Extracellular 2',3'-cAMP-adenosine pathway in proximal tubular, thick ascending limb, and collecting duct epithelial cells

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Abstract

Taken together, these investigations support the existence of a renal extracellular 2',3'-cAMP-adenosine pathway (extracellular 2',3'-cAMP → 2'-AMP + 3'-AMP → adenosine).

It is conceivable that adenosine generated by the 2',3'-cAMP-adenosine pathway helps protect kidneys from injury by promoting the proliferation of tubular epithelial cells. Indeed, in human proximal tubular epithelial cells (commercial source), the extracellular 2',3'-cAMP-adenosine pathway exists and strongly stimulates proliferation of these cells (8). However, it is unknown whether the 2',3'-cAMP-adenosine pathway exists in primary cultures of proximal tubular epithelial cells and whether the pathway exists all along the nephron except for the proximal tubule. Therefore, in the present study, we cultured primary proximal, thick ascending limb and collecting duct renal epithelial cells from freshly isolated rat nephron segments and then studied the metabolism of extracellular 2',3'-cAMP, 2'-AMP, 3'-AMP, and adenosine in these cells.

METHODS

Cell culture. Kidneys for isolation of tubular segments were obtained from adult, male Sprague-Dawley rats (Charles River, Wilmington, MA). The Institutional Animal Care and Use Committee approved all procedures. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Proximal tubules, thick ascending limbs, and collecting ducts were isolated using a Percoll solution centrifugation method previously described and validated in detail by us (10, 16). In this regard, the presence and absence of appropriate protein markers were used to validate the specificity of this isolation method (10, 16); bumetanide-sensitive cotransporter type 1 (marker specific for thick ascending limbs), thiazide-sensitive cotransporter (marker for distal tubules),...
aquaporin-2 (marker for collecting ducts), sodium-bicarbonate cotransporter type 1 (marker for proximal tubules), and sodium-hydrogen exchanger type 3 (also marker for proximal tubules). Cells were cultured from these isolated segments as previously described by us (10, 16). Briefly, freshly isolated specific tubular segments were washed in phosphate-buffered saline without calcium and magnesium and incubated for 15 min with collagenase type IV (1 mg/ml in 5 ml of DMEM F12) in a shaking water bath at 37°C. Ten milliliters of DMEM F12 with 10% fetal calf serum (FCS) were added, and the sample was centrifuged. Pellets were resuspended in DMEM F12 with 10% FCS, and 1 ml of the suspension was added to 75-cm² flasks. Before cells were added, culture flasks were preconditioned by incubating with FCS for 30 min. The medium was changed after 2 days. After 4 days, the cells were detached with trypsin/EDTA, washed, and plated with DMEM F12 with 10% FCS. All experiments were performed in these cultures once the cells reached confluence (herein defined as “primary” cultures).

**Metabolism experiments.** Cells were washed twice with HEPES-buffered Hank's balanced salt solution and then incubated for 1 h in 0.5 ml of Dulbecco’s phosphate-buffered saline with HEPES (25 mmol/l) and NaHCO₃ (13 mmol/l) in the presence and absence of 2',3'-cAMP, 5'-AMP, 3'-AMP, or 2'-AMP with or without α,β-methyleneadenosine-5'-diphosphate (AMPCP; selective inhibitor of CD73) (32), 3-isobutyl-1-methylxanthine (IBMX; broad spectrum phosphodiesterase inhibitor) (2), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX; ecto-phosphodiesterase inhibitor) (22, 29, 31), all from Sigma (St. Louis, MO). After 1-h incubation, the medium was collected, heated for 90 s at 100°C to denature enzymes, and then frozen at −80°C until assayed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**LC-MS/MS purine assay.** 2'-AMP, 3'-AMP, 5'-AMP, and adenosine were obtained from Sigma. The internal standard (13C₁₀-adenosine) was from Medical Isotopes (Pelham, NH). Purines were resolved by reversed-phase liquid chromatography (Agilent Zorbax eclipse XDB-C-18 column, 3.5-µm beads; 2.1 × 100 mm) and quantified using a triple quadrupole mass spectrometer (TSQ Quantum-Ultra, ThermoFisher Scientific, San Jose, CA) operating in the selected reaction monitoring mode with a heated electrospray ionization source as previously described in detail (14).

**Statistics.** Results were analyzed statistically using the nonparametric Kruskal-Wallis one-way ANOVA on ranks test, with post hoc comparisons using the Kruskal-Wallis multiple-comparison Z-value test. The criterion of significance was *P* < 0.05. All values in text and figures are means ± SE.

**RESULTS**

Incubation of rat proximal tubular epithelial cells with 2',3'-cAMP significantly and concentration dependently increased medium levels of 2'-AMP and 3'-AMP, but not 5'-AMP, and the increase in 2'-AMP was significantly greater than the increase in 3'-AMP at 3 and 10 µmol/l of 2',3'-cAMP (Fig. 1A). Also, 2',3'-cAMP significantly and concentration dependently increased medium levels of adenosine (Fig. 1B). Neither IBMX (broad spectrum phosphodiesterase inhibitor) (2) nor DPSPX (ecto-phosphodiesterase inhibitor) (22, 29, 31) altered the metabolism of 2',3'-cAMP to 3'-AMP (Fig. 1C) or 2'-AMP (Fig. 1D).

Incubation of rat thick ascending limb epithelial cells with 2',3'-cAMP also significantly and concentration dependently increased medium levels of 2'-AMP and 3'-AMP, but not 5'-AMP, and again the increase in 2'-AMP was significantly greater than the increase in 3'-AMP at 3 and 10 µmol/l of 2',3'-cAMP (Fig. 1E).

Fig. 1. Line graphs illustrate the concentration-dependent effects of 2',3'-cAMP on levels of 2'-AMP, 3'-AMP, and 5'-AMP (A) and adenosine (B) in the medium of cultures of rat proximal tubular cells (PTCs). Bar graphs illustrate the effects of 3-isobutyl-1-methylxanthine (IBMX; 1 mmol/l; broad spectrum phosphodiesterase inhibitor) and 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX; 1 mmol/l; ecto-phosphodiesterase inhibitor) on the metabolism of 2',3'-cAMP to 3'-AMP (C) and 2'-AMP (D) in PTCs. *P* < 0.05 compared with basal (0) and *P* < 0.05 comparing 2'-AMP levels with 3'-AMP levels at the indicated treatment concentration of 2',3'-cAMP (Kruskal-Wallis multiple-comparison Z-value test).
2',3'-cAMP (Fig. 2A). In rat thick ascending limb epithelial cells, 2',3'-cAMP significantly and concentration dependently increased medium levels of adenosine (Fig. 2B); but neither IBMX nor DPSPX inhibited the metabolism of 2',3'-cAMP to 3'-AMP (Fig. 2C) or 2'-AMP (Fig. 2D).

As with proximal tubular epithelial cells and thick ascending limb epithelial cells, incubation of rat collecting duct epithelial cells with 2',3'-cAMP significantly and concentration dependently increased medium levels of 2'-AMP and 3'-AMP, but not 5'-AMP, and the increase in 2'-AMP was significantly greater than the increase in 3'-AMP at 3 µmol/l of 2',3'-cAMP (Fig. 3A). In rat collecting duct epithelial cells, 2',3'-cAMP significantly and concentration dependently increased medium levels of adenosine (Fig. 3B). Again, neither IBMX nor DPSPX altered the metabolism of 2',3'-cAMP to 3'-AMP (Fig. 3C) or 2'-AMP (Fig. 3D).

Incubation of rat proximal tubular epithelial cells with 5'-AMP (prototypical adenosine precursor; Fig. 4A), 3'-AMP (Fig. 4B), and 2'-AMP (Fig. 4C) significantly and concentration dependently increased levels of adenosine in the medium. Similar results were obtained when 5'-AMP, 3'-AMP, and 2'-AMP were incubated with rat thick ascending limb epithelial cells (Fig. 4, D, E, and F, respectively) or rat collecting duct epithelial cells (Fig. 4, G, H, and I, respectively). AMPCP (100 µmol/l; CD73 inhibitor) had no effect on the metabolism of 5'-AMP (Fig. 5A), 3'-AMP (Fig. 5B), or 2'-AMP (Fig. 5C) to adenosine.

**DISCUSSION**

An overwhelming body of solid evidence indicates that adenosine, an endogenous nucleoside, strongly protects against acute kidney injury (AKI) (1, 5, 7). In this regard, multiple mechanisms are likely involved with the participation of multiple adenosine receptors including A1 receptors (17–20, 27), A2A receptors (3, 23–26), and A2B receptors (6). Therefore, it is important to elucidate the mechanisms contributing to renal adenosine production and disposition. Interestingly, the increase in serum creatinine induced by renal ischemia-reperfusion injury is attenuated, rather than enhanced, in mice null for either CD39 or CD73 (21) and renal histopathology induced by ischemia-reperfusion injury is not changed in CD39 knockout mice and is actually improved in CD73 knockout mice (21). CD39 (converts ATP/ADP to 5'-AMP) and CD73 (converts 5'-AMP to adenosine) are thought to be the primary mediators of adenosine production. However, the findings that renal adenosine is protective yet knockdown of CD39 or CD73 does not worsen outcome suggest that alternative metabolic pathways are also important in forming adenosine during renal ischemia-reperfusion.

Our previous studies reveal that the 2',3'-cAMP-adenosine pathway could exert biological effects via adenosine that should promote recovery of kidneys from AKI. In this regard, 2',3'-cAMP, 2'-AMP, and 3'-AMP (via metabolism to adenosine and activation of A2B receptors) inhibit the proliferation of renal vascular smooth muscle cells and glomerular mesan-
types of ecto-enzymes expressed. Consistent with our findings, 2'-AMP likely depends on the cell type and therefore the metabolism of 2',3'-AMP = 3'-AMP production exceeds 2'-AMP biosynthesis (8). Thus, whether in human proximal tubular cells (commercial source), 2'-AMP far exceeds production of 2'-AMP (8). In contrast, of 3'-AMP bond. Interestingly, our previously published results indicate -phosphodiesterases hydrolyzing the 2'-phosphoester cAMP-2'-phosphodiesterases hydrolyzing the 3'-phosphoester cAMP-3'-phosphoester 1 ways: 2',3'-cAMP-adenosine pathway could potentially promote epithelial -adenosine. Therefore, the progrowth effects of the 2'-AMP-adenosine pathway could be, yet stimulate the proliferation of renovascular endothelial cells and renal epithelial cells (8). However, the concept that the 2',3'-cAMP-adenosine pathway could be renoprotective would be even more compelling if it were the case that epithelial cells all along the nephron were capable of metabolizing 2',3'-cAMP to 2'-AMP and 3'-AMP and capable of metabolizing 2'-AMP and 3'-AMP to adenosine. In this regard, the present study shows clearly that indeed epithelial cells in the proximal tubule, in the loop of Henle, and in the collecting duct can metabolize 2',3'-cAMP to its corresponding AMPs and further metabolize the corresponding AMPs to adenosine. Therefore, the progrowth effects of the 2',3'-cAMP-adenosine pathway could potentially promote epithelial regeneration all along the nephron.

The generation of extracellular AMPs from extracellular 2',3'-cAMP potentially could involve two biochemical pathways: 1) generation of 2'-AMP via the action of ecto-2',3'-cAMP-3'-phosphodiesterases hydrolyzing the 3'-phosphoester bond; 2) generation of 3'-AMP via the action of ecto-2',3'-cAMP-2'-phosphodiesterases hydrolyzing the 2'-phosphoester bond. Interestingly, our previously published results indicate that in rat preglenular vascular endothelial cells, production of 3'-AMP far exceeds production of 2'-AMP (8). In contrast, in human proximal tubular cells (commercial source), 2'-AMP production exceeds 3'-AMP biosynthesis (8). Thus, whether metabolism of 2',3'-cAMP proceeds predominantly to 3'-AMP or 2'-AMP likely depends on the cell type and therefore the types of ecto-enzymes expressed. Consistent with our findings in human proximal tubular cells, in the present study 2',3'-cAMP was metabolized more to 2'-AMP than 3'-AMP in all three types of renal epithelial cells. We conclude therefore that in renal epithelial cells, the dominant pathway for metabolism of extracellular 2',3'-cAMP is conversion to 2'-AMP via ecto-2',3'-cAMP-3'-phosphodiesterases.

In addition to the 2',3'-cAMP-adenosine pathway, previous studies indicate that renal epithelial cells, from both proximal tubules and collecting ducts, express an extracellular 3',5'-adenosine pathway (extracellular 3',5'-cAMP → 5'-AMP → adenosine) and that the ecto-3',5'-cAMP-3'-phosphodiesterases that mediate this biochemical mechanism are inhibited by both IBMX and DPSPX (10, 16). The present studies demonstrate that unlike the extracellular 3',5'-cAMP-adenosine pathway, the extracellular 2',3'-cAMP-adenosine pathway is not inhibited by either IBMX or DPSPX, thus indicating a different set of ecto-enzymes mediating the two cAMP-adenosine pathways in renal epithelial cells.

Regarding the metabolism of 3'-AMP and 2'-AMP to adenosine, the present study demonstrates that conversion of these AMPs to adenosine in primary cultures of renal epithelial cells from rat proximal tubules, thick ascending limbs, and collecting ducts is as efficient as metabolism of 5'-AMP to adenosine, and the conversion of these AMPs to adenosine is not attenuated by the CD73 inhibitor AMPCP. This is consistent with our previous observations in human proximal tubular cells (8). Interestingly, in other cell types, including preglenular vascular smooth muscle cells, glomerular mesangial cells, preglo-

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**Fig. 3.** Line graphs illustrate the concentration-dependent effects of 2',3'-cAMP on levels of 2'-AMP, 3'-AMP, and 5'-AMP (A) and adenosine (B) in the medium of cultures of rat collecting duct cells (CDCs). Bar graphs illustrate the effects of IBMX (1 mmol/l; broad spectrum phosphodiesterase inhibitor) and DPSPX (1 mmol/l; ecto-phosphodiesterase inhibitor) on the metabolism of 2',3'-cAMP to 3'-AMP (C) and 2'-AMP (D) in CDCs. P values in panels are from Kruskal-Wallis 1-way ANOVA on ranks. *P < 0.05 compared with basal (0) and *P < 0.05 comparing 2'-AMP levels with 3'-AMP levels at the indicated treatment concentration of 2',3'-cAMP (Kruskal-Wallis multiple-comparison Z-value test).
merular vascular endothelial cells, aortic vascular smooth muscle cells, coronary artery vascular smooth muscle cells, microglia, and astrocytes, 5'-AMP generates more adenosine than do 3'-AMP or 2'-AMP (8, 9, 12, 13, 30). Also, in these other cell types, the conversion of 5'-AMP to adenosine is blocked by AMPCP, whereas the metabolism of 3'-AMP and 2'-AMP to adenosine is resistant to AMPCP (8, 9, 12, 13, 30). Thus, in renal epithelial cells, as opposed to all other examined cell types, the ecto-nucleotidase that metabolizes 5'-AMP is not CD73. Moreover, because 5'-AMP, 3'-AMP, and 2'-AMP are similarly efficacious with regard to generating adenosine in renal epithelial cells, the implication is that in renal epithelial cells 3'-AMP and 2'-AMP could be as important as 5'-AMP as precursors of adenosine.

An important issue is whether the results of our findings in epithelial cells isolated from rat kidneys can be extrapolated to human renal epithelial cells. Inasmuch as the results of the present study in rat proximal tubular epithelial cells are similar to our recent findings in human proximal tubular epithelial cells obtained from a commercial source (8), it seems likely that the results of the present study can be extended to human renal epithelial cells.

Hypoxia stimulates the adenosine system by increasing extracellular adenosine synthesis, by inhibiting the disposition of extracellular adenosine, and by increasing the expression of adenosine receptors (4). Since the 2',3'-cAMP-adenosine pathway may protect against AKI and AKI is associated with renal hypoxia, it is conceivable that hypoxia may activate enzymes involved in the metabolism of 2',3'-cAMP, and this hypothesis merits investigation.

In summary, the present results indicate that renal epithelial cells in the proximal tubule, thick ascending limb, and collecting duct can metabolize 2',3'-cAMP to 2'-AMP and 3'-AMP and can convert 2'-AMP and 3'-AMP to adenosine. Combining this information with our previous findings that the extracellular 2',3'-cAMP-adenosine pathway can stimulate the proliferation of renal epithelial cells and renovascular endothelial cells, while inhibiting the growth of renovascular smooth muscle cells and glomerular mesangial cells, suggests that the extracellular 2',3'-cAMP-adenosine pathway (which is activated by renal injury) may be important in determining renal outcomes following AKI. Definitive testing of this hypothesis must await identification of the enzymes involved in the extracellular 2',3'-cAMP-adenosine pathway so that renal outcomes following renal injury can be compared in animals with and without an intact extracellular 2',3'-cAMP-adenosine pathway.

GRANTS
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Fig. 5. Bar graphs illustrate the effects of α,β-methylene-adenosine-5’-diphosphate (AMPCP; 100 μmol/L; CD73 inhibitor) on adenosine levels in the medium of rat PTCs, TALCs, and CDCs incubated with 10 μmol/L of either 5’-AMP (A), 3’-AMP (B), or 2’-AMP (C).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: E.K.J. conception and design of research; E.K.J. and D.G.G. analyzed data; E.K.J. interpreted results of experiments; E.K.J. prepared figures; E.K.J. drafted manuscript; E.K.J. and D.G.G. edited and revised manuscript; D.G.G. analyzed data; E.K.J. interpreted results of experiments; E.K.J. prepared figures; E.K.J. and D.G.G. approved final version of manuscript; D.G.G.

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