Hypertension

ORIGINAL ARTICLE

8-Aminoguanine and Its Actions on Renal Excretory Function

Edwin K. Jackson, Delbert G. Gillespie, Zaichuan Mi

BACKGROUND: The endogenous purine 8-aminoguanine induces diuresis/natriuresis/glucosuria by inhibiting PNPase (purine nucleoside phosphorylase); however, mechanistic details are unknown.

METHODS: Here, we further explored in rats 8-aminoguanine's effects on renal excretory function by combining studies using intravenous 8-aminoguanine, intrarenal artery infusions of PNPase substrates (inosine and guanosine), renal microdialysis, mass spectrometry, selective adenosine receptor ligands, adenosine receptor knockout rats, laser doppler blood flow analysis, cultured renal microvascular smooth muscle cells, HEK293 cells expressing A_{2B} receptors and homogeneous time resolved fluorescence assay for adenylyl cyclase activity.

RESULTS: Intravenous 8-aminoguanine caused diuresis/natriuresis/glucosuria and increased renal microdialysate levels of inosine and guanosine. Intrarenal inosine, but not guanosine, exerted diuretic/natriuretic/glucosuric effects. In 8-aminoguanine-pretreated rats, intrarenal inosine did not induce additional diuresis/natriuresis/glucosuria. 8-Aminoguanine did not induce diuresis/natriuresis/glucosuria in A_{2B}-receptor knockout rats, yet did so in A_1- and A_{2A}-receptor knockout rats. Inosine's effects on renal excretory function were abolished in A_{2B} knockout rats. Intrarenal BAY 60-6583 (A_{2B} agonist) induced diuresis/natriuresis/glucosuria and increased medullary blood flow. 8-Aminoguanine increased medullary blood flow, a response blocked by pharmacological inhibition of A_{2B}, but not A_{2A}, receptors. In HEK293 cells expressing A_{2B} receptors, inosine activated adenylyl cyclase, and this was abolished by MRS 1754 (A_{2B} antagonist). In renal microvascular smooth muscle cells, 8-aminoguanine and forodesine (PNPase inhibitor) increased inosine and 3',5'-cAMP; however, in cells from A_{2B} knockout rats, 8-aminoguanine and forodesine did not augment 3',5'-cAMP yet increased inosine.

CONCLUSIONS: 8-Aminoguanine induces diuresis/natriuresis/glucosuria by increasing renal interstitial levels of inosine which, via A_{2B} receptor activation, increases renal excretory function, perhaps in part by increasing medullary blood flow.
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Key Words: A_{2B} receptor ■ diuretic ■ guanosine ■ inosine ■ purine nucleoside phosphorylase

Studies show that 8-aminoguanosine and 8-aminoguanine rapidly increase urine volume, sodium excretion and glucose excretion yet reduce potassium excretion.[1] In this regard, systemically administered 8-aminoguanosine is rapidly converted to 8-aminoguanine, which mediates most of 8-aminoguanosine's renal effects (that is 8-aminoguanosine is a prodrug).[2] 8-Aminoguanine is a naturally occurring purine that likely derives from biomolecules containing 8-nitroguanine.[3] Recent studies show that 2 additional 8-aminopurines, namely 8-aminoinosine and 8-aminohypoxanthine, also induce diuresis, natriuresis and mild glucosuria but not antikaliuresis.[4] Similar to 8-aminoguanine, 8-aminoinosine may also occur in vivo.[4]

The diuretic, natriuretic and glucosuric effects of these 8-aminopurines likely are mediated by inhibition of PNPase (purine nucleoside phosphorylase).[5] The evidence for this conclusion is that 8-aminoguanine,

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NOVELTY AND RELEVANCE

What Is New?

8-Aminoguanine, a naturally occurring purine, increases renal interstitial levels of inosine and guanosine while suppressing renal interstitial levels of hypoxanthine and guanine. Thus 8-aminoguanine rebalances the renal interstitial purine metabolome. In this regard, the largest effect of 8-aminoguanine is to increase renal interstitial levels of inosine.

8-Aminoguanine reproducibly increases urine volume and sodium and glucose excretion; also 8-aminoguanine increases renal medullary blood flow and 3',5'-cAMP production by renal microvascular smooth muscle cells. All of these effects of 8-aminoguanine are mediated by adenosine A_{2B} receptors, which are well known to stimulate adenylyl cyclase.

Like 8-aminoguanine, inosine induces diuresis/natriuresis/glucosuria and activates the A_{2B} receptor/adenylyl cyclase/3',5'-cAMP pathway.

The totality of evidence indicates that 8-aminoguanine induces diuresis/natriuresis/glucosuria at least in part by inhibiting PNPase (purine nucleoside phosphorylase), which massively increases renal interstitial levels of inosine. In turn, inosine activates the A_{2B} receptor/adenylyl cyclase/3',5'-cAMP pathway, which augments renal medullary blood flow and renal excretory function.

What Is Relevant?

Administration of 8-aminoguanine, a naturally occurring purine, reproducibly increases urine volume and sodium and glucose excretion while conserving potassium.

8-Aminoguanine affects renal excretory function even in animals that are genetically predisposed to retain sodium and challenged by a high-salt diet.

Clinical/Pathophysiological Implications?

8-Aminoguaine, a naturally occurring PNPase inhibitor, causes diuresis/natriuresis/glucosuria by rebalancing the renal interstitial purine metabolome in favor of inosine, thus resulting in A_{2B} receptor activation that increases renal medullary blood flow.

8-Aminoguanine causes diuresis/natriuresis/glucosuria by a unique mechanism that may have implications for the treatment of not only hypertension but also a broad array of vascular diseases.

Nonstandard Abbreviations and Acronyms

Dahl SS rats	Dahl salt-sensitive rats
KO	knockout
MABP	mean arterial blood pressure
PNPase	purine nucleoside phosphorylase
RMSMC	renal microvascular smooth muscle cell
WT	wild-type

8-aminoinosine, 8-aminohypoxanthine and 9-deazaguanine inhibit PNPase and induce diuresis, natriuresis and glucosuria.[4,5] Importantly, unlike 8-aminoguanine, neither 9-deazaguanine, 8-aminoinosine nor 8-aminohypoxanthine alter potassium excretion.[4,5] These findings suggest that 8-aminoguanine reduces potassium excretion by a mechanism that does not involve PNPase inhibition.

The diuretic, natriuretic and glucosuric effects of 8-aminopurines appear to be due to PNPase inhibition; however, the mechanistic details linking PNPase inhibition to effects on urine volume and sodium and glucose excretion are unknown. Accordingly, here we sought to further investigate how 8-aminoguanine induces diuresis, natriuresis and glucosuria. This objective was achieved by combining studies using ultra-performance liquid chromatography-tandem mass spectrometry, renal microdialysis, intrarenal artery infusions of PNPase substrates, selective pharmacological inhibitors, rats with specific adenosine receptors knocked out, laser doppler blood flow analysis, cultured renal microvascular smooth muscle cells, HEK293 cells expressing adenosine A_{2B} receptors and a homogeneous time resolved fluorescence assay for 3',5'-cAMP.

METHODS

Data Availability

For data and for additional information on analytical methods or study materials, contact E.K. Jackson at edj@pitt.edu.

Materials

See Supplemental Material.

Animals

Male and female wild-type (WT) Dahl salt-sensitive rats (Dahl SS rats) and Dahl salt-sensitive rats with adenosine subtype A_1, A_{2A}, and A_{2B} receptors knocked out (A_1-KO Dahl SS, A_{2A}-KO Dahl SS and A_{2B}-KO Dahl SS rats, respectively) were obtained from colonies maintained at the University of Pittsburgh. Our colonies were established from breeding pairs generated by the MCW Gene Editing Rat Resource Program (Dr Aron M. Geurts, Department of Physiology and Human Molecular Genetics Center, Medical College of Wisconsin, Milwaukee, WI). We have previously described in detail how these knockout rats were generated and how they were characterized and validated.[6] Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Because experiments with male and female WT Dahl SS, A_1-KO Dahl SS, A_{2A}-KO Dahl SS and A_{2B}-KO Dahl SS rats did not indicate sex differences with regard to the

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effects of 8-aminoguanine on renal excretory function, experiments in Sprague-Dawley rats were conducted in male rats. The Institutional Animal Care and Use Committee approved all procedures. The investigation conforms to National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animal Preparation

Rats were anesthetized (Inactin, 90 mg/kg, IP) and placed on an isothermal pad. Body temperature was continuously measured with a rectal probe thermometer and was maintained at 37 °C by adjusting the distance of a heat lamp from the surgical preparation. The trachea was cannulated with polyethylene (PE)-240 tubing, and the carotid artery was cannulated with PE-50 tubing, which was connected to a digital blood pressure analyzer (Micro-Med, Inc, Louisville, KY) for continuous monitoring of mean arterial blood pressure (MABP). Either a PE-50 cannula was positioned in the jugular vein for intravenous (IV) administration of test agents or a 30-guage needle connected to a cannula was placed in the left renal artery for intrarenal artery infusions of test agents. Hemodynamic stability was maintained by an infusion of 0.9 % saline (25 µL/min). The left ureter was cannulated with PE-10 tubing for timed collections of urine. Total renal blood flow was measured by placing on the left renal artery a 1-mm transit-time flow probe connected to a transit-time flowmeter (model T-206; Transonic Systems, Ithaca, NY). In some experiments, a microdialysis probe (MD 2310, IV-10, 30 kDa cutoff with 10 mm membrane; BASi, West Fafayette, IN) was inserted into the renal cortex of the left kidney and into the renal medulla of the right kidney. In these experiments, microdialysate (0.9% saline) was infused into the probe inlet at 2 µL/min and collected at the probe outlet. Also, in some experiments, a laser doppler needle probe (diameter, 0.48 mm) was inserted into the medulla of the left kidney and connected to a doppler flowmeter (model ALF21; Transonic Systems) for measurement of medullary blood flow. After instrumentation, animals were allowed a rest period of approximately one hour.

Analysis of Purines in Microdialysate

Purines in microdialysate samples were analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using multiple reaction monitoring. We recently published an updated description of our UPLC-MS/MS purine assays.[7]

Analysis of Sodium, Potassium and Glucose in Urine

Concentrations of sodium and potassium in urine were measured by flame photometry (Model IL-943; Instrumentations Laboratory, Lexington, MA). Glucose concentrations in urine were measured using the Cayman Chemical (Ann Arbor, MA) Glucose Colorimetric Assay Kit (catalog number 10009582).

Culture of Renal Microvascular Smooth Muscle Cells

Renal microvascular smooth muscle cells (RMSMCs) were cultured from WT and A_{2B}-KO Dahl SS rats as previously described.[8]

Inosine Signaling Via A_{2B} Receptors

The effects of inosine on signaling by A_{2B} receptors was examined in cultured HEK293 cells expressing human A_{2B} receptors (NCBI Reference Sequence: NP_000667.1). For details, see Supplemental Material.

Statistics

Statistical analysis was conducted using NCSS Statistical Software version 19.0.2 (Kaysville, Utah). $P<0.05$ was considered statistically significant. For details, see Supplemental Material.

RESULTS

Protocol 1. Effects of 8-Aminoguanine on Renal Excretory Function and Renal Microdialysis Levels of Purines

Timed collections of urine (from left ureter) and renal cortical and medullary microdiaysate were obtained from anesthetized Sprague-Dawley rats from 0 to 30 minutes (Period 1), 40 to 70 minutes (Period 2) and 85 to 115 minutes (Period 3) into the protocol. Rats received an IV bolus injection (1 mL/kg) of either vehicle (0.9% saline containing 0.03 N HCl; control group) or 8-aminoguanine (33.5 µmol/kg) immediately after Period 1. Vehicle had little or no effect on all measured variables. By contrast, by Period 3 8-aminoguanine had increased urine volume ≈4-fold ($P=0.0026$; Figure 1A), sodium excretion ≈26-fold ($P<0.0001$; Figure 1B) and glucose excretion ≈12-fold ($P<0.0001$; Figure 1C); yet had decreased potassium excretion by ≈60% ($P=0.0094$; Figure 1D). Basal levels of 8-aminoguanine in renal cortical (Figure S1A) and medullary (Figure S1B) microdialysates were at or near the detection limit of our assay system; yet by Period 2 IV 8-aminoguanine had increased both cortical (Figure S1A) and medullary (Figure S1B) microdialysate levels of 8-aminoguanine to ≈30 µmol/L. 8-Aminoguanine did not significantly affect renal cortical levels of adenosine (Figure S1C), and only mildly (50%) and transiently (Period 2 only) increased renal medullary adenosine (Figure S1D). In contrast to adenosine and consistent with the fact that inosine is a PNPase substrate, by Period 2 8-aminoguanine had augmented cortical ($P<0.0001$; Figure 1E) and medullary ($P<0.0001$; Figure 1F) microdialysate levels of inosine by 5- to 10-fold. High levels of inosine were maintained throughout Period 3. In contrast to inosine and consistent with the fact that hypoxanthine is a product of PNPase, by Period 3 8-aminoguanine had reduced cortical ($P=0.0010$; Figure S2A) and medullary ($P=0.0400$; Figure S2B) microdialysate levels of hypoxanthine by ≈60% and ≈30%, respectively. Guanosine, like inosine, is a substrate for PNPase that is converted to guanine. Therefore, one would predict that the qualitative effects of 8-aminoguanine on cortical and medullary levels of guanosine would be similar to the effects of 8-aminoguanine on inosine and that the qualitative effects of 8-aminoguanine on guanine would be similar to the effects of 8-aminoguanine on hypoxanthine.

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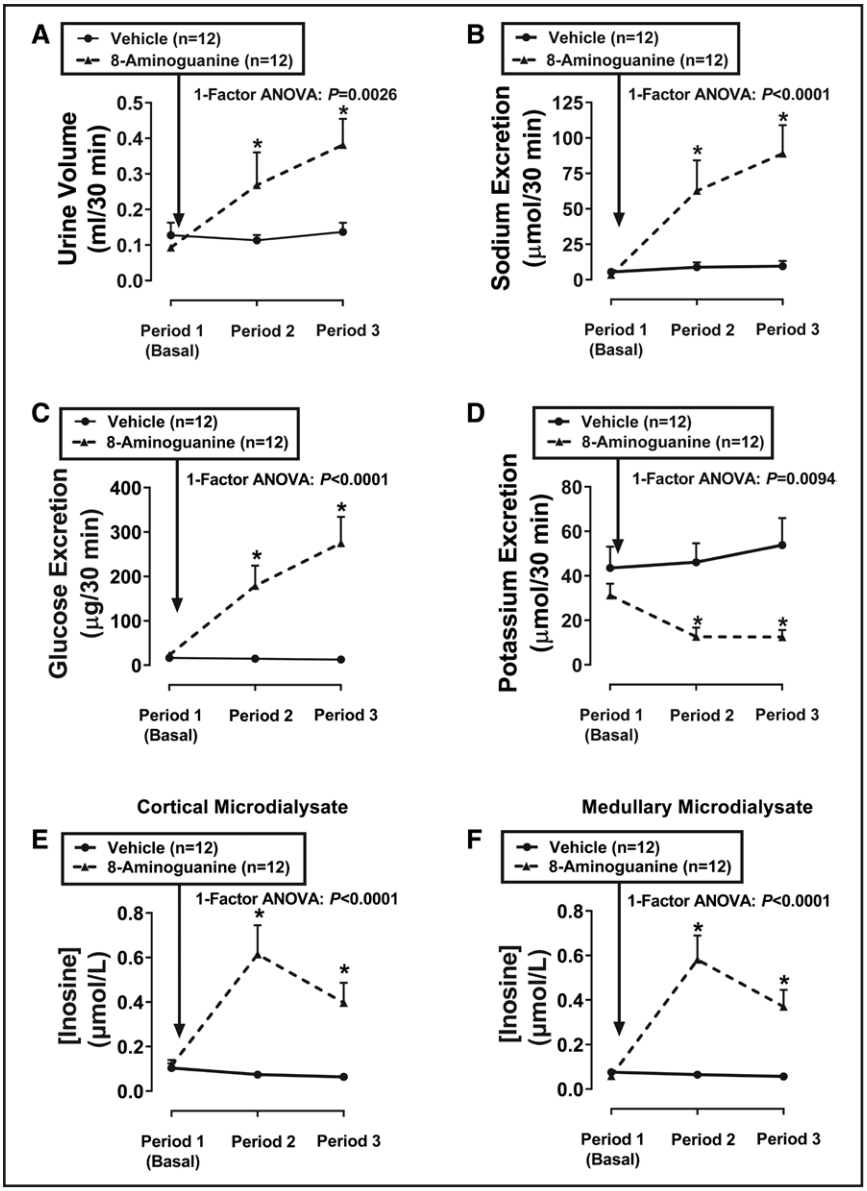

Figure 1. Effects of 8-aminoguanine on renal excretory function and renal cortical and medullary microdialysate levels of inosine.
Timed collections of urine from the ureter and renal cortical and medullary microdialysates were obtained from anesthetized rats from 0 to 30 minutes (min; Period 1), 40 to 70 minutes (Period 2), and 85 to 115 minutes (Period 3) into the protocol. Rats received an IV injection of 8-aminoguanine (33.5 µmol/kg) immediately after Period 1. Urine volumes (**A**), excretion rates of sodium (**B**), glucose (**C**), and potassium (**D**), and cortical (**E**) and medullary (**F**) concentrations of inosine were determined. Values are means and SEMs for the indicated sample size (n). *P<0.05 vs Period 1.

Indeed, 8-aminoguanine did increase cortical (P<0.0001; Figure S2C) and medullary (P<0.0001; Figure S2D) levels of guanosine and decreased cortical (P=0.0007; Figure S2E) and medullary (P=0.0414; Figure S2F) levels of guanine. Hemodynamically, rats were stable throughout the protocol. At the end of the experiments, MABPs were similar in the 2 groups (111±3 versus 112±2 mm Hg, control group versus 8-aminoguanine group, respectively; mean±SEM), as were renal blood flows (9.2±0.9 versus 8.4±0.8 mL/min, control group versus 8-aminoguanine group, respectively).

Protocol 2. Effects of Intrarenal Artery Infusions of Inosine and Guanosine on Renal Excretory Function

Because 8-aminoguanine profoundly increased renal interstitial levels of inosine and guanosine, next we examined the effects of these 2 purines on renal excretory function. To avoid systemic effects, inosine or guanosine, dissolved in 0.9% saline, was infused directly into the left renal artery of anesthetized Sprague Dawley rats at increasing doses (0 [basal; 0.9% saline only], 0.01, 0.1, and 1 µmol/kg per minute). In preliminary experiments, we showed that the medium dose (0.1 µmol/kg per minute) of inosine or guanosine achieved levels of inosine (0.5±0.3 µmol/L; n=6) or guanosine (0.6±0.3 µmol/L; n=6), respectively, in the renal cortical microdialysate of the infused kidney that were comparable to the levels achieved by IV administration of 33.5 µmol/kg of 8-aminoguanine. Each dose of inosine or guanosine was administered for 30 minutes and timed collections of urine were obtained from the left ureter between 10 and 30 minutes after initiating a given dose of inosine or guanosine. Inosine significantly increased urine volume (P=0.0039; Figure 2A), sodium excretion (P=0.0137; Figure 2C) and glucose

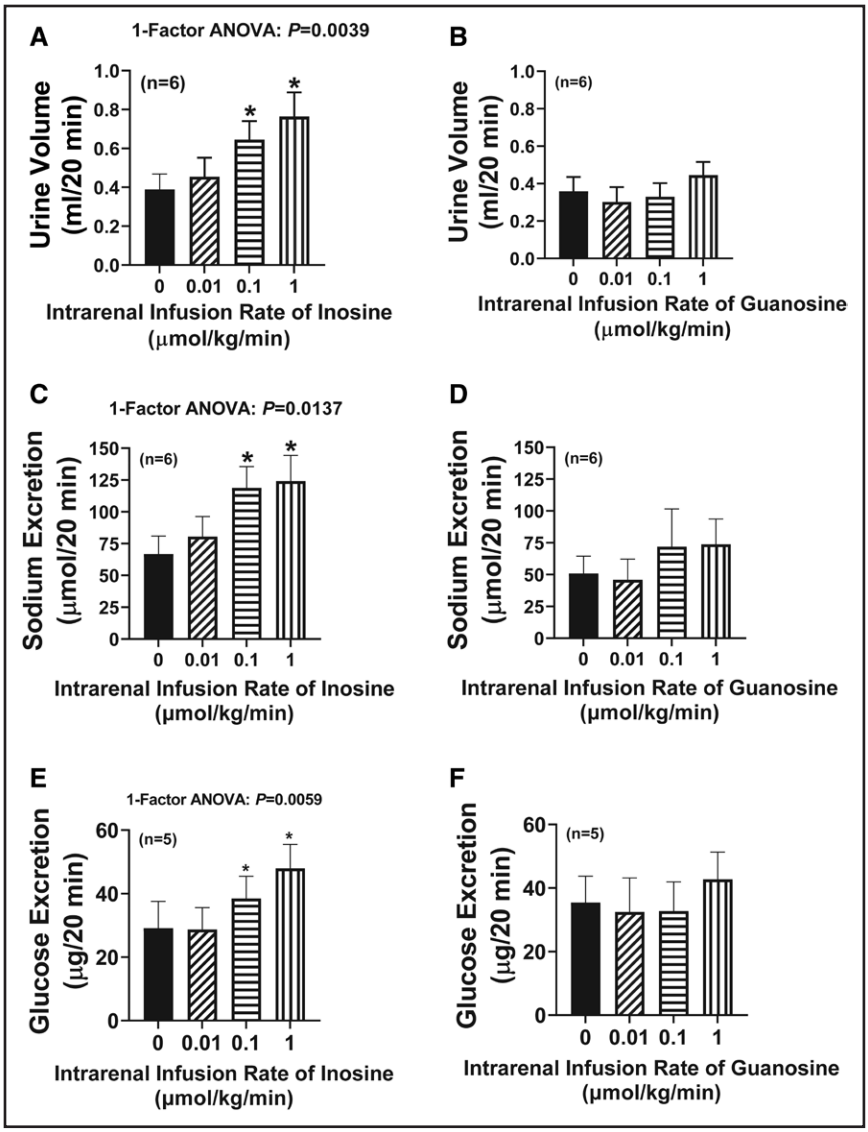

Figure 2. Effects of intrarenal artery infusions of inosine and guanosine on renal excretory function.
Inosine (**A**, **C**, **E**) or guanosine (**B**, **D**, **F**) was infused directly into the renal artery of anesthetized rats at increasing doses (0 [vehicle only; basal], 0.01, 0.1 and 1 µmol/kg per minute [min]). Each dose of inosine or guanosine was administered for 30 minutes, timed collections of urine were obtained from the ureter between 10 and 30 minutes after initiating a given dose of inosine or guanosine, and urine volumes (**A** and **B**) and excretion rates of sodium (**C** and **D**) and glucose (**E** and **F**) were determined. Values are means and SEMs for the indicated sample size (n). *P<0.05 vs 0 (vehicle; basal).

excretion (P=0.0059, Figure 2E); whereas guanosine had little or no effect on urine volume (Figure 2B), sodium excretion (Figure 2D) or glucose excretion (Figure 2F). Neither inosine (Figure S3A) nor guanosine (Figure S3B) affected potassium excretion. We also examined the effects of intrarenal infusions of inosine on renal excretory function in rats pretreated with IV 8-aminoguanine (33.5 µmol/kg) to increase basal levels of endogenous inosine. As expected, 8-aminoguanine increased urine volume (P=0.0473, Figure S4A), sodium excretion (P=0.0074, Figure S4B) and glucose excretion (P=0.0252, Figure S4C), and tended to decrease potassium excretion (Figure S4D). In rats pretreated with 8-aminoguanine, intrarenal artery infusions of inosine (0.01, 0.1, and 1 µmol/kg per minute) did not affect, relative to the 8-aminoguanine baseline, urine volume (Figure S4A), sodium excretion (Figure S4B), glucose excretion (Figure S4C) or potassium excretion (Figure S4D). If hypoxanthine decreases renal excretory function, decreases in hypoxanthine levels could theoretically participate in the ability of 8-aminoguanine to increase renal excretory function. Therefore, we also examined the effects of intrarenal artery infusions of hypoxanthine on renal excretory function both in naïve and 8-aminoguanine-pretreated rats. In naïve and 8-aminoguanine-pretreated rats, at the highest dose, hypoxanthine actually increased urine volume (P=0.0076, Figure S5A; P=0.0008, Figure S6A, respectively) and sodium excretion (P=0.0002, Figure S5B; P=0.0005, S6B, respectively), but did not affect glucose excretion (Figures S5C and S6C, respectively). Hypoxanthine did not affect potassium excretion in naïve rats (Figure S5D) but did increase potassium excretion in 8-aminoguanine-pretreated rats (P=0.0003, Figure S6D). As expected, 8-aminoguanine pretreatment increased urine volume (P=0.0011, Figure S6A), sodium excretion (P=0.0002, Figure S6B) and glucose excretion (P=0.0161, Figure S6C) and tended to decrease potassium excretion (Figure S6D). Hemodynamically, rats were stable throughout the

protocol. At the end of the experiments, MABPs (mm Hg; mean±SEM) were similar in the 5 groups (inosine group, 121±8.5; guanosine group, 111±7.5; hypoxanthine group, 122±5.2; inosine group pretreated with 8-aminoguanine, 108±5.3; and hypoxanthine group pretreated with 8-aminoguanine, 114±6.4). Also, renal blood flows (mL/min; mean±SEM) were similar in the 5 groups (inosine group, 10.1±0.7; guanosine group, 10.3±1.2; hypoxanthine group, 9.1±0.7; inosine group pretreated with 8-aminoguanine, 9.1±1.4; and hypoxanthine group pretreated with 8-aminoguanine, 9.9±0.9).

Protocol 3. Effects of 8-Aminoguanine on Renal Excretory Function in WT Dahl SS Rats Versus A_1-KO Dahl SS, A_{2A}-KO Dahl SS and A_{2B}-KO Dahl SS Rats

Our objectives here were to determine whether 8-aminoguanine has natriuretic effects in a model of sodium retention and whether the renal excretory effects are mediated by adenosine receptors. Here, male and female WT, A_1-KO, A_{2A}-KO and A_{2B}-KO Dahl SS rats were placed on a high-salt diet (4% NaCl) for 1 week. Next, rats were anesthetized, and timed urine collections were obtained from the left ureter from 0 to 30 minutes (Period 1), 40 to 70 minutes (Period 2), and 85 to 115 minutes (Period 3) into the protocol. Rats received an IV injection of 8-aminoguanine (33.5 µmol/kg) immediately after Period 1. In WT, A_1-KO and A_{2A}-KO Dahl SS rats, 8-aminoguanine increased urine volume (Figure 3A, Figure S7A and S7B, respectively; P=0.0168, P=0.0757 [near significant] and P=0.0082, respectively), sodium excretion (Figure 3C, Figure S7C and S7D, respectively; P=0.0350, P=0.0330 and P=0.0132, respectively) and glucose excretion (Figure 3E, Figure S7E and S7F, respectively; P=0.0003, P=0.0198 and P=0.0006, respectively). By contrast, in A_{2B}-KO Dahl SS rats, 8-aminoguanine did not affect urine volume (Figure 3B), sodium excretion (Figure 3D) or glucose excretion (Figure 3F). The 8-aminoguanine-induced changes (Period 3 minus Period 1) in urine volume, sodium excretion and glucose excretion for the 4 groups were calculated and compared. Notably, 8-aminoguanine induced similar changes in urine volume (Figure S8A), sodium excretion (Figure S8B) and glucose excretion (Figure S8C) in WT, A_1-KO and A_{2A}-KO Dahl SS rats. By contrast, in A_{2B}-KO Dahl SS rats, 8-aminoguanine-induced changes in urine volume (Figure S8A), sodium excretion (Figure S8B) and glucose excretion (Figure S8C) were significantly suppressed relative to WT-KO Dahl SS rats. Indeed, in A_{2B}-KO Dahl SS rats, the 8-aminoguanine-induced changes in urine volume (Figure S8A) and sodium excretion (Figure S8B) were abolished. 8-Aminoguanine decreased potassium excretion similarly in all 4 genotypes (Figure S8D). Hemodynamically, rats were stable throughout the protocol. However, as previously reported,[6] MABPs differed among the 4 genotypes. At the end of the experiments, MABPs (mm Hg; mean±SEM) were: WT, 150±5; A_1-KO, 112±5.4; A_{2A}-KO, 140±12; and A_{2B}-KO, 115±8. Renal blood flows (mL/min; mean±SEM) were: WT, 4.3±0.9; A_1-KO, 5.1±0.8; A_{2A}-KO, 4.9±0.7; and A_{2B}-KO 7.7±0.9.

Protocol 4. Effects of Intrarenal Artery Infusions of Inosine on Renal Excretory Function in WT Dahl SS Rats Versus A_{2B}-KO Dahl SS Rats

Since A_{2B} receptors appear to mediate the renal excretory effects (with the exception of potassium excretion) of 8-aminoguanine and since inosine appears to be involved in the renal excretory effects of 8-aminoguanine, here, we sought to determine whether the renal excretory effects of inosine are mediated by A_{2B} receptors. Inosine was infused at increasing doses (0 [basal], 0.01, 0.1 and 1 µmol/kg per minute] directly into the renal artery of either anesthetized WT Dahl SS or A_{2B}-KO Dahl SS rats. Each dose of inosine was administered for 30 minutes and timed collections of urine were obtained from the left ureter between 10 and 30 minutes after initiating a given dose of inosine. In WT Dahl SS rats, inosine significantly increased urine volume (P=0.0002; Figure S9A) and sodium excretion (P=0.0038; Figure S9C). By contrast, in A_{2B}-KO Dahl SS rats, direct intrarenal artery infusions of inosine did not alter either urine volume (Figure S9B) or sodium excretion (Figure S9D). Inosine did not affect potassium excretion in either WT or A_{2B}-KO Dahl SS rats, and the stimulatory effects of inosine on glucose excretion were abolished in A_{2B}-KO Dahl SS rats (data not shown). Hemodynamically, rats were stable throughout the protocol. At the end of the experiments, MABPs (mm Hg; mean±SEM) were similar in the 2 groups (WT Dahl SS rats, 124±4; A_{2B}-KO Dahl SS rats, 126±5).

Protocol 5. Effects of 8-Aminoguanine on Renal Excretory Function and Renal Medullary Blood Flow in Naïve Rats Versus Rats Pretreated With an A_{2A} Receptor Antagonist, an A_{2B} Receptor Antagonist or Both

To further test the role of adenosine receptors in the renal effects of 8-aminoguanine, we examined the effects of adenosine receptor antagonists on the renal actions of 8-aminoguanine. Anesthetized Sprague Dawley rats were pretreated intravenously with either vehicle, 10 mg/kg of ZM 241385 (selective A_{2A} receptor antagonist[9]), 30 mg/kg of PSB 1115 (selective A_{2B} receptor antagonist[10]) or ZM 241385 plus PSB 1115, and 15 minutes later timed (30 minutes) urine

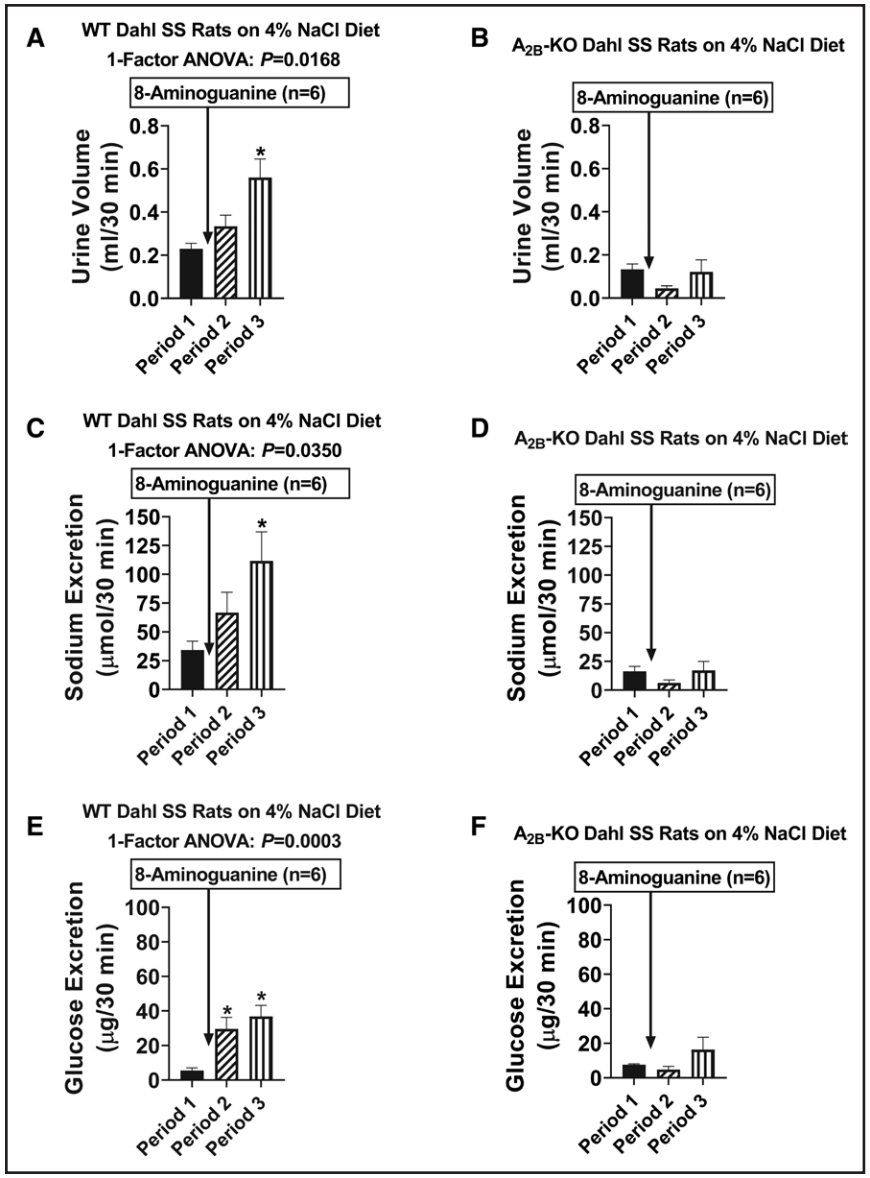

Figure 3. Effects of 8-aminoguanine in wild-type (WT) Dahl salt-sensitive rats (Dahl SS rats) versus Dahl salt-sensitive rats with knockout of A_{2B} receptors (A_{2B}-KO Dahl SS rats). WT Dahl SS rats (**A**, **C**, **E**) and A_{2B}-KO Dahl SS rats (**B**, **D**, **F**) were placed on a high salt diet (4% NaCl) for 1 week. Next, rats were anesthetized, and timed urine collections were obtained from the ureter from 0 to 30 minutes (min; Period 1), 40 to 70 minutes (Period 2), and 85 to 115 minutes (Period 3) into the protocol. Rats received an IV injection of 8-aminoguanine (33.5 µmol/kg) immediately after Period 1. Urine volumes (**A** and **B**) and sodium (**C** and **D**) and glucose (**E** and **F**) excretion rates were determined. Values are means and SEMs for the indicated sample size (n). *P<0.05 vs Period 1.

collections were obtained from the left ureter. Next, rats received an IV injection of 8-aminoguanine (33.5 µmol/kg), and after ≈1 hour 30-minute urine collections were again obtained. As expected, in naïve rats 8-aminoguanine increased urine volume (P=0.0195; Figure S10A) and sodium excretion (P=0.0055; Figure S10B). Notably, in naïve rats 8-aminoguanine also increased medullary blood flow (MBF) (P=0.0216; Figure 4A). In rats pretreated with an A_{2A} antagonist, 8-aminoguanine still increased urine volume (P=0.0485; Figure S10C), tended to increase sodium excretion (P=0.0779; Figure S10D) and still increased MBF (P=0.0320; Figure 4B). Treatment of rats with an A_{2B} antagonist abolished the effects of 8-aminoguanine on urine volume (Figure S10E), sodium excretion (Figure S10F) and MBF (Figure 4C). Co-pretreatment of rats with both an A_{2B} antagonist plus an A_{2A} antagonist also abolished the effects of 8-aminoguanine on urine volume (Figure S10G), sodium excretion (Figure S10H) and MBF (Figure 4D). The 8-aminoguanine-induced changes in urine volume, sodium excretion and MBF were calculated and compared. The changes in urine volume (Figure S11A) and sodium excretion (Figure S11B) were not different in naïve versus A_{2A} antagonist-treated rats; however, pretreatment with an A_{2B} antagonist abolished the 8-aminoguanine-induced changes in urine volume (P=0.0006; Figure S11A) and sodium excretion (P=0.0462; Figure S11B). Likewise, pretreatment with the combination of an A_{2B} plus A_{2A} antagonist abolished the 8-aminoguanine-induced changes in urine volume (P=0.0124; Figure S11A) and sodium excretion (P=0.0048; Figure S11B). The 8-aminoguanine-induced increases in MBF were similar in naïve rats versus rats pretreated with an A_{2A} antagonist but were reduced in rats pretreated with an A_{2B} antagonist or the combination of an A_{2B} plus A_{2A}

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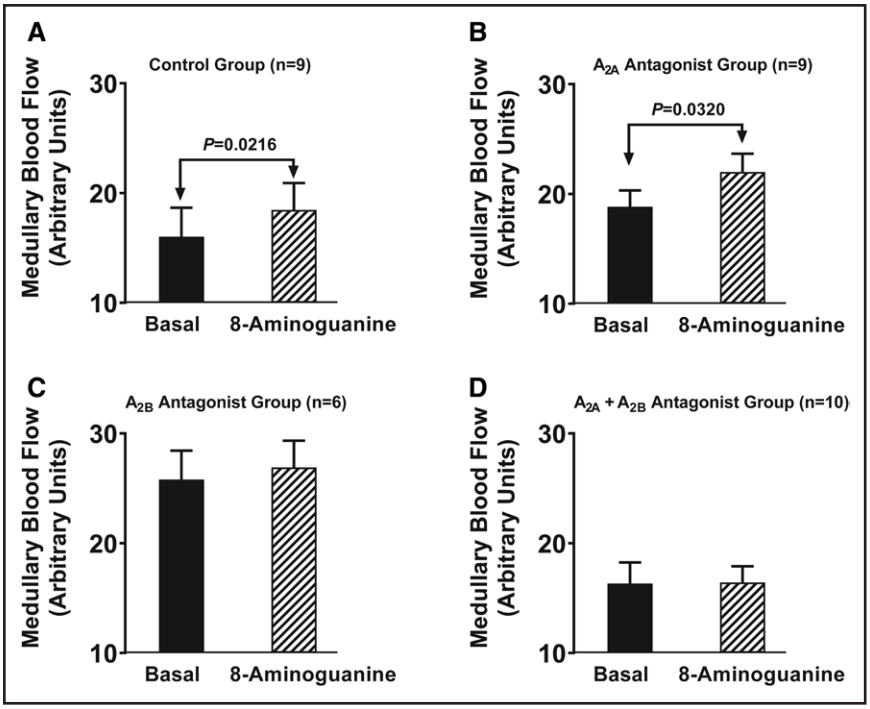

Figure 4. Effects of 8-aminoguanine on medullary blood flow (MBF) in rats pretreated with an A_{2A} receptor antagonist, an A_{2B} receptor antagonist or both an A_{2A} and A_{2B} receptor antagonist.
Anesthetized rats were pretreated intravenously with either vehicle (**A**; Control Group), 10 mg/kg of ZM 241385 (**B**; A_{2A} Antagonist Group), 30 mg/kg of PSB 1115 (**C**; A_{2B} Antagonist Group) or ZM 241385 plus PSB 1115 (**D**; A_{2A} + A_{2B} Antagonist Group), and 15 minutes later MBF was averaged over 30 minutes (Basal). Next, rats received an IV injection of 8-aminoguanine (33.5 µmol/kg), and after ≈1 hour MBF was averaged over 30 minutes (8-Aminoguanine). *P* values are from paired *t*-tests. Values are means and SEMs for the indicated sample size (n).

antagonist (*P*=0.0298; Figure S11C). 8-Aminoguanine reduced potassium excretion in all groups (Figure S12A through S12D) and the 8-aminoguanine-induced reductions in potassium excretion were similar among the 4 groups (Figure S12E). Hemodynamically, rats were stable throughout the protocol. At the end of the experiments, MABPs (mm Hg; mean±SEM) were similar in the 4 groups (naïve group, 113±4; A_{2A} antagonist pretreated group, 117±4; A_{2B} antagonist pretreated group, 126±6; combination A_{2A} plus A_{2B} antagonist pretreated group, 115±4).

Protocol 6. Effects of BAY 60-6583 on Renal Excretory Function and MBF

Because the effects of 8-aminoguanine appeared to be mediated by A_{2B} receptors, we next tested the effects of the selective A_{2B} receptor agonist, BAY 60-6583,[11] on renal excretory function and MBF. To avoid systemic effects, BAY 60-6583, dissolved in 0.9% saline, was infused directly into the renal artery of anesthetized rats at increasing doses (0 [0.9% saline only; basal], 1 and 3 nmol/kg per minute). Each dose of Bay 60-6583 was administered for 40 minutes and timed collections of urine were obtained from the left ureter between 10 and 40 minutes after initiating a given dose of BAY 60-6583. Similar to 8-aminoguanine, BAY 60-6583 increased urine volume (*P*<0.0001; Figure 5A), sodium excretion (*P*<0.0001; Figure 5B) and MBF (*P*=0.0174; Figure 5D). In contrast to 8-aminoguanine, BAY 60-6583 increased, rather than decreased, potassium excretion (*P*=0.0257; Figure 5C).

Protocol 7. Effects of 8-Aminoguanine and Forodesine on 3',5'-cAMP and Inosine in WT Versus A_{2B}-KO RMSMCs

RMSMCs were obtained from WT and A_{2B}-KO Dahl SS rats and were grown to confluence in 6-well plates. Cells were incubated for 30 minutes with vehicle or forskolin to sensitize adenylyl cyclase to receptor-mediated activation. Experiments were performed in the absence and presence of either 8-aminoguanine (100 µmol/L) or forodesine (10 µmol/L; alternative PNPase inhibitor). Medium was collected and analyzed for 3',5'-cAMP and inosine by UPLC-MS/MS. In WT RMSMCs, addition of either 8-aminoguanine (*P*=0.0003; Figure S13A) or forodesine (*P*=0.0004; Figure S13A) to forskolin-treated cells significantly increased levels of 3',5'-cAMP. However, in A_{2B}-KO RMSMCs, neither 8-aminoguanine (Figure S13B) nor forodesine (Figure S13B) affected 3',5'-cAMP levels in forskolin-treated cells. In both WT (Figure S11C) and A_{2B}-KO (Figure S11D) RMSMCs both 8-aminoguanine and forodesine increased inosine levels.

Protocol 8. Effects of Inosine on A_{2B} Receptor Signaling Induced by 5'-*N*-Ethylcarboxamidoadenosine

Next, we sought to determine whether inosine is a positive allosteric modulator of A_{2B} receptors. Human A_{2B} receptors were expressed in HEK293 cells and stimulated by the A_{2B} receptor agonist 5'-*N*-ethylcarboxamidoadenosine (NECA).[12] Receptor activation was monitored by measuring intracellular 3',5'-cAMP (as an index of

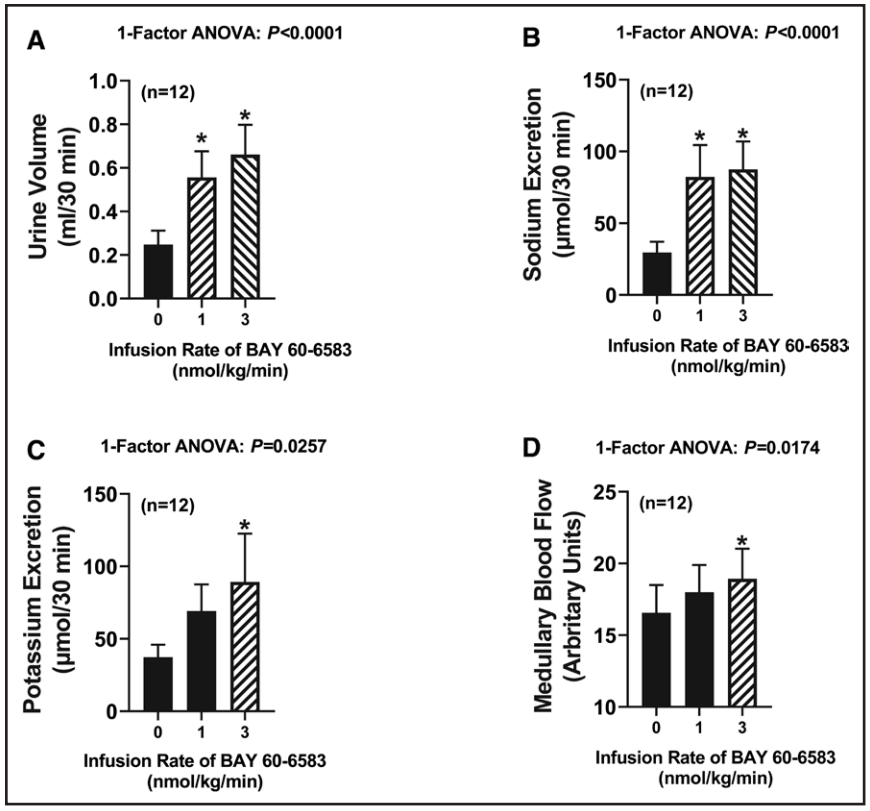

Figure 5. Effects of BAY 60-6583 on renal excretory function.
BAY 60-6583 was infused directly into the renal artery of anesthetized rats at increasing doses (0 [vehicle; basal], 1 and 3 nmol/kg per minute [min]). Each dose of Bay 60-6583 was administered for 40 minutes and timed collections of urine were obtained from the ureter between 10 and 40 minutes after initiating a given dose of BAY 60-6583, and urine volumes (**A**) and excretion rates of sodium (**B**) and potassium (**C**) were determined. Also shown are medullary blood flows during the infusions (**D**). Values are means and SEMs for the indicated sample size (n). *P<0.05 vs 0 (vehicle; basal).

adenylyl cyclase activity) using a highly sensitive assay (homogeneous time resolved fluorescence). The concentration-response curve to NECA (performed in duplicate) was similar in cells incubated without inosine (Figure 6A) versus with either 30 µmol/L (Figure 6B) or 100 µmol/L (Figure 6C) of inosine; thus, indicating that inosine is not a positive allosteric modulator of A_{2B} receptors. Although inosine did not act as a positive allosteric modulator of A_{2B} receptors, inosine per se activated adenylyl cyclase (see shift in basal adenylyl cyclase activity in Figure 6B and 6C versus Figure 6A). Figure 6D directly compares the effects of inosine (0 [basal], 30, and 100 µmol/L) on activation of adenylyl cyclase in HEK293 cells expressing A_{2B} receptors. Inosine markedly and significantly (P<0.0001) increased adenylyl cyclase activity.

Protocol 9. Concentration-Response Relationship for Agonism of A_{2B} Receptors by Inosine

Because inosine per se appeared to activate A_{2B} receptors, next we examined the full concentration-response relationship of inosine on adenylyl cyclase activity in HEK293 cells expressing human A_{2B} receptors. These experiments were performed both in the presence and absence of MRS 1754 (1 µmol/L; highly selective A_{2B} receptor antagonist[13]). Inosine caused a concentration-dependent stimulation of adenylyl cyclase with an activation threshold of 3 to 10 µmol/L (Figure 6E). The ability of inosine to activate adenylyl cyclase was abolished by MRS 1754 (Figure 6F) suggesting that the activation of adenylyl cyclase by inosine was mediated by activation of A_{2B} receptors.

DISCUSSION

Previously, we observed that 8-aminoguanine, a naturally occurring 8-aminopurine,[3] rapidly alters renal excretory function.[1] Specifically, 8-aminoguanine increases urine volume and sodium and glucose excretion while decreasing potassium excretion.[1] The diuretic, natriuretic and glucosuric effects of 8-aminoguanine appear to be mediated by inhibition of PNPase,[5] while the antikaliuretic actions of 8-aminoguanine are independent of PNPase inhibition.[4,5] The goal of the present study was to investigate how inhibition of PNPase by 8-aminoguanine induces diuresis, natriuresis and glucosuria.

Our first experimental series, which employed a large sample size (n=12), confirmed our conclusion that 8-aminoguanine is a diuretic, natriuretic, glucosuric and antikaliuretic agent. We view these results as critically important because they demonstrate the reproducibility of our previous findings. In addition to confirming our previous results, we used this opportunity to examine, via renal microdialysis combined with UPLC-MS/MS, how a diuretic/natriuretic/glucosuric dose (ie, an effective dose) of 8-aminoguanine affects renal interstitial levels of purines. The in vivo recovery rate of an analyte into microdialysate depends on a

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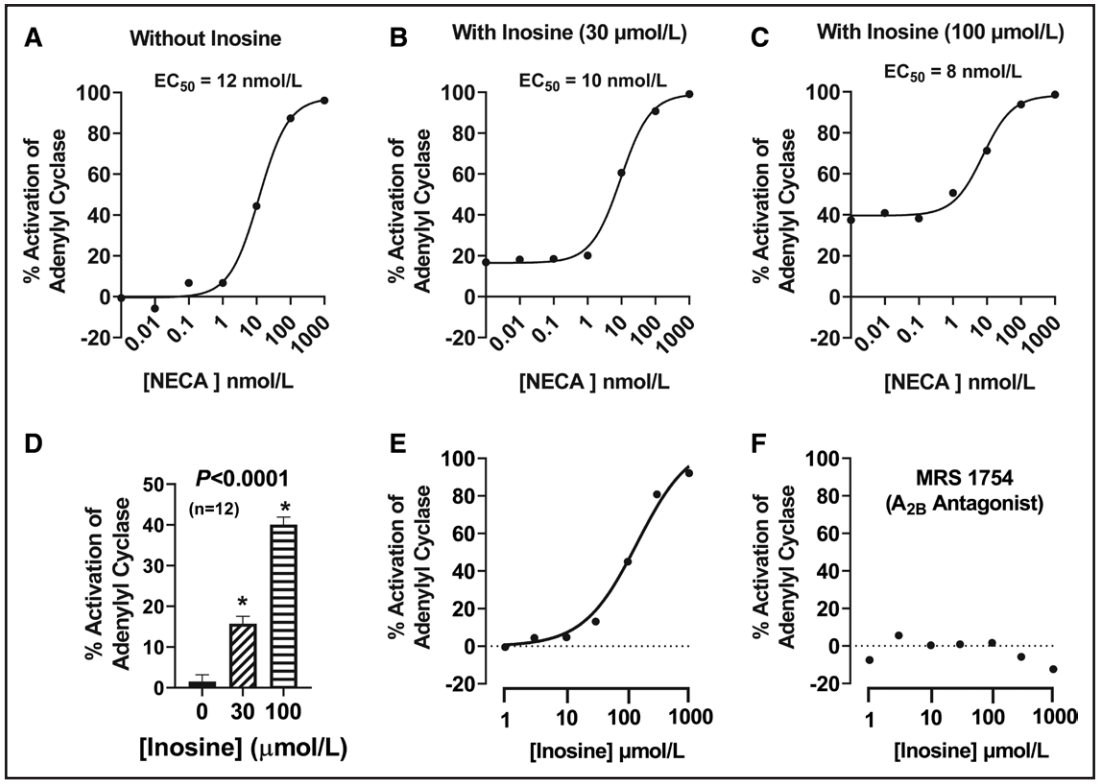

Figure 6. Effects of inosine on A_{2B} receptor signaling.
Human A_{2B} receptors were expressed in HEK293 cells and intracellular 3',5'-cAMP was measured using a highly sensitive assay (homogeneous time resolved fluorescence). In some experiments (in duplicate), cells were treated with a range of concentrations of the A_{2B} receptor agonist 5'-*N*-ethylcarboxamidoadenosine (NECA) without (**A**) or with inosine (either 30 [**B**] or 100 [**C**] µmol/L). Also shown are the shifts in basal levels of 3',5'-cAMP induced by inosine at 30 and 100 µmol/L (**D**). In additional experiments (in duplicate), cells were treated with a range of concentrations of inosine in the absence (**E**) and presence (**F**) of MRS 1754 (1 µmol/L; highly selective A_{2B} receptor antagonist). **P*<0.05 vs 0 (no inosine). **D**, Values are means and SEMs for the indicated sample size (n).

number of conditions[14] and is a fraction of the actual concentration of the analyte in the interstitium.[14] Thus, microdialysate levels of purines should not be interpreted as absolute levels of purines in the interstitial compartment. Nonetheless, using a standardized protocol, microdialysate levels of purines provide an accurate assessment of the directional and fold changes in interstitial purine levels induced by an intervention.

Importantly, our first experimental series revealed that an effective IV dose of 8-aminoguanine increased 8-aminoguanine levels in the renal microdialysate to ≈30 µmol/L. Of note, we recently showed that the Ki of 8-aminoguanine for recombinant human PNPase is approximately 2.8 µmol/L.[4] Thus, in this study, the renal interstitial levels of 8-aminoguanine after administration of an effective dose of 8-aminoguanine were undoubtably sufficient to inhibit PNPase. Consistent with this conclusion, an effective dose of 8-aminoguanine increased by several fold renal interstitial levels of PNPase substrates (ie, inosine and guanosine), yet decreased renal interstitial levels of PNPase products (ie, hypoxanthine and guanine). 8-Aminoguanine had no detectable effects on adenosine levels in the renal cortex and caused only a slight and brief increase in adenosine in the renal medulla. Mammalian PNPase exists in several different multimeric complexes,[15] most of which do not process adenosine to adenine.[15] However, there is evidence in rats that under some conditions a form of PNPase can convert adenosine to adenine.[16] There is also evidence that guanosine can increase extracellular levels of adenosine by blocking the disposition of adenosine from the extracellular compartment.[17] Nonetheless, given the limited (in location, magnitude and time) effects of 8-aminoguanine on renal interstitial levels of adenosine, it is unlikely that the renal excretory effects of PNPase inhibition are mediated by adenosine.

Because 8-aminoguanine caused large fold increases in inosine and guanosine, we entertained the hypothesis that the diuretic/natriuretic/glucosuric effects of 8-aminoguanine were mediated by one or both of these purines. To test this, we infused directly into the renal artery doses of inosine or guanosine that provided renal microdialysate levels that were comparable to those achieved by an effective dose of 8-aminoguanine. Here, we noted that inosine, but not guanosine, increased urine volume, sodium excretion and glucose excretion, without affecting potassium excretion. We view this as strong evidence that 8-aminoguanine induces diuresis, natriuresis and

glucosuria by elevating, as a consequence of PNPase inhibition, renal interstitial levels of inosine. That potassium excretion was not affected by inosine is consistent with our previous findings that PNPase inhibition does not mediate the antikaliuretic effects of 8-aminoguanine.[5] If inosine mediates the effects of 8-aminoguanine on renal excretory function, exogenous inosine should have little or no effect on renal excretory function in animals pretreated with an effective dose of 8-aminoguanine that elevates endogenous inosine levels. Consistent with this prediction, inosine did not affect renal excretory function in rats pretreated with an effective dose of 8-aminoguanine.

Because hypoxanthine induces oxidative stress[18] that could compromise renal excretory function,[19] it is conceivable that the reduction in renal hypoxanthine levels by 8-aminoguanine contributes to the renal excretory effects of 8-aminoguanine. If so, exogenous hypoxanthine should decrease renal excretory function. However, intrarenal artery infusions of hypoxanthine did not reduce renal excretory function in naïve rats or rats pretreated with 8-aminoguanine to reduce endogenous hypoxanthine levels. Thus, it is unlikely that 8-aminoguanine's effects on renal excretory function are mediated by reductions in renal hypoxanthine levels.

Next, we hypothesized that adenosine receptors mediate the renal excretory effects of 8-aminoguanine. This hypothesis was motivated by published evidence that inosine may directly, or indirectly, activate A_1,[20–22] A_{2A},[23,24] or A_{2B}[25–27] receptors. To test this hypothesis, we examined the effects of 8-aminoguanine on renal excretory function in WT, A_1-KO, A_{2A}-KO and A_{2B}-KO rats that were generated by Dr. Aron M. Geurts (Department of Physiology, Medical college of Wisconsin), with colonies maintained at the University of Pittsburgh.[6] These knockout rats were generated on a Dahl SS background, which provided the opportunity to not only test the role of adenosine receptors in the renal effects of 8-aminoguanine, but also to test whether 8-aminoguanine is an effective diuretic/natriuretic in an animal model that has a genetic predisposition to retain sodium. Importantly, 8-aminoguanine was an effective diuretic/natriuretic/glucosuric/antikaliuretic agent in WT Dahl SS rats on a 4% NaCl diet. This confirms the utility of 8-aminoguanine as a diuretic/natriuretic in a challenging model of salt retention, thus underscoring the potential of 8-aminoguanine in salt-retaining states. Equally important, we observed that renal excretory responses to 8-aminoguanine were similar in WT versus A_1-KO versus A_{2A}-KO rats; however, in A_{2B}-KO rats, 8-aminoguanine did not increase urine volume or sodium or glucose excretion. As expected, however, the antikaliuretic effects of 8-aminoguanine were maintained in A_1-KO, A_{2A}-KO and A_{2B}-KO rats. To confirm the mechanistic links between PNPase inhibition, increased inosine levels and A_{2B} receptor activation, we also compared the effects of intrarenal artery infusions of inosine in WT versus A_{2B}-KO rats. Notably, inosine increased renal excretory function in WT, but not A_{2B}-KO, rats.

To further test the concept that A_{2B} receptors mediate the effects of 8-aminoguanine on renal excretory function, we also examined the effects of 8-aminoguanine on renal excretory function in Sprague-Dawley rats before and after administration of a selective A_{2A} receptor antagonist, a selective A_{2B} receptor antagonist or both. Consistent with the results in knockout rats, the ability of 8-aminoguanine to induce diuresis and natriuresis was abolished by blockade of A_{2B}, but not A_{2A}, receptors; however, once again the antikaliuretic effects of 8-aminoguanine were not affected by suppressing A_{2B} receptors.

Although compelling, our proposed mechanistic pathway of PNPase inhibition leading to increased renal interstitial levels of inosine with subsequent activation of A_{2B} receptors was still incomplete in 2 aspects. First, there was the question as to whether 8-aminoguanine and inosine activate A_{2B} receptor signaling. Indeed, the literature regarding the effects of inosine on A_2 receptors is inconsistent. For example, Fredholm et al[20] reported that inosine did not activate either A_{2A} or A_{2B} receptors expressed in CHO cells; however, Welihinda et al reported that inosine activated A_{2A}, but not A_{2B}, receptors expressed in CHO-K1 cells and that this effect of inosine was enhanced by positive allosteric modulation.[23,24] Valada et al[28] observed that inosine did not displace radioligand binding to the orthosteric site of A_{2A} (^{3}H-CGS21680) or A_{2B} (^{3}H-NECA) receptors expressed in CHO cells. By contrast, inosine was found to attenuate spontaneous activity in the rat neurogenic bladder via an A_{2B} receptor-mediated pathway,[25,26] and da Rocha Lapa et al[27]reported that A_{2B} receptors mediate in part the anti-inflammatory effects of inosine. The complexity of A_{2B} receptor pharmacology is underscored by the recent findings of Voss et al[29]showing that A_{2B} receptors couple to multiple G-proteins in an agonist specific manner, which suggests that inosine-induced activation of A_{2B} receptors may depend on the precise G-protein environment of the receptor.

In the present study, we tested, using a model system in which A_{2B} receptors were expressed in HEK293 cells, the ability of inosine to function as a positive allosteric modulator of A_{2B} receptors. In this system, inosine did not alter the concentration-response (ie, adenylyl cyclase activity) relationship to NECA but did per se activate adenylyl cyclase. To follow-up on this observation, we examined the full concentration-response relationship to inosine on A_{2B} receptor-mediated activation of adenylyl cyclase. Here, we observed that inosine at a threshold of 3 to 10 µmol/L stimulated adenylyl cyclase, and at higher concentrations fully activated the A_{2B}-adenylyl cyclase axis. This conclusion was confirmed by the observation that a selective A_{2B} receptor antagonist abolished the ability of inosine to activate adenylyl cyclase. Further, we observed that 8-aminoguanine and

forodesine (a structurally different PNPase inhibitor) increased extracellular levels of inosine and stimulated adenylyl cyclase activity in RMSMCs isolated from WT rats. In RMSMCs lacking A_{2B} receptors, both 8-aminoguanine and forodesine increased inosine levels, yet both compounds failed to activate adenylyl cyclase in A_{2B}-KO RMSMCs. These finding support the conclusion that PNPase inhibition increases inosine levels, which then activate the A_{2B}-adenylyl cyclase axis. Although it is possible that inosine somehow indirectly activates the A_{2B}-adenylyl cyclase axis, it is also conceivable that inosine activates A_{2B} receptors by direct binding to A_{2B} receptors. For example, there could be more than one independent agonist orthosteric binding site on A_{2B} receptors such that inosine does not compete with NECA binding but still activates the A_{2B} receptor via its own orthosteric binding domain.

Another knowledge gap in our proposed mechanism relates to the question of how activation of A_{2B} receptors could increase renal excretory function. Here, we proposed that 8-aminoguanine via A_{2B} receptor activation leads to an increase in MBF, which in turn increases renal excretory function. This hypothesis is based on reports by (1) Zou et al[30] who showed that A_2 receptors increase MBF and sodium excretion; (2) Grenz et al[31] who demonstrated that within the kidney A_{2B} receptors are expressed predominantly in the renal vasculature; (3) Feng and Navar[32] and Cooper et al[33] who showed that A_{2B} receptors are the A_2 receptor subtype that mediates renal vasodilation; and (4) reports by many investigators that decreases or increases in MBF decrease or increase, respectively, renal excretory function.[34–36] In support of this aspect of our proposed mechanism, we observed that 8-aminoguanine increases MBF and that this response is blocked by antagonism of A_{2B}, but not A_{2A}, receptors. Our hypothesis is further corroborated by the finding that intrarenal artery infusions of BAY 60-6583 (a selective partial agonist for A_{2B} receptors) increase urine volume, sodium excretion and MBF.

It is possible that changes in MBF do not fully account for the renal excretory effects of 8-aminoguanine and that A_{2B} receptor activation engages direct tubular mechanisms that contribute to 8-aminoguanine-induced effects on urine volume and sodium and glucose excretion. Although MBF does affect sodium excretion, and sodium and glucose reabsorption are linked in proximal tubules by SGLT2,[37] a relationship between MBF and glucose excretion has not been established. Also, Rajagopal and Pao demonstrated that A_{2B} receptors in renal inner medullary collecting duct epithelium promote chloride excretion via cystic fibrosis transmembrane conductance regulator chloride channels and suggested that this is a mechanism for enhancing urine NaCl excretion.[38] In addition, Battistone et al[39] reported that A_{2B} receptors stimulate vacuolar ATPase-dependent proton secretion in renal medullary type A intercalated cells. Together, the evidence suggests that increases in MBF may account for some, but not all, of the renal excretory effects of 8-aminoguanine.

The mechanism by which 8-aminoguanine reduces potassium excretion remains unclear. 8-Aminoguanine, 8-aminoinosine, 8-aminohypoxanthine and 9-deazaguanine inhibit PNPase and induce diuresis, natriuresis and glucosuria, yet only 8-aminoguanine reduces potassium excretion.[4,5] This indicates that the antikaliuretic effects of 8-aminoguanine are not due to inhibition of PNPase. We have observed that 8-aminoguanine is a weak inhibitor of Rac1 and that Nsc23766 (blocks activation of Rac1) mimics the antikaliuretic effects of 8-aminoguanine.[5] Because Rac1 activates mineralocorticoid receptors,[40,41] this mechanism could explain, in part, 8-aminoguanine's antikaliuretic effects. However, since 8-aminoguanine is not a potent Rac1 inhibitor and the effects of Nsc23766 on potassium excretion could be due to off target actions of this Rac1 inhibitor, it is possible that 8-aminoguanine affects potassium excretion mostly by additional mechanisms that are yet to be discovered. These additional mechanisms may also contribute to the effects of 8-aminoguanine on other aspects of renal excretory function.

Perspectives

Taken together, the results of this investigation support the concept that inhibition of PNPase by 8-aminoguanine induces diuresis/natriuresis/glucosuria by increasing renal interstitial levels of inosine which, via A_{2B} receptor activation, increases renal excretory function, perhaps in part by increasing MBF. Previously, we reported that 8-aminoguanine exerts antihypertensive activity in DOCA/salt-induced hypertension[1]; we have also found that chronic administration of 8-aminoguanine attenuates salt-induced hypertension and strokes in Dahl SS rats (unpublished results). Likely the mechanisms revealed here mediate, at least in part, the antihypertensive actions of 8-aminoguanine. We have also observed that 8-aminoguanine has beneficial effects in other diseases including the metabolic syndrome,[42] pulmonary hypertension,[43] sickle cell disease,[44] age-associated bladder dysfunction,[45] age-associated retinal degeneration[46] and RHO-associated retinitis pigmentosa.[46] Likely, the mechanisms of action of 8-aminoguanine in these other diseases will share some common elements.

ARTICLE INFORMATION

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Disclosures

None.

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