Angiotensin II Stimulates Calcium and Nitric Oxide Release From Macula Densa Cells Through AT₁ Receptors

Ruisheng Liu, A. Erik G. Persson

Abstract—A fluorescent nitric oxide (NO) indicator, 4,5-diaminofluorescein diacetate, and the calcium indicator, indo-1, with 488 nm and 364 nm UV confocal laser scanning microscopy were used to detect NO and calcium concentration in rabbit macula densa (MD) cells challenged by angiotensin II (Ang II). Glomeruli with attached thick ascending limbs with the MD plaque were isolated and perfused. Ang II concentration from 10⁻⁸ to 10⁻⁴ progressively increased MD cell calcium and NO to peak values at 10⁻⁶ and 10⁻⁷, respectively. Ang II (10⁻⁶ M) caused the cytosolic calcium concentration ([Ca²⁺]) to increase by 125.8±16.3 nM (n=17) from the bath and by 52.3±11.5 nM (n=18) from the lumen. AT₁ agonist CV-11974 (10⁻⁶ M) blocked the Ang II-induced calcium responses from bath and lumen, but AT₂ antagonist PD-123319 (10⁻⁶ M) did not. AT₂ agonist CGP-42112A (10⁻⁶ M) did not affect [Ca²⁺], in MD cells from either side. Ang II (10⁻⁶ M) increased the NO production by 16%±3.4% (n=26) from the bath and by 18%±3.1% (n=24) from the lumen. CV-11974 (10⁻⁶ M) blocked the NO responses from both sides, but PD-123319 (10⁻⁶ M) did not on either side. CGP-42112A (10⁻⁶ M) had no effect on NO in MD cells. In calcium-free experiments there was no difference from the result in normal calcium solutions. In conclusion, we found that Ang II increased [Ca²⁺], and stimulated NO production in MD cells from the basolateral and luminal sides through AT₁ receptors. (Hypertension. 2004;43:649-653.)

Key Words: angiotensin • calcium • nitric oxide • juxtaglomerular apparatus

Macula densa (MD) cells form the specialized epithelium in the distal tubule in the region in contact with the afferent and efferent arterioles. MD cells have the function of monitoring the tubular NaCl concentration ([NaCl]), whereby they elicit two important changes. First, they send a signal to regulate glomerular vascular tone and thus alter the glomerular capillary plasma flow and glomerular filtration rate.1,2 Second, changes in [NaCl] at the MD have the effect of altering the secretion of renin from granular cells.3,4 The findings of a constitutive isoform of nitric oxide (NO) synthase, namely neuronal NO synthase (nNOS),5,6 and the angiotensin AT₁ receptor7 in MD cells have raised speculations about their possible relationship. It is well documented that angiotensin II (Ang II) can potently sensitize the tubular NaCl receptor8–10

Studies have shown that Ang II can stimulate the nNOS pathway measured by the grade of immunostaining for nNOS in the MD of the rat kidney11 and total renal nNOS mRNA levels.12 However, there have been no reports hitherto of changes in the cytosolic calcium concentration ([Ca²⁺]) and in NO production in MD cells stimulated by Ang II. In the present study, the calcium indicator indo-1 and the NO indicator, 4,5-diaminofluorescein diacetate (DAF-2 DA) were used to detect [Ca²⁺], and NO production, respectively, in MD cells challenged by Ang II, using a confocal laser scanning microscopy.

Methods

Experimental Preparation and Measurements

Glomeruli with attached cortical thick ascending limbs and containing the MD plaque were isolated and micropерfused using a method similar to that described.13 The cortical thick ascending limbs was cannulated and perfused with the 35 mmol/L NaCl buffer solution. The preparation was bathed continuously in a 135 mmol/L NaCl buffer solution (containing in mM: 135 NaCl, 1.3 CaCl₂, 1 MgSO₄, 1.6 K₂HPO₄, 5 glucose, and 20 HEPES, with pH adjusted to 7.4 and an osmolality of 290 mOsm).

The preparation was loaded with 10 μM cell-permeable fluorescent Ca²⁺ indicator indo-1 AM, 0.5% dimethyl sulfoxide plus 0.1% pluronic acid from the lumen for 30 to 40 minutes. Indo-1–loaded MD-glomerular preparations were studied in the confocal system (Narom) with UV laser. A Nikon ×60/1.2 water-immersion objective lens was used to visualize MD cells. The confocal slit was set at a width of 15 nm. Indo-1 was excited at 364 nm with a UV laser. Emission was measured at 405 nm and 485 nm and transmitted to photomultiplier tubes (Figure 1). The measurements of calcium were made as described by other investigators.14,15

A cell-permeable fluorescent NO indicator, DAF-2 DA, was used to detect NO production in MD cells. The cells were loaded with 10 μmol DAF-2 DA (in 0.5% dimethyl sulfoxide) from the lumen for 40
to 50 minutes. DAF-2 was excited at 488 nm with the argon–ion laser, whereas emitted fluorescence was recorded at a wavelength of 510 nm. Relative changes in the DAF-2 concentration were calculated by a normalization procedure to obtain a “pseudo ratio” \( \frac{F - F_{\text{rest}}}{F_{\text{rest}}} \) (Figure 2).

**Experiment Protocol**

Experiments were performed at 37°C with continuous perfusion with 35 mmol/L NaCl buffer solution from the lumen and 135 mmol/L NaCl buffer solution in the bath at a rate of 6 to 7 mL/min. MD cells were loaded with fluorescent probes from the luminal side as described. The apical and basolateral effects of Ang II (10^{-6} M) were assessed by adding this agonist to the lumen or to the bath, respectively, for 2 minutes. Either in the bath or in the lumen, the AT1 antagonist CV-11974 (10^{-6} M) or AT2 antagonist PD-123319 (10^{-6} M) was perfused for 30 minutes, after which Ang II (10^{-6} M) was added for 2 minutes. The AT2 agonist CGP-42112A (10^{-6} M) was added either to the bath or to the lumen for 5 minutes. To obtain

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**Figure 1.** Microperfused thick ascending limb and indo-1–loaded macula densa (MD). A, An individual glomerulus with perfused cortical thick ascending limb and the MD plaque. B, Perfused MD cells. C, Indo-1–loaded MD and tubule recorded at 405 nm. D, Ang II (10^{-6} M) added to the bath caused indo-1 intensity to increase significantly in the MD and tubule cells as a result of calcium increase.

**Figure 2.** DAF-2–loaded MD cells. A, DAF-2–loaded fluorescence image of the MD. B, The intensity was increased when MD was challenged with Ang II (10^{-6} M) from the bath. C, Microperfused thick ascending limb with a long distal tubule. D, Microperfused MD cells.
Ca\(^{2+}\)-free solution, the Ca\(^{2+}\) was removed and 5 mmol/L EGTA was added to the lumen and bath. 7-Nitroindazole (7-NI) (100 \(\mu\)mol/L) was perfused from the bath and lumen for 30 minutes, and Ang II (10\(^{-7}\) M) was then added into the bath or lumen. Ang II was added to the bath for 2 minutes at concentrations of 10\(^{-9}\), 10\(^{-8}\), 10\(^{-7}\), 10\(^{-6}\), and 10\(^{-5}\) M. There was a 30-minute washout interval with 135 mmol/L NaCl buffer solution before the next Ang II challenge.

**NO Calibrations**

Fluorescence was detected with the same settings as in the experiments. We prepared the standard solution freshly by gassing the PBS to room temperature with 100% N\(_2\) gas for 30 minutes and subsequently with a 1.8% NO plus 98.2% N\(_2\) gas mixture for 30 minutes. The NO concentration in the standard was calculated from the solubility and partial pressure of the equilibrating NO gas. Solutions with different NO concentrations (0, 50, 100, 200, 400, and 800 nM) were made by adding different volumes of the NO standard.

**Chemicals**

Indo-1 and pluronic acid were from Molecular Probes, Eugene, Ore. 7-NI and ionomycin were obtained from Calbiochem (Schwalbach, Germany). PBS was from Biochrom KG, Germany. CV-11974 was from AstraZeneca, Molndal, Sweden. All other chemicals were from Sigma, St. Louis, Mo.

**Statistics**

A paired or unpaired \(t\) test (two-tail) and 1-way ANOVA were used when appropriate. The level of significance was set at \(P<0.05\). Data are presented as mean\(\pm\)SE.

**Results**

**NO Calibrations**

Addition of solutions with stepwise increased NO concentrations caused a corresponding increase in DAF-2 intensity. A linear relationship was found between DAF-2 intensity and NO concentration (Figure 3).

**Calcium Responses**

The static cytosolic calcium concentration ([Ca\(^{2+}\)]) in MD was 116.5\(\pm\)9.2 nM. Ang II (10\(^{-6}\) M) caused an increase in [Ca\(^{2+}\)], increase by 125.8\(\pm\)16.3 nM (n=17) from bath and by 52.3\(\pm\)11.5 nM (n=18) from the luminal side. The AT\(_1\) antagonist CV-11974 (10\(^{-6}\) M) blocked the calcium responses to Ang II from the bath (n=5) and from the luminal side (n=4), but the AT\(_2\) antagonist PD-123319 (10\(^{-6}\) M) did not have this effect on either side (bath n=6, lumen n=7). The AT\(_2\) agonist CGP-42112A (10\(^{-6}\) M) had no effect on [Ca\(^{2+}\)], in MD cells either from the bath (n=17) or lumen (n=14). The results in experiments with calcium-free solutions (n=19) showed no significant differences from those with normal Ca\(^{2+}\) solutions (Figure 4).

**NO Responses**

Compared with unloaded cells, the MD cells loaded with DAF-2 showed stronger fluorescence intensity (Figure 2), which might reflect the basal NO production in MD cells. We found that there was usually stronger DAF-2 intensity at the cutting edge of distal tubule, which may have been caused by the large amount of NO released by damaged cells. This strong intensity could have influenced the fluorescence intensity of MD cells if they were too close. So in this series of experiments, a slightly longer distal tubule was dissected and kept (Figure 2C). The results showed that Ang II (10\(^{-6}\) M) increased the NO production by 16.1\(\pm\)3.4% (n=26) from the bath and by 17.9\(\pm\)3.1% (n=24) from the luminal side. The AT\(_1\) antagonist CV-11974 (10\(^{-6}\) M) blocked the NO response from both sides, but the AT\(_2\) antagonist PD-123319 (10\(^{-6}\) M) did not have this effect on either side. The AT\(_2\) agonist CGP-42112A (10\(^{-6}\) M) had no effect on NO in MD cells (bath n=15, lumen n=19). In calcium-free experiments (n=12), no significant differences were found compared with the results in normal Ca\(^{2+}\) solutions (Figure 5). Both from the bath and lumen, 7-NI totally inhibited the NO production caused by challenge with Ang II (bath 0.5\%\(\pm\)1.7%, n=9; lumen 0.3\%\(\pm\)1.2%, n=12).

**Dose Response of Ang II**

Parallel increases in [Ca\(^{2+}\)], (n=6) and NO (n=5) were observed in MD cells challenged with Ang II at concentrations from 10\(^{-9}\) to 10\(^{-5}\) M. The peak response of [Ca\(^{2+}\)], to Ang II occurred at 10\(^{-6}\) M, and that of NO occurred at 10\(^{-7}\) (Figure 6).

**Discussion**

The present study showed that an increasing concentration of Ang II from 10\(^{-9}\) M increased the intracellular calcium concentration and NO release. The peak release of Ca\(^{2+}\) was found at Ang II concentration of 10\(^{-6}\) M and that of NO at 10\(^{-7}\) M for NO release (Figure 6). Our results also showed that Ang II caused a [Ca\(^{2+}\)], increase both from the bath and lumen. The response characteristics are in conformity with the scenario of agonist-induced increases in [Ca\(^{2+}\)], for G-protein–coupled receptors. There is a peak elevation in [Ca\(^{2+}\)], caused by Ca\(^{2+}\) mobilization. This is followed by a lower sustained increase in [Ca\(^{2+}\)], that is caused, at least in part, by Ca\(^{2+}\) entry. Also, in our studies, there was no significant difference in the increase in [Ca\(^{2+}\)], obtained with addition of Ang II between solutions with and without the presence of Ca\(^{2+}\). This finding supports our conclusion that the most important contribution to the rapid and maximal increase in [Ca\(^{2+}\)], was from Ca\(^{2+}\) mobilization through the phospholipase C-IP3 (PLC-IP3) pathway and not through Ca\(^{2+}\) entry mechanisms through calcium channels. From the bath and luminal side, the AT\(_1\) antagonist CV-11974 blocked
the calcium responses caused by Ang II, but the AT$_2$ antagonist PD-123319 and AT$_2$ agonist CGP-42112A had no effect on [Ca$^{2+}$], in MD cells. This indicates that the [Ca$^{2+}$] mobilization occurs through the AT$_1$ and not the AT$_2$ receptor. The earlier findings that the AT$_1$ receptor was present on the basolateral and luminal sides of MD cells also strengthen our conclusion. We found that there were significant differences in [Ca$^{2+}$], when the MD cells were challenged by Ang II from the bath and from the luminal side. It has been reported that the Ang II concentration is much higher in the tubule than in the plasma and kidney. It is therefore possible that the sensitivities to Ang II on the basolateral and luminal side and/or the density of AT$_1$ receptors located on these sides are different. Thus the responses from the different sides to the same Ang II concentration might also differ.

DAF-2 DA is a newly developed indicator for measurement of NO. DAF-2 selectively traps NO between two amino groups in its molecule and yields triazolofluorescein (DAF-2T), which emits green fluorescence when excited at 490 to 495 nm. DAF-2T is not formed in the absence of NO. However, the fluorescence intensity of DAF-2 is pH-dependent. The fluorescence of DAF-2T is comparatively stable in media with a pH above 7, but its fluorescence strikingly decreases below pH 7. Earlier studies have found that Ang II stimulated apical Na:H exchanger and elevated intracellular pH in MD cells through AT$_1$ receptors. So in the present study when Ang II was used, the intracellular pH value in MD cells would be expected to increase. Because of the increased pH, the influence of pH on the DAF-2T intensity could be disregarded. Thus, in the present study, the DAF-2T intensity will reflect the NO concentration in MD cells.

NO production in MD cells stimulated by Ang II was measured directly with DAF-2 DA using confocal microscopy. We found that Ang II, from the basolateral and luminal side, increased NO production significantly. There was no significant difference in the Ang II-induced increase in the NO concentration in the presence and absence of extracellular Ca$^{2+}$. Ca$^{2+}$ and calmodulin regulate nNOS in MD at the constitutive level. This latter finding indicated that the most important regulatory effect of Ca$^{2+}$ on nNOS is exerted through the intracellular calcium pools, and not through the Ca$^{2+}$ entry through the membrane. Furthermore, it seems likely that the increase in MD cell Ca$^{2+}$ by Ang II activates the nNOS to increase the production rate of NO in a dose-dependent way. However, the [Ca$^{2+}$] response in MD cells differed significantly when challenged by Ang II from the bath and perfusate. The response of the NO concentration, however, showed no significant difference between basolateral and luminal Ang II stimulation. The reason for this might be that besides the Ca$^{2+}$ regulation, other factors such as kinin and intracellular pH probably also affect the activity of nNOS. The AT$_1$ antagonist CV-11974 blocked the NO responses both from the bath and luminal side, but the AT$_2$
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antagonist PD-123319 and AT\textsubscript{3} agonist CGP-42112A had no effect on the NO concentration in MD cells. These data suggest that the NO release occurs through the AT\textsubscript{1} and not the AT\textsubscript{2} receptor.

7-NI, a selective nNOS inhibitor, totally inhibited the NO production caused by Ang II. This indicates that the generation of NO takes place through activation of nNOS located in MD cells.

It is a well-described phenomenon that administration of Ang II to the systemic circulation or the peritubular capillaries can augment the TGF response in control and volume-expanded animals.\textsuperscript{25} Recently, it has been found that Ang II can enhance the TGF response via activation of luminal AT\textsubscript{1} receptors;\textsuperscript{26} furthermore, it is known that an increased Ang II concentration can enhance the NHE2 and NHE4 activities and the Na-2Cl-K cotransporter in the MD cells.\textsuperscript{22,27} It has been suggested that increased electrolyte transport into the MD cells may increase the TGF sensitivity, resulting in a larger response from the TGF system at a lower load than normal. However, Kovacs et al\textsuperscript{27} found that a high dose of Ang II (10\textsuperscript{-8}) had no effect on the transport rate from the MD cells, although a dose of 10\textsuperscript{-9} did have such an effect. In the present study, there was no evidence of inhibition at a high concentration of Ang II, but rather a dose-dependent release of calcium that became maximal at an Ang II concentration of 10\textsuperscript{-7}. The MD cells show high expression of calcium-dependent nNOS and accordingly the increase in intracellular calcium in the MD cells was associated with an increased production rate of NO until it became maximal at an Ang II concentration of 10\textsuperscript{-7}. NO can potently reduce TGF sensitivity, and it is known that NO dampens the effect of Ang II on the afferent arteriole.\textsuperscript{28}

In many physiological situations, Ang II through AT\textsubscript{1} receptor activation has an opposite effect on NO. This is true in the juxtaglomerular apparatus and the afferent arteriole, where Ang II sensitizes the TGF mechanism while NO desensitizes this mechanism; furthermore, Ang II has a strong contractile effect on the afferent arteriole while NO dilates it. The observation in the present study is that Ang II also releases its opponent NO, which will modulate not only its contractile response but also its sensitizing effects on the TGF mechanism.

In conclusion, we found that Ang II could increase [Ca\textsuperscript{2+}], and thereby stimulate NO production through nNOS activation in MD cells from the basolateral and luminal sides. These effects are mediated through AT\textsubscript{1} receptors and not through AT\textsubscript{2} receptors.

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