ONLINE SUPPLEMENT

8-Aminoguanine and Its Actions on Renal Excretory Function

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Running title: **8-Aminoguanine on Renal Excretory Function**

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METHODS

Materials. 8-Aminoguanine, ZM 241385, PSB 1115, BAY 60-6583, MRS 1754, 5'-*N*-ethylcarboxamidoadenosine (NECA) and $^{13}C_5$-3',5'-cAMP were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada); adenosine, inosine, hypoxanthine, xanthine, guanosine, guanine, forodesine, forskolin and 3',5'-cAMP were obtained from Sigma-Aldrich (St. Louis, MO); $^{13}C_{10}$-adenosine, $^{13}C_2$,^{15}N-8-aminoguanine, $^{13}C_{10}$,$^{15}N_5$-guanosine and $^{13}C_2$,^{15}N-guanine were sourced from Medical Isotopes (Pelham, NH); $^{13}C_5$-hypoxanthine and $^{15}N_4$-inosine were purchased from Cambridge Isotope Laboratories (Tewksbury, MA).

Animals. Male and female wild-type and A_1, A_{2A} and A_{2B} knockout Dahl salt-sensitive rats (WT Dahl SS, 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.[1] 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 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, i.p.) and placed on an isothermal pad. Body temperature was continuously measured with a rectal probe thermometer and was maintained at 37ºC 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 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 (RBF) 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 (MBF). After instrumentation, animals were allowed a rest period of approximately one hour.

Analysis of Purines in Microdialysate. Microdialysate samples were diluted in water (1:1 dilution) and stable-isotope internal standards (see Materials for specific compounds) were added to the samples. Without further manipulation purines in 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 including instruments, instrument settings, columns, mobile phases, gradient conditions and mass transitions.[2]

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 (RMSMCs). RMSMCs were cultured from WT and A_{2B}-KO Dahl SS rats as previously described.[3]

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). HEK293 cells expressing recombinant human A_{2B} receptors were grown prior to the test in media without antibiotic and detached by gentle flushing with PBS-EDTA (5 mM EDTA), recovered by centrifugation and resuspended in assay buffer (5 mM KCl, 1.25 mM $MgSO_4$, 124 mM NaCl, 25 mM HEPES, 13.3 mM glucose, 1.25 mM KH_2PO_4, 1.45 mM $CaCl_2$, 0.5 g/l BSA, supplemented with 25 µM rolipram). Concentration-response curves were performed in duplicate in a 96-well format. To test whether inosine is a positive allosteric modulator of A_{2B} receptors, 12 µl of cells were mixed with 6 µl of inosine at increasing concentrations (0, 30 or 100 µmol/L), and then incubated for 10 min. Thereafter, 6 µl of NECA (non-selective A_{2B} agonist [4]) at increasing concentrations (0.01, 0.1, 1, 10, 100 and 1000 nmol/L) were added. To test whether inosine activates A_{2B} receptors independent of other agonists, HEK293 cells expressing A_{2B} receptors were treated with increasing concentrations of inosine (1, 3, 10, 30, 100, 300 and 1000 µmol/L) in the absence and presence of the selective A_{2B} receptor antagonist MRS 1754[5] (1 µmol/L). The plates were then incubated for 30 min at room temperature. 3',5'-cAMP was measured by homogeneous time resolved fluorescence (HTRF) using the Cisbio - cAMP-Gs Dynamic Kit (Cisbio; Bedford, MA). After addition of the lysis buffer containing the detection reagents cAMP-d2 (FRET acceptor) and anti-cAMP cryptate (FRET donor), plates were incubated for 1-hour at room temperature, and fluorescence ratios (665 nm/620 nm = delta F) were measured according to the manufacturer's specification. The percent activation of adenylyl cyclase was calculated using the delta F in buffer (corresponding to the basal level or 0 activation) and the delta F for the EC100 of NECA (corresponding to 100% activation).

Statistics. Multiple groups were compared with repeated measures 1-factor analysis of variance (ANOVA) followed by a Fisher's LSD test for multiple comparisons if the ANOVA showed significant differences among the groups. For these analyses, if necessary, data were log or Box-Cox transformed to improve homoscedasticity so as to conform to the assumptions of ANOVA. Unpaired t-tests (with equal or unequal variances) or paired t-tests, as appropriate, were employed to compare two groups. Adenylyl cyclase activation by inosine was fitted, using non-linear regression, to a three-parameter concentration-response curve using GraphPad Prism version 9.2.0 for Windows (GraphPad Software, San Diego, CA). Statistical analysis was conducted using NCSS Statistical Software version 19.0.2 (Kaysville, Utah). $P<0.05$ was considered statistically significant.

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3. Zhu X, Jackson EK. RACK1 regulates angiotensin II-induced contractions of SHR preglomerular vascular smooth muscle cells. *Am J Physiol Renal Physiol*. 2017;312:F565-F576. doi: 10.1152/ajprenal.00547.2016

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5. Ji X, Kim YC, Ahern DG, Linden J, Jacobson KA. [3H]MRS 1754, a selective antagonist radioligand for A_{2B} adenosine receptors. *Biochem Pharmacol*. 2001;61:657-663. doi: 10.1016/s0006-2952(01)00531-7

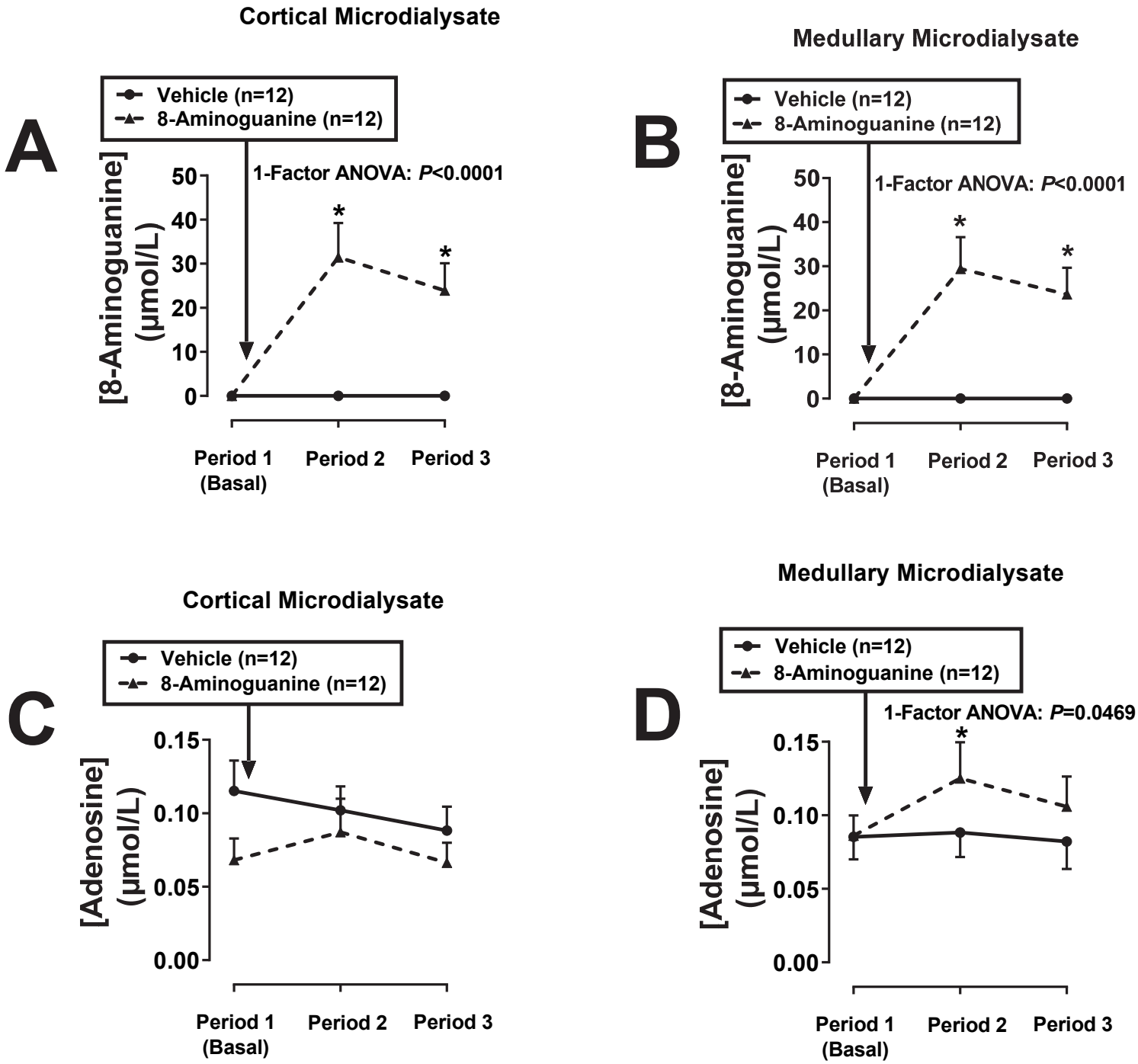

A
Cortical Microdialysate
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P<0.0001
[8-Aminoguanine] (µmol/L)
50
40
30
20
10
0
*
*
Period 1 (Basal)
Period 2
Period 3
B
Medullary Microdialysate
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P<0.0001
[8-Aminoguanine] (µmol/L)
50
40
30
20
10
0
*
*
Period 1 (Basal)
Period 2
Period 3
C
Cortical Microdialysate
Vehicle (n=12)
8-Aminoguanine (n=12)
[Adenosine] (µmol/L)
0.15
0.10
0.05
0.00
Period 1 (Basal)
Period 2
Period 3
D
Medullary Microdialysate
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P=0.0469
*
[Adenosine] (µmol/L)
0.15
0.10
0.05
0.00
Period 1 (Basal)
Period 2
Period 3

Figure S1

Figure S1. Effects of 8-aminoguanine on renal cortical and medullary microdialysate levels of 8-aminoguanine and adenosine. Timed samples of microdialysate were collected from microdialysis probes positioned in the renal cortex (A,C) and medulla (B,D). Samples were collected from anesthetized rats from 0-30 min (Period 1), 40-70 min (Period 2) and 85-115 min (Period 3) into the protocol, and rats received an intravenous injection of either vehicle or 8-aminoguanine (33.5 μmoles/kg) immediately after Period 1. 8-Aminoguanine (A,B) and adenosine (C,D) levels were measured in microdialysate by UPLC-MS/MS. Vehicle had little or no effect on cortical or medullary microdialysate levels of 8-aminoguanine or adenosine. 8-Aminoguanine significantly increased cortical (P<0.0001) and medullary (P<0.0001) microdialysate levels of 8-aminoguanine. 8-Aminoguanine also modestly and briefly (Period 2 only) increased medullary (P=0.0469), but not cortical, microdialysate levels of adenosine. Values are means and SEMs for the indicated sample size (n). ANOVA, analysis of variance; *P<0.05 vs Period 1.

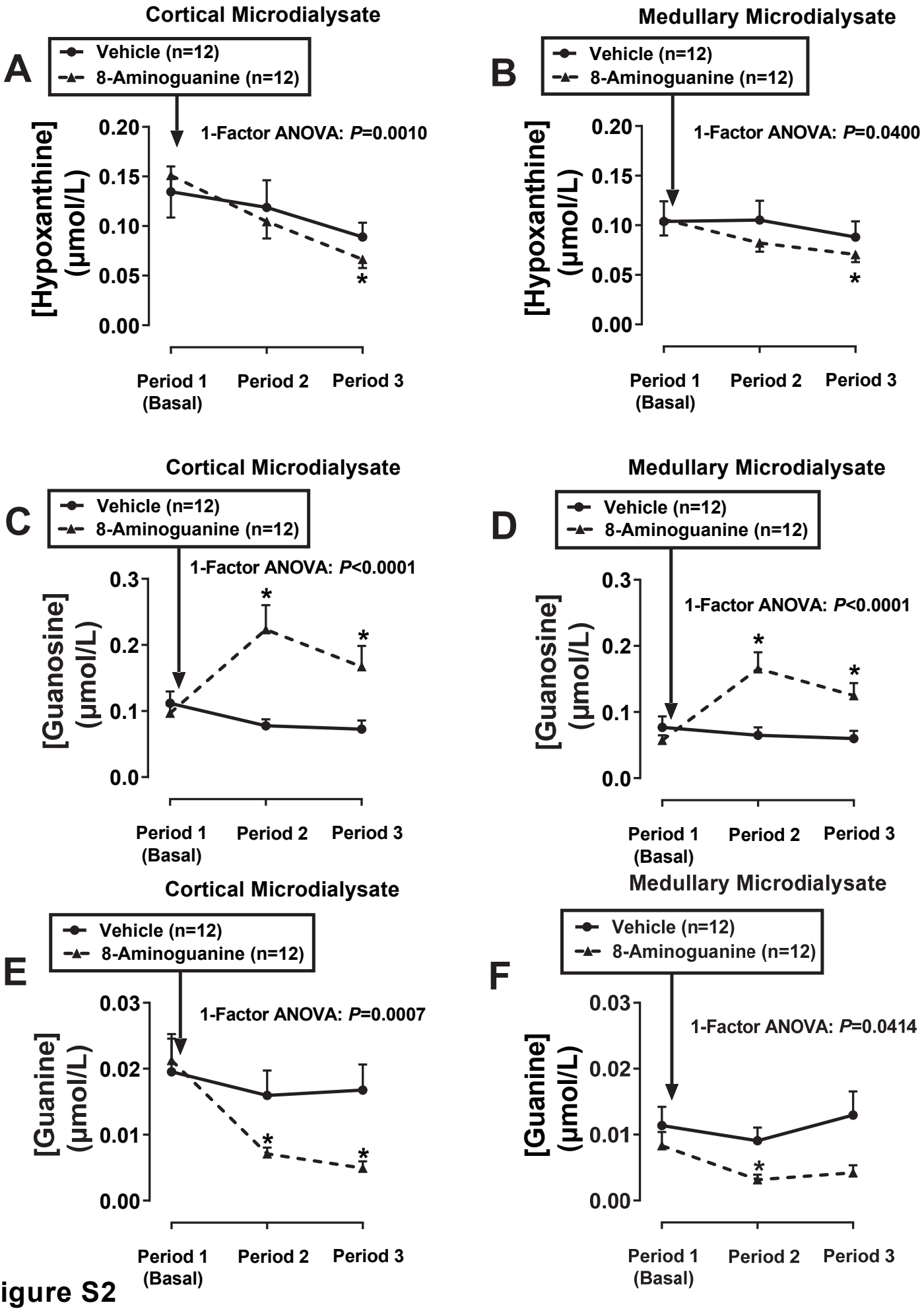
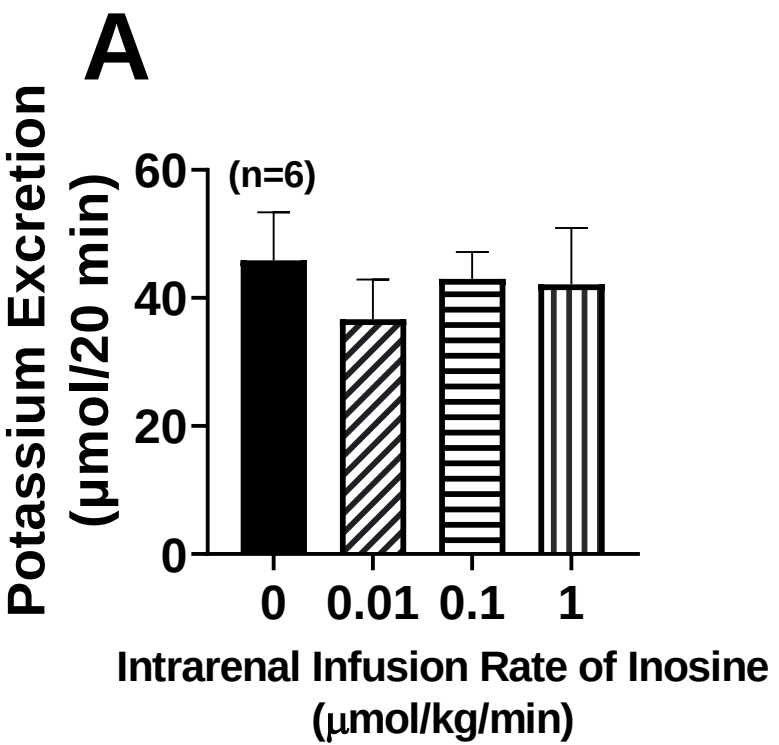

Cortical Microdialysate
A
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P=0.0010
[Hypoxanthine] (µmol/L)
0.20
0.15
0.10
0.05
0.00
*
Period 1 (Basal)
Period 2
Period 3
Medullary Microdialysate
B
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P=0.0400
[Hypoxanthine] (µmol/L)
0.20
0.15
0.10
0.05
0.00
*
Period 1 (Basal)
Period 2
Period 3
Cortical Microdialysate
C
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P<0.0001
[Guanosine] (µmol/L)
0.3
0.2
0.1
0.0
*
*
Period 1 (Basal)
Period 2
Period 3
Medullary Microdialysate
D
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P<0.0001
[Guanosine] (µmol/L)
0.3
0.2
0.1
0.0
*
*
Period 1 (Basal)
Period 2
Period 3
Cortical Microdialysate
E
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P=0.0007
[Guanine] (µmol/L)
0.03
0.02
0.01
0.00
*
*
Period 1 (Basal)
Period 2
Period 3
Medullary Microdialysate
F
Vehicle (n=12)
8-Aminoguanine (n=12)
1-Factor ANOVA: P=0.0414
[Guanine] (µmol/L)
0.03
0.02
0.01
0.00
*
Period 1 (Basal)
Period 2
Period 3

Figure S2

Figure S2. Effects of 8-aminoguanine on renal cortical and medullary microdialysate levels of hypoxanthine, guanosine and guanine. Timed collections of renal cortical and medullary microdialysates were obtained from anesthetized rats from 0-30 min (Period 1), 40-70 min (Period 2) and 85-115 min (Period 3) into the protocol. Rats received an intravenous injection of 8-aminoguanine (33.5 µmoles/kg) immediately after Period 1. Concentrations of hypoxanthine (A,B), guanosine (C,D) and guanine (E,F) in the microdialysate were determined. Values are means and SEMs for the indicated sample size (n). ANOVA, analysis of variance; **P*<0.05 vs Period 1.


A
Potassium Excretion
(µmol/20 min)
60
40
20
0
(n=6)
0
0.01
0.1
1
Intrarenal Infusion Rate of Inosine
(µmol/kg/min)

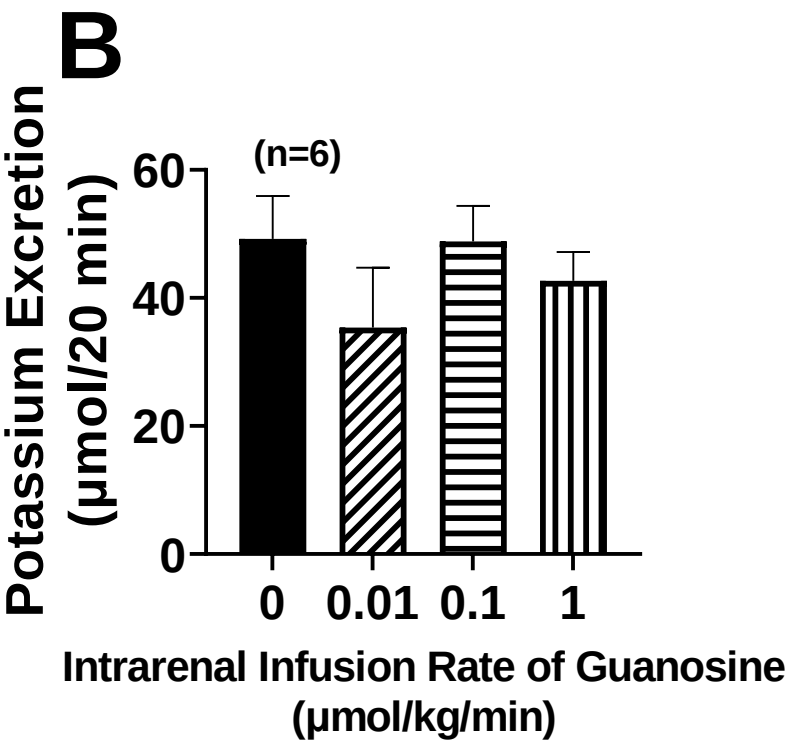

B
Potassium Excretion
(µmol/20 min)
60
40
20
0
(n=6)
0
0.01
0.1
1
Intrarenal Infusion Rate of Guanosine
(µmol/kg/min)

Figure S3

Figure S3. Effects of intrarenal artery infusions of inosine and guanosine on potassium excretion. Inosine (A) or guanosine (B) 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/min]. Each dose of inosine or guanosine was administered for 30 min, timed collections of urine were obtained from the ureter between 10 and 30 min after initiating a given dose of inosine or guanosine and excretion rates of potassium were determined. Values are means and SEMs for the indicated sample size (n). ANOVA, analysis of variance; *P<0.05 vs 0 (vehicle; basal).

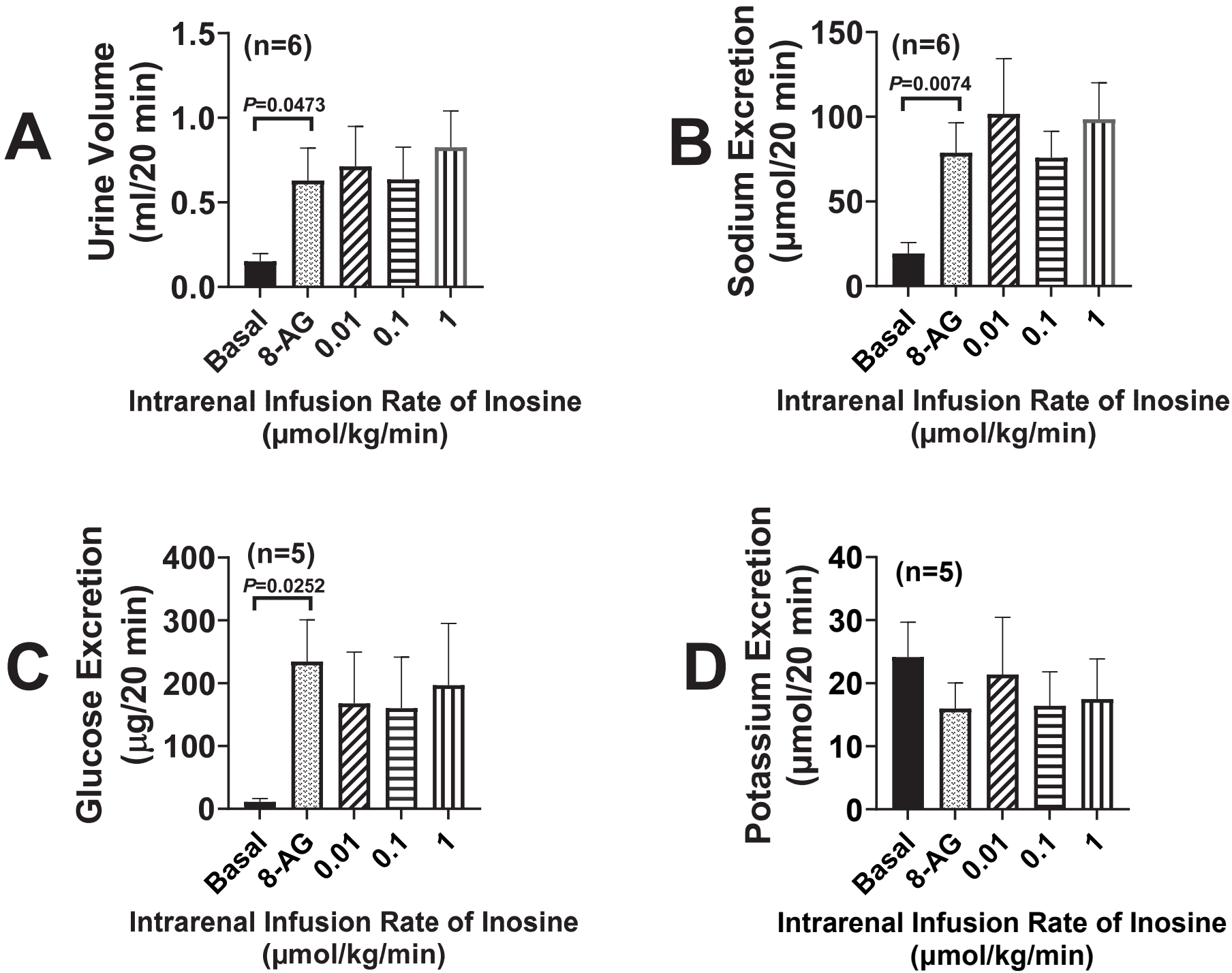

A
Urine Volume
(ml/20 min)
1.5
1.0
0.5
0.0
(n=6)
P=0.0473
Basal
8-AG
0.01
0.1
1
Intrarenal Infusion Rate of Inosine
(μmol/kg/min)
B
Sodium Excretion
(μmol/20 min)
150
100
50
0
(n=6)
P=0.0074
Basal
8-AG
0.01
0.1
1
Intrarenal Infusion Rate of Inosine
(μmol/kg/min)
C
Glucose Excretion
(μg/20 min)
400
300
200
100
0
(n=5)
P=0.0252
Basal
8-AG
0.01
0.1
1
Intrarenal Infusion Rate of Inosine
(μmol/kg/min)
D
Potassium Excretion
(μmol/20 min)
40
30
20
10
0
(n=5)
Basal
8-AG
0.01
0.1
1
Intrarenal Infusion Rate of Inosine
(μmol/kg/min)

Figure S4

Figure S4. Effects of intrarenal artery infusions of inosine on renal excretory function in rats pretreated with 8-aminoguanine. Urine was collected for 20 min (Basal) from the ureter of anesthetized rats. Rats then received an intravenous injection of 8-aminoguanine (33.5 µmoles/kg). One hour later another 20-min urine collection was obtained (8-AG). Next, inosine was infused directly into the renal artery at increasing doses (0.01, 0.1 and 1 µmol/kg/min). Each dose of inosine was administered for 30 min, timed collections of urine were obtained from the ureter between 10 and 30 min after initiating a given dose of inosine, and urine volumes (A) and excretion rates of sodium (B), glucose (C) and potassium (D) were determined. 8-Aminoguanine increased urine volume (P=0.0473), sodium excretion (P=0.0074), and glucose excretion (P=0.0252), and tended to decrease potassium excretion. However, compared to the 8-aminoguanine baseline, inosine did not alter urine volume or excretion of sodium, glucose or potassium. Values are means and SEMs for the indicated sample size (n).

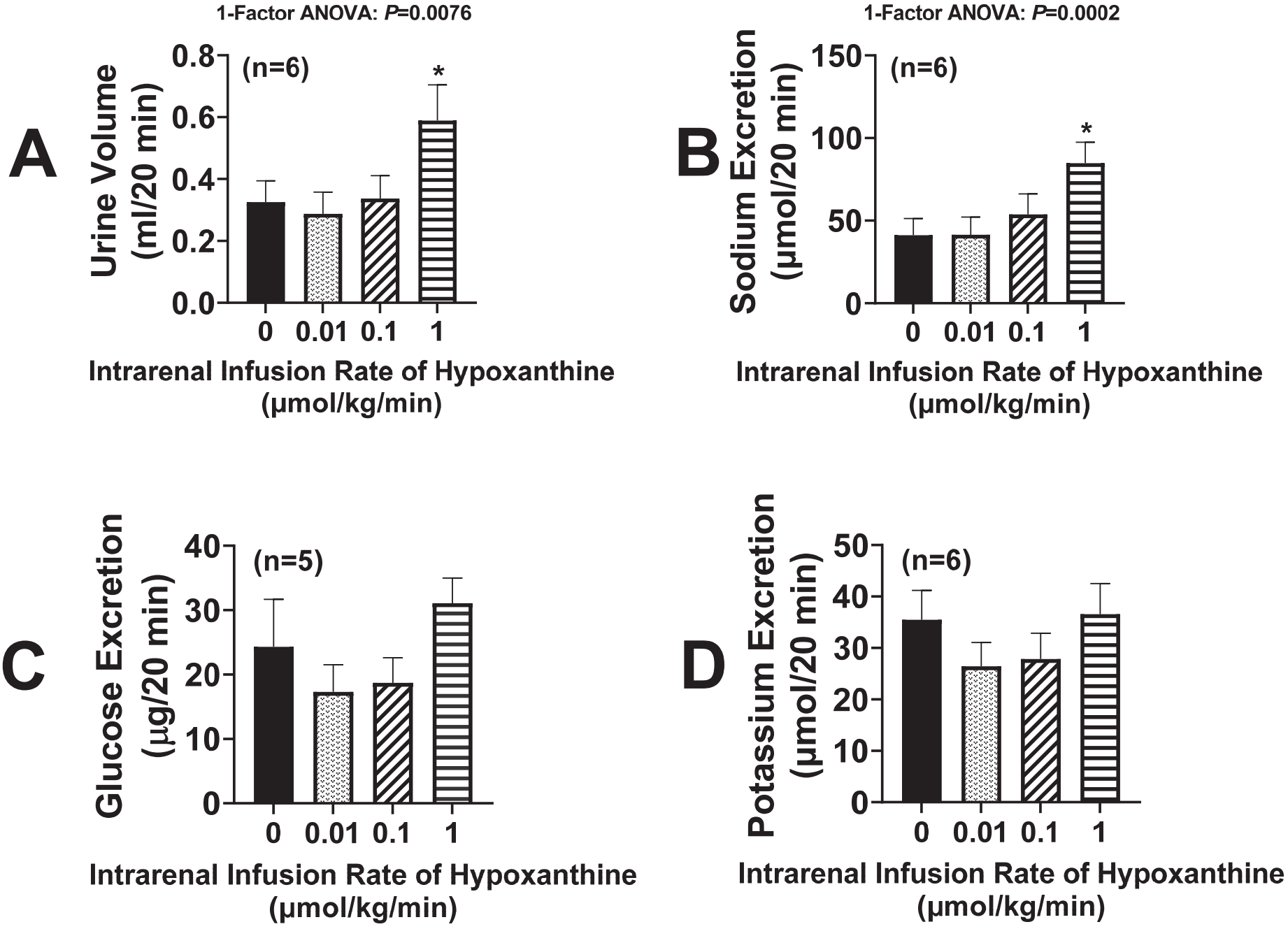

A
1-Factor ANOVA: P=0.0076
Urine Volume (ml/20 min)
0.8
0.6
0.4
0.2
0.0
(n=6)
*
0
0.01
0.1
1
Intrarenal Infusion Rate of Hypoxanthine (µmol/kg/min)
B
1-Factor ANOVA: P=0.0002
Sodium Excretion (µmol/20 min)
150
100
50
0
(n=6)
*
0
0.01
0.1
1
Intrarenal Infusion Rate of Hypoxanthine (µmol/kg/min)
C
Glucose Excretion (µg/20 min)
40
30
20
10
0
(n=5)
0
0.01
0.1
1
Intrarenal Infusion Rate of Hypoxanthine (µmol/kg/min)
D
Potassium Excretion (µmol/20 min)
50
40
30
20
10
0
(n=6)
0
0.01
0.1
1
Intrarenal Infusion Rate of Hypoxanthine (µmol/kg/min)

Figure S5

Figure S5. Effects of intrarenal artery infusions of hypoxanthine on renal excretory function. Hypoxanthine 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/min]. Each dose of hypoxanthine was administered for 30 min, timed collections of urine were obtained from the ureter between 10 and 30 min after initiating a given dose of hypoxanthine, and urine volumes (A) and excretion rates of sodium (B), glucose (C) and potassium (D) were determined. At the highest dose, hypoxanthine increased urine volume (P=0.0076) and sodium excretion (P=0.0002). Values are means and SEMs for the indicated sample size (n).

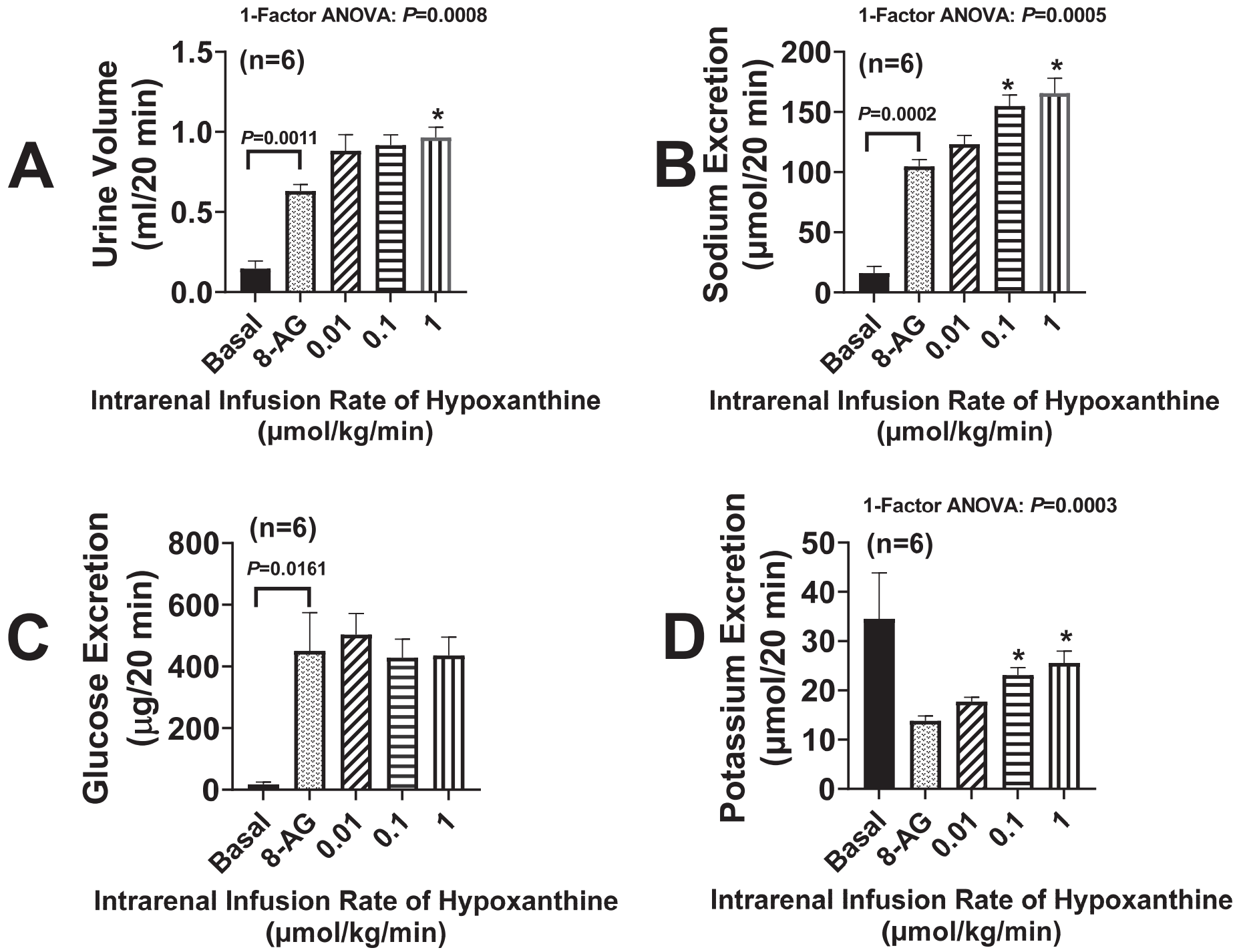
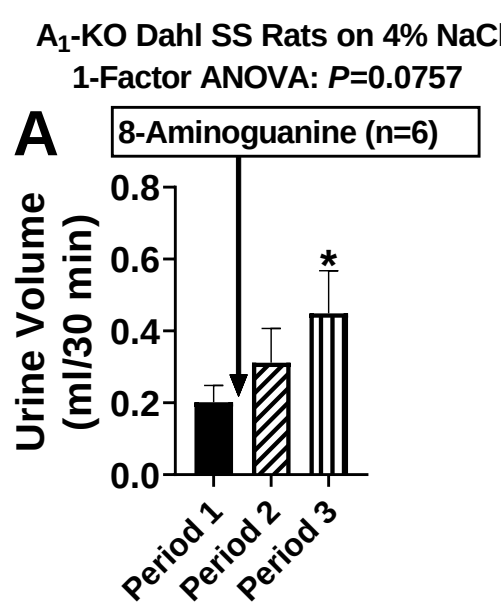
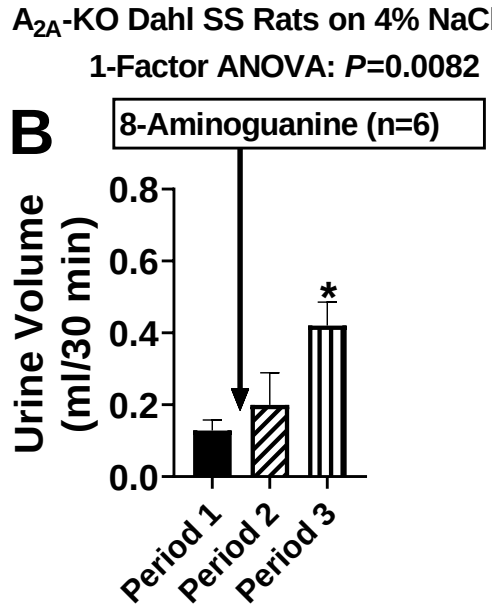
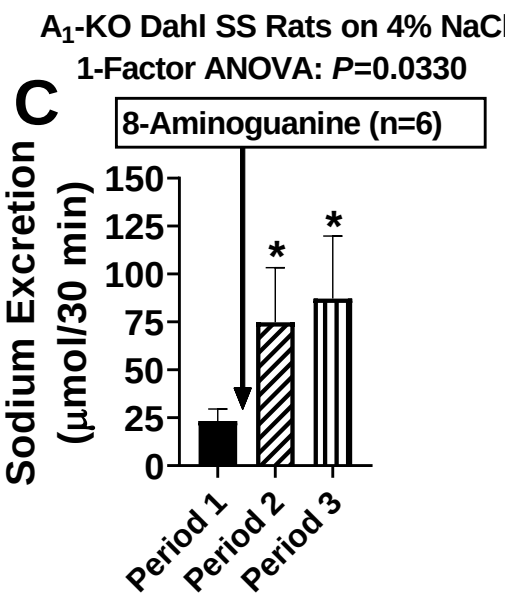

A
1-Factor ANOVA: P=0.0008
Urine Volume (ml/20 min)
(n=6)
P=0.0011
1.5
1.0
0.5
0.0
Basal
8-AG
0.01
0.1
1
Intrarenal Infusion Rate of Hypoxanthine (µmol/kg/min)
B
1-Factor ANOVA: P=0.0005
Sodium Excretion (µmol/20 min)
(n=6)
P=0.0002
200
150
100
50
0
Basal
8-AG
0.01
0.1
1
Intrarenal Infusion Rate of Hypoxanthine (µmol/kg/min)
C
Glucose Excretion (µg/20 min)
(n=6)
P=0.0161
800
600
400
200
0
Basal
8-AG
0.01
0.1
1
Intrarenal Infusion Rate of Hypoxanthine (µmol/kg/min)
D
1-Factor ANOVA: P=0.0003
Potassium Excretion (µmol/20 min)
(n=6)
50
40
30
20
10
0
Basal
8-AG
0.01
0.1
1
Intrarenal Infusion Rate of Hypoxanthine (µmol/kg/min)

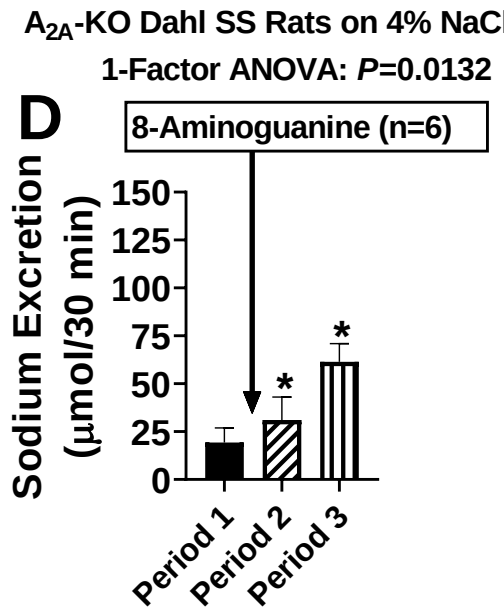
Figure S6

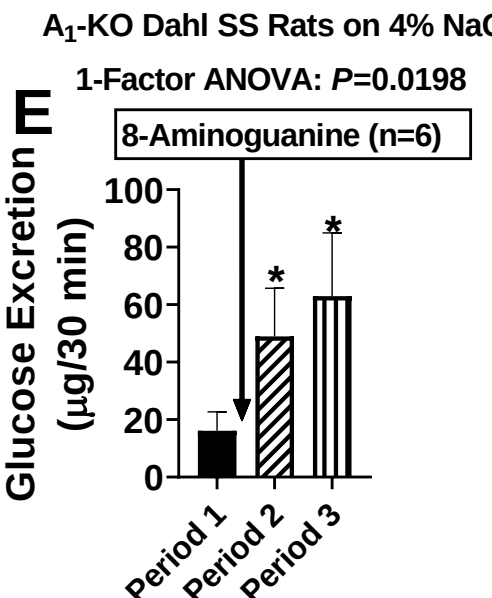
Figure S6. Effects of intrarenal artery infusions of hypoxanthine on renal excretory function in rats pretreated with 8-aminoguanine. Urine was collected for 20 min (Basal) from the ureter of anesthetized rats. Rats then received an intravenous injection of 8-aminoguanine (33.5 µmoles/kg). One hour later another 20-min urine collection was obtained (8-AG). Next, hypoxanthine was infused directly into the renal artery at increasing doses (0.01, 0.1 and 1 µmol/kg/min). Each dose of hypoxanthine was administered for 30 min, timed collections of urine were obtained from the ureter between 10 and 30 min after initiating a given dose of hypoxanthine, and urine volumes (A) and excretion rates of sodium (B), glucose (C) and potassium (D) were determined. 8-Aminoguanine increased urine volume (P=0.0011), sodium excretion (P=0.0002), and glucose excretion (P=0.0161), and tended to decrease potassium excretion. Compared to the 8-aminoguanine baseline, hypoxanthine increased urine volume (P=0.0008) and excretion of sodium (P=0.0005) and potassium (P=0.0003), but not glucose. Values are means and SEM for the indicated sample size (n).


A1-KO Dahl SS Rats on 4% NaCl Diet
1-Factor ANOVA: P=0.0757
A
8-Aminoguanine (n=6)
Urine Volume (ml/30 min)
0.0
0.2
0.4
0.6
0.8
*
Period 1
Period 2
Period 3


A2A-KO Dahl SS Rats on 4% NaCl Diet
1-Factor ANOVA: P=0.0082
B
8-Aminoguanine (n=6)
Urine Volume (ml/30 min)
0.0
0.2
0.4
0.6
0.8
*
Period 1
Period 2
Period 3


A1-KO Dahl SS Rats on 4% NaCl Diet
1-Factor ANOVA: P=0.0330
C
8-Aminoguanine (n=6)
Sodium Excretion (µmol/30 min)
0
25
50
75
100
125
150
*
*
Period 1
Period 2
Period 3


A2A-KO Dahl SS Rats on 4% NaCl Diet
1-Factor ANOVA: P=0.0132
D
8-Aminoguanine (n=6)
Sodium Excretion (µmol/30 min)
0
25
50
75
100
125
150
*
*
Period 1
Period 2
Period 3


A1-KO Dahl SS Rats on 4% NaCl Diet
1-Factor ANOVA: P=0.0198
E
8-Aminoguanine (n=6)
Glucose Excretion (µg/30 min)
0
20
40
60
80
100
*
*
Period 1
Period 2
Period 3

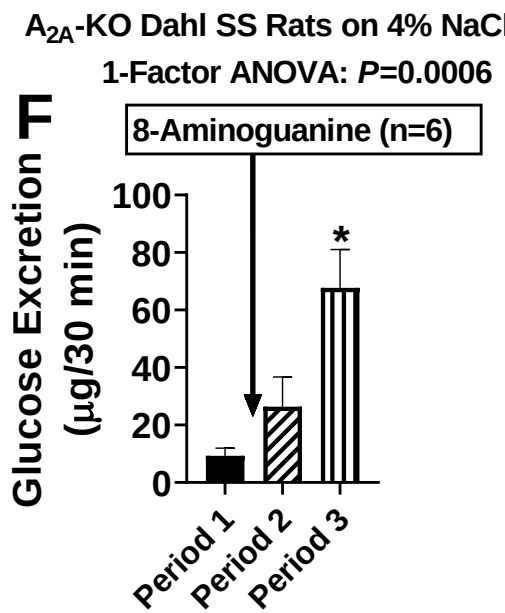

A2A-KO Dahl SS Rats on 4% NaCl Diet
1-Factor ANOVA: P=0.0006
F
8-Aminoguanine (n=6)
Glucose Excretion (µg/30 min)
0
20
40
60
80
100
*
Period 1
Period 2
Period 3

Figure S7

Figure S7. Effects of 8-aminoguanine in Dahl salt sensitive rats with knockout of A_1 (A_1-KO Dahl SS rats) or A_{2A} (A_{2A}-KO Dahl SS rats) receptors. A_1-KO Dahl SS rats (A,C,E) and A_{2A}-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 0-30 min (Period 1), 40-70 min (Period 2) and 85-115 min (Period 3) into the protocol. Rats received an intravenous injection of 8-aminoguanine (33.5 µmoles/kg) immediately after Period 1. Urine volumes (A,B) and sodium (C,D) and glucose (E,F) excretion rates were determined. Values are means and SEMs for the indicated sample size (n). ANOVA, analysis of variance; *indicates $P<0.05$ vs Period 1.

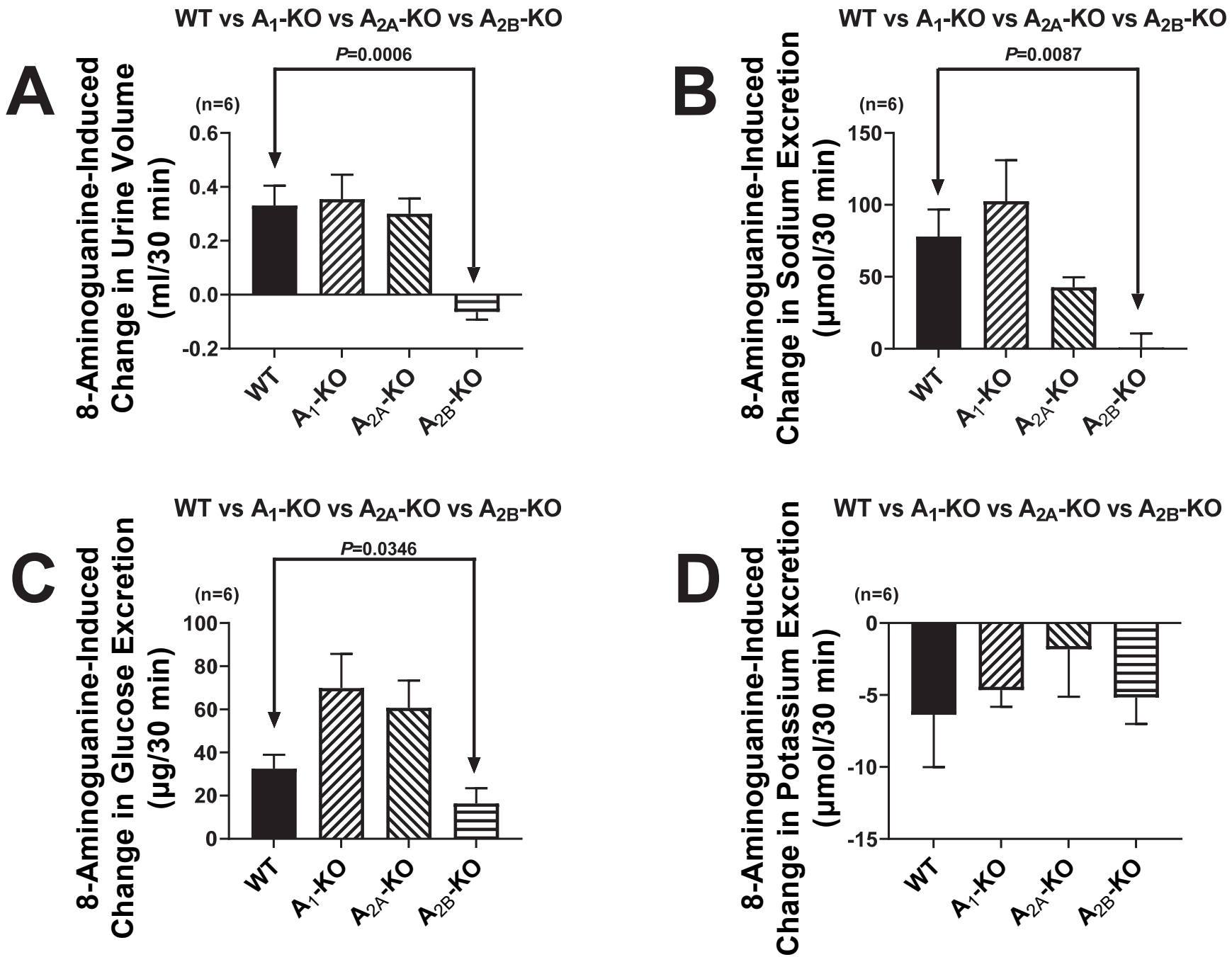

A
WT vs A1-KO vs A2A-KO vs A2B-KO
P=0.0006
(n=6)
8-Aminoguanine-Induced Change in Urine Volume (ml/30 min)
0.6
0.4
0.2
0.0
-0.2
WT
A1-KO
A2A-KO
A2B-KO
B
WT vs A1-KO vs A2A-KO vs A2B-KO
P=0.0087
(n=6)
8-Aminoguanine-Induced Change in Sodium Excretion (µmol/30 min)
150
100
50
0
WT
A1-KO
A2A-KO
A2B-KO
C
WT vs A1-KO vs A2A-KO vs A2B-KO
P=0.0346
(n=6)
8-Aminoguanine-Induced Change in Glucose Excretion (µg/30 min)
100
80
60
40
20
0
WT
A1-KO
A2A-KO
A2B-KO
D
WT vs A1-KO vs A2A-KO vs A2B-KO
(n=6)
8-Aminoguanine-Induced Change in Potassium Excretion (µmol/30 min)
0
-5
-10
-15
WT
A1-KO
A2A-KO
A2B-KO

Figure S8

Figure S8. Effects of 8-aminoguanine on renal excretory function in wild-type Dahl salt-sensitive rats (WT Dahl SS rats) versus Dahl salt sensitive rats with knockout of either A_1, A_{2A} or A_{2B} receptors (A_1-KO Dahl SS, A_{2A}-KO Dahl SS and A_{2B}-KO Dahl SS rats, respectively). 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 ureter from 0-30 min (Period 1), 40-70 min (Period 2) and 85-115 min (Period 3) into the protocol, and urine volumes and excretion rates of sodium, glucose and potassium were determined. Rats received an intravenous injection of 8-aminoguanine (33.5 μmoles/kg) immediately after Period 1. Shown are the changes (Period 3 minus Period 1) in urine volumes (A) and excretion rates of sodium (B), glucose (C) and potassium (D) induced by 8-aminoguanine in the four groups. 8-Aminoguanine-induced changes in urine volume and sodium excretion rate were similar in WT, A_1-KO and A_{2A}-KO Dahl SS rats. In contrast, 8-aminoguanine-induced changes in urine volume and sodium excretion were abolished (P=0.0006 and P=0.0087, respectively) in A_{2B}-KO Dahl SS rats, and the 8-aminoguanine-induced change in glucose excretion was significantly suppressed (P=0.0346) in A_{2B}-KO Dahl SS rats. As expected, 8-aminoguanine-induced changes in potassium excretion were similar among the four groups. Values are means and SEMs for the indicated sample size (n). P-values are from unpaired t-tests; *indicates $P<0.05$ vs Period 1.

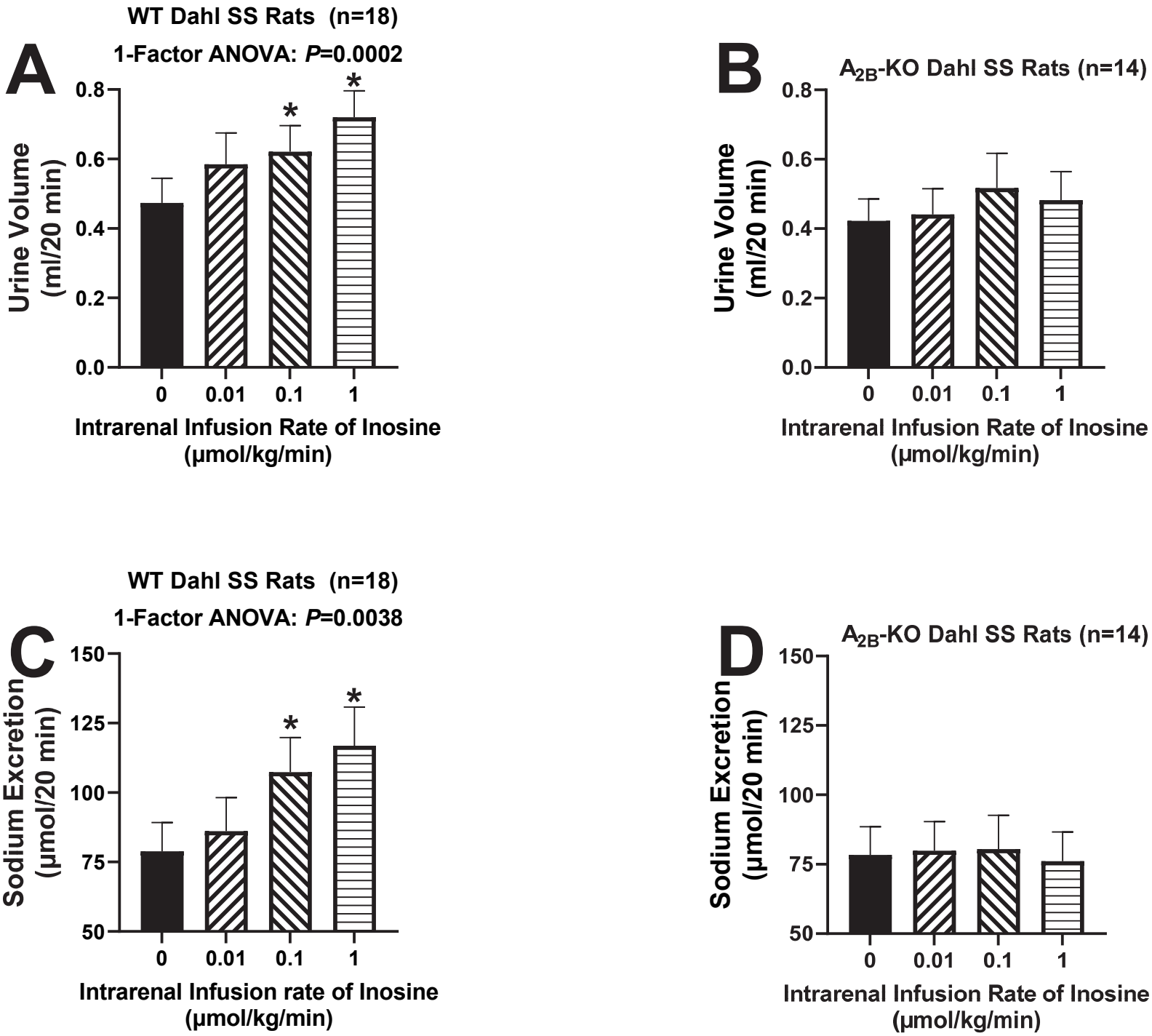

A
WT Dahl SS Rats (n=18)
1-Factor ANOVA: P=0.0002
Urine Volume (ml/20 min)
0.0
0.2
0.4
0.6
0.8
*
*
0
0.01
0.1
1
Intrarenal Infusion Rate of Inosine (µmol/kg/min)
B
A2B-KO Dahl SS Rats (n=14)
Urine Volume (ml/20 min)
0.0
0.2
0.4
0.6
0.8
0
0.01
0.1
1
Intrarenal Infusion Rate of Inosine (µmol/kg/min)
C
WT Dahl SS Rats (n=18)
1-Factor ANOVA: P=0.0038
Sodium Excretion (µmol/20 min)
50
75
100
125
150
*
*
0
0.01
0.1
1
Intrarenal Infusion rate of Inosine (µmol/kg/min)
D
A2B-KO Dahl SS Rats (n=14)
Sodium Excretion (µmol/20 min)
50
75
100
125
150
0
0.01
0.1
1
Intrarenal Infusion Rate of Inosine (µmol/kg/min)

Figure S9

Figure S9. Effects of intrarenal artery infusions of inosine on urine volume and sodium excretion rate in wild-type Dahl salt-sensitive rats (WT Dahl SS rats) versus Dahl SS rats with knockout of A_{2B} receptors (A_{2B}-KO Dahl SS rats). Inosine was infused directly into the renal artery of anesthetized WT Dahl SS rats (A,C) or A_{2B}-KO Dahl SS rats (B,D) at increasing doses [0 (vehicle only; basal), 0.01, 0.1 and 1 µmol/kg/min]. Each dose of inosine was administered for 30 min, timed collections of urine were obtained from the ureter between 10 and 30 min after initiating a given dose of inosine, and urine volumes (A,B) and sodium excretion rates (C,D) were determined. Inosine increased urine volume (P=0.0002) and sodium excretion (P=0.0038) in WT, but not A_{2B}-KO, Dahl SS rats. Values are means and SEM for the indicated sample size (n). ANOVA, analysis of variance; *P<0.05 vs 0 (vehicle only; basal).

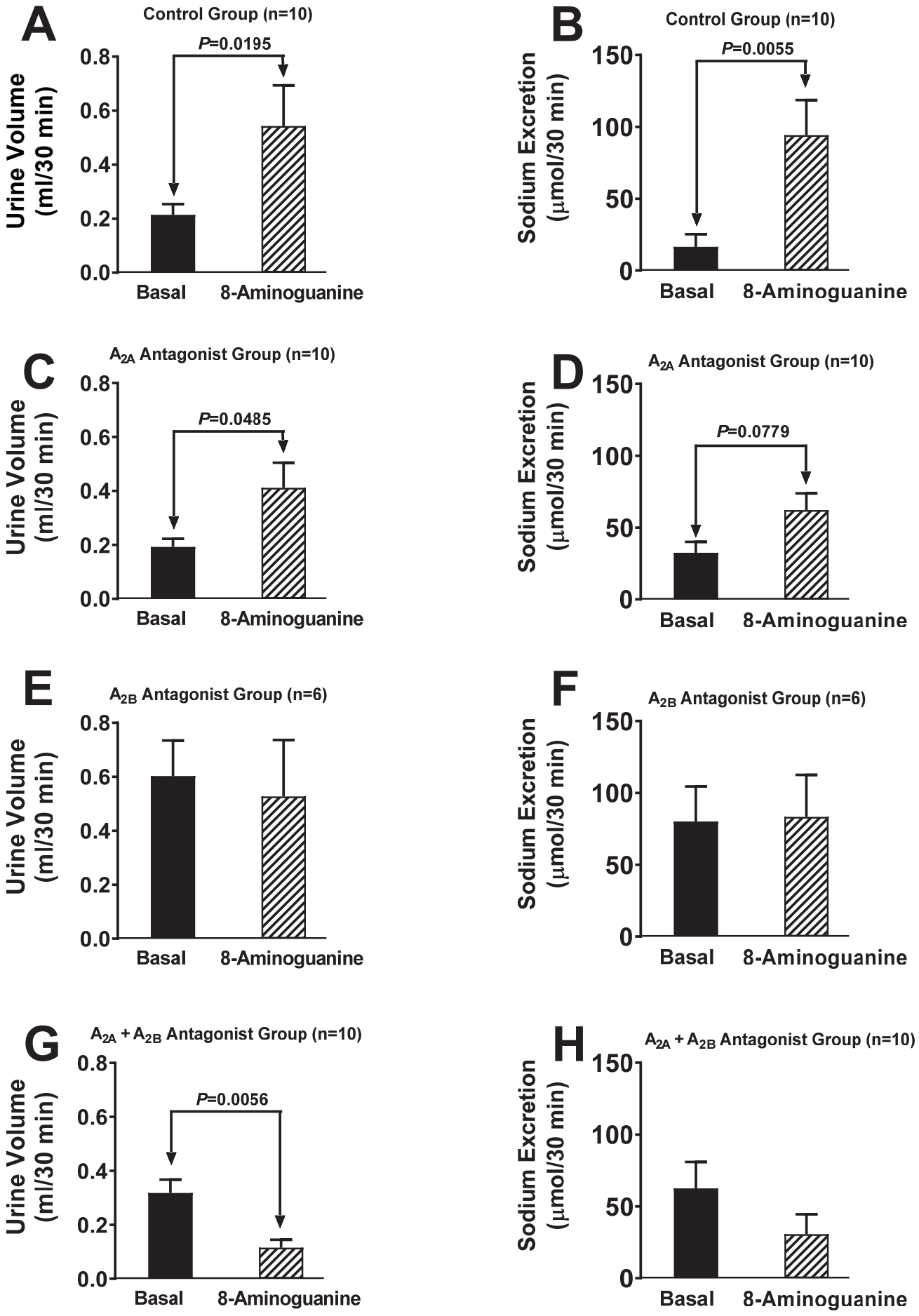

A
Control Group (n=10)
P=0.0195
Urine Volume (ml/30 min)
0.8
0.6
0.4
0.2
0.0
Basal
8-Aminoguanine
B
Control Group (n=10)
P=0.0055
Sodium Excretion (μmol/30 min)
150
100
50
0
Basal
8-Aminoguanine
C
A2A Antagonist Group (n=10)
P=0.0485
Urine Volume (ml/30 min)
0.8
0.6
0.4
0.2
0.0
Basal
8-Aminoguanine
D
A2A Antagonist Group (n=10)
P=0.0779
Sodium Excretion (μmol/30 min)
150
100
50
0
Basal
8-Aminoguanine
E
A2B Antagonist Group (n=6)
Urine Volume (ml/30 min)
0.8
0.6
0.4
0.2
0.0
Basal
8-Aminoguanine
F
A2B Antagonist Group (n=6)
Sodium Excretion (μmol/30 min)
150
100
50
0
Basal
8-Aminoguanine
G
A2A + A2B Antagonist Group (n=10)
P=0.0056
Urine Volume (ml/30 min)
0.8
0.6
0.4
0.2
0.0
Basal
8-Aminoguanine
H
A2A + A2B Antagonist Group (n=10)
Sodium Excretion (μmol/30 min)
150
100
50
0
Basal
8-Aminoguanine

Figure S10

Figure S10. Effects of 8-aminoguanine on urine volume and sodium excretion 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,B; Control Group], 10 mg/kg of ZM 241385 (C,D; A_{2A} Antagonist Group), 30 mg/kg of PSB 1115 (E,F; A_{2B} Antagonist Group) or ZM 241385 plus PSB 1115 (G,H; A_{2A} + A_{2B} Antagonist Group), and 15 min later timed (30-min) urine collections were obtained from the ureter (Basal). Next, rats received an intravenous injection of 8-aminoguanine (33.5 μmoles/kg), and after approximately 1 hr 30-min urine collections were again obtained (8-Aminoguanine). Urine volumes (A,C,E,G) and sodium excretion rates (B,D,F,H) were determined. *P*-values are from paired t-tests. Values are means and SEMs for the indicated sample size (n).

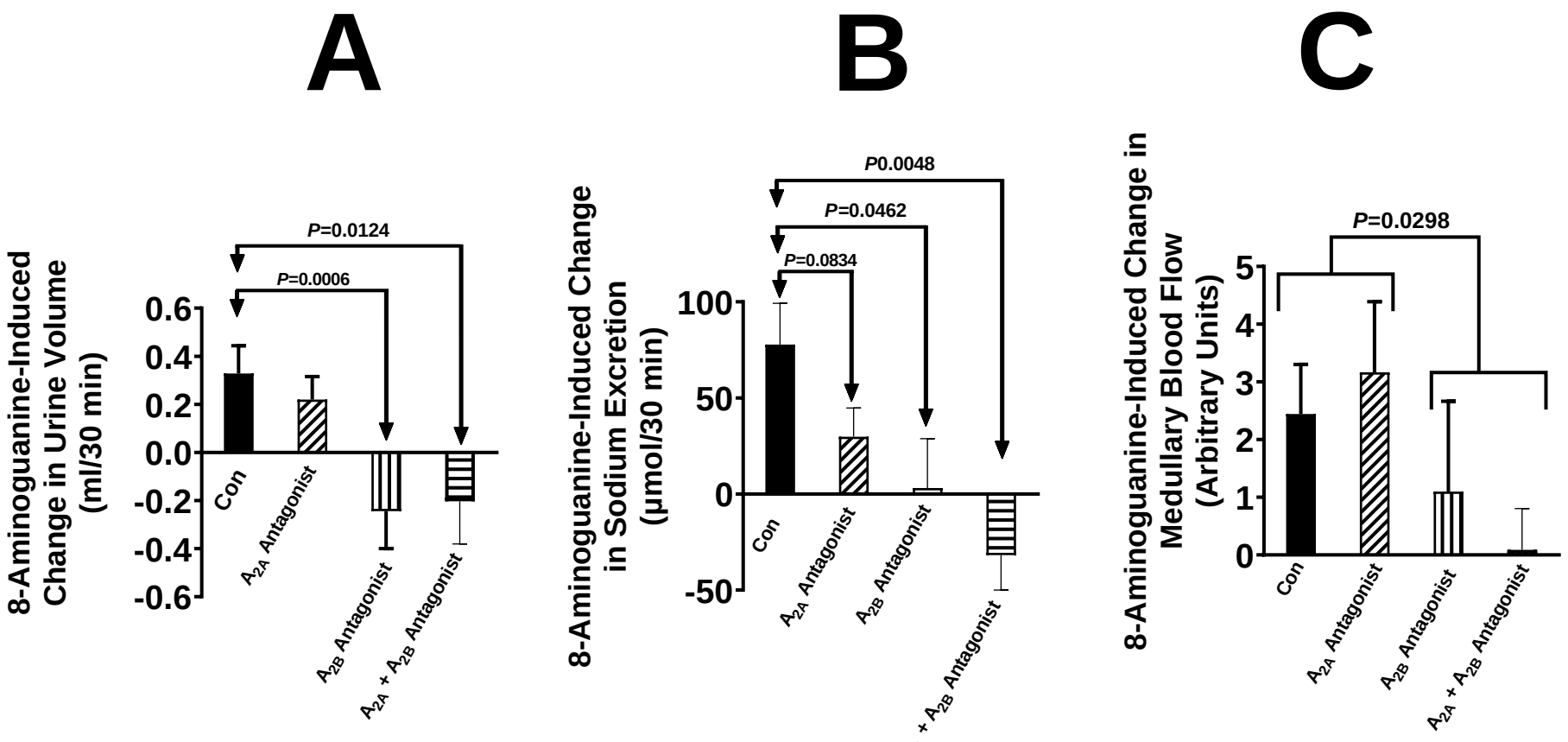

A
B
C
8-Aminoguanine-Induced Change in Urine Volume (ml/30 min)
P=0.0124
P=0.0006
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
Con
A2A Antagonist
A2B Antagonist
A2A + A2B Antagonist
8-Aminoguanine-Induced Change in Sodium Excretion (µmol/30 min)
P0.0048
P=0.0462
P=0.0834
100
50
0
-50
Con
A2A Antagonist
A2B Antagonist
A2A + A2B Antagonist
8-Aminoguanine-Induced Change in Medullary Blood Flow (Arbitrary Units)
P=0.0298
5
4
3
2
1
0
Con
A2A Antagonist
A2B Antagonist
A2A + A2B Antagonist

Figure S11

Figure S11. Effects of 8-aminoguanine on renal excretory function and medullary blood flow 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 [Control (Con) Group], 10 mg/kg of ZM 241385 (A_{2A} Antagonist Group), 30 mg/kg of PSB 1115 (A_{2B} Antagonist Group) or ZM 241385 plus PSB 1115 (A_{2A} + A_{2B} Antagonist Group), and 15 min later timed (30 min) urine collections were obtained from the left ureter. Next, rats received an intravenous injection of 8-aminoguanine (33.5 µmoles/kg), and after approximately 1 hr 30-min urine collections were again obtained. Shown are the changes in urine volume (A), sodium excretion rate (B) and medullary blood flow (C) induced by 8-aminoguanine in the four groups. 8-Aminoguanine-induced changes in urine volume, sodium excretion rate and medullary blood flow were abolished in the A_{2B} Group and A_{2A} + A_{2B} Group, but not in the A_{2A} Group. Values are means and SEMs for the indicated sample size (n). *P*-values are from unpaired t-tests.

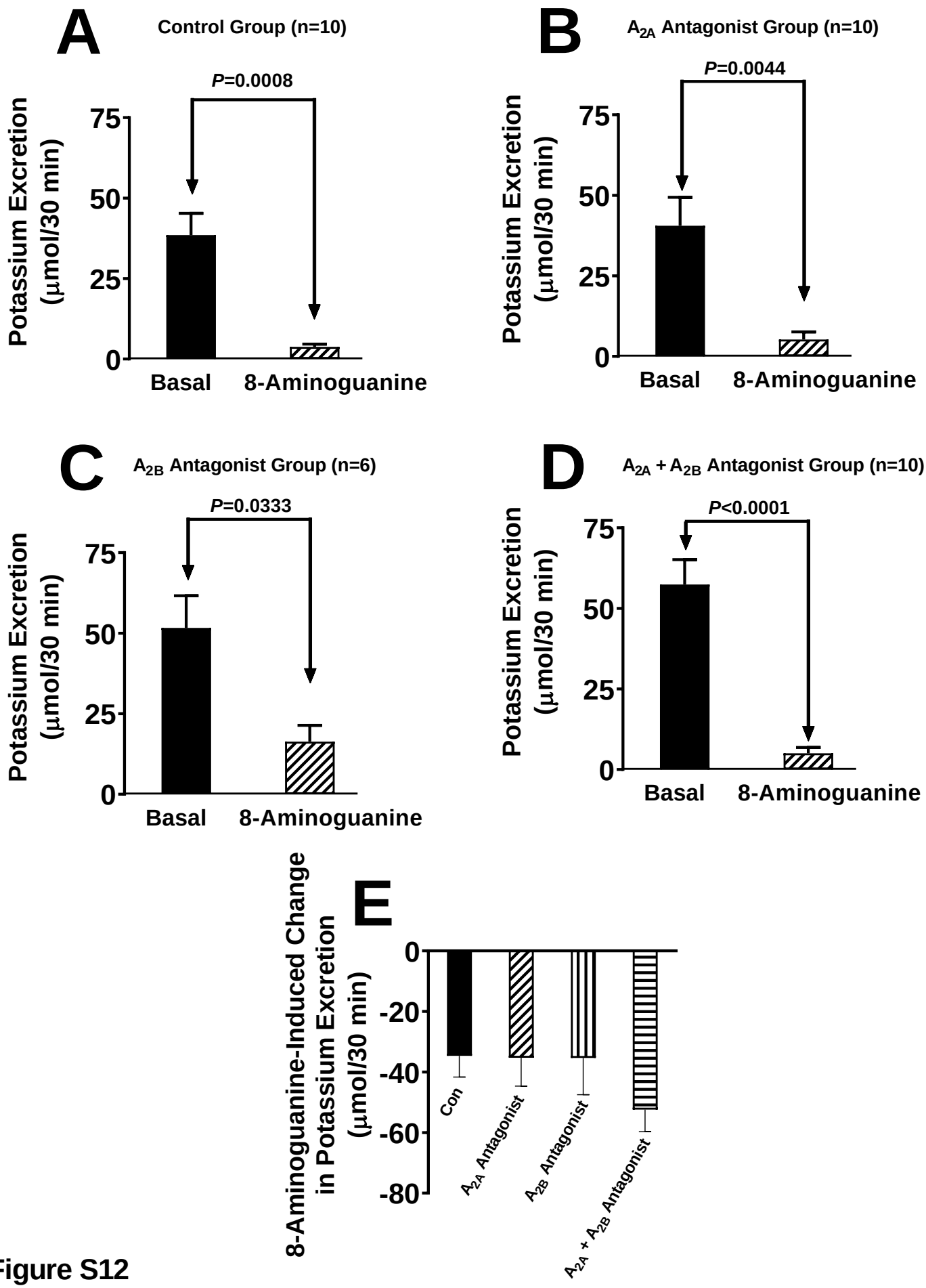

A
Control Group (n=10)
P=0.0008
Potassium Excretion (μmol/30 min)
75
50
25
0
Basal
8-Aminoguanine
B
A2A Antagonist Group (n=10)
P=0.0044
Potassium Excretion (μmol/30 min)
75
50
25
0
Basal
8-Aminoguanine
C
A2B Antagonist Group (n=6)
P=0.0333
Potassium Excretion (μmol/30 min)
75
50
25
0
Basal
8-Aminoguanine
D
A2A + A2B Antagonist Group (n=10)
P<0.0001
Potassium Excretion (μmol/30 min)
75
50
25
0
Basal
8-Aminoguanine
E
8-Aminoguanine-Induced Change in Potassium Excretion (μmol/30 min)
0
-20
-40
-60
-80
Con
A2A Antagonist
A2B Antagonist
A2A + A2B Antagonist

Figure S12

Figure S12. Effects of 8-aminoguanine on potassium excretion rates 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 (Con) 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 min later timed (30 min) urine collections were obtained from the ureter and potassium excretion rates were determined (Basal). Next, rats received an intravenous injection of 8-aminoguanine (33.5 µmoles/kg), and approximately 1 hr later 30 min urine collections were again obtained for measurement of potassium excretion rates (8-Aminoguanine). Also shown are the changes in potassium excretion rates induced by 8-aminoguanine in the four groups (E). 8-Aminoguanine decreased potassium excretion in the Control Group (P=0.0008), the A_{2A} Antagonist Group (P=0.0044), the A_{2B} Antagonist Group (P=0.0333) and the A_{2A} + A_{2B} Antagonist Group (P<0.0001). Also, the 8-aminoguanine-induced change in potassium excretion was similar among the four groups. P-values are from paired t-tests. Values are means and SEMs for the indicated sample size (n).

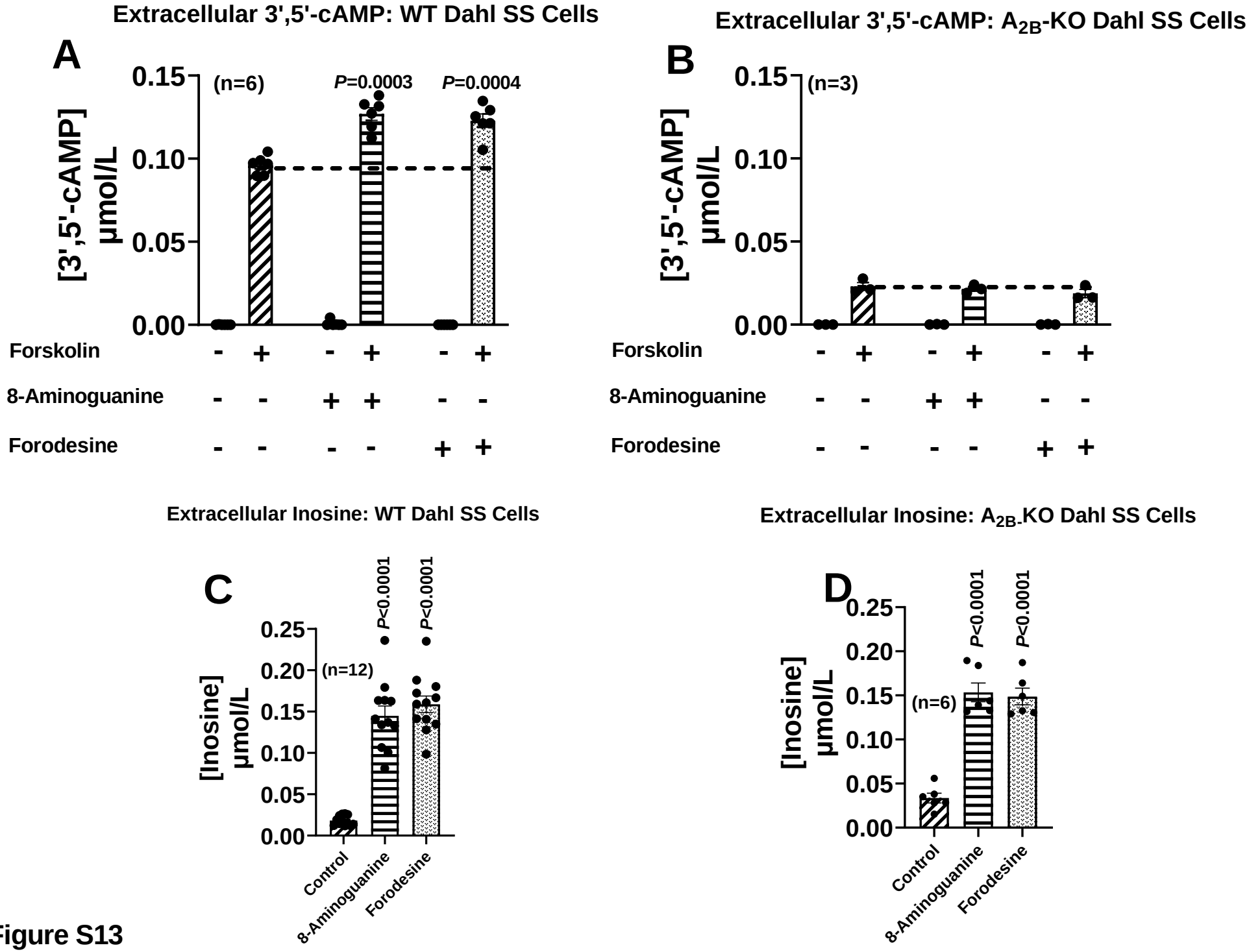

Extracellular 3',5'-cAMP: WT Dahl SS Cells
A
[3',5'-cAMP] µmol/L
0.15
0.10
0.05
0.00
(n=6)
P=0.0003
P=0.0004
Forskolin - + - + - +
8-Aminoguanine - - + + - -
Forodesine - - - - + +
Extracellular 3',5'-cAMP: A2B-KO Dahl SS Cells
B
[3',5'-cAMP] µmol/L
0.15
0.10
0.05
0.00
(n=3)
Forskolin - + - + - +
8-Aminoguanine - - + + - -
Forodesine - - - - + +
Extracellular Inosine: WT Dahl SS Cells
C
[Inosine] µmol/L
0.25
0.20
0.15
0.10
0.05
0.00
(n=12)
P<0.0001
P<0.0001
Control
8-Aminoguanine
Forodesine
Extracellular Inosine: A2B-KO Dahl SS Cells
D
[Inosine] µmol/L
0.25
0.20
0.15
0.10
0.05
0.00
(n=6)
P<0.0001
P<0.0001
Control
8-Aminoguanine
Forodesine

Figure S13

Figure S13. Effects of 8-aminoguanine and forodesine on 3',5'-cAMP and inosine levels in renal microvascular smooth muscle cells (RMSMCs) from wild-type Dahl salt-sensitive rats (WT Dahl SS rats) versus Dahl SS rats with knockout of A_{2B} receptors (A_{2B}-KO Dahl SS rats). 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 min with vehicle (-) or forskolin (+; sensitizes 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 the presence of forskolin, both 8-aminoguanine (P=0.0003) and forodesine (P=0.0004) increased levels of 3',5'-cAMP in WT RMSMCs, but not in A_{2B}-KO RMSMCs. However, regardless of presence or absence of forskolin, both 8-aminoguanine and forodesine increased levels of inosine in both WT(P<0.0001) and A_{2B}-KO (P<0.0001) RMSMCs. P-values are from unpaired t-tests. Values are means and SEMs for the indicated sample size (n).