Effects of adenosine stimulation on the mRNA expression of *CLCNKB* in the basolateral medullary thick ascending limb of the rat kidney

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Abstract. Adenosine is a molecule produced by several organs within the body, including the kidneys, where it acts as an autoregulatory factor. It mediates ion transport in several nephron segments, including the proximal tubule and the thick ascending limb (TAL). Ion transport is dictated in part by anionic chloride channels, which regulate crucial kidney functions, including the reabsorption of Na^+ and Cl^-, urine concentration, and establishing and maintaining the corticomedullary osmotic gradient. The present study investigated the effects of adenosine on the mRNA expression of chloride voltage-gated channel Kb (*CLCNKB*), a candidate gene involved in hypertension, which encodes for the ClC-Kb channel. Medullary thick ascending limb (mTAL) tubules were isolated from the rat kidney, and primary cultures of mTAL cells from the mTAL tubules were established. The cells were treated with adenosine and the mRNA expression of *CLCNKB* was detected by reverse transcription-quantitative polymerase chain reaction. The cells were also treated with pathways inhibitors (H8 and AACOCF3), and the protein expression of cyclic adenosine 3',5'-monophosphate (cAMP)-protein kinase A (PKA) and phospholipase A_2 (PLA_2) by were analyzed by western blotting. The findings indicated that adenosine increased the mRNA expression of *CLCNKB* in primary cultures of medullary TAL cells, and this stimulatory effect was regulated by the cAMP-PKA and PLA_2-arachidonic acid (AA) pathways. The present study showed that adenosine affected the mRNA expression of *CLCNKB*, initially through the cAMP-PKA pathway and then the PLA_2-AA pathway.

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Introduction

The kidney is crucial in maintaining homeostasis within the body by regulating the excretion of water and electrolytes according to the requirements of the body. The maintenance of homeostasis by the kidney is controlled at the neural and humoral levels, however, it is also mediated through autoregulation, which has a critical effect on function. The kidney can produce a multitude of local, active substances, including adenosine triphosphate, adenosine and angiotensin II (1-7).

Previous studies have shown that, in addition to cardiac and skeletal muscle undergoing autoregulation by adenosine, kidney function may be controlled in the same manner (8-10). Under physiological conditions, adenosine has been shown to control the release of renin (11-13), renal blood flow, glomerular filtration rate (14), glomerulotubular balance and tubuloglomerular feedback (15,16). It is also involved in regulating ion transport in several nephron segments, including the proximal tubule and the thick ascending limb (TAL) (6,14,17-19).

The degree of NaCl transport has a direct effect on the formation of the renal medulla hypertonic gradient, which ultimately affects urine dilution and concentration. Furthermore, NaCl transport is affected significantly by chloride channel activity. Our previous studies showed that arachidonic acid (AA), a local and active substance, inhibits the activities of potassium and chloride channels. Additionally, adenosine was found to affect the activity of potassium channels in the medullary TAL (mTAL). Thus, elucidating the regulatory mechanism of adenosine on chloride channels has the potential not only to improve current understanding of Na^+, K^+ and Cl^- transport in the mTAL, but also offers novel insights for developing high performance diuretics and therapies for the clinical treatment of hypertension. The current study investigated the effects of adenosine stimulation on the expression of *CLCNKB* mRNA in the basolateral mTAL of the rat kidney, and the pathways involved in th effects. This study provides insight into the regulation and mechanisms of kidney function, and a potential new target for the clinical treatment of kidney disease.

Materials and methods

Animals. Pathogen-free Sprague-Dawley rats (male and female; 50-60 g; n=20) were obtained from the Animal Center of the Second Affiliated Hospital of Harbin Medical University (Harbin, China) and were maintained with standard rat chow and access to tap water *ad libitum*. The study was approved by the ethics committee of Harbin Medical University.

Isolation of mTAL tubules and cells. The method for the preparation of mTAL suspensions was established on the basis of the methods described in several previous studies (20-22). The rats were sacrificed by cervical dislocation and the kidneys were removed immediately for future dissection of the mTAL cells. The inner stripe of the outer medulla was carefully excised and minced with a sterile blade. It was then sequentially incubated and shaken at 37˚C in HEPES buffer solution containing 0.01% collagenase type IA (1 mg/ml; Sigma-Aldrich; Thermo Fisher Scientific, Inc., Walthlam, MA, USA) and 140 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 10 mM HEPES (pH 7.4) for 5 min. The suspension was precipitated on ice, and then mixed with HEPES buffer solution again. Finally, the supernatant containing the crude suspension of tubules was collected. The undigested tissues were subjected to three treatments with collagenase (5 min each), and the combined supernatants were suspended in HEPES buffer solution and then filtered through 180 and 50 μm nylon mesh membranes. The tubules retained on the 50 μm mesh were collected with centrifugation (1,000 x *g*, 5 min, 4˚C) and suspended in HEPES buffer solution. The suspension, which contained the mTAL tubules, was used to establish primary cultures of mTAL cells. The mTAL tubules ($5x10^5$/ml) were cultured at 37˚C in 90-mm-diameter Petri dishes using Renal Epithelial Cell culture medium (Dulbecco's modified Eagle's medium/F12; 1:1; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), which contained 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% streptomycin-penicillin (100 U/ml), rhEGF (Peprotech, Inc., Rocky Hill, NJ, USA), insulin, hydrocortisone, amphotericin B and epinephrine (Nanjing KGI Biological Technology Development Co., Ltd., Nanjing, China). After 6-7 days, the monolayer of cells was 80-90% confluent, and used in the subsequent experiments.

Cell treatments. When the cells were in the logarithmic growth phase, the medium was changed to serum-free medium. Initially, 25.6 μM adenosine was added to the cells and incubated for 6, 12, 24 and 48 h to find the greatest effect time. Subsequently, different concentrations of adenosine (1.6, 3.2, 6.4, 12.8, 25.6 and 51.2 μM) were added to indicate the best concentration to use. The mRNA expression of *CLCNKB* was analyzed using the chosen duration and concentration of adenosine treatment. Finally, 5 μM AACOCF3 or 5 μM H8 were used to treat cells for 6 h, then 25.6 μM adenosine was added 24 h to determine whether the cAMP-PKA and PLA2-AA pathways affected the mRNA expression of *CLCNKB* in the presence of adenosine.

Western blot analysis. When the cells were in logarithmic growth phase, the medium was completely removed, the cells were washed twice in ice-cold PBS and lysed with 400 μl cell lysis buffer (1% sodium deoxycholate; 10 mM Tris-Cl, pH 8; 1 nM EDTA-Na, pH 8; 0.5 mM PMSF; 1.5 μM aprotinin; 154 nM pepstatin; 50 μM phloroglucinol) per dish. Cells were scraped off the dish and transferred into eppendorf tubes, and maintained on ice for 15 min. The samples were sonicated for 3-5 sec. Protein concentrations were determined by bicinchoninic acid assay (Thermo Fisher Scientific, Inc.) The protein samples (50 μg) from the primary cultures of mTAL cells were separated by electrophoresis using 10% SDS-PAGE and transferred onto nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA). The membranes were blocked with 5% nonfat dry milk in 0.1% Tween-Tris-buffered saline (TBS-T), and then washed with 0.1% TBS-T. The membranes were incubated with primary antibody overnight at 4˚C (rabbit anti-rat $cPLA_2$, 1:1,000; rabbit anti-rat PKA, 1:1,000; rabbit anti-rat β-actin, 1:1,000). Following incubation, the membranes were washed four times (10 min per wash) with 0.1% TBS-T, following which the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; OriGene Technologies, Inc., Rockville, MD, USA) for 1 h at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.) was used to detect the protein bands. The protein bands were quantified by densitometry using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was purified from the primary cultures of the mTAL cells in the logarithmic growth phase using a Protein and RNA Extraction kit for mammalian cells (Takara Biotechnology Co., Ltd., Dalian, China). RNA was then quantified using spectrophotometry and reverse transcribed into cDNA using RT-PCR kit (Takara Biotechnology Co., Ltd.). The cDNA was amplified using a master mix containing Taq polymerase (Takara Biotechnology Co., Ltd.) and the following primers from AuGCT DNA-SYN Biotechnology Co., Ltd. (Beijing, China): *CLCNKB*, sense 5'-CTGTTCCGT GTGGGTGAG-3' and antisense 5'-GGGTACACGGTCCAA GAG-3'; *β-actin*, sense 5'-AGATCCTGACCGAGCGTGGC-3' and antisense 5'-CCAGGGAGGAAGAGGATGCG-3'. Each primer was used at a final concentration of 20 μM. The thermocycling steps were as follows: *β-actin*; 94˚C for 5 min, then 94˚C for 30 sec, 56˚C annealing for 30 sec and 72˚C extension for 25 sec (25 cycles), and 72˚C for 7 min; *CLCNKB*: 94˚C for 5 min, then 94˚C for 30 sec, 56˚C annealing for 30 sec, 72˚C extension for 25 sec (40 cycles), and 72˚C for 7 min The PCR products were separated on a 3% agarose gel and stained with ethidium bromide to identify fragments of *CLCNKB* and *β-actin*. The gene fragments were quantified by densitometry using Quantity One software (Bio-Rad Laboratories, Inc.).

Chemicals. The antibodies for β-actin (cat. no. sc-7210), PKA (cat. no. sc-28892) and $cPLA_2$ (cat. no. sc-438) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Reagent-grade chemicals, adenosine, AACOCF3 (an antagonist of PLA_2) and H8 (an antagonist of PKA) were obtained from Sigma-Aldrich; Thermo Fisher Scienfiic, Inc. The prestained protein ladder was obtained from Fermentas; Thermo Fisher Scientific, Inc.

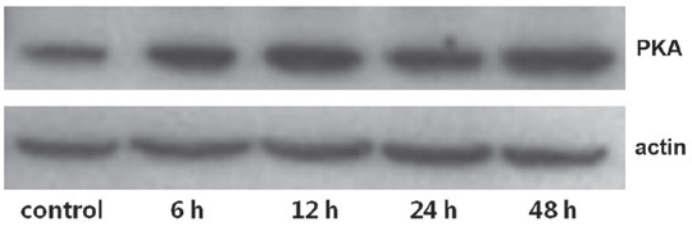

Figure 1. Effects of adenosine (25.6 μM) on the expression of PKA in the primary cultures of medullary thick ascending limb cells. Expression of PKA was determined via western blot analysis. PKA, protein kinase A.

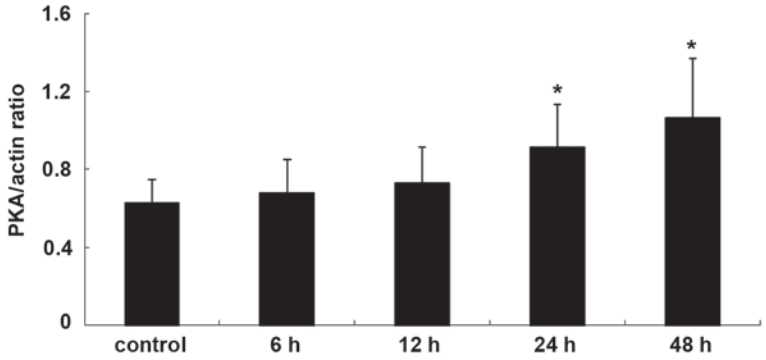

Figure 2. Effects of adenosine (25.6 μM) on the expression of PKA in the primary cultures of medullary thick ascending limb cells. Data are presented as the mean + standard deviation (n=3). *P<0.05, vs. control group. PKA, protein kinase A.

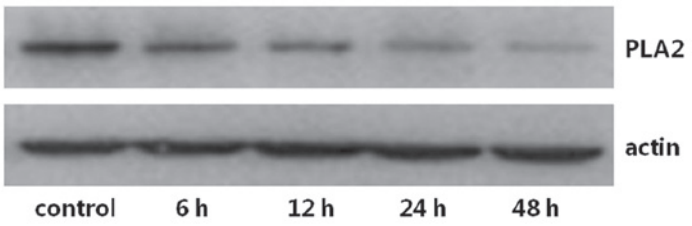

Figure 3. Effects of adenosine (25.6 μM) on the expression of PLA_2 in the primary cultures of medullary thick ascending limb cells. Expression was detected using western blot analysis. PLA_2, phospholipase A_2.

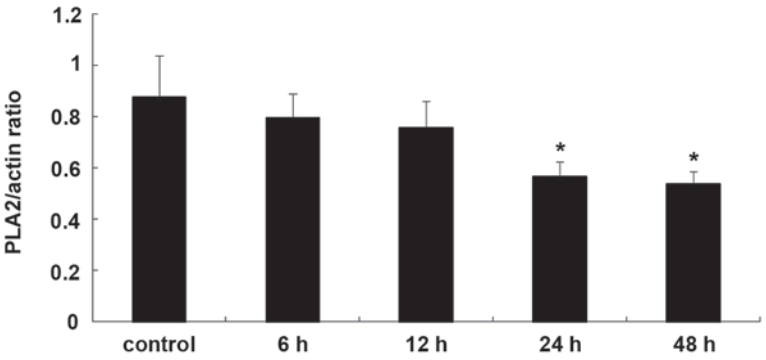

Figure 4. Effects of adenosine (25.6 μM) on the expression of PLA_2 in the primary cultures of medullary thick ascending limb cells. Data are presented as the mean + standard deviation (n=3). *P<0.05, vs. control group. PLA_2, phospholipase A_2.

Statistical analysis. Data are shown as the mean ± standard deviation. Paired Student's *t*-tests were used to determine the significance of differences between the control and experimental groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of PKA and PLA_2 in primary cultures of mTAL cells. Upon adenosine binding to its receptors, target cells are typically affected through the activation or inhibition

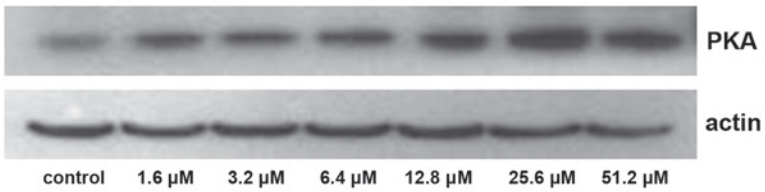

Figure 5. Effects of adenosine (24 h) on the expression of PKA in the primary cultures of medullary thick ascending limb cells. Expression was detected using western blot analysis. PKA, protein kinase A.

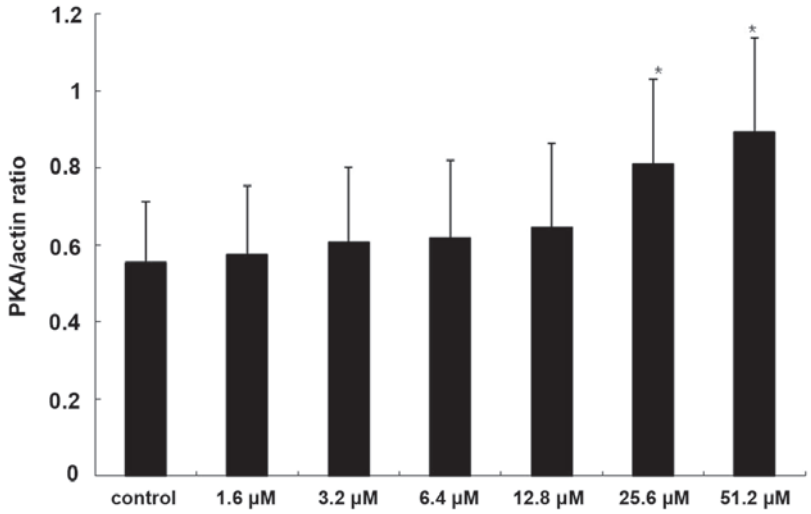

Figure 6. Effects of adenosine (24 h) on the expression of PKA in the primary cultures of medullary thick ascending limb cells. Data are presented as the mean + standard deviation (n=3). *P<0.05, vs. control group. PKA, protein kinase A.

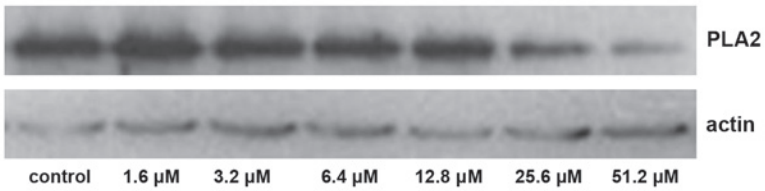

Figure 7. Effects of adenosine (24 h) on the expression of PLA_2 in the primary cultures of medullary thick ascending limb cells. Expression was detected using western blot analysis. PLA_2, phospholipase A_2.

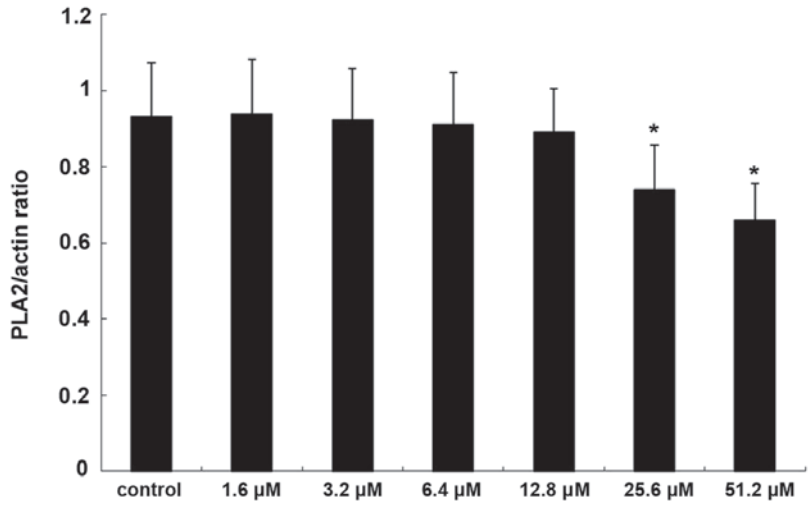

Figure 8. Effects of adenosine (24 h) on the expression of PLA_2 in the primary cultures of medullary thick ascending limb cells. Data are presented as the mean + standard deviation (n=3). *P<0.05, vs. control group. PLA_2, phospholipase A_2.

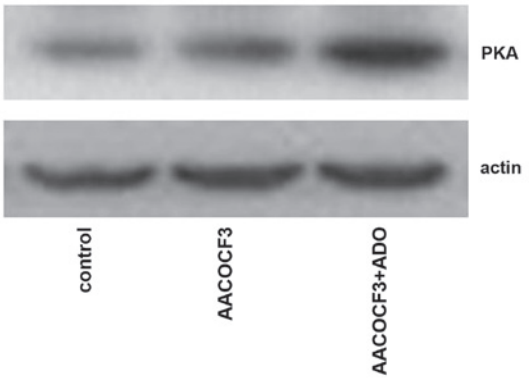

Figure 9. Effects of adenosine (25.6 μM) on the expression of PKA in the primary cultures of medullary thick ascending limb cells pretreated with AACOCF3 (5 μM). Expression was detected using western blot analysis. PKA, protein kinase A; ADO, adenosine.

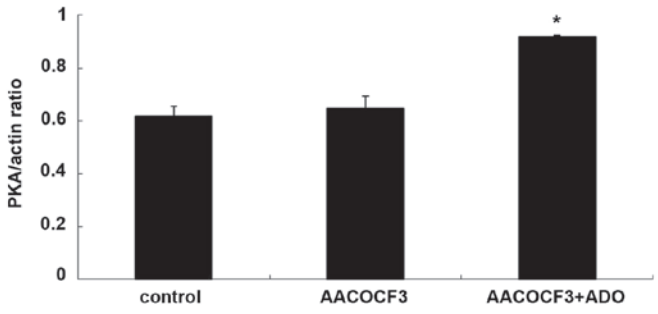

Figure 10. Effects of adenosine (25.6 μM) on the expression of PKA in the primary cultures of medullary thick ascending limb cells pretreated with AACOCF3 (5 μM). Data are presented as the mean + standard deviation (n=3). *P<0.05, vs. control group. PKA, protein kinase A; ADO, adenosine.

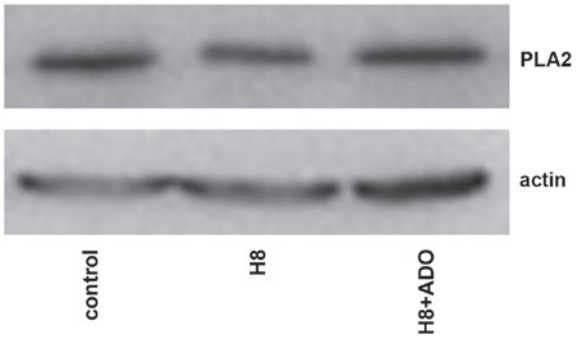

Figure 11. Effects of adenosine (25.6 μM) on the expression of PLA_2 in the primary cultures of medullary thick ascending limb cells pretreated with H8 (5 μM). Expression was detected using western blot analysis. PLA_2, phospholipase A_2.

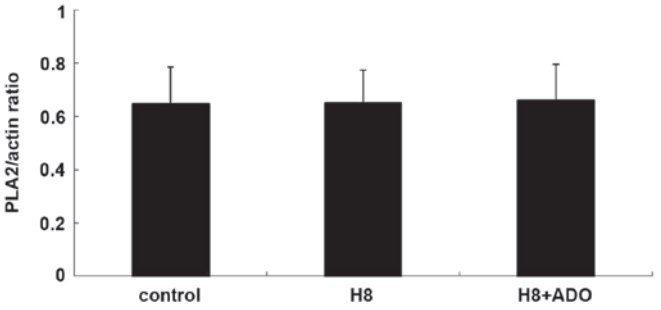

Figure 12. Effects of adenosine (25.6 μM) on the expression of PLA_2 in the primary cultures of medullary thick ascending limb cells, which were pretreated with H8 (5 μM). Data are presented as the mean + standard deviation (n=3). PLA_2, phospholipase A_2.

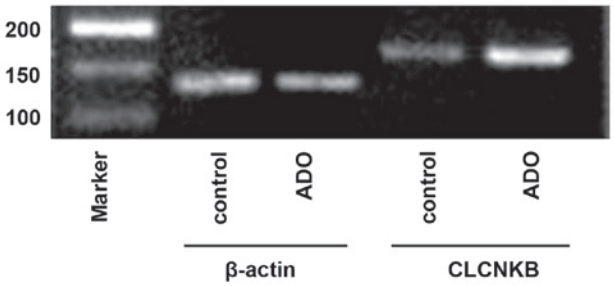

Figure 13. Effects of adenosine (25.6 μM; 24 h) on the mRNA expression of *CLCNKB* in the primary cultures of medullary thick ascending limb cells. Expression was detected using reverse transcription-polymerase chain reaction analysis. *β-actin* was used as a positive control. Ethidium bromide was used to observe the products for *CLCNKB* (173 bp) and *β-actin* (138 bp). *CLCNKB*, chloride voltage-gated channel Kb; ADO, adenosine.

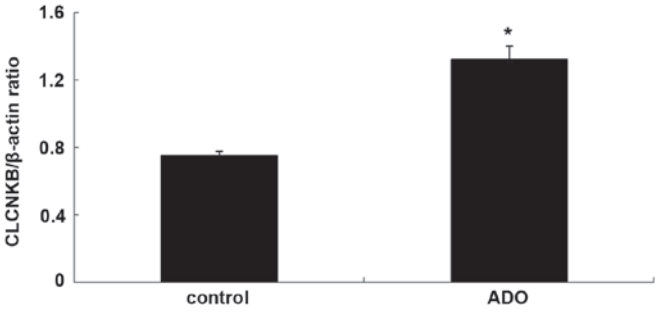

Figure 14. Effects of adenosine (25.6 μM, 24 h) on the mRNA expression of *CLCNKB* in the primary cultures of medullary thick ascending limb cells. Data are presented as the mean + standard deviation (n=3). *P<0.05, vs. control group. *CLCNKB*, chloride voltage-gated channel Kb; ADO, adenosine.

of the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA), phospholipase A_2-arachidonic acid (PLA_2-AA) and phospholipase C-protein kinase C pathways (23). The expression levels of PKA and PLA_2 in primary cultures of mTAL cells were determined using western blot analysis. The expression levels of PKA and PLA_2 were detected over time and in the presence of increasing concentrations of adenosine. Compared with the control group, the expression of PKA increased significantly at 24 and 48 h post-adenosine treatment (P<0.05; Figs. 1 and 2), whereas the expression of PLA_2 decreased significantly at 24 and 48 h post-adenosine treatment (P<0.05; Figs. 3 and 4). No change was observed at 6 or 12 h.

The expression of PKA increased significantly following treatment with 25.6 and 51.2 μM adenosine (P<0.05; Figs. 5 and 6), whereas the expression of PLA_2 decreased significantly following treatment with 25.6 and 51.2 μM adenosine (P<0.05; Figs. 7 and 8).

Interaction of the cAMP-PKA and PLA_2-AA pathways. As shown in Figs. 9 and 10, adenosine treatment significantly

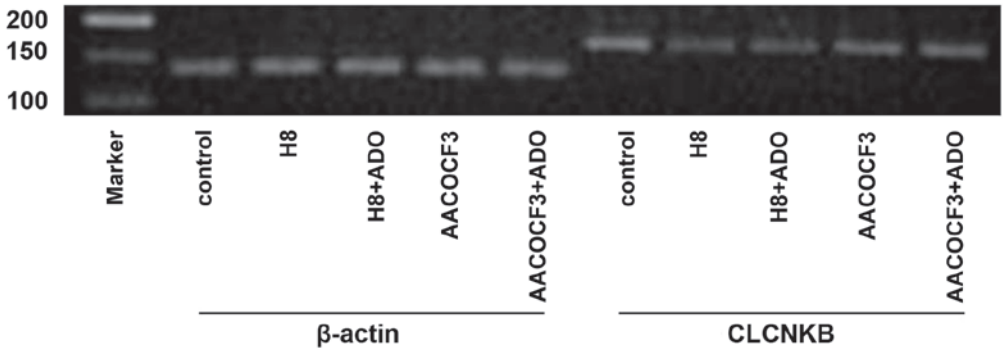

Figure 15. Effects of adenosine (25.6 μM, 24 h) on the mRNA expression of *CLCNKB* following H8 and AACOCF3 treatments in the primary cultures of medullary thick ascending limb cells. Expreesion was detected using reverse transcription-polymerase chain reaction analysis. *β-actin* was used as a positive control. Ethidium bromide was used to observe the products for *CLCNKB* (173 bp) and *β-actin* (138 bp). *CLCNKB*, chloride voltage-gated channel Kb; ADO, adenosine.

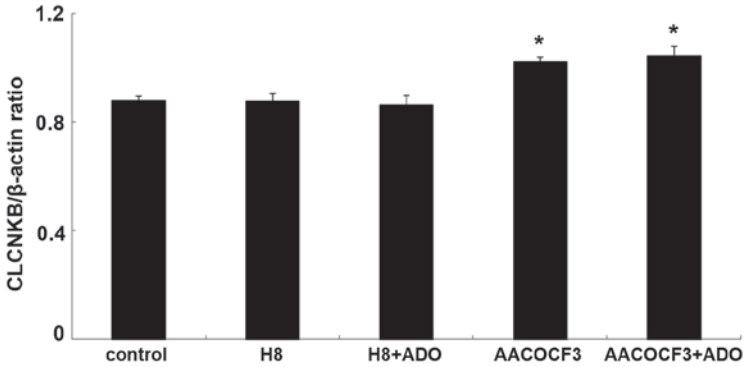

Figure 16. Effects of adenosine (25.6 μM; 24 h) on the mRNA expression of *CLCNKB* following H8 and AACOCF3 treatments in the primary cultures of medullary thick ascending limb cells. Data are presented as the mean + standard deviation (n=3). *P<0.05, vs. control group. *CLCNKB*, chloride voltage-gated channel Kb.

increased the expression of PKA in primary cultures of mTAL cells (P<0.05), which were pretreated with AACOCF3 for 6 h prior to treatment with adenosine for 24 h. However, as shown in Figs. 11 and 12, adenosine treatment did not alter the expression of PLA_2 in the primary cultures of the mTAL cells, which had been pretreated with H8 for 6 h prior to treatment with adenosine for 24 h.

mRNA expression levels of CLCNKB in primary cultures of mTAL cells. Several studies have confirmed that the mRNA expression of *CLCNKB* is highest in the TAL of the loop of Henle and the distal convoluted tubule (24-26). In the present study, total RNA was isolated from primary cultures of mTAL cells, and the mRNA expression level of *CLCNKB* was detected using RT-qPCR analysis.

The mRNA expression of *CLCNKB* increased significantly following treatment with adenosine (P<0.05; Figs. 13 and 14). To determine whether the cAMP-PKA and PLA_2-AA pathways affected the mRNA expression of *CLCNKB* in the presence of adenosine, the cells were pretreated with H8 and AACOCF3 for 6 h and then stimulated with adenosine for 24 h. It was found that H8 and AACOCF3 treatment inhibited the adenosine-induced changes in the mRNA expression of *CLCNKB* (Figs. 15 and 16). Thus, it was concluded that adenosine affected the mRNA expression of *CLCNKB* through the cAMP-PKA and PLA_2-AA pathways.

Discussion

The findings of the present study demonstrated that adenosine treatment increased the mRNA expression of *CLCNKB* in the primary cultures of mTAL cells. Furthermore, it was found that the stimulatory effect of adenosine was mediated by the cAMP-PKA and PLA_2-AA pathways. This was supported by the observation that the inhibition of PKA and PLA_2 eliminated the stimulatory effect of adenosine on the mRNA expression of *CLCNKB*. The present study also found that adenosine treatment resulted in increased expression of PKA and decreased expression of PLA_2. Sequential crosstalk was observed between these two pathways, in that adenosine first affected the mRNA expression of *CLCNKB* through the cAMP-PKA pathway, followed by the PLA_2-AA pathway. These results led to the conclusion that the cAMP-PKA pathway was upstream of the PLA_2-AA pathway. This was supported by the observation that adenosine stimulated the expression of PKA following inhibition of PLA_2 with AACOCF3, but failed to affect the expression of PLA_2 following inhibition of PKA with H8.

In the kidney, ~20 to 25% of the Na^+ and Cl^- in the renal filtrate are actively reabsorbed. This indicates that the TAL is key in mediating the renal medulla hypertonic gradient, the concentration and dilution of urine, and salt capacity. Chloride channels are important anion channels in the human body (27), and Cl^- transport is a crucial moderator of

Na^+-$2Cl^-$-K^+ cotransport. Utilizing patch-clamp and molecular biology techniques, six categories of chloride channels have been identified, and are encoded by several unrelated gene families (28). These include the CLC family, cystic fibrosis transmembrane conductance regulators (CFTRs), intracellular chloride channels (CLICs), calcium activated channels (CaCCs), volume-regulated anion channels (VRACs) and glycine or γ-GABA-activated chloride channels. CLCs, CFTRs, CaCCs and VRACs are chloride channels specific to the kidney (29). Using *in situ* hybridization and immunohistochemical staining, studies have confirmed that CLC-K2 is predominantly expressed in the basolateral mTAL of the rat kidney (24-26,30). The human ortholog, ClC-Kb, which has ~90% sequence homology and ~80% homology with the rat ortholog (31), is also located in the basolateral mTAL, and is encoded for by *CLCNKB*, which has been listed as one of the candidate genes involved in hypertension (32,33). ClC-Kb is crucial, not only for the reabsorption of Na^+ and Cl^- in the medullary and cortical portions of the distal tubule, but also in urine concentration, and in the establishment and maintenance of the corticomedullary osmotic gradient (34).

Previous studies have shown that the concentration of adenosine and mRNA expression of *CLCNKB* increase when NaCl transport is upregulated, and that activation of the ClC-Kb channel enhances NaCl transport (35,36). A mutation in the *CLCNKB* gene can result in Bartter syndrome type III, (predominantly from reduced reabsorption of Na^+ and Cl^- in the TAL and distal convoluted tubule, and chronic hypotension) and salt-sensitive hypertension (35). These studies indicate that changes in chloride channel activity may be one of the factors affecting blood pressure. As already mentioned, adenosine is important in regulating kidney function through binding to adenosine receptors. There are four types of adenosine receptors: A1, A2a, A2b and A3 (37). Each of these is widely expressed in the kidney and it has been confirmed that the adenosine receptors have different binding affinities to adenosine (38). When the concentration of adenosine in the extracellular fluid is low (~50-200 nM) (23), adenosine binds primarily to the A1 receptor. Under these conditions, kidney function is protected by the inhibition of NaCl transport in the TAL, and decreased consumption of energy and O_2 (18). By contrast, under certain pathophysiological conditions, including ischemia and hypoxia, cells release an increased quantity of adenosine into the extracellular fluid (>1 μM) (39,40). A high concentration of adenosine primarily binds to the A_2 receptor, leading to the overtransportation of NaCl and overconsumption of O_2 (18). Therefore, these pathophysiological conditions damage kidney function.

It has been previously established that the effects of adenosine are mediated by several pathways, including the cAMP-PKA and PLA_2-AA pathways (37,41). A previous study also showed that a high-salt diet can increase the production of adenosine in mIMCD-K2 cells, and stimulate Cl^- secretion by binding to the A2 receptor and activating the CFTR channel through the cAMP-PKA pathway (42). In addition, our previous study demonstrated that adenosine and its analog, N6-cyclohexyladenosine, enhance apical and basolateral potassium channel activity in the TAL through the cAMP-PKA pathway, which promotes K^+ outflow and regulates Cl^- secretion (43-45). It was found that 5 μM AA inhibits the activity of the 50 pS potassium channel and the 10 pS chloride channel, which are located in the basolateral mTAL. These effects were also achieved by 20-HETE, which is generated via the cytochrome P-450 monooxygenase pathway (46).

In conclusion, the data obtained in the present study showed the effects of adenosine on the mRNA expression of *CLCNKB* in the basolateral mTAL of the rat kidney. As adenosine assists in the regulation of salt balance in the blood, it is a potential therapeutic target for certain diseases, including salt-sensitive hypertension. However, the results of the present study are limited as the mechanisms underlying the effect of adenosine on chloride channels remain to be fully elucidated. Therefore, additional investigations are required to assess the effects of adenosine on chloride channels and other channels under conditions of a high-salt diet.

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