Extracellular ATP Stimulates NO Production in Rat Thick Ascending Limb

Guillermo Silva, William H. Beierwaltes, Jeffrey L. Garvin

Abstract—NO produced by NO synthase (NOS) 3 acts as an autacoid to regulate NaCl absorption in the thick ascending limb. ATP induces NO production by NOS 3 in endothelial cells. We hypothesized that extracellular ATP activates NOS in thick ascending limbs through P2 receptors. To test this, we measured intracellular NO production using the NO-selective fluorescent dye DAF-2 in suspensions of rat medullary thick ascending limbs. We found that ATP increased DAF-2 fluorescence in a concentration-dependent manner, reaching saturation at \( \approx 200 \) \( \mu \)mol/L with an EC\(_{50}\) of 37 \( \mu \)mol/L. The increase was blunted by 74\% by the nonselective NOS inhibitor l-\( \omega \)-nitro-arginine-methyl-ester (2 \( \mu \)mol/L; 60\% versus 16\% arbitrary fluorescence units; \( P<0.02; n=5 \)). In the presence of the P2 receptor antagonist suramin (300 \( \mu \)mol/L), ATP-induced NO production was reduced by 64\% (101\% versus 37\% arbitrary fluorescence units; \( P<0.002; n=5 \)). Blocking ATP hydrolysis with a 5’-ectonucleotidase inhibitor, ARL67156 (30 \( \mu \)mol/L) enhanced the response to ATP and shifted the EC\(_{50}\) to 0.8 \( \mu \)mol/L. In the presence of ARL67156, the EC\(_{50}\) of the P2X-selective agonist \( \beta_\gamma \)-methylene-adenosine 5’-triphosphate was 4.8 \( \mu \)mol/L and the EC\(_{50}\) for the P2Y-selective agonist UTP was 40.4 \( \mu \)mol/L. The maximal responses for both agonists were similar. Taken together, these data indicate that ATP stimulates NO production in the thick ascending limb primarily through P2X receptor activation and that ATP hydrolysis may regulate NO production. (Hypertension. 2006;47[part 2]:563-567.)

Key Words: nitric oxide ■ kidney ■ receptors, purinergic ■ signal transduction

The thick ascending limb of Henle’s loop is a water-impermeable segment of the nephron. It generates the corticomedullary osmotic gradient necessary for urine concentration and absorbs 30\% of the filtered NaCl load. NaCl absorption by the thick ascending limb is regulated by several factors, including NO. NO decreases NaCl absorption by inhibiting the Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter. NO synthase (NOS) 3 is the primary source of NO in the thick ascending limb. Factors known to enhance NOS 3 activity in the thick ascending limb also stimulate NOS 3 activity in endothelial cells. These include endothelin 1, \( \alpha_\_\)-adrenergic receptor agonists, and luminal flow.

ATP is a paracrine/autocrine factor released by several different cells in response to many stimuli. The effects of extracellular ATP are mediated by a family of transmembrane purinergic (P2) receptors including P2X and P2Y receptors. P2 receptors are expressed in several types of cells in different structures in the kidney, including epithelial cells along the nephron and in the microvasculature. They are involved in basic functions such as ion transport and regulation of afferent arteriole diameter. Stimulation of both P2X\(_{18}\) and P2Y\(_{19}\) receptors has been shown to induce NO production. The thick ascending limb expresses P2 receptors, but their role in NO production is unknown.

The effects of extracellular ATP are limited via hydrolysis by 5’-ectonucleotidases. These enzymes are expressed in the plasma membrane of several nephron segments. This family of enzymes has a high affinity for ATP and other nucleotides. Thus, the expression and activity of 5’-ectonucleotidases in the thick ascending limb could modify the response to ATP.

We hypothesized that extracellular ATP stimulation in the thick ascending limb stimulates NO production by activation of P2 receptors, and this response is modified by the 5’-ectonucleotidases expressed by this nephron segment.

Methods

Medullary Thick Ascending Limb Tubule Suspensions

This study was approved by the Henry Ford Hospital review committee. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats weighing 200 to 250 g (Charles River Breeding Laboratories, Kalamazoo, MI) were fed a diet containing 0.22\% sodium and 1.1\% potassium (Purina) for \( \geq 7 \) days and then anesthetized with ketamine (100 mg/kg body weight IP) and xylazine (20 mg/kg body weight IP). Medullary thick ascending limb suspensions were prepared according to Herrera and Garvin. In brief, kidneys were perfused via retrograde perfusion of the aorta with 40 mL of HEPES-buffered physiological saline.
containing (in mmol/L): 130 NaCl, 4 KCl, 2.5 NaH2PO4, 1.2 MgSO4, 2 calcium dilactate, 5.5 glucose, 6 D-alanine, 1 trisodium citrate, and 10 HEPES plus 0.1% collagenase A (Sigma-Aldrich, St. Louis, MO) and 100 U of heparin. The inner stripe of the outer medulla was cut from coronal slices of the kidney, minced, and incubated at 37°C for 30 minutes in 0.1% collagenase A and was agitated and gassed with 100% O2 every 5 minutes. Tissue was pelleted by centrifugation at 100g for 2 minutes, resuspended in cold HEPES-buffered physiological saline, and stirred on ice for 30 minutes to release the tubules. The suspension was filtered through a 250-μm nylon mesh and centrifuged at 100g for 2 minutes. The pellet was washed, centrifuged again, and resuspended in 1 mL of cold HEPES-buffered physiological saline.

Measurement of Intracellular NO by DAF-2 DA

Intracellular NO production by thick ascending limbs was measured using the NO-selective fluorescent dye DAF-2 DA (Calbiochem). Suspensions of medullary thick ascending limbs were loaded by adding 10 μmol/L DAF-2 DA to the suspension and incubating them at 37°C for 20 minutes. Tubules were spun at 100g for 2 minutes; the pellet was washed 3 times and resuspended in 1 mL of HEPES-buffered physiological saline gassed with 100% oxygen at 37°C. All experiments were performed in the presence of L-arginine (100 μmol/L), the substrate for NO synthase. Once loaded, the tubules were placed in the chamber of a fluorometer at 37°C. After 20 minutes of equilibration, measurements were taken for 5 minutes. The dye was excited with a low-pressure mercury arc lamp using a 488-nm filter. Emitted fluorescence was filtered using a 510-nm high-pass filter and measured with a photomultiplier tube. Data were collected using DATAQ Acquisition acquisition software v1.46 and an analogue chart recorder.

Statistics

Data are reported as mean±SEM. Differences in means were analyzed using an unpaired t test. 95% CIs were calculated for EC50 and maximum responses of the concentration-response curves for UTP and β,γ-methylene-adenosine 5'-triphosphate (β,γ-Me-ATP). Statistical analysis was performed by the Department of Biostatistics and Epidemiology of Henry Ford Hospital.

Results

To investigate whether ATP stimulates NO production in the thick ascending limb, we first examined concentration dependence. Treatment of medullary thick ascending limb suspensions with ATP (Sigma-Aldrich) at concentrations of 0.5, 20, 50, 100, 250, and 500 μmol/L increased DAF-2 fluorescence in a concentration-dependent manner (Figure 1). The response showed saturation at 200 μmol/L and an EC50 of 37 μmol/L. The vehicle effect was subtracted from the effect of each concentration.

To show that the effect on DAF-2 fluorescence was caused by an increased NO production, we tested the ability of the NO synthase inhibitor, L-ω-nitro-arginine-methyl-ester (L-NAME; 2 mmol/L; Sigma-Aldrich) to block the increase caused by ATP. Medullary thick ascending limb suspensions were pretreated with L-NAME for 20 minutes. In the absence of L-NAME, ATP (100 μmol/L) increased DAF-2 fluorescence by 60±7 arbitrary fluorescence units (AFUs), corrected for the vehicle effect. In the presence of L-NAME, ATP increased DAF-2 fluorescence by only 16±6 AFU (p<0.02 versus control; n=5), an inhibition of 74% (Figure 2). Taken together, these data indicate that ATP induces NO production in the thick ascending limb by activating NOS.

Because ATP is a selective ligand of P2 receptors, we investigated the possible role of P2 receptor activation in ATP-induced NO production. For this, we tested the ability of ATP to stimulate NO production in the presence of a generic P2 receptor antagonist, suramin (Sigma-Aldrich). In the presence of suramin (300 μmol/L), ATP (100 μmol/L) increased DAF-2 fluorescence by 56±7 arbitrary fluorescence units (AFUs), corrected for the vehicle effect. In the presence of suramin, ATP increased DAF-2 fluorescence by only 16±6 AFU (p<0.002 versus control; n=5), an inhibition of 74% (Figure 3). Taken together, these data indicate that ATP induces NO production in the thick ascending limb by activating NOS.
absence of suramin, ATP (100 µmol/L) increased NO production by 101±12 AFU. After treating medullary thick ascending limb suspensions with suramin (300 µmol/L) for 20 minutes, we found that ATP only increased NO production by 37±5 AFU (P<0.002 versus control; n=5), a decrease of 65% (Figure 3). These data indicate that ATP-induced NOS activation is primarily mediated by P2 receptors.

To investigate which P2 receptor mediates ATP-stimulated NO production, we first tested whether ATP was undergoing hydrolysis, because the P2Y-selective agonist UTP could also be hydrolyzed. We measured NO production induced by ATP concentrations of 0, 0.5, 1, 10, and 50 µmol/L in the presence of a selective 5′-ectonucleotidase inhibitor, 6-mercaptopurine-N,N-diethyl-β-γ-dibromomethylene-adenosine-5-triphosphate (ARL67156; Sigma-Aldrich; 30 µmol/L). When hydrolysis was blocked, saturation was reached at ≈5 µmol/L with an EC50 of 0.8 µmol/L (Figure 4). The EC50 value obtained was >40 times more sensitive than the EC50 value observed during hydrolysis (Figure 1), indicating that ATP is hydrolyzed by 5′-ectonucleotidases in the extracellular space of the thick ascending limb. The maximal responses obtained between both concentration-response curves were not statistically different.

Because of this endogenous hydrolysis, we ran the remaining experiments in the presence of the inhibitor ARL67156.

The kidney expresses both P2X and P2Y receptors. To determine which receptor mediates ATP-induced NO production, in the presence of ARL67156 we examined the relative efficacy of a P2X-selective agonist β,γ-Me-ATP (Sigma-Aldrich) and a P2Y-selective agonist (UTP, Sigma-Aldrich) by generating concentration–response curves for both agonists and maximum responses. We found that the EC50 for β,γ-Me ATP was 4.8 µmol/L, with a 95% CI of 2.8 to 6.8 µmol/L. The maximum response was 79 AFU with a 95% CI of 74 to 84 AFU. In contrast, the EC50 for UTP was 40.4 µmol/L with a 95% CI of 26 to 54 µmol/L. The maximum response was 78 AFU with a 95% CI of 70 to 86 AFU (Figure 5). These data indicate that ATP stimulates NO production primarily by activating the P2X receptors in the thick ascending limb.

Discussion

ATP has been shown to stimulate NO production in several cell types. We have reported previously that several factors that stimulate NO production in endothelial cells also enhance NO generation in thick ascending limbs. Consequently, we investigated whether ATP stimulates NO production by thick ascending limbs and which receptors are involved. We found that ATP enhanced thick ascending limb NO production in a concentration-dependent manner as measured by the NO-sensitive dye DAF-2. The EC50 was 37 µmol/L, and saturation occurred at ≈200 µmol/L. To show that the change in fluorescence was because of activation of NOS by ATP, we added the NO synthase inhibitor L-NAME, which blunted the effect of ATP on NO production by 74%. These data indicate that ATP stimulates NOS activity in thick ascending limbs.

In the thick ascending limb, NOS is activated by endothelin-1, α-adrenergic receptor agonists, and luminal flow. Interestingly, these same stimuli have been demonstrated to produce similar effects in other cells, including chondrocytes and vascular endothelial cells. Given that ATP stimulates NOS in neurons and vascular endothelium, it is not surprising that ATP would also stimulate NOS in the thick ascending limb.

The actions of ATP are limited via hydrolysis by 5′-ectonucleotidases. To determine which P2 receptor mediates the effects of ATP in the thick ascending limb, we first investigated whether ATP was being hydrolyzed by 5′-ectonucleotidases, because the selective P2Y agonist (UTP) could also be hydrolyzed by these enzymes. For this, we generated a second concentration–response curve in the presence of the 5′-ectonucleotidase inhibitor ARL67156. We found that the ATP concentration–response curve was shifted to lower concentrations of the agonist. When 5′-ectonucleotidases were inhibited, the EC50 was 0.8 µmol/L, and saturation occurred at ≈5 µmol/L. These data indicate that ATP is hydrolyzed by
5′-ectonucleotidases in the plasma membrane of thick ascending limbs.

ATP usually acts by binding to P2 receptors. This family of receptors is composed of both P2X and P2Y receptors. To study whether P2 receptors are responsible for NOS activation in the thick ascending limb, we measured the effect of ATP on intracellular NO production in the presence of the P2 receptor antagonist suramin and observed a 65% decrease in NO production. These data indicate that extracellular ATP stimulates the P2 receptors expressed by the thick ascending limb and mediates NOS activation.

Both P2X and P2Y receptors are expressed in the thick ascending limb.\(^1\)\(^2\)\(^2\)\(^0\) Pharmacological characterization of the P2 receptors involved in NO production is possible because there are selective agonists for both receptors. UTP has been shown to be a selective P2Y receptor agonist.\(^3\)\(^0\) On the other hand, \(\beta\gamma\)-Me-ATP has been shown to be highly selective for P2X receptors.\(^3\)\(^1\)\(^3\)\(^3\) The dissociation constant (\(K_D\)) of \(\beta\gamma\)-Me-ATP for P2X receptors is \(\approx 10 \mu M\).\(^3\)\(^3\)\(^3\) In contrast the \(K_D\) of UTP for these receptors is \(\approx 10\)-fold greater. However, UTP has a \(K_D\) of \(\approx 3 \mu M\) for P2Y receptors.\(^3\)\(^4\)\(^5\)\(^6\) Our data show that the \(EC_50\) for \(\beta\gamma\)-Me-ATP in stimulating NO production is \(\approx 5 \mu M\), whereas that for UTP is \(\approx 40 \mu M\). The maximum response caused by both agents was similar. These data indicate that ATP enhances NO production primarily by activating P2X receptors.

ATP regulates epithelial transport in the lung,\(^6\) gastro-intestinal tract,\(^7\) and kidney.\(^8\)\(^9\)\(^10\) We found that ATP acting via the P2X receptor stimulates NO production in the thick ascending limb. Because we reported previously that NO inhibits NaCl absorption in this segment,\(^3\)\(^5\)\(^3\)\(^8\)\(^9\)\(^3\)\(^9\) ATP may also inhibit NaCl absorption in the thick ascending limb. This hypothesis is supported by data showing that in MDCK cells, ATP inhibits Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport.\(^4\)\(^0\)

Although the effect of ATP on NaCl absorption has not been investigated in the medullary thick ascending limb, it has been studied in other nephron segments. In vivo microperfusion experiments showed that ATP inhibited bicarbonate reabsorption in the proximal tubule.\(^1\)\(^5\) These effects may be mediated by NO, which likewise reduced bicarbonate absorption in this segment.\(^2\) ATP also reduces transport in collecting duct cells, inhibiting sodium absorption via activation of P2Y\(^4\)\(^1\)\(^–\)\(^4\)\(^3\) or both P2X and P2Y receptors.\(^4\)\(^4\)

In conclusion, we found that ATP acts as a paracrine factor in the thick ascending limb of Henle’s loop, inducing NO production via activation of P2X receptors. ATP is hydrolyzed in the plasma membrane by 5′-ectonucleotidases, indirectly regulating the amount of NO produced in the thick ascending limb. ATP may be an important regulator of NaCl absorption by the thick ascending limb.

**Perspectives**

The effect of ATP on NO production is likely to reduce NaCl absorption.\(^3\) The ability of ATP to stimulate NO production may be significant when interstitial ATP concentrations are elevated. This occurs during autoregulation of renal blood flow\(^4\)\(^5\) and is at least partly attributable to release of ATP by the macula densa during tubuloglomerular feedback.\(^4\)\(^6\) ATP levels may also increase during the early stages of diabetes, because glucose enhances ATP release by mesangial cells.\(^4\)\(^7\) Additionally, ATP may mediate cross-talk between the vasa recta and thick ascending limbs, because endothelial cells release ATP in response to many stimuli.\(^8\)\(^–\)\(^1\)\(^0\) Finally, ATP may be released from the renal nerves.\(^4\)\(^8\)

**References**


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