Adenosine Triphosphate Inhibits Endothelin-1 Production by Rat Inner Medullary Collecting Duct Cells

ALISA K. HUGHES, PETER K. STRICKLETT, BELLAMKONDA K. KISHORE, AND DONALD E. KOHAN[1]

Division of Nephrology, University of Utah Health Sciences Center, Salt Lake City, Utah 84132; and Salt Lake Veterans Affairs Medical Center, Salt Lake City, Utah 84148

Adenosine triphosphate (ATP) and endothelin (ET)-1 inhibit vasopressin-stimulated water reabsorption in the inner medullary collecting duct (IMCD). Because both ATP and ET-1 are released by the IMCD and can act in an autocrine manner to regulate IMCD water transport, we sought to determine whether these factors can modulate the other's production. To begin such studies, the effect of ATP on IMCD ET-1 production was examined. ATP caused a dose-dependent inhibition of ET-1 release and inhibited ET-1 mRNA levels in primary cultures of rat IMCD cells. This effect was first evident after 4 hrs of exposure to ATP and persisted for at least 24 hrs. The 50% inhibitory concentration for ATP inhibition of ET-1 production was approximately 1 μ*M,* and the maximal response was observed at 25–100 μ*M.* ATP acted, at least in part, through the P2Y2 receptor because its effect was mimicked by UTP, but not by the P2X agonist, α,ß-methylene–ATP. *N*-methyl-L-arginine, or indomethacin, did not block the ATP inhibitory effect. In summary, these data demonstrate that ATP inhibits IMCD ET-1 protein and mRNA accumulation, that this is mediated *via* P2Y receptors, and that the ATP effect is independent of cyclooxygenase or nitric oxide synthase metabolites. These findings suggest that although ATP and ET-1 both antagonize vasopressin action in the IMCD, they may have a complex interaction that ultimately determines the degree to which they each participate in modulating collecting duct function. Exp Biol Med 231:1006–1009, 2006

Key words: endothelin-1; purinergic; P2Y; ATP; collecting duct

Introduction

Studies suggest that both adenosine triphosphate (ATP) and endothelin (ET)-1 inhibit Na and water transport by the renal collecting duct; however, the interaction between these two substances in this nephron segment is unknown. ET-1 inhibits arginine vasopressin (AVP)-stimulated cyclic adenosine monophosphate (cAMP) accumulation (1) and water flux (2) in the collecting duct *in vivo*, whereas collecting duct–specific knockout of ET-1 results in an impaired ability to excrete a water load associated with enhanced collecting duct AVP responsiveness (3). *In vitro*, ET-1 reduces hormone-stimulated Na reabsorption in the cortical collecting duct (4) and decreases Na/K-ATPase activity in the inner medullary collecting duct (IMCD; Ref. 5); whereas collecting duct–specific knockout of ET-1 causes reduced excretion of a Na load associated with elevated systemic blood pressure (6). Although the role of ATP in regulating collecting duct Na and water transport has not been examined in intact animals, *in vitro* studies suggest that ATP also inhibits collecting duct salt and water reabsorption. ATP inhibits AVP-stimulated cAMP accumulation and osmotic water permeability in acutely isolated IMCD (7). In addition, ATP reduces amiloride-sensitive Na transport in a cortical collecting duct cell line (8). Taken together with the fact that both ET-1 and ATP are released in abundance by the IMCD (7, 9), these studies raise the question of whether these two IMCD-derived natriuretic and diuretic factors interact to modulate one another's activity.

Relatively little is known regarding interactions between the purinergic and ET systems. Purinergic receptor activation seems to have stimulatory or inhibitory effects on ET-1 synthesis or activity, depending on the system studied. ATP, *via* P1 receptor activation, increases ET-1 secretion by rat thyroid cells (10), whereas ET-1 and ATP (ATP through the P2Y receptor) can have synergistic mitogenic effects on vascular smooth-muscle cells (11). In contrast, ATP induces heterologous desensitization of ET-1–evoked calcium increases in astrocytes (12). Similarly, and most relevant to the current study, ATP inhibited ET-1–induced calcium

This research was supported in part by the National Institutes of Health grant DK 96392 (D.E.K.).

[1] To whom correspondence should be addressed at Division of Nephrology, University of Utah Health Sciences Center, 1900 East, 30 North, Salt Lake City, UT 84132. E-mail: donald.kohan@hsc.utah.edu

Received September 6, 2005.
Accepted November 16, 2005.

1535-3702/06/2316-1006$15.00

increases in the acutely isolated rat outer medullary collecting duct (13). Thus, although ATP and ET-1 may have similar natriuretic and diuretic effects in the collecting duct, previous studies indicate that they may have inhibitory or stimulatory effects on one another. Consequently, the current study was undertaken to explore the effect of ATP on ET-1 production by collecting duct cells.

Materials and Methods

Reagents. ATP-γS was purchased from Calbiochem (La Jolla, CA), cell culture media from Cambrex (Walkersville, MD), and collagenase from Worthington (Lakewood, NJ). All other reagents were purchased from Sigma (St. Louis, MO), unless stated otherwise.

Cell Culture. Sprague Dawley rats weighing 150–250 g were used for all experiments. Rat inner medullae were dissected free from the kidney, finely minced with a razor blade, and incubated with 4 ml of 1 mg/ml collagenase (Type 2) and 0.1 mg/ml DNase I for 30–45 mins at 37°C. Cells were water-shocked with 6 ml water to destroy contaminating red blood cells and endothelial cells. Cells were then washed in 10% bovine serum albumin and centrifuged at 800 rpm. The pellets were plated in 24-well plates in renal epithelial cell growth media containing epidermal growth factor, insulin, hydrocortisone, gentamicin, amphotericin, 0.5% fetal bovine serum, epinephrine, triiodothyronine, and transferrin (concentrations are proprietary). Once cells reached confluence, the media was changed to Dulbecco's modified Eagle's medium (DMEM):F12 alone for 16–24 hrs before study.

ET-1 Assay. Cells were incubated in DMEM:F12 and inhibitors (indomethacin or *N*-methyl-L-arginine [L-NMMA]), when appropriate, for 1 hr. Subsequently, the media was changed, and agonist (ATP-γS, UTP, ADP, or α,ß-methylene–ATP) and fresh inhibitor (if appropriate) were added in 300 μl DMEM:F12 for 4 hrs or 24 hrs. All of the media was dried, resuspended in phosphate-buffered saline, and assayed for ET-1 using the QuantiGlo human ET-1 chemiluminescent immunoassay (R&D Systems, Minneapolis, MN). Samples were read on a Dynex Technologies MLX Luminometer. Cells were solubilized in 0.1 *M* NaOH and total cell protein determined by the Bradford assay. ET-1 was standardized to total cell protein.

Determination of mRNA Levels. Total RNA was prepared from rat IMCD cell culture using acid phenol, electrophoresis was performed, and RNA was transferred to a nylon membrane. For probe, complementary DNA made from rat IMCD cell total RNA was used as a template for polymerase chain reaction amplification using primers to rat ET-1 (GenBank accession no. NM_012548): forward primer 5′-GGC TTT CCA AGG AGC TCC AGA-3′ and reverse primer 5′-ATC AAC TTC TGG TCT CTG TAG AG-3′, which yield a product size of 552 base pairs. The product was purified, sequenced, inserted into a cloning vector, and the antisense riboprobe was made by [^{32}P]UTP incorporation with either T7 or SP6 RNA polymerase. The probe was added to hybridization solution and incubated overnight. Blots were washed and subjected to autoradiography and densitometry. All blots were stripped and reprobed for U1 small nuclear ribonucleoprotein (snRNP) mRNA (mRNA size is 0.75 kb) using a template provided by Dr. Andrew Thorburn at the University of Utah (14). All mRNA results were determined as the ratio of ET-1 to snRNP mRNA levels.

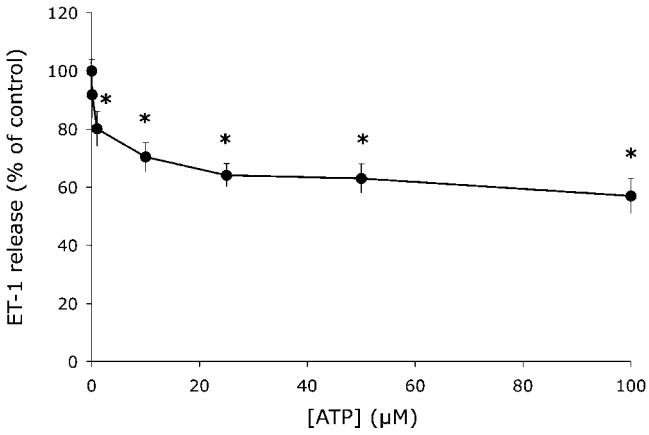

Figure 1. Dose-dependent effect of ATP-γS (ATP) on ET-1 release by cultured rat IMCD cells. Cells were incubated with ATP for 4 hrs; $n = 10$ for each data point. Data are expressed as percent of control ET-1 release (no added ATP). Control ET-1 release was 613 ± 23 pg ET-1/mg total cell protein ($n = 10$). *$P < 0.001$ versus control.

Statistics. Comparisons between control and ATP-γS–treated cells for RNA levels were analyzed by the unpaired Student's *t* test. All other comparisons were made using one-way analysis of variance with a Bonferroni correction. $P < 0.05$ was taken as significant. Data are expressed as mean ± SEM.

Results

The rat IMCD cell cultures were all studied at confluence and after 1 day in growth-arrest media (DMEM:F12 alone). These primary cell cultures have been extensively described; they consist of only IMCD cells (15). Primary cultures of rat IMCD cells release abundant ET-1 after 1 day of growth arrest (613 ± 23 pg ET-1/mg total cell protein; $n = 10$). Addition of ATP-γS, the relatively stable ATP analog, for 4 hrs, significantly inhibited ET-1 release from these cells; the 50% inhibitory concentration was approximately 1 μ*M*, with the maximal effect (62 ± 4% of control; $n = 10$; $P < 0.001$ vs. control) evident at 25–100 μ*M* (Fig. 1). No inhibitory effect of ATP-γS on ET-1 release was evident after 1 hr of incubation, whereas 24 hrs of incubation with ATP-γS caused a comparable decrease in ET-1 production (data not shown). ATP-γS (4 hrs exposure) also decreased ET-1 mRNA levels in IMCD cells (70 ± 8% of control, as determined by densitometry; $n = 4$ per data point, $P < 0.01$ vs. control; Fig. 2A and B). Similarly, 24-hr

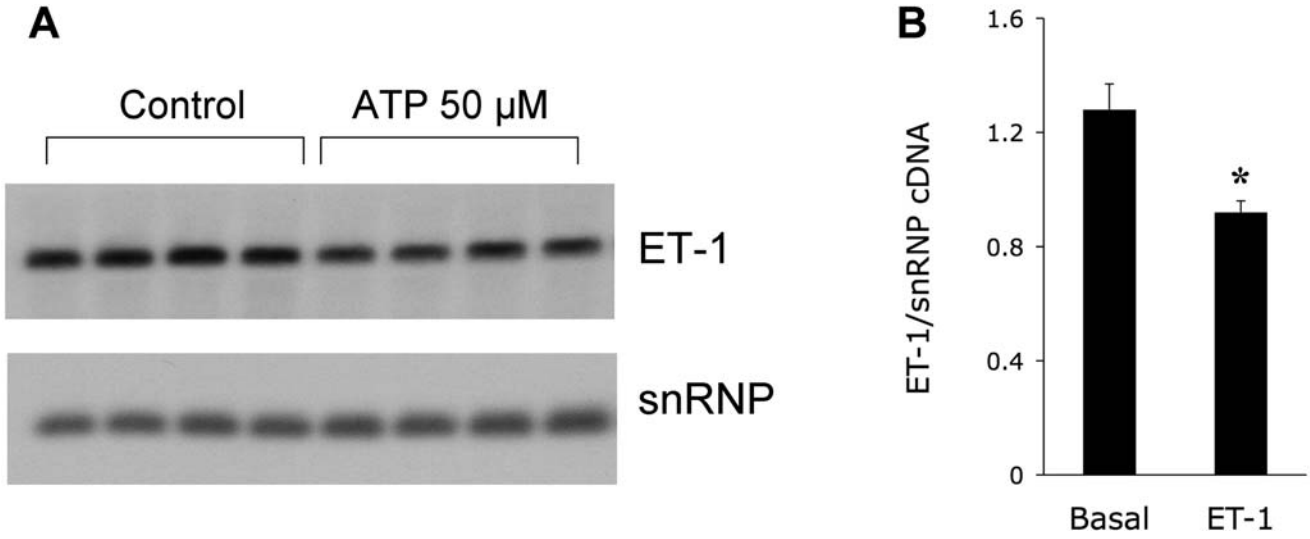

Figure 2. Effect of 4-hr exposure to 50 μ*M* ATP-γS on ET-1 mRNA levels in cultured rat IMCD cells. ET-1 and snRNP riboprobes were used for Northern analysis; $n = 4$ for each condition. (A) Blot; (B) densitometry. *$P < 0.01$ versus basal.

exposure to ATP-γS decreased ET-1 mRNA levels (data not shown).

To determine which purinergic receptors mediated the ATP-γS response, IMCD cells were incubated with UTP, ADP, or α,ß-methylene–ATP for 4 hrs. UTP and ADP had similar inhibitory effects as ATP-γS, indicating that the response was not idiosyncratic to ATP-γS and that P2Y receptors are involved (Fig. 3). In contrast, α,ß-methylene–ATP did not alter IMCD ET-1 release, indicating that stimulation of at least some P2X receptors does not affect ET-1 production by rat IMCD cells.

Because both prostaglandins and nitric oxide (NO) are important mediators of ATP action (16, 17), the effect of blocking prostaglandin and NO production on ATP-γS inhibition of ET-1 release by IMCD cells was examined. As shown in Figure 4, neither indomethacin (nonspecific cyclooxygenase blockade) nor L-NMMA (NO blockade) at maximal inhibitory doses had any effect on ATP-γS inhibition of ET-1 production by IMCD cells, indicating that the ATP response is independent of NO synthase or cyclooxygenase metabolites. Notably, these concentrations of L-NMMA and indomethacin completely block all NO and PGE2 production, respectively, in IMCD cells (data not shown).

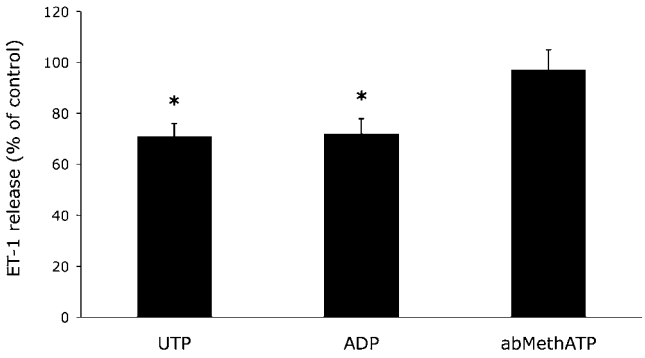

Figure 3. Effect of 50 μ*M* UTP, 50 μ*M* ADP, or 50 μ*M* α,ß-methylene–ATP on ET-1 release by cultured rat IMCD cells. Cells were incubated with agonists for 4 hrs; $n = 12$ for each data point. Data are expressed as percent of control ET-1 release (no added ATP). *$P < 0.001$ versus control.

Discussion

The current study examined the interaction between ATP and ET-1 in renal epithelium for the first time, to our knowledge. We describe ATP-γS inhibition of IMCD ET-1 protein and mRNA levels. As discussed in the Introduction, this observation is in agreement with previous studies showing ATP reduction of ET-1 signaling in astrocytes (12) and outer medullary collecting duct cells (13). However, to our knowledge, there are no previous reports of ATP inhibition of ET-1 production. The only study examining ATP regulation of ET-1 secretion described a stimulatory effect in thyroid cells (10). Thus, the field of purinergic regulation of ET-1 is in its infancy, and much further investigation is required.

IMCD cells express different P2Y and P2X receptor isoforms (7). The findings in the current study suggest that P2Y receptors mediate the inhibitory effect of ATP-γS on ET-1 synthesis by IMCD cells. However, because α,ß-methylene–ATP does not activate all P2X receptors, the role of these receptors in mediating ATP actions cannot be completely excluded. Based on numerous reports of P2Y2 receptors mediating many of the effects of ATP in the IMCD (7), it is tempting to speculate that this is the primary purinergic receptor involved in this response. However, recent studies indicate that other P2Y receptors with high UTP affinity may be expressed in the nephron (7). Notably, the one report of a stimulatory effect of ATP on ET-1 production implicated activation of P1 receptors (10). Thus,

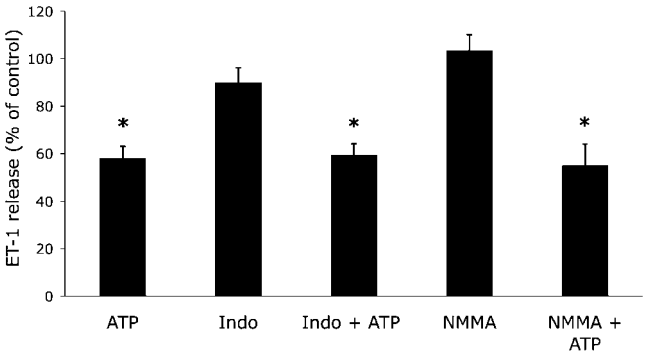

Figure 4. Effect of 10 μM indomethacin or 100 μM L-NMMA on the inhibitory effect of 50 μM ATP-γS on ET-1 release by cultured rat IMCD cells. Cells were incubated with inhibitors for 1 hr before addition of ATP for 4 hrs; $n = 10$ for each data point. Data are expressed as percent of control ET-1 release (no added ATP). $^*P < 0.001$ versus control.

ATP regulation of ET-1 production may depend on which purinergic receptors are predominantly involved.

The mechanisms by which ATP-γS regulates ET-1 production in the IMCD are unknown. The current study ruled out a role for cyclooxygenase or NO synthase metabolites, however, many other possible pathways remain. ATP activates a wide variety of intracellular signaling mechanisms, including protein kinase C, mitogen-activated protein kinases, phosphodiesterases, calmodulin, and others (7). Another important unanswered question is the physiologic relevance of ATP inhibition of IMCD ET-1 production. Because both ATP and ET-1 inhibit water reabsorption by the IMCD, our original hypothesis was that ATP would increase ET-1 production. That the opposite effect occurs suggests that this system serves as a negative feedback, limiting ongoing reduction of water transport. One could argue that this response is only relevant at pharmacologically high concentrations of ATP, however, the same concentrations of ATP that inhibit water transport or stimulate prostaglandin E2 production in the IMCD also reduce ET-1 production (17, 18). It is also interesting to note that although ADP had a similar inhibitory effect on ET-1 production as did ATP and UTP in isolated perfused IMCD, UTP and ATP (but not ADP) inhibit water transport (18). Thus, ATP-γS inhibition of ET-1 production may involve, at least in part, different purinergic receptors than those involved in inhibition of water reabsorption. Why such a system exists, and the ultimately importance of this interaction to renal regulation of salt and water excretion, is an area in need of further study.

1. Tomita K, Nonoguchi H, Marumo F. Effects of endothelin on peptide-dependent cyclic adenosine monophosphate accumulation along the nephron segments of the rat. J Clin Invest 85:2014–2018, 1990.
2. Oishi R, Nonoguchi H, Tomita K, Marumo F. Endothelin-1 inhibits AVP-stimulated osmotic water permeability in rat medullary collecting duct. Am J Physiol 261:F951–F956, 1991.
3. Ge Y, Ahn D, Stricklett P, Hughes A, Yangisawa M, Verbalis J, Kohan D. Collecting duct-specific knockout of endothelin-1 alters vasopressin regulation of urine osmolality. Am J Physiol 288:F912–F920, 2005.
4. Tomita K, Nonguchi H, Terada Y, Marumo F. Effects of ET-1 on water and chloride transport in cortical collecting ducts of the rat. Am J Physiol 264:F690–F696, 1993.
5. Zeidel ML, Brady HR, Kone BC, Gullans SR, Brenner BM. Endothelin, a peptide inhibitor of Na^+-K^+-ATPase in intact tubular epithelial cells. Am J Physiol 257:C1101–C1107, 1989.
6. Ahn D, Ge Y, Stricklett P, Gill P, Taylor D, Hughes A, Yanagisawa M, Miller L, Nelson R, Kohan DE. Collecting duct-specific knockout of endothelin-1 causes hypertension and sodium retention. J Clin Invest 114:504–511, 2004.
7. Schwiebert E, Kishore B. Extracellular nucleotide signaling along the renal epithelium. Am J Physiol 280:F946–F963, 2001.
8. Thomas J, Deetjen P, Ko WH, Jacobi C, Leipziger J. P2Y2 receptor-mediated inhibition of amiloride-sensitive short circuit current in M-1 mouse cortical collecting duct cells. J Membr Biol 183:115–124, 2001.
9. Kohan DE. Endothelin synthesis by rabbit renal tubule cells. Am J Physiol 261:F221–F226, 1991.
10. Vainio M, Saijonmaa O, Fyhrquist F, Tornquist K. Purinergic agonists stimulate the secretion of endothelin-1 in rat thyroid FRTL-5 cells. J Cell Physiol 169:538–543, 1996.
11. Erlinge D. Extracellular ATP: a growth factor for vascular smooth muscle cells. Gen Pharmacol 31:1–8, 1998.
12. Cho YJ, Lee SY, Kim YS, Lee EJ, Seo MS, Yeon G, Lee KH, Lee KJ, Jo YK, Rha HK. Adenosine triphosphate-induced heterologous desensitization of endothelin-1- and glutamate-evoked calcium increases in cultured rat cortical astrocytes. Neurosci Lett 286:33–36, 2000.
13. Cha SH, Sekine T, Endou H. P2 purinoceptor localization along rat nephron and evidence suggesting existence of subtypes P2Y1 and P2Y2. Am J Physiol 274:F1006–1014, 1998.
14. Thorburn J, Frost J, Thorburn A. Mitogen-activated protein kinases mediate changes in gene expression, but not cytoskeletal organization associated with cardiac muscle cell hypertrophy. J Cell Biol 126:1565–1572, 1994.
15. Kohan DE, Padilla E. Regulation of endothelin-1 synthesis by rat renal inner medulla. J Am Soc Nephrol 3:439, 1992.
16. Cary SP, Winger JA, Marletta MA. Tonic and acute nitric oxide signaling through soluble guanylate cyclase is mediated by nonheme nitric oxide, ATP, and GTP. Proc Natl Acad Sci U S A 102:13064–13069, 2005.
17. Welch BD, Carlson NG, Shi H, Myatt L, Kishore BK. P2Y2 receptor-stimulated release of prostaglandin e2 by rat inner medullary collecting duct preparations. Am J Physiol Renal Physiol 285:F711–21, 2003.
18. Kishore B, Chou C-L, Knepper M. Extracellular nucleotide receptor inhibits AVP-stimulated water permeability in inner medullary collecting duct. Am J Physiol 269:F863–F869, 1995.