Adenosine formed by 5′-nucleotidase mediates tubuloglomerular feedback

Scott Thomson,[1] Dingjiu Bao,[1] Aihua Deng,[1] and Volker Vallon[2]

[1]Department of Medicine, University of California San Diego and San Diego Veterans Affairs Medical Center, San Diego, California, USA
[2]Department of Pharmacology, University of Tuebingen, Tuebingen, Germany

Address correspondence to: Scott Thomson, Division of Nephrology/Hypertension, University of California San Diego and San Diego Veterans Affairs Medical Center, 3350 La Jolla Village Drive, San Diego, California 92161-9151, USA. Phone: (858) 552-8585 xt 3445; Fax: (858) 552-7549; E-mail: sthomson@ucsd.edu.

Received for publication October 22, 1999, and accepted in revised form May 30, 2000.

Nephron function is stabilized by tubuloglomerular feedback (TGF). TGF operates within the juxtaglomerular apparatus, sensing changes in tubular flow and eliciting compensatory changes in single nephron GFR (SNGFR). The mediator(s) of TGF remains unconfirmed. One theory is that ATP consumed in active transport by the macula densa leads to formation of adenosine, which causes glomerular vasoconstriction. We performed micropuncture in rats to test this hypothesis. Adenosine activity was manipulated by microperfusing nephrons with adenosine A1 receptor blocker, A1-agonist, or 5′-nucleotidase inhibitor. Effects on TGF were characterized by changes in TGF efficiency (the compensation for small perturbations in tubular flow) and by changes in the maximum range over which TGF can cause SNGFR to change. These data were further applied to generate TGF profiles [SNGFR versus late proximal flow (V_{LP})]. TGF efficiency was significantly reduced by blocking A1-receptors. TGF efficiency, TGF range, and the slope of the TGF profile ($\Delta SNGFR/\Delta V_{LP}$) were all significantly reduced by blocking 5′-nucleotidase. When adenosine activity was clamped by combining 5′-nucleotidase inhibitor with A1-agonist to determine whether TGF requires adenosine to be present or to fluctuate, the TGF slope was reduced by 83%, indicating that adenosine activity must fluctuate for normal TGF to occur and that adenosine is a mediator of TGF.

J. Clin. Invest. **106**:289–298 (2000).

Introduction

Glomerular filtration is coupled to tubular reabsorption by a system of tubuloglomerular feedback (TGF). TGF operates within the juxtaglomerular apparatus (JGA) of each nephron, where changes in the salt content of tubular fluid at the end of Henle's loop are sensed and transmitted to the glomerular microvasculature to evoke compensatory changes in single nephron GFR (SNGFR). The molecular mediator(s) of TGF has not been confirmed, but one of the main theories to account for TGF is the so-called adenosine hypothesis. According to the adenosine hypothesis, the consumption of ATP during NaCl transport across the macula densa causes adenosine to accumulate in the region of the macula densa, from where it diffuses to the vascular pole of the glomerulus and causes vasoconstriction of the preglomerular arteriole (1, 2). This hypothesis has teleologic appeal because linking SNGFR to the ATP/ADP ratio in the tubule will protect the tubule against accruing negative energy balance regardless of whether energy balance is threatened by an increase in SNGFR or by a decrease in the ability of the tubule to generate ATP. In organs other than the kidney (especially the coronary circulation), adenosine is well established as an endogenous vasodilator that ensures blood flow to match metabolic demand. The adenosine hypothesis of TGF postulates a role for adenosine that is analogous to other organs in the sense of providing a link between blood flow and metabolism. However, the adenosine hypothesis of TGF requires adenosine to cause renal vasoconstriction, not vasodilation. This is because blood flow in the kidney is a main determinant of metabolic work.

There are published data consistent with the adenosine hypothesis of TGF. For example, activation of adenosine A1 receptors with exogenous agonists causes vasoconstriction of the preglomerular arterioles (3) and postischemic renal vasoconstriction can be blocked with A1-receptor antagonists (4). Furthermore, endogenous adenosine must be present for TGF to function normally, as A1 receptors blockers can prevent the TGF–mediated decline in glomerular capillary pressure that normally occurs when the loop of Henle is perfused at supraphysiologic flow rates with artificial tubular fluid (2). However, the fact that adenosine must be present in order to elicit the maximum TGF–mediated change in glomerular capillary pressure need not imply that adenosine mediates TGF. For example, it is possible for TGF to affect SNGFR independent of glomerular capillary pressure (5, 6), and there are other vasoconstrictors, most notably angiotensin II, that enhance the TGF–mediated change in glomerular capillary pressure but do not mediate the TGF response (6–8).

The present studies were performed to test the adenosine hypothesis more definitively. A variety of in vivo micropuncture approaches were used to measure the incremental TGF response to small perturbations in ambient tubular flow and to assess the maximum range over which SNGFR can be made to change by manipulating TGF. To exclude the possibility that adenosine is a mere background requirement of the TGF system, drugs were administered at the single nephron level in various combinations to block adenosine A1 receptors, prevent adenosine formation by 5′-nucleotidase, or clamp adenosine activity. The results of these experiments confirm that adenosine is a mediator of TGF.

Methods

Overview. Micropuncture experiments were performed in hydropenic male Wistar and Wistar-Froemter rats. The adenosine axis was manipulated at the single nephron level by infusing drug(s) into the tubular lumen. In most experiments, perfusion was orthograde from the late proximal tubule. In some experiments, perfusion was retrograde from the early distal tubule. Some experiments were performed in free-flowing nephrons to study the behavior of TGF near to the natural operating point of the nephron. In other experiments, tubular flow was interrupted by inserting a wax block into the tubule, and then flow into the loop of Henle was regulated by microperfusion. These wax-block experiments enabled us to determine the maximum range of the TGF response and to assess the contributions of proximal tubule and loop of Henle reabsorption to the apparent TGF response. Animal experimentation was conducted in accord with NIH standards for the care and use of laboratory animals.

Surgical preparation for micropuncture. Studies were performed in male Wistar rats purchased from Harlan-Sprague-Dawley (Indianapolis, Indiana, USA) and in adult male Wistar-Froemter rats from a breeding colony at the University of Tuebingen. Inactin anesthesia, surgical preparation, equilibration, and monitoring during micropuncture were performed according to protocols as described previously (9). Hydropenia was maintained by infusing Ringer's saline at 2.0 mL/h. [^{3}H]Inulin (80 μCi/mL) was added as a marker of GFR. Tubular perfusion was performed with Hampel nanoliter pumps (University of Tuebingen) and artificial tubular fluid (ATF). ATF for orthograde perfusion was of the following composition: 130 mM NaCl, 10 mM $NaHCO_3$, 4 mM KCl, and 2 mM $CaCl_2$, 45 mg/dl urea, and 0.1% FD&C green (pH 7.4). For retrograde perfusion, ATF was altered to resemble native early distal tubular fluid (see later discussion here).

Theoretical paradigm. The conceptual framework for TGF has been described in publications from this laboratory (10–14). Briefly, the TGF system operates as a negative feedback loop in which two parameters [SNGFR and late proximal flow (V_{LP})] are linked by TGF and glomerulotubular balance (GTB). This can be depicted graphically by two curves that intersect at a single point in a plane defined by V_{LP} and SNGFR. This single point of intersection defines the natural operating point of the nephron. These relationships are represented by the following two equations in two unknowns, *SNGFR* and V_{LP}:

(Equation 1)

$$SNGFR = SNGFR_0\text{-}f_1(V_{LP})$$

(Equation 2)

$$V_{LP} = f_2(SNGFR)$$

where $SNGFR_0$ denotes SNGFR during zero flow past the macula densa; f_1 is a function of V_{LP} denoting the impact of TGF on SNGFR; and f_2 is a function of SNGFR that denotes GTB. The effect of TGF on SNGFR is nonlinear and saturable. In logistical models, the TGF curve, f_1, is represented by a sigmoid curve (15). Over the range of physiologically relevant SNGFR, f_2 approximates a straight line.

The tendency for the TGF system to stabilize nephron function increases with the product of the slopes of f_1 and f_2 at the operating point. This can be demonstrated by imagining the response to a perturbation in proximal tubular flow. When V_{LP} is perturbed, TGF will mediate a reciprocal change in SNGFR that will be reflected along the proximal tubule and back to the site where the perturbation was applied. The efficiency of TGF is defined by the fractional compensation, $C = -\Delta V_M/\Delta V_H$, where V_H is the applied perturbation and V_M is the flow immediately upstream from the perturbation. The system is now described by three equations in three unknowns, *SNGFR*, V_{LP}, and V_M:

(Equation 3)

$$SNGFR = SNGFR_0\text{-}f_1(V_{LP})$$

(Equation 4)

$$V_{LP} = f_2(SNGFR) + V_H$$

(Equation 5)

$$V_M = f_2(SNGFR)$$

Differentiation and substitution lead to the following expression for *C* in terms of f_1' and f_2':

(Equation 6)

$$C = \frac{-dV_M}{dV_H} = \frac{f'_1(V_{LP}) \cdot f'_2(SNGFR)}{1 + f'_1(V_{LP}) \cdot f'_2(SNGFR)}$$

A more detailed derivation of the relationships between *C*, *SNGFR*, V_{LP}, TGF, and GTB has been published (10).

The behavior of TGF was characterized in four steps. The first step involved studies analogous to the hypothetical case discussed in the derivation of Eq. 6. Flow was perturbed in free-flowing nephrons, and the TGF response was used to determine the product, $f_1'f_2'$. This reveals the behavior of TGF near the natural operating

point. Second, SNGFR and V_{LP} were measured during orthograde perfusion of Henle's loop in wax-blocked nephrons. Measurements were made in each nephron during high and low rates of loop perfusion. These data served two purposes. First, they established a range for f_1. Second, they permitted calculation of f_2'. Data from these two sets of experiments were combined to calculate the slope of the TGF curve, f_1', near the natural operating point. Third, experiments were performed to ascertain to what extent apparent changes in f_1' could result from changes in loop of Henle reabsorption. This was done by measuring net reabsorption of sodium, chloride, potassium, and water from Henle's loop during orthograde perfusion of the loop. Fourth, SNGFR was measured by proximal collection while manipulating salt concentration at the macula densa by retrograde perfusion with solutions containing different amounts of salt. The purpose of these experiments was to confirm that effects of drugs delivered by orthograde perfusion could be reproduced in experiments not susceptible to confounding by a requirement to vary the rate of drug delivery.

Experiments in free-flowing nephrons. A technique for testing the effects on TGF efficiency of drugs delivered at the single nephron level was used as described previously (11). Ambient proximal flow and the fractional compensation for ± 5 nL/min perturbations in ambient flow were measured in free-flowing late proximal nephrons before and during pharmacologic manipulation of the adenosine axis. Tubular flow was measured by a noninvasive optical technique (videometric flow velocitometry; VMFV) as described later here. Two Hampel nanoliter pumps were used, one to deliver drugs and the other to perturb flow. Both pumps were placed into the free-flowing late proximal tubule with the drug pump 30–50 μm downstream. V_M (see Eq. 5) was measured immediately upstream from the pipettes as V_H was applied by adding or subtracting fluid. Measurements were made before and during tubular infusion of drug(s). Predrug measurements were made for V_H(nL/min) = –5, 0, and +5. Then the drug pump was started at 5 nL/min. After 5 minutes, the perturbation cycle was repeated with the perturbation pump adjusted to compensate for the volume from the drug pump. Each perturbation was for 3 minutes, and data were collected over the last 50 seconds.

Videometric flow velocitometry. This technique was used to measure flow in unobstructed nephrons as described in several publications previously (5, 10–14). Small boluses of ATF containing rhodamine B dextran as a fluorescent marker were injected into the proximal tubule by a pneumatic microinjection pump. The dye was excited by a laser reflected onto the kidney surface. The magnified image of the kidney surface was filtered to maximize resolution of emitted fluorescence and monitored with a videomicroscope. The intensity of the video image was monitored at two points downstream along the nephron. The time required for fluid to traverse the two points was calculated by cross-correlating the videodensities. Geometry of the tubular segment was measured from digitized images. Tubular fluid flow rate, V_M, was calculated from the cross-correlations and geometry.

Orthograde perfusion in wax-blocked nephrons. These experiments were performed to achieve three objectives. The first objective was to establish the role of adenosine as a determinant of the maximum range over which TGF can cause SNGFR to change. The second objective was to determine the slope of the GTB function, f_2', so that the TGF slope, f_1', could be extracted from the fractional compensation (see Eq. 6). The third objective was to assess whether changes in f_1 resulted from events within the JGA rather than within the loop of Henle. The first two objectives were achieved with a single set of experiments. A microperfusion pipette containing ATF or ATF + drug(s) was placed in the most downstream visible segment of the proximal tubule. A wax block was inserted immediately upstream from the perfusion pipette. Perfusion was begun at 38 nL/min. After 4 minutes, a timed collection was made upstream from the wax block to determine SNGFR by [^{3}H]inulin clearance, and V_{LP} by volumetric assessment. Then, the perfusion rate was reduced to 4 nL/min, 2 minutes allowed for equilibration, and a second collection performed. When fluid was not being collected for measurement, the late proximal tubule was vented into another pipette to prevent stasis in the proximal tubule. The maximum effect of TGF on SNGFR was taken as the increase in SNGFR when loop of Henle perfusion was reduced from 38 to 4 nL/min. The GTB slope, f_2', was analyzed by correlating changes in V_{LP} with changes in SNGFR between the first and second collections in each nephron.

In other experiments, measurements were made of the volume, sodium, and potassium (microflame photometry; ref. 16), and chloride content (microelectrometric titration method; Microtitre ET-1, World Precision Instruments, Sarasota, Florida, USA; ref. 17) of fluid collected from the early distal nephron during orthograde perfusion of Henle's loop at 16 nL/min with ATF ± various drugs. This was done to confirm that the apparent effects of these drugs on TGF were not attributable to changes in loop reabsorption.

Retrograde perfusion experiments. These studies were performed to confirm that certain results obtained during orthograde perfusion of were not due to different amounts of drug reaching the macula densa at the different perfusion rates. A wax block was inserted into the early distal nephron, and Hampel microperfusion apparatus were used to deliver low-salt distal ATF ± drugs or high-salt distal ATF ± drugs by retrograde perfusion upstream from the wax block. Low-salt distal ATF contained 10 mM NaCl, 15 mM urea, and 0.1% FD&C green (pH 7.3). High-salt distal ATF contained 50 mM NaCl, 15 mM urea, and 0.1% FD&C green (pH 7.3). All perfusions were at 12.5 nL/min. A second wax block was placed in the proximal tubule. Perfusate was vented with a pipette distal to this wax block. SNGFR

was measured by timed collection upstream from this wax block. Measurements were made in each nephron during high-salt and low-salt perfusion.

Drug perfusion protocols. Adenosine (ADO) activity was manipulated by delivering the following drugs directly into the tubule: (a) adenosine A1-receptor agonist (cyclohexyladenosine [CHA] at 10–50 μM); (b) 5′-nucleotidase blocker (α, β methylene adenosine diphosphate [MADP], 1 mM in ATF; this drug blocks conversion of AMP to adenosine); (c) adenosine A1-receptor blocker (KW3902, 400 μM in ATF at 5 nL/min for free-flow studies, 40 μM in ATF for wax-blocked studies); (d) ADO clamp (MADP [1 mM] combined with CHA [10–50 μM for experiments in free-flowing nephrons, 2 μM for orthograde perfusion in wax-blocked nephrons, and 0.5–1.0 μM for retrograde perfusion]).

Calculations and statistical analyses. For data from free-flowing nephrons, fractional compensation was calculated for each nephron by linear regression of the tubular flow, V_M, against the applied perturbation, V_H.

For orthograde perfusion in wax-blocked nephrons, a maximum TGF response was calculated for each nephron as $\Delta SNGFR$ = SNGFRmax – SNGFRmin where SNGFRmax is the SNGFR obtained during loop perfusion at 4 nL/min and SNGFRmin is the SNGFR obtained during perfusion at 38 nL/min. Corresponding calculations were made for the change in V_{LP} (ΔV_{LP}) and the change in APR (ΔAPR). For each nephron, SNGFRmean was also calculated as SNGFRmean = 0.5 × (SNGFRmax + SNGFRmin). The TGF curve is commonly represented by a symmetric sigmoid (15). SNGFRmean corresponds to the value of SNGFR at the inflection point of that sigmoid curve. Intergroup comparisons were made by ANOVA with post hoc Tukey test for ΔSNGFR and for ΔSNGFR/SNGFRmean. (Normalizing the TGF response to SNGFRmean is more appropriate than the common practice of normalizing to SNGFRmax because the latter approach will introduce an apparent positive correlation between ΔSNGFR and SNGFRmax as an artifact of the random error in measuring SNGFRmax. Another justification for normalizing to SNGFRmean is that nephrons naturally operate near the TGF inflection point [5, 10], such that SNGFRmean is close to the starting point for changes in SNGFR that are mediated by TGF under natural circumstances.)

For each group, a composite slope for the GTB curve was obtained by dividing mean ΔV_{LP} by mean ΔSNGFR. The SE for the GTB slope was calculated from the first term of the Taylor series expansion of the GTB slope in ΔSNGFR and ΔV_{LP}. A composite inflection-point TGF slope was computed for each group by rearranging Eq. 6 to express f_1' as a function of C and f_2'. The SEs for f_1' were calculated by Taylor series expansion of this function.

This method for computing SE for a function of random variables has been described previously (10). The basic formula is:

(Equation 7)

$$\sigma_\psi^2 \cong \left(\frac{\partial \psi}{\partial x}\,\sigma_x\right)^2 + \left(\frac{\partial \psi}{\partial y}\,\sigma_y\right)^2 + 2r_{xy}\frac{\partial \psi}{\partial x}\frac{\partial \psi}{\partial y}\sigma_y\sigma_x$$

where ψ is a function of two random variables, x and y; σ_v^2 is the mean square error for some random variable, v; and r_{xy} is the correlation between the random variables x and y. For the slope of the GTB function (f_2'), x = ΔV_{LP}, y = ΔSNGFR, and ψ = x/y. After computing group means and SEs for f_2', these results were combined with the fractional compensation data to calculate group means and SEs for the slope of the TGF curve at the operating point, f_1'. Eq. 7 was applied again, by rearrangement of Eq. 6, with $x = f_2'$, y = C, and

(Equation 8)

$$\Psi \equiv f_1' = \left(\frac{1}{f_2'}\right)\left(\frac{C}{1-C}\right)$$

Because C and f_2' were measured in separate experiments, it is not possible to quantify their correlation. However, based on the mathematical dependence of C on f_2', it was assumed that they should not be negatively correlated within an experimental group. Furthermore,

(Equation 9)

$$\frac{\partial \psi}{\partial f_2'} = \frac{-1}{(f_2')^2} \le 0 \quad \text{and} \quad \frac{\partial \psi}{\partial C} = \frac{1}{(1-C)^2} \ge 0$$

Hence, when Eq. 7 is used to calculate SEs for f_1', the final term in Eq. 7 should be nonpositive. Therefore, calculating SEs of f_1' based on the assumption that f_2' and C are not correlated will yield values greater than or equal to the true SEs.

The composite group means ± SEM generated in this way were used in intergroup comparisons by Student's t test with Bonferroni correction. In these comparisons, the degrees of freedom were calculated conservatively, based on the smaller of two sample sets (free-flow or wax-blocked) that contributed to the calculation of f_1'. Because these statistical tests used maximum estimates for error (as shown above) and conservative numbers for the degrees of freedom, the probability of falsely discarding the null hypothesis is less than the P values generated by the t tests.

For the retrograde perfusion experiments, the effect of clamping ADO activity on the TGF response was tested by repeated-measures ANOVA.

Results

Data were obtained from 50 rats weighing 255–346 g. Mean arterial blood pressure was 110 ± 3 mmHg.

Experiments in free-flowing nephrons. Paired perturbation cycles (control ATF followed by ATF + drug) were completed in 43 free-flowing nephrons from 17 rats (Figures 1 and 2). Of these, eight nephrons were tested for effects of CHA, ten nephrons were tested for effects of KW3902, 13 nephrons were tested for effects of MADP, and 12 nephrons were tested for effects of MADP + CHA (ADO clamp). Baseline V_{LP} before

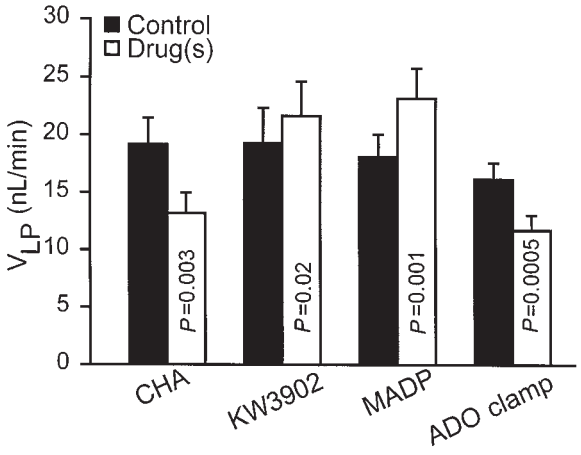

Figure 1
Ambient tubular flow before and during addition of various drugs to the free-flowing late proximal nephrons (mean ± SEM). Fluid was drawn from the tubule directly upstream from the pump delivering the drug(s) to correct for the volume required to deliver the drug(s). *P* values refer to the effect of each drug by repeated measures ANOVA. CHA, adenosine A1 receptor agonist (cyclohexyladenosine); KW3902, adenosine A1 receptor blocker; MADP, inhibitor of 5′-nucleotidase (α, β methylene adenosine diphosphate); ADO clamp, MADP + CHA.

administration of drug(s) was 18.1 ± 1.1 nL/min and was not different between groups ($P = 0.7$ for the effect of group on baseline V_{LP} by ANOVA). Baseline TGF compensation among the 43 nephrons was 0.713 ± 0.061, with some variability between groups mostly attributable to high baseline compensation among nephrons in the KW3902 group.

The effects of manipulating the adenosine system on V_{LP} and fractional compensation (C) were tested by repeated measures ANOVA. During perfusion with CHA, V_{LP} declined by 29 ± 7% ($P = 0.003$), whereas C was unaffected ($P = 0.5$). During perfusion with, KW3902, V_{LP} increased by 17 ± 5% ($P = 0.02$) and C declined by 44 ± 18% ($P = 0.013$). During perfusion with MADP, ambient V_{LP} increased by 30 ± 8% ($P = 0.001$) and C declined by 24 ± 6% ($P = 0.005$).

MADP + CHA were perfused together with the intent of maintaining ambient tubular flow constant. However, during these perfusions, ambient V_{LP} declined by 27 ± 6% ($P = 0.0005$), indicating that the perfusate contained too much CHA to achieve an adenosine clamp at the original level of activity. This maneuver tended to diminish fractional compensation, although the statistical significance was less clear-cut than for MADP alone or for KW3902.

Within each group, and across all groups, there was a significant correlation of compensation with V_{LP}. (When VMFV data are obtained over a range of V_H and fit with a polynomial, peak compensation and V_{LP} are uncorrelated [4]. When compensation is calculated by linear regression based on a finite perturbation, this will underestimate the true maximum value, and this underestimation will be exaggerated at lower V_{LP}. Hence, the C and V_{LP} appear correlated in the present case.) Correcting for this confounding effect of V_{LP} revealed a tendency for the ADO clamp to reduce the fractional compensation ($P = 0.098$). Also, as discussed earlier here, there was intergroup heterogeneity for predrug compensation that disappeared when the A1-blocker group was excluded from the analysis. Pooling the remaining controls and comparing these to the clamp by unpaired *t* test confirmed that compensation was reduced during adenosine clamp ($P = 0.02$).

Orthograde perfusion in wax-blocked nephrons. SNGFR and V_{LP} were measured during minimum and maximum TGF stimulation in multiple nephrons from 17 rats (Figures 3–5). Collections were made during perfusion at 38 and 4 nL/min in 84 nephrons. Data from six nephrons were excluded because tubular fluid to plasma inulin ratios were not consistent with late proximal collections. The remaining 78 nephrons included 19 perfused with MADP, 19 perfused with MADP + CHA, and 40 controls. These experiments were performed after it was known that the 10–50 μM CHA used in the free-flow clamp studies reduced ambient tubular flow. For these studies, the clamp was performed with 1 mM MADP and 2 μM CHA.

Maximum TGF responses. To test for adenosine as a determinant of the maximum TGF response, intergroup comparisons were performed for both absolute changes in SNGFR (ΔSNGFR) and normalized changes in SNGFR (ΔSNGFR/SNGFRmean). For perfusion with ATF, SNGFRmean was 23.8 ± 1.6 nL/min, ΔSNGFR was 14.5 ± 1.3 nL/min, and ΔSNGFR/SNGFRmean was 0.70 ± 0.06. In nephrons perfused with the 5′-nucleotidase inhibitor, MADP,

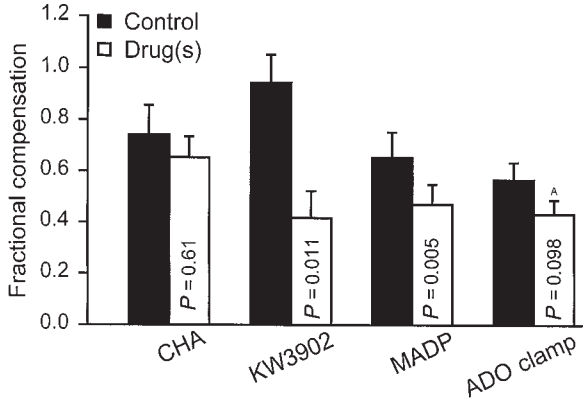

Figure 2
Fractional compensation for ± 5 nL/min perturbations in V_{LP} applied before and during addition of various drugs to the free-flowing late proximal nephrons (mean ± SEM). *P* values refer to the effect of each drug by repeated measures ANOVA. The adenosine A1 agonist, CHA, had no effect. The blocking adenosine A1 receptors with KW3902 or inhibiting 5′-nucleotidase with MADP clearly reduced fractional compensation. The effect of the ADO clamp was equivocal by repeated measures, although fractional compensation during the ADO clamp was significantly less than a control value pooled from all groups. $^AP = 0.02$ by unpaired Student's *t* test versus homogeneous pooled controls.

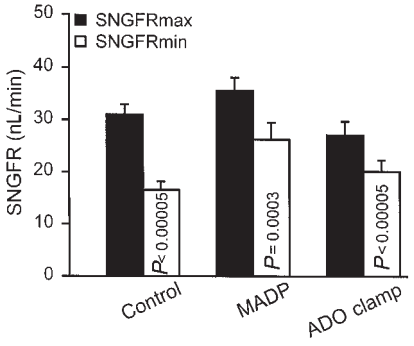

Figure 3
SNGFR during loop of Henle perfusion at 4 nL/min (SNGFRmax) versus flow during loop perfusion at 38 nL/min (SNGFRmin); mean ± SEM. Stimulation of TGF through loop of Henle perfusion caused SNGFR to decline in all cases.

SNGFRmean was 30% greater than control (P = 0.045 by ANOVA with Tukey test for multiple group comparisons). At the same time, ΔSNGFR was less by 36% (P = 0.089) and ΔSNGFR/SNGFRmean was less by 48% (P = 0.01) than in control nephrons. In these ADO-clamp experiments, the goal was achieved of not affecting SNGFRmean (P = 0.99 versus control). However, although SNGFRmean remained unchanged, the ADO clamp reduced ΔSNGFR by 52% (P = 0.008) and reduced ΔSNGFR/SNGFRmean by 52% (P = 0.004). These results are depicted in Figures 3 and 4.

Analysis of GTB. Values for ΔAPR and ΔV_{LP} were calculated for each nephron along with values for ΔSNGFR. The relationship between these parameters and ΔSNGFR were quantified for the nephrons in each group. As expected, both ΔAPR and ΔV_{LP} correlated strongly with ΔSNGFR. The slopes of the GTB curves (ΔV_{LP}/ΔSNGFR) were 0.60 ± 0.05, 0.88 ± 0.11, and 0.99 ± 0.13 in control, MADP-perfused, and clamped nephrons, respectively. After Bonferroni correction for multiple group comparisons, MADP and ADO-clamped nephrons differed from control at P = 0.052 and P < 0.02, respectively. These results are depicted in Figure 5.

Loop of Henle reabsorption. Nephrons were perfused from the late proximal tubule at 16 nL/min with ATF followed by ATF + CHA (n = 10), ATF + MADP (n = 10), or ATF (time controls, n = 9). The time-control experiments confirmed a stable preparation and drug perfusion experiments revealed no effect of CHA or MADP on reabsorption of water, sodium, chloride, or potassium from the loop of Henle (Table 1).

Effects on the TGF slope. From the fractional compensation and GTB slopes, the mean ± SEM operating-point slope of the TGF curve was calculated for each group to yield slopes of 4.2 ± 1.2, 1.0 ± 0.3, and 0.7 ± 0.2 for control, MADP, and ADO clamp, respectively. After Bonferroni correction for multiple group comparisons, both MADP and ADO clamp differed significantly from control ($P \leq 0.02$ for each comparison). In other words, the steepness of the slope of the TGF curve was markedly reduced by blocking 5′-nucleotidase, and there was no tendency for the TGF slope to be restored when enough adenosine agonist was added back to reverse the effect of MADP on SNGFRmean. TGF curves derived by matching ΔSNGFR and ΔV_{LP}/ΔSNGFR from the orthograde perfusions with fractional compensation measured in free-flowing nephrons are depicted in Figure 6. To generate these curves, it was assumed that the fractional compensation reflected the behavior of TGF at the inflection point.

Retrograde perfusion experiments. SNGFR was measured in each nephron during perfusion with low-salt distal ATF (minimum TGF) and high-salt distal ATF (maximum TGF) in 32 nephrons from six rats (15 control nephrons, 17 ADO-clamp nephrons) (Figure 7). The ADO-clamp perfusate contained 0.5 μM CHA + 1 mM MADP in five nephrons and 1.0 μM CHA + 1 mM MADP in 12 nephrons. Values for SNGFRmean were calculated for each nephron analogous to those calculated for the orthograde perfusion studies (see earlier discussion here). There was a slight tendency for the clamp solution with the higher CHA content to manifest a lower SNGFRmean (21 ± 1 vs. 23 ± 2 nL/min; P = NS). Overall, the ADO clamp achieved the intent of not altering SNGFRmean (22 ± 1 vs. 23 ± 2 nL/min, clamp versus control, P = NS.) However, ADO clamp eliminated the change in SNGFR achieved changing salt content of the perfusate (ΔSNGFR = –0.1 ± 1.6 vs. 11.2 ± 2.6 nL/min, clamp versus control, P = 0.001). In other words, clamping ADO activity abolished the TGF–mediated change in SNGFR during manipulation of macula densa salt by retrograde perfusion.

Table 1
Content of early distal fluid during orthograde perfusion of Henle's loop at 16 nL/min with artificial tubular fluid

	V_{ED} (nL/min)	Na_{ED} (mEq/L)	K_{ED} (mEq/L)	Cl_{ED} (mEq/L)	FR-volume (%)	FR-Na (%)	FR-K (%)	FR-Cl (%)
Basal	7.4 ± 0.7	25 ± 2	1.7 ± 0.2	21 ± 2	54 ± 4	91 ± 1	82 ± 1	93±1
Time control	7.0 ± 0.5	28 ± 2	1.9 ± 2	22 ± 1	56 ± 3	91 ± 1	81±1	93±1
Basal	7.8 ± 0.4	22 ± 2	2.0 ± 0.7	24 ± 2	51 ± 3	92 ± 1	77 ± 7	91±1
CHA	7.5 ± 0.4	24 ± 2	2.0 ± 0.7	26 ± 2	53 ± 3	92 ± 1	79 ± 7	91±1
Basal	6.1 ± 0.8	19 ± 2	1.9 ± 0.3	15 ± 1	62 ± 5	95 ± 1	82 ± 4	95±1
MADP	5.7 ± 0.6	21 ± 4	2.1 ± 0.4	18 ± 2	65 ± 4	95 ± 1	81 ± 4	95±1

ED, early distal; V, volume; FR, fractional reabsorption; CHA, cyclohexyladenosine; MADP, α, β methylene adenosine diphosphate. Results are expressed as mean ± SEM.

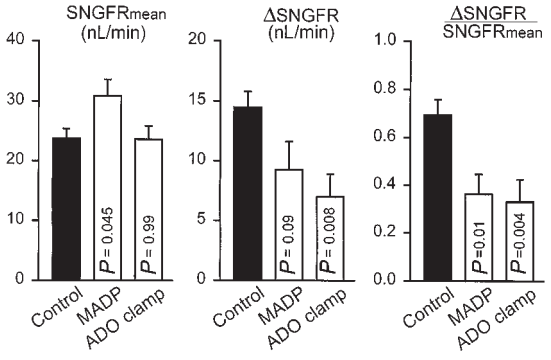

Figure 4
Effects of MADP and ADO clamp on SNGFR at the TGF inflection point (SNGFRmean), the maximum amount by which SNGFR can be made to change via activation of TGF (ΔSNGFR), and ΔSNGFR normalized to SNGFRmean (mean ± SEM). *P* values shown are comparisons to control by ANOVA with Tukey adjustment for post hoc intergroup comparisons.

Discussion

The present studies imply that adenosine exerts a tonic influence on the glomerular microvasculature and contributes to the compensatory changes in SNGFR that occur when flow is altered in the tubule. Evidence that 5′-nucleotidase activity influences basal glomerular hemodynamics is provided by two sets of experiments. First, delivering the 5′-nucleotidase inhibitor, MADP, into the free-flowing late proximal tubule caused baseline tubular flow to increase, suggesting that SNGFR had increased. A similar response was obtained with the adenosine A1 receptor antagonist, KW3902, and an opposite response was obtained with the adenosine A1 agonist, CHA. The role of 5′-nucleotidase activity as a determinant of SNGFR was confirmed in a second set of studies in which SNGFR was measured directly and found to be increased during perfusion of the loop of Henle with MADP, irrespective of the perfusion rate (Figures 3 and 4).

In addition to influencing basal SNGFR, adenosine formed by 5′-nucleotidase was confirmed to participate in TGF. Inhibiting 5′-nucleotidase with MADP reduced the homeostatic efficiency of TGF as manifest by a lesser fractional compensation for small perturbations in V_{LP}. Fractional compensation is the integrated result of glomerulotubular balance and TGF. After adjusting for the contribution of GTB to overall TGF efficiency (Figure 5), MADP reduced the maximum slope of the TGF curve by more than 80% (Figure 6). MADP also reduced the maximum range of the TGF response by 35–45% (Figure 4).

Autacoid or paracrine substances that impinge on TGF may either mediate or modulate TGF. To qualify as a TGF mediator, a substance must meet the following two criteria. First, its activity must change in response to changes in delivery of NaCl to the luminal macula densa, and these changes must occur within the minute-to-minute time frame of the TGF response. Second, assuming there is only one TGF mediator, preventing the activity of that mediator from changing in response to signals from the macula densa will abolish the TGF response. In contrast, a TGF modulator does not actually cause the TGF response but operates in the background to condition the response. The activity of a modulator might or might not be linked to changes in macula densa NaCl, but if such a link is present, interrupting the link will not abolish the TGF response. It is common to think of a modulator as something that reacts more slowly than the system it modulates. However, a system that responds rapidly to changes in macula densa NaCl is not excluded from being a TGF modulator as long as there is some level of activity at which the putative modulator can be clamped without suppressing the TGF response.

In practice, it is simpler to demonstrate that a substance modulates TGF than it is to demonstrate that a substance mediates TGF, and several candidate mediators have been reduced to modulators. For example, several characteristics of the renin-angiotensin system make angiotensin II a candidate mediator for TGF. However, it has been demonstrated by these criteria that angiotensin II is an important TGF modulator but does not mediate TGF (18). Similarly, the abundance of nitric oxide synthase in the macula densa combined with the vasodilatory influence of nitric oxide on the glomerular microvasculature suggests that TGF might be mediated by an inverse dependence of nitric oxide generation on macula densa NaCl. However, it has also been demonstrated that macula densa nitric oxide is a modulator, not a mediator, of TGF (reviewed in ref. 19).

In the present case, inhibiting 5′-nucleotidase reduced both the slope of the TGF curve and the maximum range of the TGF response. This is sufficient to prove that 5′-nucleotidase impinges on the TGF system, but it is not sufficient to determine whether

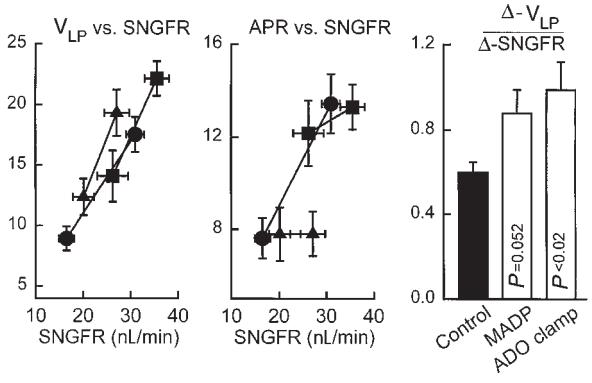

Figure 5
Glomerular tubular balance. The TGF system was used as a tool to manipulate SNGFR. Values are shown corresponding to paired determinations of SNGFR and V_{LP} or absolute proximal reabsorption (APR) during minimal and maximal stimulation of TGF. Controls are indicated by circles; MADP, by squares; ADO clamp, by triangles. The right-hand panel depicts the slopes of the lines in the left-hand panel that correspond to f_2' in Eq. 2 (see text). *P* values shown refer to comparisons with control by Student's *t* test with Bonferroni correction for multiple group comparisons.

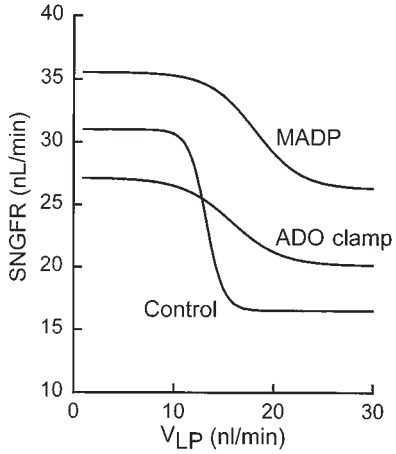

Figure 6
TGF curves generated from the present data. Four parameters are required to generate each curve. These include SNGFRmean, ΔSNGFR, V_{LP} at the inflection point, and f_1' at the inflection point. To generate these curves, it was assumed that f_1' is a symmetric sigmoid and that nephrons in the perturbation experiments were operating near the inflection points of their respective TGF curves. All other features of the curves are obtained directly from the present data.

adenosine is a mediator or a modulator of TGF. In fact, the effects of MADP could be accounted for if adenosine were merely operating in the background to intensify the response to some other vasoconstrictor or vasodilator. To test the hypothesis that adenosine is an actual mediator of TGF, experiments were performed to determine whether restoring adenosine activity to pre-MADP levels with exogenous adenosine A1 agonist would reverse the effects of MADP on the TGF response. This was the idea behind the ADO-clamp experiments in which the adenosine A1 receptor agonist, CHA, was administered along with the 5′-nucleotidase inhibitor, MADP. If the effects of MADP on TGF were reversed by addition of CHA, then adenosine would be an unlikely mediator of TGF. On the other hand, if the effects of MADP on the TGF response were not reversed by adding CHA, this would be evidence that the mere presence of adenosine is not sufficient to account for its role in TGF. Instead, adenosine activity must be allowed to fluctuate along with changes in tubular flow for TGF to operate normally, as would be expected if adenosine were a mediator of TGF.

In practice it is not possible to clamp adenosine activity perfectly at pre-MADP levels because that level will be different in every nephron. Furthermore, it is not possible to know the degree to which MADP prevents adenosine from fluctuating when the TGF signal is manipulated unless MADP were to eliminate TGF completely. However, to prove that the actions of TGF can be suppressed by clamping adenosine activity, it is not essential that the clamp be perfect or that adenosine activity be identical to baseline. To achieve positive proof that adenosine mediates TGF, the model must meet two less-stringent conditions. The first condition is that the clamp must be tight enough to alter the TGF response measurably. The second condition is that the clamped adenosine activity should not be less than baseline but must not be so high as to potentially overwhelm the vascular response to other unknown TGF mediators. In the present experiments, the first condition was clearly met, as the ADO clamp had effects on the behavior of TGF that were similar to the effect of MADP alone (Figures 2 and 4).

The second condition also appears to have been met, although this is less obvious. As surrogate measurements of adenosine activity in the different experiments, measurements were made of unperturbed V_{LP} or SNGFRmean. In the free-flow experiments, enough CHA was given to reduce V_{LP} by approximately 4 nL/min during the ADO clamp (Figure 1). Therefore, it cannot be argued that too little CHA was given in these experiments. However, it is possible that too much CHA was given and that the associated decrease in ambient tubular flow caused the ambient flow to shift from a steep to a flat portion of the TGF curve. Alone, this could account for the apparent loss of fractional compensation, as fractional compensation depends on the slope of the TGF curve over the range in which the nephron operates during the perturbation experiment (see Eq. 6). However, when CHA was added alone, V_{LP} decreased by 6 nL/min, yet fractional compensation was unaffected (Figures 1 and 2). Therefore, it is unlikely that the ADO clamp lessened the fractional compensation purely by altering the position of the operating point along the TGF curve.

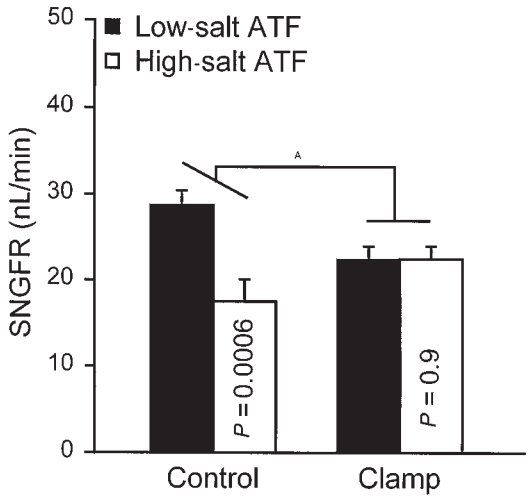

Figure 7
SNGFR by paired proximal collections during retrograde perfusion of Henle's loop from the early distal tubule. Perfusion was at 12.5 nL/min with artificial tubular fluid (ATF) containing either 10 or 50 mM NaCl to effect minimum or maximum stimulation of TGF. To clamp adenosine activity, 5′-nucleotidase blocker and cyclohexyladenosine were added to tubular ATF. *P* values refer to effect of changing salt concentration. [A]By two-way ANOVA, the adenosine clamp reduced the TGF response ($P = 0.0006$) but had no effect on SNGFR independent of TGF ($P = 0.75$).

In the wax-block experiments, relatively less CHA was used. In these experiments, SNGFRmean was used as the surrogate assay for the underlying adenosine activity. MADP alone caused SNGFRmean to increase significantly, and this effect was completely reversed by the ADO clamp (Figure 4). Therefore, adenosine activity achieved in these experiments met the conditions required to prove that TGF will not function normally unless adenosine activity is allowed to fluctuate from minute to minute. The impact of clamping ADO activity on the TGF response was even more apparent during retrograde perfusion, which provided a shorter path for delivering drugs to the macula densa. In this case, clamping ADO activity appeared to abolish TGF altogether, although the applied stimulus (10–50 mM NaCl variation) should dissipate somewhat before reaching the macula densa and is less than the range of macula densa NaCl variation expected during 4–38 nL/min perfusion from the late proximal tubule (11).

It was not the main purpose of this study to delineate the role of 5′-nucleotidase or adenosine in normal function of the proximal tubule. However, to extract the slope of the TGF curve from the response to flow perturbations in free-flowing nephrons, it was necessary to know how glomerular tubular balance was affected when MADP or MADP + CHA was perfused through the loop of Henle. Both MADP and MADP + CHA somewhat reduced the dependence of proximal reabsorption on SNGFR, and the addition of CHA to MADP reduced proximal reabsorption relative to MADP alone (Figure 5). From the current experiments, it cannot be determined whether the effects of MADP and CHA on the proximal tubule were direct or indirect. Because of the experimental design, the drugs could only arrive at the early proximal tubule circuitously and proximal reabsorption might have been affected by differences in peritubular oncotic pressure arising from impacts of CHA and/or MADP on glomerular filtration fraction. Nonetheless, the difference in proximal reabsorption between nephrons perfused with MADP alone and those perfused with MADP + CHA suggests that adenosine, acting through A1 receptors can reduce proximal reabsorption. Furthermore, the finding of impaired glomerular tubular balance in nephrons perfused with either MADP or MADP + CHA suggests that the modulation of local adenosine concentration by 5′-nucleotidase might be necessary for normal glomerular tubular balance. However, there is no evidence from the present findings that proximal tubular reabsorption is autoregulated by transport-dependent adenosine formation (Figure 5, center panel). Whereas proximal reabsorption was affected by adenosine analogs delivered into the loop of Henle, there was no effect of these drugs on loop of Henle reabsorption, per se.

The current data imply that adenosine that is generated by 5′-nucleotidase mediates a compensatory change in SNGFR when flow is altered beyond the late proximal tubule. However, these data do not prove that the substrate for this 5′-nucleotidase comes from ATP hydrolyzed by macula densa cells. In fact, it is known that macula densa cells exhibit relatively little Na-K-ATPase activity relative to other cells in the neighboring thick ascending limb (20). This raises the possibility that tubular-glomerular interactions mediated by adenosine do not involve the macula densa in the same way as usually envisioned for TGF. On the other hand, relative changes in the abundance AMP that result from changing rates of ATP hydrolysis depend strongly on the ATP/AMP ratio and not merely on the absolute rate of ATP hydrolysis. This can be demonstrated mathematically as follows.

In cells, ATP, ADP, and AMP are maintained in equilibrium by the following reaction (Rxn 1), which is mediated by adenylate kinase (21): $ATP + AMP \leftrightarrow 2ADP$. Accordingly,

(Equation 10)

$$[AMP] = k[ADP]^2 \cdot [ATP]^{-1}$$

where k is the equilibrium constant. If the system is essentially closed with respect to total nucleotide content while the number of high-energy phosphate bonds is allowed to change through hydrolysis and formation of ATP, then ATP, ADP, and AMP can change, but

(Equation 11)

$$\Delta ATP + \Delta ADP + \Delta AMP = 0$$

When ATP is hydrolyzed to ADP, some of this ADP will be converted to ATP + AMP according to Rxn 1. Therefore, AMP formation will track ATP hydrolysis and a gain for this process can be defined as:

(Equation 12)

$$Gain = -\left(\frac{\Delta AMP}{AMP}\right) \cdot \left(\frac{\Delta ATP}{ATP}\right)^{-1}$$

Expanding Eq. 11 and substituting from Eq. 10 and Eq. 11 gives

(Equation 13)

$$\frac{\Delta AMP}{\Delta ATP} = \frac{1}{\Delta ATP}\left(\frac{\partial AMP}{\partial ATP} \cdot \Delta ATP + \frac{\partial AMP}{\partial ADP} \cdot \Delta ADP\right)$$

$$= \frac{-k(ADP)^2}{(ATP)^2} + \frac{\Delta ADP}{\Delta ATP} \cdot \frac{2kADP}{ATP}$$

$$= \frac{-AMP}{ATP} - 2 \cdot \sqrt{\frac{AMP}{ATP}} - 2 \cdot \frac{\Delta AMP}{\Delta ATP} \cdot \sqrt{\frac{AMP}{ATP}}$$

Rearranging Eq. 13 and dividing through by AMP/ATP gives

(Equation 14)

$$Gain = \left(1 + 2 \cdot \sqrt{\frac{ATP}{AMP}}\right)\left(1 + 2 \cdot \left(\sqrt{\frac{ATP}{AMP}}\right)^{-1}\right)^{-1}$$

This is a monotonically increasing function of ATP/AMP. Therefore, fractional changes in AMP are a more sensitive signal of changes in ATP in cells where the starting ATP/AMP ratio is high. Therefore, the transduction of changes in luminal NaCl delivery into

changes in AMP formation might have high gain in the macula densa, even though the absolute transport rate by these cells is low.

Adenosine is generated in the JGA by 5′-nucleotidase and exerts a tonic influence on single nephron glomerular filtration rate and on the process of TGF. Both the maximum range over which TGF can cause SNGFR to change and the strength of the TGF response to small perturbations in ambient tubular flow are reduced when adenosine formation is suppressed or when adenosine activity is clamped. These findings establish adenosine as a mediator of TGF.

Acknowledgments

This work was performed with funds provided by the Department of Veterans Affairs Research Service and by the NIH (National Institute of Diabetes and Digestive and Kidney Diseases, RO1 DK-28602). V. Vallon was supported by grants from the Deutsche Forschungsgemeinschaft (Va 118/3-2) and the Federal Ministry of Education and Research (BMBF 01 EC 9405 and 01 KS 9602). KW-3902 was kindly provided by H. Osswald (Department of Pharmacology, University of Tuebingen, Tuebingen, Germany).

1. Osswald, H., Muhlbauer, B., and Schenk, F. 1991. Adenosine mediates tubuloglomerular feedback response: an element of metabolic control of kidney function. *Kidney Int.* **39**:S128–S131.
2. Schnermann, J., Weihprecht, H., and Briggs, J. 1990. Inhibition of tubuloglomerular feedback during adenosine-1 receptor blockade. *Am. J. Physiol.* **258**:F553–F561.
3. Weihprecht, H., Lorenz, J.N., Briggs, J.P., and Schnermann, J. 1994. Synergistic effects of angiotensin and adenosine in the renal microvasculature. *Am. J. Physiol.* **266**:F227–F239.
4. Pflueger, A.C., Schenk, F., and Osswald, H. 1995. Increased sensitivity of the renal vasculature to adenosine in streptozotocin-induced diabetes mellitus rats. *Am. J. Physiol.* **269**:F529–F535.
5. Thomson, S., Vallon, V., and Blantz, R.C. 1996. Asymmetry of tubuloglomerular feedback effector mechanism with respect to ambient tubular flow. *Am. J. Physiol.* **271**:F1123–F1130.
6. Huang, W.C., Bell, P.D., Harvey, D., Mitchell, K.D., and Navar, L.G. 1988. Angiotensin influences on tubuloglomerular feedback mechanism in hypertensive rats. *Kidney Int.* **34**:631–637.
7. Schnermann, J., and Briggs, J.P. 1990. Restoration of tubuloglomerular feedback in volume-expanded rats by angiotensin II. *Am. J. Physiol.* **259**:F565–F572.
8. Mitchell, K.D., and Navar, L.G. 1988. Enhanced tubuloglomerular feedback during peritubular infusions of angiotensins I and II. *Am. J. Physiol.* **255**:F383–F390.
9. Blantz, R.C., and Tucker, B.J. 1978. Measurements of glomerular dynamics. In *Methods in pharmacology.* M. Martinez-Maldonado, editor. Plenum Publishing Co. New York, New York, USA. 141–163.
10. Thomson, S.C., and Blantz, R.C. 1993. Homeostatic efficiency of tubuloglomerular feedback in hydropenia, euvolemia, and acute volume expansion. *Am. J. Physiol.* **264**:F930–F936.
11. Vallon, V., and Thomson, S.C. 1995. Inhibition of local nitric oxide synthase increases the homeostatic efficiency of tubuloglomerular feedback. *Am. J. Physiol.* **38**:F892–F899.
12. Vallon, V., Blantz, R.C., and Thomson, S. 1995. Homeostatic efficiency of tubuloglomerular feedback is reduced in established diabetes mellitus in rats. *Am. J. Physiol.* **38:**F876–F883.
13. Thomson, S.C., Blantz, R.C., and Vallon, V. 1996. Increased tubular flow induces resetting of tubuloglomerular feedback in euvolemic rats. *Am. J. Physiol.* **270**:F461–F468.
14. Thomson, S.C., Vallon, V., and Blantz, R.C. 1997. Reduced proximal reabsorption resets tubuloglomerular feedback in euvolemic rats. *Am. J. Physiol.* **273**:R1414–R1420.
15. Schnermann, J., and Briggs, J. 1985. Function of the juxtaglomerular apparatus: local control of glomerular hemodynamics. In *The kidney: physiology and pathophysiology.* D.W. Seldin and G. Giebisch, editors. Raven Press. New York, New York, USA. 669–697.
16. Vallon, V., Osswald, H., Blantz, R.C., and Thomson, S. 1997. Potential role of luminal potassium in tubuloglomerular feedback. *J. Am. Soc. Nephrol.* **8**:1831–1837.
17. Vallon, V., Richter, K., and Blantz, R.C. Thomson, S., and Osswald, H. 1999. Glomerular hyperfiltration in experimental diabetes mellitus: potential role of tubular reabsorption. *J. Am. Soc. Nephrol.* **10**:2569–2576.
18. Schnermann, J., and Briggs, J. 1984. Restoration of tubuloglomerular feedback in volume-expanded rats by angiotensin II. *Am. J. Physiol.* **247**:F246–F251.
19. Welch, W.J., Wilcox, C.S., and Thomson, S.C. 1999. Nitric oxide and tubuloglomerular feedback. *Semin. Nephrol.* **19**:251–262.
20. Schnermann, J., and Marver, D. 1986. ATPase activity in macula densa cells of the rabbit kidney. *Pflugers Arch.* **407:**82–86.
21. Mayes, P. 1979. Bioenergetics. In *Review of physiological chemistry.* H.A. Harper, V.W. Rodwell, and P.A. Mayes, editors. Lange Medical Publications. Los Altos, California, USA. 260–265.