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Possible mechanism of efferent arteriole (Ef-Art) tubuloglomerular feedback

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Adenosine triphosphate (ATP) is liberated from macula densa cells in response to increased tubular NaCl delivery. However, it is not known whether ATP from the macula densa is broken down to adenosine, or whether this adenosine mediates efferent arteriole (Ef-Art) tubuloglomerular feedback (TGF). We hypothesized that increased macula densa Ca^{2+}, release of ATP and degradation of ATP to adenosine are necessary for Ef-Art TGF. Rabbit Ef-Arts and adherent tubular segments (with the macula densa) were simultaneously microperfused *in vitro* while changing the NaCl concentration at the macula densa. The Ef-Art was perfused orthograde through the end of the afferent arteriole (Af-Art). In Ef-Arts preconstricted with norepinephrine (NE), increasing NaCl concentration from 10 to 80 mM at the macula densa dilated Ef-Arts from 7.5 ± 0.7 to 11.1 ± 0.3 μm. Buffering increases in macula densa Ca^{2+} with the cell-permeant Ca^{2+} chelator BAPTA-AM diminished Ef-Art TGF from 3.1 ± 0.3 to 0.1 ± 0.2 μm. Blocking adenosine formation by adding α-β-methyleneadenosine 5′-diphosphate (MADP) blocked Ef-Art TGF from 2.9 ± 0.5 to 0.1 ± 0.2 μm. Increasing luminal NaCl at the macula densa from 10 to 45 mM caused a moderate Ef-Art TGF response, 1.3 ± 0.1 μm. It was potentiated to 4.0 ± 0.3 μm by adding hexokinase, which enhances conversion of ATP into adenosine. Our data show that *in vitro* changes in macula densa Ca^{2+} and ATP release are necessary for Ef-Art TGF. ATP is broken down to form adenosine, which mediates signal transmission of Ef-Art TGF.

Kidney International (2007) **71,** 861–866. doi:10.1038/sj.ki.5002161; published online 7 March 2007

KEYWORDS: macula densa; glomerular hemodynamics; adenosine; microperfusion

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Received 25 August 2006; revised 4 January 2007; accepted 9 January 2007; published online 7 March 2007

Glomerular filtration is a function of intraglomerular pressure, the surface area available for filtration, plasma flow rate, plasma oncotic pressure, Bowman's capsule pressure and K_f.[1] Because the afferent arteriole (Af-Art), glomerulus and efferent arteriole (Ef-Art) are arranged in series, they regulate inflow and outflow of blood through the glomeruli, and thus their dynamics are closely interrelated. Changes in Af- and/or Ef-Art resistance could influence glomerular filtration by altering glomerular capillary pressure. Thus understanding how the Af- and Ef-Arts are regulated is fundamental to explaining how glomerular filtration rate (GFR) is regulated under specific physiological and pathological conditions.

Macula densa cells are located within the thick ascending limb, and their basolateral membranes come into contact with extraglomerular mesangial cells, which in turn are contiguous with smooth muscle cells of the Af- and Ef-Art. Their function is to sense tubular fluid NaCl concentration and send a signal that regulates Af- and Ef-Art resistance, a process known as tubuloglomerular feedback (TGF).[2,3] Although there have been numerous studies of Af-Art TGF, much less attention has been paid to investigating directly whether an analogous process might also modulate resistance in the Ef-Art. We have obtained direct evidence that preconstricted Ef-Arts relax via activation of the adenosine A_2 receptor when the NaCl concentration at the macula densa is increased.[3] However, the mechanism of Ef-Art TGF is unknown.

Recent studies have shown that adenosine triphosphate (ATP) is liberated from macula densa cells in response to increased tubular NaCl delivery *in vitro*.[4] Additionally, ATP levels in the interstitium of the renal cortex are increased by maneuvers that cause a TGF response.[5] Extracellular ATP may exert biological effects directly via its P_2 purinergic receptor, or indirectly via hydrolysis by ectonucleotidases, forming adenosine that binds to adenosine receptors. We and others have shown that adenosine constricts Af-Arts and dilates Ef-Arts via activation of the A_1 and A_2 receptor, respectively.[6] Adenosine has been implicated in the Af-Art TGF response via activation of the adenosine A_1 receptor.[7,8] We have also shown that ATP released from the macula densa is broken down to form adenosine monophosphate (AMP) in the extracellular space, which in turn is degraded by ecto-5′-nucleotidases to adenosine that mediates signal transmission

of the Af-Art TGF response.[9] Thus adenosine, which may be produced in the interstitial space, mediates both Af-Art and Ef-Art TGF. We tested the hypothesis that changing luminal NaCl from 10 to 80 mM increases intracellular Ca^{2+} in the macula densa, which stimulates the release of ATP. ATP is further hydrolyzed to adenosine to induce vasodilatation.

RESULTS

Ef-Art TGF response

We first studied the effect of increasing NaCl at the macula densa in preconstricted Ef-Art, and found that increasing NaCl concentration at the macula densa induces vasodilatation in all the arteriole studied (Table 1).

Ef-Art TGF during removal of intracellular Ca^{2+} from the macula densa

To study the mechanism of Ef-Art TGF, we first investigated intracellular calcium in the macula densa and its role in regulating Ef-Art TGF, adding the Ca^{2+} chelator BAPTA-AM (25 μm) to the lumen. When Ef-Arts were preconstricted with norepinephrine (NE) to ~70% of resting diameter (from 12.4 ± 0.5 to 8.1 ± 0.4 µm; $P = 0.001$), changing the perfusion medium from 10 to 80 mM NaCl caused the Ef-Art to dilate from 8.1 ± 0.4 to 11.3 ± 0.5 μm ($P < 0.001$). Adding BAPTA-AM did not alter Ef-Art diameter when the macula densa was perfused with 10 mM NaCl, but inhibited dilatation when the perfusate was switched to 80 mM NaCl; diameter remained unchanged, 8.15 ± 0.4 vs 8.2 ± 0.4 μm (Figure 1). In time controls, Ef-Art diameter increased from 7.5 ± 0.7 to 11.1 ± 0.3 μm ($P < 0.05$) when the perfusate was first changed from 10 to 80 mM NaCl. When we repeated the process, diameter increased from 6.9 ± 1.1 to 11.2 ± 0.3 μm ($P < 0.05$; $n = 3$). Time controls showed that the Ef-Art dilatation induced by 80 mM NaCl was reproducible (Figure 2).

TGF during blockade of adenosine formation

We next investigated whether Ef-Art TGF requires adenosine by adding α-β-methyleneadenosine 5′-diphosphate (MADP) (10^{-4} M) to the bath to block 5′-nucleotidase and prevent adenosine formation. During the control period, increasing NaCl at the macula densa dilated the preconstricted Ef-Art from 9.3 ± 0.4 to 12.1 ± 0.5 μm ($n = 6$; $P < 0.05$). Adding MADP to the bath did not alter Ef-Art diameter when the macula densa was perfused with 10 mM NaCl, but inhibited dilatation when the perfusate was changed to 80 mM NaCl; 9.1 ± 0.4 vs 8.9 ± 0.4 μm (Figure 3).

Ef-Art TGF during increasing ATP hydrolysis

To determine whether increasing hydrolysis of ATP to adenosine enhances Ef-Art TGF, we added hexokinase (20 μ/ml), which facilitates hydrolysis of ATP after it is released from the macula densa and thereby increases adenosine formation. We first tested whether adding hexokinase to the bath would enhance the Ef-Art TGF induced by 80 mM NaCl. When the perfusate was changed from 10 to 80 mM NaCl, Ef-Art diameter increased from

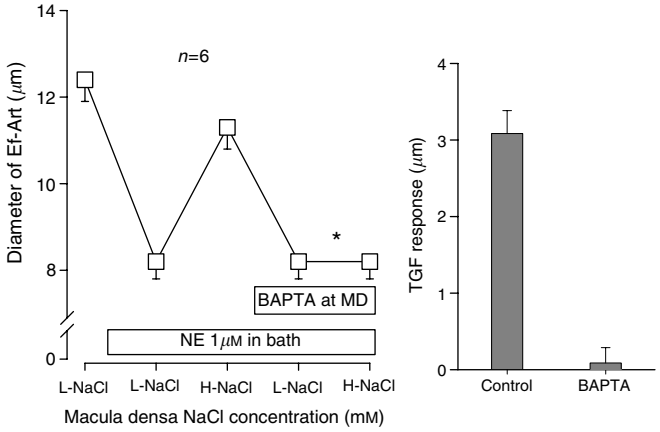

Figure 1 | Orthograde perfusion of the efferent arteriole (Ef-Art), showing the effect of varying NaCl concentration at the macula densa (MD) on Ef-Art diameter in the controls and in samples treated with the calcium chelator BAPTA-AM. Chelation of MD intracellular calcium inhibited Ef-Art tubuloglomerular feedback (TGF). *$P < 0.05$, with vs without BAPTA-AM.

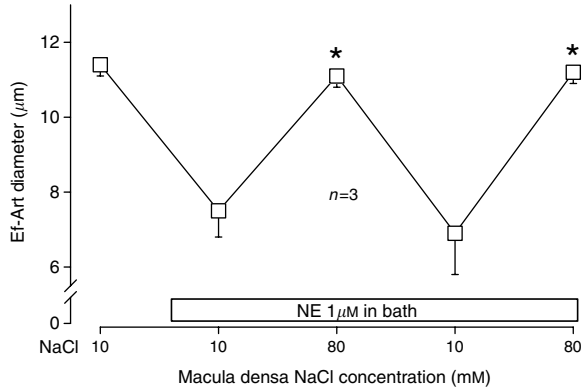

Figure 2 | Orthograde perfusion of the Ef-Art, showing the effect of varying NaCl concentration at the MD on Ef-Art diameter with norepinephrine (NE) preconstriction. When Ef-Arts were preconstricted by adding 1 μm NE to the bath, increasing the NaCl concentration at the MD caused the Ef-Art to dilate (*$P < 0.05$, low vs high NaCl).

Table 1 | Ef-Art TGF response

	Ef-Art diameter (μm)		
	Basal	*NE preconstriction before TGF*	*NE preconstriction during TGF*
1	11.6	7.4	10.7
2	11.8	8.7	11.6
3	10.8	6.3	10.9
4	11.1	7.9	10.8
5	13.1	7.5	11.7
6	13	9.7	13.4
Mean ± s.d.	11.9 ± 0.4	7.9 ± 0.5	11.5 ± 0.4

*Perfusion rate in the macula densa was about 15 nl/min. Pressure at the end of Af-Art (inside the glomeruli) was 50 mmHg.
NE, norepinephrine; TGF, tubuloglomerular feedback; s.d., standard deviation.

9.0 ± 0.5 to 12.5 ± 0.7 μm. After adding hexokinase to the bath, increasing NaCl dilated Ef-Arts from 8.8 ± 0.4 to 12.7 ± 0.5 μm, an increase not different from control (Figure 4). This is not surprising, as high NaCl-induced Ef-Art dilatation had already reached a maximum. To avoid this, we used a smaller increase in NaCl concentration at the macula densa to induce Ef-Art TGF. As shown in Figure 5, during the control period increasing NaCl from 10 to 45 mM increased Ef-Art diameter from 8.8 ± 0.6 to 10.2 ± 0.6 μm. In the presence of hexokinase, when the perfusate was changed from 10 to 45 mM the TGF response was enhanced; diameter increased from 8.1 ± 0.6 to 12.1 ± 0.5 μm ($n = 6$; $P < 0.05$ with vs without hexokinase) (Figure 5).

Ef-Art TGF during inhibition of the P_2 receptor

To determine whether ATP released from the macula densa binds to another cell type and activates Ef-Art TGF, we examined the TGF response before and after blocking the P_2 receptors. During the control period, when NaCl was increased from 10 to 80 mM NaCl, TGF increased Ef-Art diameter from 8.3 ± 1.1 to 11.0 ± 1.0 μm. Adding suramin (10^{-4} M) to the bath and lumen did not alter the Ef-Art TGF response. When macula densa NaCl was changed from 10 to 80 mM, TGF increased diameter to the same degree as control (from 7.7 ± 1.0 to 11.1 ± 1.0 μm; $n = 5$) (Figure 6).

DISCUSSION

We have previously provided direct evidence that preconstricted Ef-Arts relax when the NaCl concentration at the macula densa is increased.[10] In the present study, we investigated the possible mechanism of Ef-Art TGF. Our findings suggest that the mechanism of Ef-Art TGF is similar to Af-Art TGF, save that intracellular Ca^{2+} in the macula densa needs to be increased to initiate the signaling cascade. Increased macula densa Ca^{2+} in response to increased NaCl stimulates the release of ATP, which is then rapidly

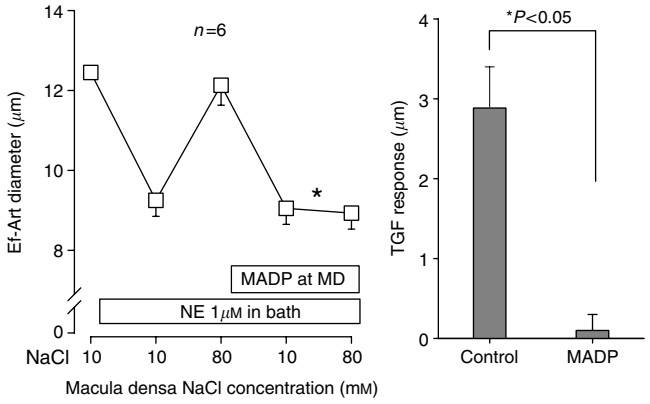

Figure 3 | Orthograde perfusion of the Ef-Art, showing the effect of varying NaCl concentration at the MD on Ef-Art diameter in the controls and in samples treated with α-β-methyleneadenosine 5′-diphosphate (MADP). Blocking adenosine formation with MADP suppressed Ef-Art TGF. *$P < 0.05$, with vs without MADP.

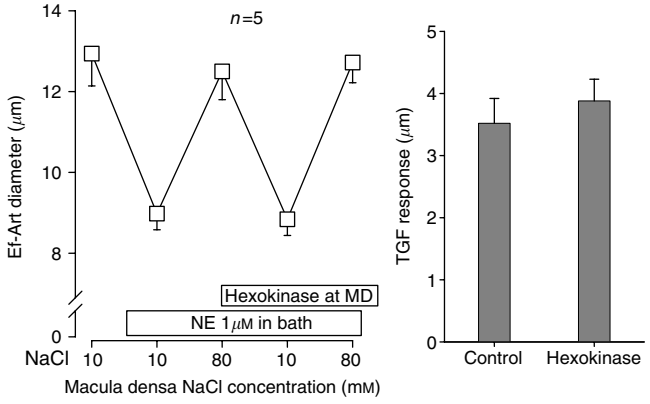

Figure 4 | Orthograde perfusion of the Ef-Art, showing the effect of varying NaCl concentration at the MD (from 10 to 80 mM) on Ef-Art diameter in the controls and in samples treated with hexokinase. Enhancing conversion of ATP did not augment maximal Ef-Art TGF.

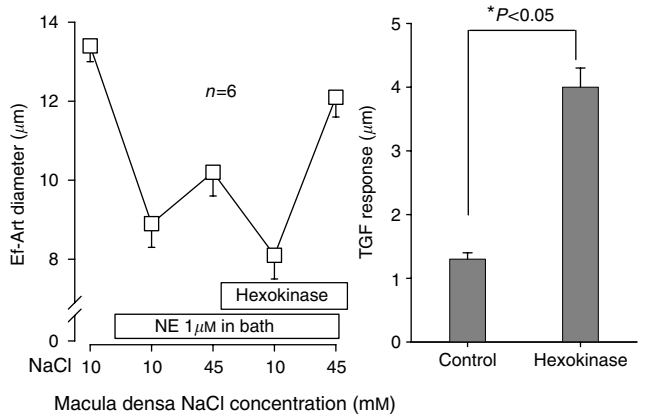

Figure 5 | Orthograde perfusion of the Ef-Art, showing the effect of varying NaCl concentration at the MD (from 10 to 45 mM), which induced modest Ef-Art TGF, in the controls and in samples treated with hexokinase. Enhancing conversion of ATP augmented Ef-Art TGF. *$P < 0.05$, with vs without hexokinase.

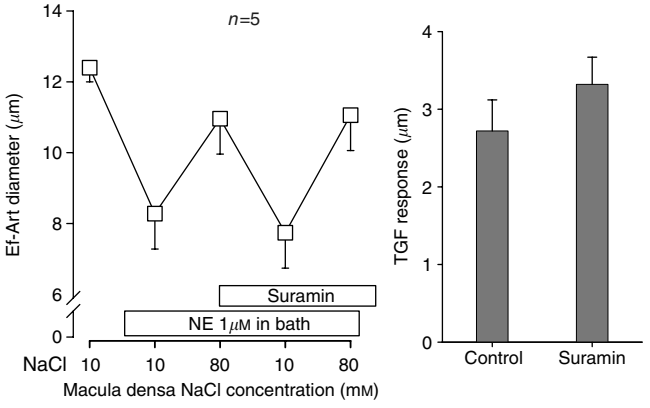

Figure 6 | Orthograde perfusion of the Ef-Art, showing the effect of varying NaCl concentration at the MD on Ef-Art diameter in the controls and in samples treated with suramin. Adding suramin to the bath and lumen did not alter Ef-Art TGF.

metabolized to ADP, AMP, and ultimately adenosine by the cell surface ectonucleotidases involved in the Ef-Art TGF response. This is supported by the fact that when we prevented or enhanced adenosine formation, the TGF response was abolished or augmented. Moreover, blocking the P_2 receptor with suramin did not influence Ef-Art TGF.

We have obtained direct evidence that preconstricted Ef-Arts relax when the NaCl concentration at the macula densa is increased. In micropuncture studies using the stop-flow technique, Schnermann and Briggs[11] reported that when the rate of perfusion through the loop of Henle was increased, single-nephron GFR (SNGFR) decreased; whereas stop-flow pressure (SFP) remained essentially unchanged, suggesting that the change in SNGFR was primarily due to a reduction in ultrafiltration coefficient (K_f). Because SFP did not change, Af-Art and Ef-Art resistance were either unaltered or changed in the same direction. A similar conclusion was reached by Davis,[12] using a low dose of the calcium channel blocker nitrendipine. They found that during blockade of the calcium channel, TGF-induced decreases in SNGFR were not accompanied by simultaneous changes in P_{GC}, suggesting parallel alterations in Af-Art and Ef-Art resistance. Thus collectively these studies do not support our conclusion that during TGF changes in Ef-Art resistance are opposite from those in the Af-Art. However, most of these evidences supporting participation of the Ef-Art in TGF have been indirect, depending on calculated values. Briggs and Wright[13] further examined stellate vessel pressure and single-nephron plasma flow as well as SNGFR and SFP (as indicators of P_{GC}), while changing the perfusion rate from 16 to 40 nl/min; they found that both SNGFR and SFP decreased but stellate vessel pressure did not change, and concluded that the feedback response to increased flow through the loop of Henle is probably mediated primarily by Af-Art vasoconstriction. As stellate vessel pressure did not change despite decreasing P_{GC}, one possible explanation is that Ef-Arts dilate during TGF, decreasing resistance and improving transmission of pressure to the stellate vessels. More recently, Deng *et al.*[14] studied renal hemodynamics during early TGF resetting. They found that during 1 h of persistent TGF stimulation, renal blood flow increases toward normal but GFR does not. This requires predominant dilatation of the Ef-Art, an interpretation consistent with our findings.

We and other investigators have shown that increased intracellular Ca^{2+} in the macula densa is essential for transmission of Af-Art TGF.[10,15] To test whether this is also true for Ef-Art TGF, we buffered increases in intracellular Ca^{2+} by adding the cell-permeant Ca^{2+} chelator BAPTA-AM. Our data clearly demonstrate that like Af-Art TGF, Ef-Art TGF also depends on macula densa intracellular Ca^{2+}, because chelating Ca^{2+} with BAPTA-AM blocked Ef-Art TGF. Therefore, increasing intracellular Ca^{2+} in the macula densa is a key step in initiating Af-Art as well as Ef-Art TGF.

Recently, Peti-Peterdi[16] studied the calcium wave during TGF and found that activation of TGF by increasing tubular flow rate at the macula densa rapidly produced a significant elevation in intracellular Ca^{2+} concentration in extraglomerular mesangial cells, and rapid cell-to-cell propagation of the calcium wave was observed to originate from the macula densa and travel upstream toward proximal segments of the Af-Art and adjacent glomeruli, which was abolished by inhibition of P_2 purinergic receptors. The signal transduction pathways involved in the TGF response remain poorly understood; however, both ATP release from macula densa cells [4,17] and local formation of adenosine may play important roles in this process.[18–24] We and others have reported that ATP is released from macula densa cells into the interstitium of the extraglomerular mesangium, where it is broken down by nucleoside triphosphate diphosphohydrolases to AMP.[4,25] The latter is converted into adenosine by ecto-5′-nucleotidase to activate the adenosine A_1 receptor, causing Af-Art constriction.[9,26] This enzyme is widely distributed in the kidney and is expressed in all nephron segments, including macula densa cells.[27–29] A recent study using gene targeting provided further evidence for this mechanism by showing that Af-Art TGF was impaired in ecto-5′-nucleotidase knockout mice.[30] More recently, a study by Huang *et al.*[31] found that adenosine generated by both ecto-5′-nucleotidase-dependent and -independent mechanisms participates in the mediation of TGF *in vivo*. We and others have shown that adenosine constricts the Af-Art and dilates the Ef-Art via activation of the A_1 and A_2 receptor, respectively.[32,33,6] We have also shown that increasing hydrolysis of ATP by adding hexokinase to the bath significantly enhanced Af-Art TGF.[9] In a previous study, we also showed that increasing NaCl at the macula densa dilated the Ef-Art via the A_2 receptor.[3] On the basis of these findings, it is reasonable to propose that Ef-Art TGF shares the same mediator as Af-Art TGF, which is adenosine acting as a mediator of Ef-Art TGF. Although we cannot exclude the possibility that direct release of adenosine from macula densa cells has an effect on TGF, our results demonstrate that decreasing adenosine formation by blocking 5′-nucleotidase inhibits Ef-Art TGF, whereas increasing adenosine formation by enhancing ATP hydrolysis augments Ef-Art TGF.

Because adenosine is a major metabolic end product and is known to elicit vasoactive effects, it has been viewed as one possible paracrine factor that transmits signals from the macula densa to mediate the TGF response. On the other hand, abundant evidence indicates that extracellular ATP exerts its biological effects by activating members of the P_2 class of purinoceptors. P_2X and P_2Y receptors are expressed on various segments of the renal vessels as well as the microvasculature,[31,34–37] and P_2 receptors mediate autoregulatory behavior.[38,37] To find out whether P_2 receptors are also involved in Ef-Art TGF, we tested a P_2 receptor antagonist (suramin). Blocking the purinergic P_2 receptor with suramin did not alter Ef-Art TGF, suggesting that activation of the P_2 receptor may not play a very important role in Ef-Art TGF. In agreement with our findings, *in vitro* studies of isolated blood-perfused juxtamedullary nephrons showed that Ef-Arts were nonresponsive to ATP stimulation.[39] The lack of

Ef-Art responsiveness suggests that this segment does not possess sufficient P_2 receptors to modulate vascular function.

In conclusion, it appears that increasing macula densa intracellular Ca^{2+} and adenosine formation in response to increased NaCl at the macula densa is a common pathway for both Af-Art and Ef-Art TGF. Increasing macula densa cell Ca^{2+} stimulates ATP release. ATP is hydrolyzed to adenosine and activates the A_2 adenosine receptors to induce Ef-Art dilatation, whereas it activates the A_1 adenosine receptors to induce Af-Art constriction. Thus the distribution of A_1 and A_2 adenosine receptors may be responsible for the differing responses between Af- and Ef-Arts during TGF.

MATERIALS AND METHODS

We used a method similar to that described previously to isolate and microperfuse Ef-Arts with the macula densa attached[40] from the superficial cortex of the rabbit kidney.[3,40] Young male New Zealand white rabbits were fed standard chow (Ralston Purina, St Louis, MO, USA) and given tap water *ad libitum*. They were anesthetized with ketamine (50 mg/kg, intramascularly) and given heparin intravenously (500 U). The kidneys were removed and sliced along the corticomedullary axis. Slices were placed in ice-cold MEM (Gibco, Grand Island, NY, USA) containing 5% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO, USA) and dissected under a stereomicroscope (Olympus SZH, Tokyo, Japan). A single superficial Ef-Art, Af-Art, and glomerulus from each rabbit were microdissected together with adherent tubular segments consisting of portions of the thick ascending limb, macula densa, and early distal tubule. Using a pipette, the microdissected complex was transferred to a temperature-regulated chamber mounted on an inverted microscope (model IMT-2, Olympus) with Hoffmann modulation.

Oxygenated MEM with room air containing 5% BSA was used to perfuse the Ef-Art via the Af-Art. As Ef-Art resistance is influenced by the upstream Af-Art, to eliminate the hemodynamic influences of the Af-Art, the Af-Art was cut short ($\cong 50\,\mu$m). The perfusion pipette was advanced to the end of the Af-Art. The tip of the pressure pipette was placed just beyond the distal end of the Af-Art, maintaining intraluminal pressure at 50 mmHg by adjusting perfusion flow accordingly. The macula densa was perfused with 10, 45 or 80 mM NaCl in a solution containing (in mM): 10 HEPES; 0.5 K_2HPO_4; 4 $KHCO_3$; 1.2 $MgSO_4$; 1.0 $CaCO_3$; 0.5 sodium acetate; 0.5 sodium lactate, and 5.5 glucose, adding 1 M NaCl to achieve the desired final NaCl concentration. The driving force to maintain the tubular perfusion rate (about 15 nl/min) was provided by hydrostatic pressure. The bath had a volume of 1 ml and was exchanged continuously at a rate of 1 ml/min with MEM containing 0.15% BSA. Microdissection and cannulation were completed within 90 min at 8°C and the bath was then gradually warmed to 37°C for the rest of the experiment. Once the temperature was stable, a 30-min equilibration period was allowed before taking any measurements. Images were displayed at magnifications up to $\times 1980$ and recorded with a Sony video system consisting of a camera (DXC-755), monitor (PVM1942), and video recorder (EDV-9500). Ef-Art diameter was measured with an image analysis system (Universal Imaging, Fryer, Carpentersville, IL, USA). On the basis of Edwards' study of the segmental effects of vasoconstrictors on isolated renal microvessels, which showed that contractile responses of the Ef-Art were limited to the first 50–75 μm,[41] we took all our measurements within 50 μm of the glomeruli.

As the Ef-Art has no myogenic tone during perfusion *in vitro*, vasodilator responses are absent during TGF; thus we preconstricted Ef-Arts with NE to induce basal tone. The protocol consisted of four experimental periods: (1) a 30-min equilibration period, first perfusing the macula densa with 10 mM NaCl and then adding NE to the bath to preconstrict the Ef-Art for 5 min; (2) perfusing the macula densa with 80 mM NaCl solution for 5 min; (3) perfusing the macula densa with 10 mM NaCl for 15 min while adding drugs directly to the macula densa lumen, vascular lumen or bath for 15 min; and (4) perfusing the macula densa with 80 mM NaCl for 5 min while continuing to constrict the Ef-Art with NE and/or other drugs. In experiments studying the effect of hexokinase, 45 mM NaCl was used rather than 80 mM NaCl.

Statistics

Values are expressed as mean $\pm$ s.e.m. Analysis of variance for repeated measures was used to examine the data. Comparisons of first to second, second to third, and third to fourth experimental periods were of primary interest and were examined using paired *t*-tests. Significance was judged using Holm's method for multiple comparisons. The adenosine receptors were analyzed further by comparing the changes between third and fourth experimental periods and between treated and control groups. This component of interaction was assessed using a two-sample *t*-test of the differences, taking $P < 0.05$ as significant.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grant HL-28982.

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