²⁺ concentration ([Ca²⁺]) was calibrated from maximum and minimum fura 2 signals at the end of each experiment. Specifically, the tissue bath solution was exchanged to 5 µmol/L of the Ca²⁺ ionophore 4-bromo-A23187 (Molecular Probes Inc) with 2.5 mmol/L Ca²⁺ and 5 µmol/L EGTA (Sigma) and 5 µmol/L of the NO-specific dye DAF-2T (Molecular Probes Inc) with 2.5 mmol/L Ca²⁺. DAF-2T was excited at 480 nm and collected through a 535/40-nm bandpass emission filter at 3.5-second intervals. Preliminary data showed that the excitation of the dye in this condition did not bleach the DAF-2T fluorescence during the course of the experiment. To compare the intracellular NO production between IMCDs during the experiment, the change in DAF-2T fluorescence was measured continuously for 500 seconds after exchanging the bath solution with the agonist vehicle, 100 mmol/L AVP, 20 µmol/L of the NO donor DET-A-NO (Daiichi Chemical Co), and 5 µmol/L of the Ca²⁺-ionophore 4-bromo-A23187. The agonists remained in the bathing solution during the 500-second time course of fluorescence measurement. To confirm DAF-2 NO detection specificity, all experiments were also performed with 100 µmol/L of the NO scavenger carboxy-PTIO (Dojindo Molecular Technologies Inc) added to the preincubation buffer for 15 minutes after dye loading but before agonist stimulation and also added to the agonist-containing experimental buffers. NO measurements were expressed as relative units of fluorescence intensity under constant conditions of microscopic signal acquisition between experiments.

NO Measurement in IMCDs

NO production in the IMCDs was measured by using the NO-sensitive dye DAF-2DA, as established by Kojima et al.® DAF-2DA is a cell-permeable molecule and is hydrolyzed to an impermeable nonfluorescent active form, DAF-2, in cells by the action of esterases. On reaction with NO, DAF-2 is then converted to a cell-impermeable fluorescent form, DAF-2T, whose presence can be measured as a specific increase in fluorescence signal.© IMCDs were incubated on coverslips in 10 µmol/L of the NO-specific dye DAF-2DA (Calbiochem-Novabiochem Co) for 60 minutes at room temperature and then washed and incubated for another 15 minutes in the experimental buffer. DAF-2T was excited at 480 nm and collected through a 535/40-nm bandpass emission filter at 3.5-second intervals. Preliminary data showed that the excitation of the dye in this condition did not bleach the DAF-2T fluorescence during the course of the experiment. To compare the intracellular NO production between IMCDs during the experiment, the change in DAF-2T fluorescence was measured continuously for 500 seconds after exchanging the bath solution with the agonist vehicle, 100 mmol/L AVP, 20 µmol/L of the NO donor DET-A-NO (Daiichi Chemical Co), and 5 µmol/L of the Ca²⁺-ionophore 4-bromo-A23187. The agonists remained in the bathing solution during the 500-second time course of fluorescence measurement. To confirm DAF-2 NO detection specificity, all experiments were also performed with 100 µmol/L of the NO scavenger carboxy-PTIO (Dojindo Molecular Technologies Inc) added to the preincubation buffer for 15 minutes after dye loading but before agonist stimulation and also added to the agonist-containing experimental buffers. NO measurements were expressed as relative units of fluorescence intensity under constant conditions of microscopic signal acquisition between experiments.

Statistical Analysis

Data are expressed as mean±SE. The time responses of [Ca²⁺], and NO production were evaluated with a 1-way ANOVA. For [Ca²⁺], the Dunn multiple range test was carried out as a post hoc test to baseline, peak, and 500 seconds after AVP stimulation. The Bonferroni multiple range test was carried out as a post hoc test for increase in NO production. To examine the differences in response to vehicle and agonists, t tests were used. The level of significance was set at a value of P<0.05.

Results

Intracellular Ca²⁺ Response to AVP

Intracellular Ca²⁺ responses of the IMCDs were measured after 100 mmol/L AVP stimulation. AVP produced a statistically significant peak increase in [Ca²⁺] (344.5±53 mmol/L) 25 seconds after stimulation, which was followed by a sustained increase, as represented by the average value at 500 seconds (126.2±45 mmol/L) compared with that of the control vehicle (Figure 1).
NO Response of IMCDs to AVP and Effects of NO Scavenger Carboxy-PTIO

The intracellular NO production ([NO]i) brought about by AVP (100 nmol/L) in IMCDs is summarized in Figure 2. [NO], was determined by changes of DAF-2T intensity 10 seconds before AVP and 500 seconds after AVP stimulation. AVP administration (100 nmol/L) in the presence of 1 mmol/L l-arginine significantly increased the NO production to an average of 34±7 U (Figure 2A). This increase was similar to that produced by 20 nmol/L of the slowly releasing NO donor DETA-NONOate. To confirm the presence of Ca2+-dependent NOS activity in IMCDs, we examined the [NO]i response to increased [Ca2+]i by using 5 nmol/L of the Ca2+ ionophore 4-bromo-A23187 and found a significant increase of [NO]i compared with [NO]i in the control vehicle (P<0.05). To confirm DAF-2DA [NO]i specificity, [NO]i responses of IMCDs to the agonists in the presence of the NO scavenger carboxy-PTIO were measured. This compound completely abolished the changes in [NO]i, in response to 100 nmol/L AVP, 20 nmol/L DETA-NONOate, and 5 nmol/L of the Ca2+ ionophore 4-bromo-A23187 (Figure 2B).

Time Course of Intracellular Ca2+ and NO Response of IMCDs to AVP

To determine the temporal relationships between the averaged sequential increases in [Ca2+]i, and the elevations of [NO]i, the[Ca2+]i, and [NO] signals were combined on a single time line (Figure 3). [Ca2+]i showed a rapid peak increase (466.4±70 nmol/L) within a few seconds and a sustained increase (248.1±51 nmol/L) lasting 500 seconds. The rise of [NO], followed the peak increase in [Ca2+]i, and achieved a maximum plateau response in 300 to 500 seconds.

Abolition of NO Production by Depletion of [Ca2+]i

To examine whether increased [Ca2+]i was responsible for increased NO production in IMCDs, we depleted [Ca2+]i, with the Ca2+-ATPase inhibitor thapsigargin (5 μmol/L) in Ca2+-free buffer. This fully abolished the increase in [Ca2+]i, to 100 nmol/L of AVP and abolished increases in [NO], by 100 nmol/L AVP stimulation while preserving the NO donor response (Figure 4).

Discussion

The present study addressed 2 issues: (1) whether AVP could increase intracellular NO in IMCDs and (2) whether production of NO in IMCDs by AVP stimulation was mediated by the intracellular Ca2+ signal transduction pathway.
Changes of intracellular Ca$^{2+}$ by AVP V$_2$ receptors can result in the dilation of renal microvascular beds (MBF) and hypertension. AVP-mediated stimulation of renal medullary production of NO was blunted by reducing the Ca$^{2+}$-free buffer concentration. This response was shown to be mediated by NO production, especially in the medullary vasculature, which has the highest NOS activity in IMCDs.

**Direct Evidence of Intracellular NO Increase by AVP Stimulation in IMCDs**

Previous studies in which urinary cGMP concentrations were measured have suggested that AVP can increase the renal production of NO. It has also been shown that stimulation of vasopressin V$_2$ receptors can result in the dilation of renal blood vessels, especially in the medullary vasculature. These responses were shown to be mediated by NO production. Recently, studies from our laboratory found that if the medullary production of NO was blunted by reducing the Ca$^{2+}$-free buffer concentration, even small elevations of circulating levels of AVP produced sustained reductions of medullary blood flow (MBF) and hypertension. AVP-mediated stimulation of medullary NO production thereby protects against AVP-induced hypertension. However, the specific sites and the mechanisms of NO production by AVP stimulation in the medulla have remained unclear. Moreover, the mRNA for vasopressin V$_2$ receptors appears absent in renal arteries, arterioles, and the vasa recta. Because NO enzyme activity is highest in the inner medullary collecting duct relative to other tubular or vascular segments and because IMCDs contain abundant vasopressin V$_2$ receptors, which are known to stimulate NO production, it was logical to search for these responses in the IMCDs. The results of the present study provide direct evidence that AVP produces an increase of [NO]$_i$ in IMCDs.

**Ca$^{2+}$-Dependent Pathway of NO Production After AVP Stimulation**

Star et al. have reported that Ca$^{2+}$ and cAMP both serve as second messengers for vasopressin in the rat inner medullary collecting duct. Because of the identification of vasopressin receptors in IMCDs, a number of studies have shown that AVP increases intracellular Ca$^{2+}$ in IMCDs by V$_2$-like receptors. However, the physiological responses of these changes of intracellular Ca$^{2+}$ caused by AVP stimulation are not fully known. Because IMCDs are rich in Ca$^{2+}$/calmodulin-dependent constitutive NOS isoforms and exhibit the highest levels of NOS activity within the nephrons, we hypothesized that AVP would stimulate NO through a Ca$^{2+}$-dependent signal transduction pathway. The results of the present study show that an increase of [Ca$^{2+}$]$_i$ brought about by AVP was followed by NO production. NO production was brought about by increases in [Ca$^{2+}$], alone with the Ca$^{2+}$-ionophore 4-bromo-A23187. Abolition of the [Ca$^{2+}$]$_i$ increases in response to AVP stimulation by IMCD Ca$^{2+}$ depletion with thapsigargin in Ca$^{2+}$-free buffer also abolished the NO response. These results, combined with the knowledge derived from our previous studies of the presence of Ca$^{2+}$/calmodulin-dependent constitutive NOS isoforms in IMCDs, suggest a Ca$^{2+}$-dependent pathway for NO production with AVP stimulation in IMCDs.

**NO Detection by NO-Sensitive Dye DAF-2 DA**

NO production in IMCDs was measured by using an NO-sensitive dye, DAF-2DA, as established by Kojima et al. In the present study, the dye was loaded into IMCDs, and it enabled the detection of changes in [NO]$_i$ with AVP stimulation. The highest level of basal DAF-2T fluorescence intensity was observed in IMCDs compared with the surrounding tissue, consistent with our previous report, which found the highest NOS activity in IMCDs. The DAF-2DA technique enabled the characterization of the NO responses and the temporal relationships related to Ca$^{2+}$ responses but did not enable absolute quantification of NO, because the dye-loading condition may differ between the cell types and experimental conditions. For example, DAF-2T fluorescent signal responses to equal molar concentrations of the NO donor DETA-NONOate were ~8-fold higher for experiments performed in the Ca$^{2+}$-free buffer compared with the 1.5 mmol/L Ca$^{2+}$-containing buffer. We assume that these different experimental treatments of the cells modified the dye-loading condition, leading to the differences in baseline NO sensitivity of DAF-2. Therefore, we hesitated to compare the absolute change in DAF-2 intensity between experimental groups, and instead, we compared the DAF-2 response from AVP stimulation or other agonists with that of the NO donor response in the same experimental conditions. DAF-2T has a long half-life and is not reversible, so the increase in DAF-2T signal shows the production of NO, and it is these responses that were evaluated in the present study.

**Time-Dependent Relationship of Intracellular Ca$^{2+}$ Changes and NO Production**

[Ca$^{2+}$]$_i$ and [NO]$_i$ were determined from IMCDs by loading 2 different fluorescent indicators, which enabled us to characterize the temporal relationship between [Ca$^{2+}$]$_i$ and [NO]$_i$. Simultaneous measurements of [Ca$^{2+}$]$_i$ and [NO]$_i$ were also made in several single IMCDs and revealed the same pattern of response (data not shown). The results clearly demonstrated that the rapid rise of Ca$^{2+}$ precedes the increase of [NO]$_i$, indicating that increased Ca$^{2+}$ is activating Ca$^{2+}$-dependent NOS.

**Second Pathway for AVP Signal Transduction in IMCDs**

It is well known that AVP activates adenyl cyclase via V$_2$ receptors and increases cAMP in IMCDs. This results in increased water permeability of the cell membrane and activates the aquaporin shuttle system. Recently, Chou et al. have shown that AVP-enhanced water permeability in IM-
CDs due to increased aquaporin-2 trafficking is stimulated by a Ca$^{2+}$-calmodulin pathway via Ca$^{2+}$ release from ryanodine-sensitive stores, indicating that [Ca$^{2+}$], plays a critical role in water reabsorption in IMCDs. However, long-term administration of AVP results in only a transient increase in water retention and urine osmolality because of a phenomenon called vasopressin escape. Many investigators have shown that this escape is driven by body fluid expansion called vasopressin escape. Many investigators have shown why only V1 of AVP. Taken together, these events help to explain the vasodilation via V2 receptors in conscious dogs. Am J Physiol 1993:265:R934–R942. 


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