Akt1 mediates purinergic-dependent NOS3 activation in thick ascending limbs

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Submitted 14 May 2009; accepted in final form 30 June 2009

Akt1 mediates purinergic-dependent NOS3 activation in thick ascending limbs. Am J Physiol Renal Physiol 297: F646–F652, 2009. First published July 1, 2009; doi:10.1152/ajprenal.00270.2009.—Extracellular ATP regulates many physiological processes via release of nitric oxide (NO). ATP stimulates NO in thick ascending limbs (TALs), but the signaling cascade involved in the cells of this nephron segment, as well as many other types of cells, is poorly understood. We hypothesized that ATP enhances NO synthase (NOS) activity by stimulating PI3 kinase and Akt. We measured 1) NO in TALs using the NO-sensitive dye DAF-2 DA and 2) Akt activity by fluorescence resonance energy transfer and phosphorylation of Akt isoforms. ATP (100 \( \mu \)M) stimulated NO in wild-type mice \((26 \pm 4\) arbitrary units (AU), but not in NOS3 \(-/-\) mice \((2 \pm 2\) AU; \( P < 0.04\)). In the presence of the NOS1- and NOS2-selective inhibitors 7-NI and 1400W, ATP stimulated NO by 30 \( \pm 2\) and 33 \( \pm 3\) AU, respectively (not significant vs. control). In the presence of the PI3 kinase inhibitor LY294002, ATP increased NO by 85\% \((5 \pm 2\) vs. \(28 \pm 4\) AU; \( P < 0.02\)). ATP alone increased Akt activity and this effect was significantly blocked by suramin, a P2 receptor antagonist. In the presence of an Akt-selective inhibitor, ATP-induced NO was blocked by 90 \( \pm 4\)\%. ATP significantly stimulated Akt1 phosphorylation at Ser \(^{473}\) by 91 \( \pm 13\)\%, whereas Akt2 phosphorylation remained unchanged and Akt3 phosphorylation decreased. In vivo transduction of TALs with a dominant-negative Akt1 significantly decreased ATP-induced NO by 88 \( \pm 6\)\%. We concluded that ATP increases NOS3-derived NO via Akt1 activation in the TAL.

purinergic signaling; adenosine triphosphate; Na-K-2Cl cotransporter

In the kidney, adenosine triphosphate (ATP) is released from several types of cells. Macula densa cells release ATP in response to an increase in luminal NaCl (19), while mesangial cells release ATP in response to elevated glucose (35). The cells that constitute the thick ascending limb release ATP in response to decreases in osmolality (34) and increases in luminal flow (16). The ATP released from these and other cells has pleotropic effects. In vivo studies showed that extracellular ATP inhibits fluid absorption (1). In the collecting duct extracellular ATP also decreases Na (32) and water (18) reabsorption. Increases in ATP in the renal interstitium correlate with decreases in renal blood flow (26), most likely related to afferent arterial autoregulation (14). Moreover, we recently demonstrated that extracellular ATP stimulates nitric oxide (NO) by thick ascending limbs via activation of purinergic 2X (P2X) receptors (33). Indeed, ATP has been shown to stimulate NO production in a variety of cells, including platelets (20), endothelial cells (37), and cells of the central nervous system (31). However, the mechanisms by which ATP stimulates NO production are poorly understood.

Activation of P2X receptors may lead to stimulation of several signaling cascades. These include glycogen synthase kinase (25), ERK1/2 (3), protein kinase C (38), and MAP kinase (38). In addition, activation of P2X receptors by ATP increases phosphatidylinositol (3,4,5)-trisphosphate (PIP3) production by PI3 kinase and stimulates Akt activity (10, 15). In the thick ascending limb, the PI3 kinase/Akt pathway is an important signaling cascade involved in NO synthase 3 (NOS3) activation (12, 27, 29). Therefore, it is possible that ATP also stimulates NOS via Akt in this segment.

The Akt family consists of three isoforms: Akt1, Akt2, and Akt3, all serine-threonine kinases (9). However, the physiological relevance of the individual isoforms in several types of cells, including the thick ascending limb, is still unclear. Although we showed that Akt is important in regulating thick ascending limb NO production, we did not identify the specific isoform involved, nor are we aware of any reports of isoform-specific activation of Akt family members by extracellular ATP. Therefore, we hypothesized that extracellular ATP stimulates NOS by activating PI3 kinase, which in turn activates Akt1.

METHODS

Animals. Male Sprague-Dawley rats weighing 200 to 250 g (Charles River Breeding Laboratories) were fed a diet containing 0.22% Na (Purina). Eight-week-old C57BL/6J (wild-type) and NOS3 \(-/-\) mice (Jackson Laboratories) were fed a diet containing 0.22% Na (Purina). All animals were housed for at least 7 days before the experiments. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee (IACUC).

Rat medullary thick ascending limb suspensions. Medullary thick ascending limb suspensions were prepared as we described previously (33). In brief, rats were anesthetized with ketamine (100 mg/kg body wt ip) and xylazine (20 mg/kg body wt ip). The abdominal cavity was opened and the abdominal aorta was perfused retrograde with 0.1% collagenase (Jackson Laboratories) were fed a diet containing 0.22% Na (Purina). All animals were housed for at least 7 days before the experiments. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee (IACUC).

Mouse medullary thick ascending limb suspensions. C57BL/6J (wild-type) or NOS3 \(-/-\) mice were anesthetized with ketamine (100 mg/kg body wt ip) and xylazine (20 mg/kg body wt ip) and placed under binocular magnifiers (Optivisor) with halogen side illumination. After the abdominal cavity was opened, connective tissue was excised.
from the abdominal aorta and vena cava. Both vessels were ligated before they branched into the femoral artery and vein. The abdominal aorta was cannulated using polyethylene (PE)-10 tubing (stretched at the tip) and the cannula was secured to the aorta by 2–0 silk. Kidneys were perfused from the abdominal aorta with 0.1% collagenase type I (Sigma) and 100 U heparin in HEPES-buffered physiological saline. After perfusion, the kidneys were excised, coronal slices were cut, and the inner stripe of the outer medulla was dissected under binocular magnifiers. The tissue was treated as described above for rat medullary thick ascending limb suspensions.

**Measurement of intracellular NO.** Intracellular NO in thick ascending limbs was measured using the NO-selective fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA; EMB Biosciences). Suspensions of medullary thick ascending limbs were loaded by incubating them at 37°C with 10 μM DAF-2 DA for 20 min in HEPES-buffered physiological saline. Tubules were spun at 96 g for 2 min; the pellet was washed three times and resuspended in 1 ml HEPES-buffered physiological saline at 37°C gassed with 100% oxygen. All experiments were performed in the presence of L-arginine (Sigma) and 100 U heparin in HEPES-buffered physiological saline. Tubules were spun at 96,000 g for 20 min; the pellet was washed three times and resuspended in 1 ml HEPES-buffered physiological saline at 37°C gassed with 100% oxygen. All experiments were performed in the presence of L-arginine (Sigma) and 100 U heparin in HEPES-buffered physiological saline.

**In vivo adenoviral kidney transduction.** We performed in vivo adenovirus-mediated transduction of thick ascending limbs as we described previously (34). Briefly, rats were anesthetized with ketamine (60 mg/kg ip) and xylazine (20 mg/kg ip) before surgery. The left kidney was exposed via flank incision, the fatty tissue around the renal pole was removed, and the renal artery and vein were clamped. Then, 20-μl aliquots of the virus were injected at four sites at a rate of 20 μl/min using a syringe pump (Harvard Apparatus). The renal artery was unclamped after 8 min, the kidney was returned to the abdominal cavity, and the incision was sutured. Previously, we showed that at least 80% of the thick ascending limbs can be efficiently transduced using this technique (28).

**Measurements of Akt activity by FRET.** Thick ascending limb suspensions were generated from the virus-transduced kidney as described above and a 100-μl aliquot was placed in a custom-made temperature-controlled chamber at 37°C under an inverted microscope (Nikon Eclipse TE-2000-U). Data were acquired and quantified using laser-scanning confocal microscopy (VisiTech International). The flow rate of the bath was 0.3 ml/min. Suspensions were equilibrated for 20 min. A single tubule was chosen and two regions of interest were selected. During the control period, the ratio of emissions from cyan (CFP) and yellow fluorescent protein (YFP) was obtained by exciting CFP at 442 nm once a minute for 5 min and measuring CFP and YFP emissions at 480 ± 20 (CFP) and 540 ± 20 nm (YFP). Then, ATP (100 μM) was added to the bath and increases in CFP and decreases in YFP fluorescence intensities were observed. Then, CFP/YFP ratios were obtained once every minute for 15 min. The means of the 5-min control period and the last 5 min of the experimental period were compared. Increases in CFP/YFP ratio were taken as a measure of Akt activity. Drugs were added to the bath during the equilibration period. Control experiments were performed to show that emissions from YFP were due to FRET (12). The same microscope settings (laser intensity, contrast, brightness, resolution, and exposure time) were used to acquire these data.

**Determination of protein content.** Total protein content was determined using Coomassie Plus reagent (Pierce), based on Bradford’s colorimetric method.

**Statistics.** Data are reported as means ± SD. Differences in means were analyzed using either Student’s t-test for paired experiments or unpaired t-test applying Hochberg’s adjustment when appropriate to determine significance. Statistical analysis was performed by the Henry Ford Hospital Department of Biostatistics and Epidemiology.

**RESULTS.** First, we examined which NOS isoform was activated by ATP using NOS3 −/− mice and selective inhibitors for NOS1 and NOS2. Figure 1A shows a representative tracing of the experiment. Treatment of medullary thick ascending limbs from the Howard Hughes Medical Institute at the University of California, San Diego (21). The dominant-negative Akt1 was generated and provided by Dr. K. Walsh from Boston University School of Medicine. Two point mutations at Thr108 and Ser273 were inserted into the full-length Akt1, preventing it from being activated by phosphorylation (23).

Recombinant replication-deficient adenoviruses encoding the FRET Akt activity reporter (AdBKAR), dominant-negative Akt1 (Ad-dn-Akt1), or LacZ (encoding for β-galactosidase; Ad-control) under control of a cytomegalovirus (CMV) promoter were constructed by ViraQuest as we described previously (34). The complete DNA sequence encoding for BKAR, the full length of dominant-negative Akt1, or control DNA was inserted into a specific shuttle plasmid (pQVAdSCMVK-Npa) provided by ViraQuest. These plasmids contain the CMV promoter, a cloning site for insertion of a heterologous gene, and a polyadenylation signal flank by adenosinoviral sequences 5′ and 3′. The final viral titer was 3 × 1012 particles/ml for Ad-BKAR and 1 × 1012 for Ad-dn-Akt1 and Ad-control.

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C57BL/6J (wild-type) mice with ATP (100 μM) increased NO by 26 ± 4 arbitrary fluorescent units (AFU). In contrast, ATP did not increase NO in medullary thick ascending limbs from NOS3/−/− mice compared with wild-type C57BL/6J. Extracellular ATP stimulated NO in the presence and absence of the NOS1- and NOS2-selective inhibitors 7-NI and 1400W, respectively.

Because increased PI3 kinase activation stimulates Akt phosphorylation and activation (9), we investigated the role of Akt in ATP-induced NO. First, we measured Akt activity using a FRET probe (12, 21) in response to extracellular ATP. Figure 3A shows a representative tracing of the experiment. ATP (100 μM) increased the CFP/YFP ratio by 0.122 ± 0.030, indicating increased Akt activity. In contrast, in the presence of the generic P2 receptor antagonist suramin (300 μM), ATP did not increase CFP/YFP ratio (Δ0.021 ± 0.006; Fig. 3B; P < 0.04 vs. ATP, n = 6). Suramin alone did not increase CFP/YFP ratio (Δ0.016 ± 0.012, not significant vs. ATP).

We next tested the ability of ATP to stimulate NO in the presence of an Akt-selective antagonist, the Akt inhibitor VIII. In the absence of the inhibitor, ATP increased NO by 31 ± 3 AFU. After medullary thick ascending limb suspensions with Akt inhibitor VIII (5 μM) were treated for 10 min, ATP increased NO only 3 ± 1 AFU (P < 0.05 vs. control, n = 5; Fig. 3C), a 90% inhibition. Taken together, these data indicate that extracellular ATP stimulates Akt activity by stimulating P2 receptors and that extracellular ATP enhances NO by stimulating Akt.

There are three different Akt isoforms. To study which isoform mediated the ATP-induced NOS3 activation, we measured Akt isoform expression. We found that all Akt isoforms were expressed in medullary thick ascending limbs. Next, we measured isoform-specific Akt activation based on Akt1, Akt2, and Akt3 phosphorylation at Ser473, Ser474, and Ser472, respectively. We found that in the presence of ATP (100 μM), Akt1 phosphorylation at Ser473 was 1.98 ± 0.4 optical density units (Fig. 1C; n = 5). Taken together, these data indicate that 1) ATP stimulates NO from NOS3, but not NOS1 and NOS2, and 2) DAF-2 fluorescence is a valid indicator of NO.

To establish whether the effect of ATP on NO is mediated by PI3 kinase, we tested the ability of ATP to stimulate NO in the presence of PI3 kinase inhibitors. We found that in the absence of LY294002, ATP increased NO by 28 ± 4 AFU. In the presence of LY294002 (10 μM), ATP increased NO by only 5 ± 2 AFU (P < 0.02 vs. control, n = 5; Fig. 2), an 83% inhibition. In the presence of the PI3 kinase inhibitor wortmannin, ATP increased NO by only 4 ± 1 AFU (P < 0.03 vs. control, n = 5), an 86% inhibition. Taken together, these data indicate that ATP stimulates NO in the thick ascending limb by activating PI3 kinase.

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In the presence of suramin (300 \( \mu \)M) plus ATP, Akt1 phosphorylation at Ser473 was only 1.02 ± 0.3 ODU (Fig. 4A; \( P < 0.01 \) vs. ATP alone, \( n = 5 \)). Addition of ATP did not affect Akt2 phosphorylation at Ser474 compared with vehicle (0.86 ± 0.12 vs. 0.80 ± 0.14 ODU, \( n = 5 \)). Similarly, addition of ATP did not affect Akt2 phosphorylation compared with samples incubated with ATP plus suramin (Fig. 4B; 1.26 ± 0.28 vs. 1.11 ± 0.29 ODU, \( n = 5 \)). Addition of ATP decreased Akt3 phosphorylation at Ser472 compared with vehicle (1.99 ± 0.30 vs. 1.2 ± 0.14 ODU, \( P < 0.05 \), \( n = 5 \)). ATP also decreased Akt3 phosphorylation compared with samples incubated with ATP plus suramin (Fig. 4C; 0.96 ± 0.22 vs. 1.53 ± 0.35, \( P < 0.02 \), \( n = 5 \)). Expression of total Akt1, Akt2, and Akt3 remained unchanged after treatment. In addition, vehicle or suramin had no effect on basal phosphorylation of the Akt isoforms.

To test whether Akt1 activation was required for the ATP-induced NOS3 activity, we used a dominant-negative Akt1. We found that ATP (100 \( \mu \)M) increased NO by 26 ± 2 AFU in thick ascending limbs from kidneys transduced with virus expressing \( \beta \)-galactosidase (as a control). In contrast, ATP did not increase NO (\( \Delta 3 \pm 2 \) AFU) in thick ascending limbs expressing the dominant-negative Akt1 (Fig. 5; \( P < 0.02 \) vs. control, \( n = 6 \)). Taken together, these data indicate that extracellular ATP selectively stimulates Akt1 via P2 receptors in the thick ascending limb and that ATP-induced NOS3 activation is primarily mediated by Akt1.

**DISCUSSION**

ATP stimulates NO in endothelial cells, platelets, and the thick ascending limb. However, the signaling cascade involved in this nephron segment and other cell types is poorly understood. To begin our study of how ATP increases NO in thick ascending limbs, we first investigated the NOS isoform involved. The thick ascending limb expresses all three NOS isoforms. Because of the lack of specific NOS3 inhibitors, we first measured NO in thick ascending limbs from NOS3−/− and wild-type mice treated with ATP. We found that ATP failed to stimulate NO in thick ascending limbs from NOS3−/− mice compared with wild-type. Then, we tested whether ATP stimulated NO from NOS1−/− and NOS2-selective inhibitors 7-NI and 1400W, respectively, ATP still stimulated NO. These data
indicate that ATP stimulates NO from NOS3 rather than NOS1 or NOS2.

ATP increases PI3 kinase activity (10, 15) and in the thick ascending limb this signaling cascade increases NO production (11, 12). Therefore, we tested whether ATP stimulates NO by activating PI3 kinase. For this, we measured ATP-induced NO in the presence and absence of the PI3 kinase inhibitors LY294002 and wortmannin. We found that in the presence of these inhibitors, ATP failed to stimulate NO. This indicates that PI3 kinase is a necessary mediator for ATP-induced NO in thick ascending limbs.

Because activation of PI3 kinase in turn enhances Akt activity, we measured the effect of ATP on total Akt activity using FRET. We compared the effects of ATP in the presence of suramin to ensure any measured response was due to purinergic activation. We found that ATP stimulated Akt activity, an effect completely blocked by the P2 receptor antagonist suramin. To further test the role of Akt on ATP-induced NO, we measured NO in the presence and absence of the Akt-selective inhibitor Akt inhibitor VIII. We found that in the presence of Akt inhibitor VIII, ATP failed to stimulate NO. These data indicate that Akt mediates the stimulatory effects of ATP on NO.

To date three different Akt isoforms have been cloned. However, expression and physiological relevance of the various isoforms have not been studied in the thick ascending limb to our knowledge. We first measured Akt isoform expression in thick ascending limb suspensions and found that Akt1, Akt2,
and Akt3 were all expressed in this nephron segment. To determine whether extracellular ATP stimulates a specific Akt isoform, we then measured Akt isoform phosphorylation in response to extracellular ATP-induced P2 receptor activation. We found that Akt1 phosphorylation increased whereas Akt3 phosphorylation decreased and Akt2 phosphorylation was not affected. To our knowledge, this is the first reported demonstration that extracellular ATP exerts isoform-specific Akt activation in epithelial cells.

To show that Akt1 is required for ATP-induced NO, we used a dominant-negative Akt1 and we measured NO production in thick ascending limbs from kidneys transduced with dominant-negative Akt1. We found that in the thick ascending limb, ATP stimulates NO via activation of Akt1 rather than Akt2 or Akt3.

While it is known that ATP increases NO, the signaling cascade is still poorly understood. Our goal was to clarify a signaling pathway that remains unknown in many types of cells, including those of the thick ascending limb. In vascular endothelial cells, ATP released by physical and chemical stimuli increases endothelium-derived NO (22). Similarly, ATP released from red blood cells in the lung vasculature stimulates NO production by endothelial cells (37). However, the mechanism by which extracellular ATP increases NO in endothelial cells is unclear. Nor do we know how extracellular ATP increases NO in activated platelets (20) and neuronal cells (31). We found that in the thick ascending limb, ATP stimulates NO via the PI3 kinase/Akt1 pathway. These findings are supported by published reports that other factors that stimulate NO in the thick ascending limb, such as endothelin-1 (12) and luminal flow (27), do so by activating NOS3. Since the members of this signaling cascade are expressed in endothelial cells, platelets, and neuronal tissue, the P2 receptors/PI3 kinase/Akt1/NOS3 pathway may be common to all cell types in which ATP increases NO production.

Our data demonstrating that ATP activates the PI3 kinase/Akt signaling pathway are supported by other’s findings. In vitro experiments demonstrated that extracellular ATP directly stimulates the PI3 kinase/Akt signaling pathway by activating P2 receptors in neuronal tissue (15). Additionally, ATP and P2 receptors were shown to increase endothelial cell migration and adhesion in a PI3 kinase-dependent manner (17). Similarly, in vivo microinjections of ATP into the nucleus accumulated activated the PI3 kinase/Akt signaling pathway, leading to neuron proliferation, and this was blocked by the P2 receptor antagonist PPADS (8).

We found that ATP selectively activates Akt1, suggesting that the three Akt isoforms may play different roles in the thick ascending limb. Animal models deficient in Akt1, Akt2, or Akt3 have been generated to study the physiological relevance of these Akt isoforms. Although these kinases are 70–80% homologous, they do not seem to show any functional overlap (24). Mice lacking one allele of the gene encoding for Akt1 are able to survive; however, prenatal mortality is higher in this strain and body weight is markedly reduced (5). In addition, Akt1 plays a key role in vascular bed maturation and angiogenesis in endothelial cells but Akt2 does not (36). Similarly, erythropoietin regulates tetrahydrobiopterin biosynthesis via an Akt1-dependent pathway in endothelial cells, affording vascular protection (2). In contrast, Akt2 activation seems to activate different signaling pathways. Akt2-deficient mice display a diabetes-like syndrome with elevated plasma glucose levels, increased hepatic glucose output, and insulin resistance (5). In contrast, Akt3-deficient mice exhibit reduced neuronal cell size and neuron number, yet maintain normal glucose homeostasis (6). These studies suggest that Akt isoforms may have separate physiological functions, activate different signaling cascades, and mediate distinct cellular processes.

We found that Akt1 is responsible for ATP-induced NOS3 activation in the thick ascending limb. This has been described previously in epithelial and endothelial cells. For example, increased Akt1 activity in endothelial cells accelerates the rate of migration, which is mediated by NOS3 activation (13). Similarly, in endothelial cells from coronary arteries, deletion of Akt1 reduces NOS3 activity by blunting its phosphorylation at Ser1177, increasing the risk of atherogenesis (7). Moreover, in the thick ascending limb, both stimulation of NO by endothelin-1 and inhibition of transport occur via Akt1 (12).

The mechanism we described here could be important when renal ATP release is increased, such as during autoregulation of renal blood flow (26) or in patients with diabetes (35). In the thick ascending limb, ATP-stimulated NO could mediate several processes, including regulation of transport (30), modification of tubulovascular cross-talk with the vasa recta (4), and alteration of tubuloglomerular feedback by diffusion to the macula densa (39). In conclusion, we found that extracellular ATP selectively activates Akt1, which stimulates NO production by the thick ascending limb of the loop of Henle.

**Grants**

This work was supported in part by grants from the National Institutes of Health (HL-090550-01) to J. L. Garvin and from the American Heart Association Greater Midwest (0615718Z) to G. B. Silva.

**References**


