Hemodynamics of early tubuloglomerular feedback resetting during reduced proximal reabsorption

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Background. Carbonic anhydrase inhibition with benzolamide reduces proximal reabsorption and activates tubuloglomerular feedback (TGF). In rats, TGF activation for 30 to 60 minutes locally suppresses renin secretion and resets TGF rightward to accommodate increased late proximal flow. After 24 hours of TGF activation, there is upward resetting of GFR and increased activity of macula densa nitric oxide synthase I (NOS I).

Methods. We studied renal hemodynamics during early TGF resetting with attention to the importance of renin suppression and NOS I activation. Left kidney blood flow (RBF, pulse Doppler) and glomerular filtration rate (GFR; inulin clearance or Fick method) were measured before and during benzolamide infusion (5 mg/kg bolus followed by 5 mg/kg/h IV) in Wistar rats concurrently receiving the converting enzyme inhibitor, enalaprilat (0.3 mg/kg/h IV) or NOS-I blocker S-methyl-thiocitrulline (SMTC; 2.7 mg/kg/h IV).

Results. Activating TGF initially reduced RBF and GFR in all groups as expected. During continuous benzolamide, RBF gradually increased toward baseline in control and enalaprilat-treated rats, but not in NOS-I-blocked rats. After the initial decline, GFR did not change further during one hour of benzolamide in any group.

Conclusions. During one hour of persistent TGF stimulation, RBF increases toward normal, but GFR does not. This requires an overall decrease in renal vascular resistance and a decrease in the ratio of efferent/afferent arteriolar resistance (R_e/R_a), implying a major decrease in R_e. NOS I, but not angiotensin-converting enzyme (ACE), is required for RBF to increase during TGF resetting. Although the hemodynamic changes during TGF resetting resemble the response to blocking the renin-angiotensin system, these data fail to show that the increase in RBF during early TGF resetting is mediated by renin suppression.

Key words: tubuloglomerular feedback, nitric oxide synthase, angiotensin, glomerular filtration rate, vascular resistance, efferent resistance, renin.

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The body’s internal environment is regulated, in large part, by events that occur in the juxtaglomerular apparatus (JGA) of nephrons. One role of the JGA is to mediate tubuloglomerular feedback (TGF). TGF causes single nephron glomerular filtration rate (SNGFR) to change in the opposite direction whenever there is a change in the salt concentration of tubular fluid reaching the macula densa. A TGF response operates in the time frame of several seconds. In this way, TGF provides a negative feedback control that stabilizes both SNGFR and the amount of salt reaching the distal nephron. However, due to the reciprocal relationship conferred by TGF, SNGFR and distal salt delivery can never change in parallel unless there is a resetting of the TGF response (Fig. 1). For example, in order for SNGFR to increase as it does during normal growth, pregnancy, reduced nephron number, or acute plasma volume expansion, TGF must reset in a direction that is generally rightward. Furthermore, for TGF to perform its stabilizing role after resetting, resetting must occur to the proper extent so that the ambient stimulus remains in the narrow range where TGF is most responsive. Data from prior studies suggest that control over TGF resetting is maintained within the JGA itself rather than being driven primarily by systemic neuroendocrine events. In fact, TGF resetting can be initiated by a sustained increase in delivery to the macula densa per se, such that a stimulus that initially activates TGF subsequently causes TGF to reset and to regain efficiency over the next 30 to 60 minutes [1–3].

Beyond the fact that it is initiated by a prolonged TGF stimulus and can occur within 30 to 60 minutes, the mechanism of early TGF resetting is poorly understood. For instance, it is not known what vascular elements are involved or whether renal blood flow or GFR are affected during the early stages of TGF resetting. This is because the micropuncture experiments previously performed to demonstrate the phenomenon of early TGF resetting by the JGA were designed to monitor temporal adaptation of TGF efficiency and were not amenable to monitoring temporal adaptation of glomerular hemodynamics within the 30 to 60 minute time frame [1, 2]. Also, the exact
Fig. 1. Idealized early tubuloglomerular feedback (TGF) resetting. Movement from A to B depicts immediate activation of TGF. In nature, this could occur from a spike in blood pressure, suddenly reduced proximal reabsorption, etc. Movement from B to C occurs as TGF resets to the dashed line. Whether the single nephron glomerular filtration rate (SNGFR) increases as resetting occurs is hypothetical. When at point B, TGF efficiency is lost because a subsequent disturbance in macula densa [Cl] will have no effect on SNGFR. After resetting to the new TGF curve, TGF efficiency is restored. We hypothesize that macula densa NaCl induces rightward resetting by suppressing renin or by activating nitric oxide synthase I (NOS I) in the macula densa.

Molecular mediators of TGF resetting are unknown, although indirect evidence makes local modulation of the renin-angiotensin system (RAS) or macula densa nitric oxide synthase (NOS I) likely candidates to explain the phenomenon. To investigate the renal hemodynamic aspects of early TGF resetting and to look for direct evidence that TGF resetting occurs through changes in RAS or NOS I activity, we monitored renal blood flow (RBF) and GFR while inducing TGF to reset by infusing the proximal tubular diuretic, benzolamide. To establish the roles of the RAS and NOS I in TGF resetting, animals were pretreated with respective pharmacological blockers.

METHODS

Animal experiments described herein were conducted in accordance with the NIH Guide for the Care and Use of Animals in Research. Experiments were performed in adult male Wistar rats under inactin anesthesia (100 mg/kg IP; Research Biochemicals, Natick, MA, USA). Catheters were placed in the trachea (PE 240), jugular vein (PE 50), femoral artery (PE 50), left ureter (PE 50) and urinary bladder (PE 50). Body temperature was maintained with servo-controlled heating table and rectal temperature probe. The left kidney was exposed through a flank incision and immobilized in a lucite cup [4]. Blood flow to the left kidney (RBF) was monitored by a perivascular ultrasonic transit time flow probe (Transonics T206, Ithaca, NY, USA) connected to a computer for continuous recording. In some cases a 26 gauge needle was placed in the proximal left renal vein for sampling renal venous blood. GFR was measured by urinary clearance of $^3$H-inulin administered in Ringer saline (5 μCi/mL at 1.5 mL/h) or computed from RBF, hematocrit (Hct) and filtration fraction (FF) where FF was calculated according to the Fick principle by $^3$H-inulin content of arterial and renal venous plasma:

$$FF = \left(1 - \frac{\text{renal venous inulin}}{\text{arterial inulin}}\right)$$

(Eq. 1)

After the initial surgical preparation, animals were allowed 90 minutes to recover with the Doppler flow probe in place with stable blood pressure and renal blood flow. TGF was activated, and thereby induced to reset rightward, by infusion of the carbonic anhydrase inhibitor, benzolamide. Benzolamide was administered as a 5 mg/kg bolus in Ringer saline followed by continuous infusion of 5 mg/kg/h in 300 mmol/L NaHCO$_3$ at 1.5 mL/hr. This initial bolus of benzolamide was reduced by 50% from prior studies [2] in order to mute high amplitude transient RBF oscillations noted in response to a 10 mg/kg bolus. The timing of data collection before and during benzolamide infusion is outlined in Figure 2.

Data were collected from control rats, rats in which RAS activity was blocked with enalaprilat (0.3 mg/kg/h IV; Sigma Chemical Co., St. Louis, MO, USA) begun prior to benzolamide, and rats during NOS I blockade with S-methyl-thiocitrulline (SMTC; 2.7 mg/kg/h IV; Alexis Biochemicals, San Diego, CA, USA) begun prior to benzolamide. This dose of enalaprilat was confirmed to block the blood pressure response to a 100 ng IV bolus of angiotensin I. The amount of SMTC given was the maximum dose that did not increase arterial blood pressure and was, therefore, deemed not to cross over significantly to NOS III.

Statistics

Intergroup comparisons were by repeated measures analysis of variance (ANOVA). The first statistical test was to compare the initial responses to benzolamide. The next test was to compare trajectories during the on-
going infusion of benzoalamide indicative of TGF resetting. Intergroup differences in RBF proved easy to detect. However, power analysis applied to the urinary inulin clearance revealed that N = 36 animals per group would have been required to detect proportional differences in GFR by the inulin clearance method. This could be due to inherent variability in GFR during TGF resetting or to a poor signal-to-noise ratio in the data. To distinguish between these two possibilities, additional experiments were performed to measure filtration fraction before and during benzoalamide by the Fick method as described above. These data confirmed a significant downward course in filtration fraction with a small sample size and were combined with blood flow data to compute GFR. Statistical significance is assigned for P < 0.05.

RESULTS

Renal blood flow and inulin clearance data were obtained before and during benzoalamide infusion in seven control rats, seven rats given enalaprilat, and six rats given SMTC. Serial determinations of filtration fraction were made by arteriovenous inulin differences before and during benzoalamide infusion in four additional control rats. The determination of the importance of the RAS or NOS I to TGF resetting different groups received prior treatment with enalaprilat or SMTC. Enalaprilat increased basal left kidney blood flow by approximately 20% (P = 0.003) and SMTC decreased basal left kidney blood flow by approximately 25% (P = 0.002). Neither drug caused a detectable change in baseline inulin clearance (P = 0.7 and 0.5 for enalapril and SMTC, respectively; Figs. 3 and 4). A diuretic response to benzoalamide was evident within one to two minutes in each group. The initial effects of activating TGF were ascribed to measurements obtained by averaging RBF or collecting urine for inulin clearance during the next 20 minutes. Resetting of the TGF response was assessed by comparing the measurements made during these first 20 minutes to those made during subsequent 20-minute periods out to one hour. Benzoalamide initially caused RBF to decline significantly in all groups (P < 0.001) and by a similar amount in all groups (P = 0.43 for the effect of group on the initial RBF response to benzoalamide). Likewise, benzoalamide caused left-kidney inulin clearance to decline overall (P < 0.0001) without significant intergroup differences (P = 0.34; Figs. 3 and 4).

After the initial 20 minutes of benzoalamide infusion, RBF trended significantly upward in control rats (P = 0.0006) and in those pre-treated with enalaprilat (P = 0.006), but not in those pre-treated with SMTC (P = 0.7). By two-way ANOVA there was no effect of enalaprilat on the resetting of RBF during benzoalamide (P = 0.93). By two-way ANOVA the resetting of RBF was prevented by SMTC (P = 0.0003; Fig. 3). In contrast to the time-related changes in renal blood flow, there was no significant change in inulin clearance in any group over the one hour of benzoalamide infusion (Fig. 4). In fact, the data lacked power to detect a true increase in single kidney GFR any smaller than 0.19 mL/min, raising the possibility that important resetting of
GFR might have gone undetected. Furthermore, power analysis applied to these data suggested that an untenably large data set would be required to exclude GFR resetting by inulin clearance. This could reflect either true heterogeneity in GFR during TGF resetting or an unfavorable signal-to-noise ratio for inulin clearance calculated from brief urine collections. To distinguish between these alternative explanations, additional experiments were performed using serial arteriovenous inulin differences in four control rats to overcome problems with dead space inherent in brief timed urine collections. As calculated directly from the arteriovenous difference in plasma inulin concentration, the filtration fraction was unaffected during the initial activation of TGF by benzolamide. However, during the ensuing 30 to 60 minutes filtration fraction declined significantly ($P = 0.004$) for the effect of time on filtration fraction by repeated measures (Fig. 5). Combining these inulin-extraction data with the RBF measurements as an alternate method for calculating GFR yielded similar results as obtained for GFR by inulin clearance. (Fig. 6). The data obtained by inulin clearance were compared also to those obtained by inulin extraction for the ability of filtration fraction or GFR after 10 minutes of benzolamide to predict the respective values after 50 minutes of benzolamide. The results are shown in Table 1. Whether determined by inulin clearance or inulin extraction, filtration fraction at 10 minutes strongly correlated with filtration fraction at 50 minutes. However, inulin clearance at 10 minutes correlated weakly with inulin clearance at 50 minutes even though these were the same data used to calculate the highly-correlated filtration fractions. This implies that much of the inter-animal variability in the course taken by GFR during early TGF resetting results from true variability in the physiologic response (not from measurement error), and that the hemodynamics of early TGF resetting are characterized by a predictable increase in RBF, a predictable decrease in filtration fraction, and a variable change in GFR.

Since the ability to detect a trend in filtration fraction in control animals by the ratio of inulin clearance to RBF was substantiated by inulin extraction experiments, the time course also was calculated for the ratio of inulin clearance/RBF for the converting enzyme and NOS I blocked rats. Only enalapril rats manifested a decline in inulin clearance/RBF at the onset of TGF activation ($P < 0.03$). In each group, a stronger correlation of the 10 and 50 minute data was obtained for inulin clearance/RBF than for inulin clearance (Table 1). However, in the enalapril rats, the correlation was negative suggesting regression to the mean. During TGF resetting, there was a small, but significant decline in inulin clearance/RBF in SMTC rats ($P = 0.028$). An overall decrease of similar magnitude was not statistically significant for the enalapril rats, which showed more variability (Fig. 5).

### Table 1. Correlation coefficients between values obtained after 10 and 50 minutes of benzolamide

<table>
<thead>
<tr>
<th></th>
<th>RBF by flowmetry</th>
<th>GFR by inulin clearance</th>
<th>GFR/RBF</th>
<th>Filtration fraction by Fick method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.941</td>
<td>0.320</td>
<td>0.728</td>
<td>0.935</td>
</tr>
<tr>
<td>Enalaprilat</td>
<td>0.770</td>
<td>-0.142</td>
<td>-0.405</td>
<td>-0.035</td>
</tr>
<tr>
<td>SMTC</td>
<td>0.880</td>
<td>0.742</td>
<td>0.9577</td>
<td>-</td>
</tr>
</tbody>
</table>

In every case, GFR/RBF is more correlated than the GFR from which GFR/RBF is derived. Filtration fraction determined by Fick method for controls is only slightly better than GFR/RBF, but much better than GFR. These data point to an inherent variability in GFR between the time points.
DISCUSSION

A functioning tubuloglomerular feedback system has been established in publications from many laboratories. Most of this research has employed micropuncture to measure adjustments in glomerular filtration brought about within two to three minutes in order to compensate for abrupt changes in tubular flow. In such studies, the range of tubular flow rates over which TGF is maximally efficient has been shown to surround the natural tubular flow rate [5]. For the purposes of most studies, the behavior of TGF is considered to be static. However, it is obvious that TGF cannot be a static process. For example, the immediate action of TGF is to confer an inverse dependence of SNGFR on late proximal flow or tubular fluid salt concentration at the macula densa. Therefore, any physiologic circumstance where SNGFR and distal salt delivery change in the same direction also requires a change in the behavior of TGF. Typical examples of this include normal growth and development of the kidney, pregnancy, the renal response to changes in extracellular volume, changes in cardiovascular hemodynamics, the single nephron response to reduced renal mass, etc. Furthermore, TGF adaptation to changing physiological circumstances cannot be accounted for merely by increases or decreases in the maximum range of the TGF response or by changes in the TGF slope, since such changes would not allow for the preservation of TGF efficiency that usually occurs [5].

Furthermore, since there tends to be less variation between the ambient tubular flow and the TGF inflection point in a given nephron than in ambient tubular flow among a group of nephrons [5], TGF resetting can likely be mediated from within the JGA independent of systemic neurohumoral events. In previous micropuncture studies, we demonstrated that TGF resets during one hour in response to augmented tubular flow in a single nephron [1] or during systemic infusion of benzolamide where the resetting is contrary to that expected from the effects of benzolamide on volume status [2]. The evidence for TGF resetting by the JGA during benzolamide included restoring the ambient tubular flow to a steep part of the TGF curve from which it was initially displaced by TGF activation and a gradual upward relaxation in late proximal flow during TGF resetting. It can be shown by geometry of the TGF curve that this combination of effects requires the TGF curve to reset (Fig. 1). However, prior studies of early TGF resetting do not address the glomerular hemodynamic facet of TGF resetting and the micropuncture method is poorly suited to serial assessment of glomerular hemodynamics in freely flowing nephrons. Hence, the current studies were performed using whole kidney blood flow, inulin clearance, and inulin extraction.

The present data demonstrate that during one hour of a sustained reduction in proximal reabsorption, there is initial renal vasoconstriction and reduced GFR as expected from the activation of TGF. This is followed by a gradual restoration of renal blood flow without a concomitant increase in GFR. The timing of these events corresponds to the rightward resetting of the TGF curve inferred from prior micropuncture studies [1, 2]. Furthermore, the gradual increase in renal blood flow requires NOS I but is unaffected by prior blockade of angiotensin converting enzyme, which should have overridden further suppression of RAS activity by the macula densa.

Macula densa NOS I is already known to be an important endogenous modulator of TGF [6, 7] and inhibition of this enzyme has been shown to prevent rebound hyperfiltration that occurs when benzolamide is administered for 24 hours and then withdrawn [8]. Furthermore, macula densa NOS I is calcium dependent and could be activated by calcium, which enters the macula densa cell that is depolarized by apical salt flux [9], providing a link from increased distal delivery to activation of macula densa NOS. Finally, long-term suppression of NOS I leads gradually to arterial hypertension due to sodium retention that results from a tonic leftward resetting of the TGF response [10]. Hence, there was a high prior likelihood that NOS I blockade would interfere with the renal hemodynamics of TGF resetting as demonstrated by the present data.

There is also a sound basis to suspect that rightward TGF resetting is mediated by macula densa suppression of the renin angiotensin system. Macula densa salt transport is a major determinant of renin secretion [11] and, through this mechanism, renin secretion is expected to decline as distal delivery increases in response to benzolamide. Furthermore, based on the glomerular hemodynamic actions of angiotensin II [12], suppressing RAS activity should increase renal blood flow and reduce filtration fraction, which is precisely the pattern observed during TGF resetting in control animals. Therefore, the failure of enalaprilat to suppress the increase in RBF during TGF resetting comes as a surprise. Notably, enough enalaprilat was administered to increase basal renal blood flow, reduce basal filtration fraction, and prevent a hypertensive response to angiotensin I. Hence, the lack of effect on TGF resetting is not likely due to under-dosing the enalaprilat.

The present data reveal that during a sustained TGF stimulus, the system relaxes to permit an increase in RBF without restoring GFR. This finding has teleologic appeal if TGF is viewed as a system for guarding the kidney against negative energy balance [13], since increasing RBF without increasing GFR will increase oxygen delivery without increasing the energy required for tubular transport.

The significant decline in filtration fraction that occurs as the kidney vasodilation occurs during one hour of right-
ward TGF resetting reveals that the vascular events that accompany TGF resetting are not a mirror image of TGF-mediated vasoconstriction. Traditionally, the TGF response is viewed as being mediated by constriction of the afferent arteriole. If this were true and if TGF resetting merely results from fatigue of the afferent arteriole, then filtration fraction should increase during rightward resetting. However, a more accurate depiction of the TGF effector response includes constriction of the afferent arteriole across the range of stimuli combined with a biphasic effect on efferent arteriolar resistance that parallels the afferent arteriole around the usual operating point, but actually begins to dilate while the afferent arteriole further constricts during maximum stimulation [14, 15]. The present data suggest that rightward TGF resetting also involves gradual vasodilation of the efferent arteriole, since this will increase renal blood flow and reduce filtration fraction. There are two alternative explanations. The first involves afferent arteriolar vasodilation accompanied by a reduction in glomerular ultrafiltration coefficient ($K_f$), which does not seem plausible based on any known mediators of glomerular hemodynamics. Furthermore, the present experiments were conducted under hydropenic conditions where filtration equilibrium prevails, eliminating $K_f$ as a determinant of SNGFR [16–19]. The second alternative to efferent arteriolar vasodilation involves a gradual increase in tubular pressure, which prevents GFR from increasing in spite of reduce afferent arteriolar resistance. In fact, we have previously monitored the pressure in free-flowing proximal tubules during benzolamide infusion and found it to remain stable at 1.1 mm Hg above the pre-benzolamide baseline during the first 45 minutes of benzolamide infusion [2].

Because there was much variability in the trajectory for inulin clearance during TGF resetting, secondary experiments were performed to corroborate the clearance data by arteriovenous inulin differences. Indeed, the Fick method proved capable of detecting a change in filtration fraction far smaller than any proportional change in GFR response is viewed as being mediated by constriction of the afferent arteriole [2]. The present findings are sufficient to conclude that the efferent arteriole must dilate during early TGF resetting, but are not sufficient to draw clear insight about the af-
different resistance. This is illustrated in Figure 8, which shows the combination of possible changes in afferent and efferent arteriolar resistances compatible with increasing RBF but not GFR. The present studies reveal hemodynamic changes that correspond to TGF resetting with implications for energy supply/demand in the proximal tubule. However, these studies do not explicitly characterize the new TGF curve that results from resetting. A full and precise evaluation of the glomerular hemodynamics of TGF resetting by traditional glomerular micropuncture is not feasible due to the time required to gather such data. On the other hand, the present finding that GFR fails to increase along with renal blood flow during one hour of TGF resetting indicates the original observation that benzolamide activates TGF and reduces single nephron GFR, since that observation was made in micropuncture experiments lasting more than an hour [19]. The consistent decline in filtration fraction during TGF resetting also may explain why ambient late proximal flow appears to increase during this period [2], while GFR does not. This is because reducing filtration fraction will reduce oncotic pressure in the peritubular capillary, which is a driving force for proximal reabsorption.

Benzolamide has been employed as a tool for activating TGF since the 1970s [19]. Since then it has come to light that there is also carbonic anhydrase [21] and sodium-proton exchange [22] in the macula densa, which may mediate up to 20% of apical salt entry to the macula densa [23]. Since the sodium gradient for driving apical sodium-proton exchange in macula densa is small to begin with, luminal pH disequilibrium resulting from benzolamide could eliminate that portion of apical sodium entry at the macula densa. However, this would have the opposite effect of a TGF stimulus. Furthermore, we observe that TGF resetting cannot be explained by simply desensitizing the macula densa to salt. If this were the case, then TGF resetting would appear as the mirror image of TGF activation, which is clearly not the case.

In summary, renal hemodynamic correlates have been established for the process whereby the tubuloglomerular feedback system adjusts over an hour of persistent stimulation. This resetting includes a restoration of renal blood flow and a decline in filtration fraction, which requires predominant dilation of the efferent arteriole. Macula densa NOS is required for normal resetting. The present data fail to confirm a major role for macula densa renin suppression in these events.

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