Tubuloglomerular and connecting tubuloglomerular feedback during inhibition of various Na transporters in the nephron

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Am J Physiol Renal Physiol 308: F1026–F1031, 2015. First published February 25, 2015; doi:10.1152/ajprenal.00605.2014.—Afferent (Af-Art) and efferent arterioles (Ef-Art) resistance are critical regulators of glomerular capillary pressure (GCP). The GCP regulates the efficiency of glomerular filtration rate (GFR) as well as the macula densa overexpression of Na-Cl cotransporter (NKCC2) (2, 11), and benzamil blocks this (furosemide + benzamil: −1.1 ± 0.2 mmHg; P < 0.01, n = 6). We conclude that NHE in the nephron decreases PSF (Af-Art constriction) when NKCC2 and ENaC are inhibited, suggesting that in the presence of TGF and ENaC, NHE participates in Af-Art constriction. Thus, NHE is also regulated by eicosanoids released from the glomerulus (17).

In vivo, when TGF is inhibited with benzamil, TGF is potentiated causing a greater decrease in stop-flow pressure (PSF), suggesting that TGF normally antagonizes TGF (24). Also, TGF participates in TGF resetting (23). However, in vivo, even when TGF is blocked by the NKCC2 inhibitor furosemide, TGF does not cause an increase in PSF (vassodilation), suggesting that there is another mechanism(s) that could antagonize TGF. The vasodilator effect of TGF in vivo had only been proven indirectly by observing that when TGF is inhibited, it causes a potentiation of TGF as measured by a further decrease in PSF. Therefore, it is possible that other processes initiated by Na transport along the distal nephron may regulate Af-Art resistance and antagonize TGF.

Recent studies have shown that in addition to expressing NKCC2, macula densa cells functionally and immunologically express Na/H exchanger (NHE) (6, 15). The distal convoluted tubule reabsorbs NaCl via thiazide-sensitive Na-Cl cotransporters (NCC) (13, 16). Thus, NCC may also be involved in the regulation of Af-Art resistance.

We hypothesize that in addition to NKCC2, under some circumstances NHE can also mediate a vasoconstrictor mechanism that antagonizes TGF. Thus, when both NKCC2 and NHE are blocked, TGF increases PSF due to Af-Art dilation (18). To test this hypothesis, we used the nephron micropuncture technique in vivo. We measured two consecutive TGF responses by increasing the perfusion of the nephron from 0 to 40 nl/min, while adding the drugs to the tubular perfusate that blocks transporters and measuring PSF as an index of GCP. The limitation of this technique is that a decrease in PSF or GCP could be due to an Af-Art constriction and/or Ef-Art dilation and vice versa, an increase in PSF could be due to an Af-Art dilation and/or Ef-Art constriction. Thus, the data presented here were interpreted taking into consideration these dual effects on TGF.

METHODS

Male Sprague-Dawley rats weighing 314.3 ± 3.6 g were used in this study. All experiments were approved by the Henry Ford Health System Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were fed
standard rat chow and given tap water ad libitum. Micropuncture studies were performed as previously described (12, 20). The rats were anesthetized; the left kidney was exposed, placed in a Lucite cup, and immobilized by surrounding it loosely with saline-soaked cotton. After 30–45 min of equilibration, grease was injected into an early segment of a proximal tubule, and two pipettes were inserted. One pipette for perfusion was inserted downstream from the grease block and attached to the nanoliter infusion pump. A second pipette (for measuring P_{SF}), was inserted upstream from the grease block and attached to a micropressure system (model 900A; World Precision Instruments, Sarasota, FL). To generate a P_{SF} curve, the late proximal perfusion rate was incrementally increased from 0 to 10, 20, 30, and 40 nl/min while P_{SF} was measured. Each perfusion rate was maintained for 1–5 min, as required, to observe a stable P_{SF}. We performed two consecutive TGF responses while adding drugs that inhibit the transporters to the tubular perfusate. The following drugs were used: NKCC2 inhibitor furosemide, ENaC inhibitor benzamil, NCC blocker hydrochlorothiazide (HCTZ), and NHE inhibitor dimethylamiloride (DMA).

Statistics. Data are expressed as means ± SE. As the data were normally distributed, we used both Student’s two-sample t-tests and Student’s paired t-tests on repeated-data measurements. Hochberg’s step-up procedure was used to adjust the P values for multiple comparisons to control for the family-wise type I error rate, predefined as 0.05.

RESULTS

Time control TGF responses and effect of inhibiting TGF with NKCC2 blocker furosemide on P_{SF}. To study whether TGF responses varied with time, late proximal tubule perfusion was increased twice from 0 to 40 nl/min while P_{SF} was measured. We found that increasing the tubule perfusion decreased P_{SF}, reflecting Af-Art constriction and/or Ef-Art dilation. There was no difference between the first and second curves, indicating that this response was reproducible over time (Fig. 1A). As expected, this response was blocked by the addition of the NKCC2 inhibitor furosemide (10^{-4} M; Fig. 1B). These data indicate that NKCC2 in the macula densa is a major component of the TGF response.

Effect of inhibiting TGF and CTGF simultaneously on a TGF-like response. To test whether the effect of furosemide on TGF is reproducible, we generated two consecutive P_{SF} response curves in the presence of furosemide. There was no difference between the first and second curves, indicating that this response was reproducible over time (Fig. 1A). As expected, this response was blocked by the addition of the NKCC2 inhibitor furosemide (10^{-4} M; Fig. 1B). These data indicate that NKCC2 in the macula densa is a major component of the TGF response.

Effect of the NCC blocker HCTZ on P_{SF} when NKCC2 and NHE are inhibited. The increase in P_{SF} could be mainly due to a decrease in the resistance of Ef-Art during the CTGF. The increase in P_{SF} was due to CTGF, since it was blocked by the addition of benzamil to the perfusate. We also tested the effect of DMA alone. We found that increasing the tubule perfusion rate decreased P_{SF} similarly in the vehicle- and DMA-treated groups (Fig. 2D). These data suggest that NHE by itself has no effect on TGF or CTGF.

Effect of simultaneously inhibiting NKCC2 and NHE on CTGF response. In the presence of furosemide, increasing tubular perfusion caused no change in P_{SF}. However, inhibition of both NKCC2 and NHE via addition of furosemide and DMA caused P_{SF} to increase in response to increasing the nephron perfusion (Fig. 3A). These data suggest that the vasodilator effect of CTGF can be observed as an increase in P_{SF} when both NKCC2 and NHE are inhibited. The increase in P_{SF} could be mainly due to a decrease in the resistance of Af-Art and/or an increase in the resistance of Ef-Art during the CTGF. The increase in P_{SF} was due to CTGF, since it was blocked by the addition of benzamil to the perfusate (Fig. 3B).

Effect of the NCC blocker HCTZ on P_{SF} when NKCC2 and CTGF are inhibited. In the presence of furosemide and benzamil, P_{SF} decreased in response to increasing the nephron perfusion. Addition of the NCC blocker HCTZ (10^{-3} M) did not affect the decrease in P_{SF} (Fig. 4). These data suggest that nephron NCC does not participate in the control of Af-Art tone.
CTGF, we observed a decrease in PSF. These data suggest a constrictor phenomenon initiated in the nephron. However, when we perfused the nephron with furosemide plus the ENaC inhibitor benzamil to block TGF, we found that the NKCC2 inhibitor furosemide completely blocked TGF. However, when we perfused the nephron with furosemide and benzamil, adding epithelial Na channel (ENaC) blocker benzamil (benz; ●) caused PSF to decrease in response to increasing the nephron perfusion, suggesting that when NKCC2 and CTGF are both blocked there is an additional constrictor phenomenon initiated in the nephron.

We hypothesize that in addition to NKCC2, under some circumstances NHE can mediate a vasoconstrictor mechanism that antagonizes CTGF. Thus, when both NKCC2 and NHE are blocked, CTGF increases PSF due to Af-Art dilation. In contrast to TGF, CTGF is a vasodilator mechanism initiated in the CT by the ENaC, by an increase in NaCl (18). In vitro CTGF dilates Af-Arts while in vivo it antagonizes the decrease in PSF caused by TGF (18, 24). When we inhibited TGF by adding furosemide to the tubule perfusate, the reduction in PSF caused by increasing nephron perfusion was completely blocked as expected.

In vitro CTGF dilates Af-Arts while in vivo it antagonizes the decrease in PSF caused by TGF (18, 24). If TGF and CTGF were the only two mechanisms that control PSF, one would expect that blocking TGF with furosemide would reveal CTGF-induced increase in PSF in response to increasing the nephron perfusion. However, here we show that when TGF was blocked with furosemide, increasing the tubular perfusion did not increase PSF. This observation led us to hypothesize that when NKCC2 is blocked with furosemide, there is another constrictor mechanism that opposes CTGF.

DISCUSSION

We hypothesize that in addition to NKCC2, under some circumstances NHE can mediate a vasoconstrictor mechanism that antagonizes CTGF. Thus, when both NKCC2 and NHE are blocked, CTGF increases PSF due to Af-Art dilation. As expected, we found that the NKCC2 inhibitor furosemide completely blocked TGF. However, when we perfused the nephron with furosemide plus the ENaC inhibitor benzamil to block CTGF, we observed a decrease in PSF. These data suggest a novel Af-Art constrictor and/or Ef-Art dilator mechanism initiated by the nephron. We showed that this vasoconstrictor mechanism can be blocked by inhibiting NHE, but not NCC, and when both NKCC2- and NHE-mediated mechanisms are blocked, CTGF causes an increase in PSF due to Af-Art dilatation.

TGF is a constrictor mechanism initiated by apical NKCC2 in the macula densa (2, 3, 9). Loop diuretics, such as furosemide, added to the tubular perfusate can reduce renal vascular resistance by blocking the constrictor effect of TGF (4, 7, 8, 25). When we inhibited TGF by adding furosemide to the tubule perfusate, the reduction in PSF caused by increasing nephron perfusion was completely blocked as expected.

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In contrast to TGF, CTGF is a vasodilator mechanism initiated in the CT by the ENaC, by an increase in NaCl (18). In vitro CTGF dilates Af-Arts while in vivo it antagonizes the decrease in PSF caused by TGF (18, 24). If TGF and CTGF were the only two mechanisms that control PSF, one would expect that blocking TGF with furosemide would reveal CTGF-induced increase in PSF in response to increasing the nephron perfusion. However, here we show that when TGF was blocked with furosemide, increasing the tubular perfusion did not increase PSF. This observation led us to hypothesize that when NKCC2 is blocked with furosemide, there is another constrictor mechanism that opposes CTGF.
Recent studies have shown that in addition to expressing NKCC2, macula densa cells functionally and immunologically express Na/H exchanger 2 (NHE2) at the apical membrane and NHE4 at the basolateral membrane. These two isoforms likely participate in Na transport, pHi, and cell volume regulation, and may be involved in the regulation of TGF (6, 15). Thus, we suggest that when NKCC2 and NHE are both blocked, the afferent arteriole affect PSF, suggesting that the distal convoluted tubule does not participate in Na transport, pHi, and cell volume regulation, and may be involved in the regulation of TGF (6, 15). Hence, we can explain such data? In short, when luminal NaCl increases and NKCC2 is active, it mediates Na entry, intracellular Na increases, and NHE restores intracellular Na, as put forth by Bell and colleagues (6). NHE is driven in reverse mode (Na out and protons in) by the elevated intracellular Na. However, when NKCC2 is inhibited, it cannot cause an increase in intracellular Na and there is a large gradient for Na entry. This gradient drives Na entry by the NHE. NHE-mediated Na entry causes an increase in intracellular Na, just as NKCC2 would have if it was not inhibited, and the increase in intracellular Na initiates TGF just as it would have if NKCC2 had mediated its entry. Thus, the vasoconstriction caused by increasing luminal NaCl that is mediated by NHE can only be seen when NKCC2 is inhibited and it can operate in a mode that is the opposite of what happens when NKCC2 is not blocked by furosemide.

Alternatively, NHE-induced reduction in $P_{SF}$ may be initiated in a different nephron segment such as CT. Since the CT is in close proximity to the Af-Art and since the intercalated luminal perfusion, and that it can be blocked by the NHE inhibitor DMA.

Using micropuncture, one cannot definitively define the segment or the exact mechanism by which the NHE-mediated decrease in $P_{SF}$ occurs. However, the literature suggests reasonable explanation. Bell and colleagues (14) postulated that Na-K-ATPase does not play a significant role in pumping Na out of the macula densa due to low amounts of active Na-K-ATPase in the basolateral membrane of the macula densa. One may hypothesize that NHE mediates the removal of Na from the cell interior in exchange for H. Thus, intracellular pH will become relatively acidic at some point after the luminal NaCl is raised. We reported that NHE activity is necessary for an increase in luminal Na to activate nitric oxide production in the macula densa, and this blunts TGF (11), and that in vitro, in the absence of furosemide, blockade of NHE with DMA potentiates TGF (21). Now we show data that during the TGF blockade with furosemide, by inhibiting NKCC2, inhibition of NHE causes vasodilation rather than the vasoconstriction. How can one explain such data? In short, when luminal NaCl increases and NKCC2 is active, it mediates Na entry, intracellular Na increases, and NHE restores intracellular Na, as put forth by Bell and colleagues (6). NHE is driven in reverse mode (Na out and protons in) by the elevated intracellular Na. However, when NKCC2 is inhibited, it cannot cause an increase in intracellular Na and there is a large gradient for Na entry. This gradient drives Na entry by the NHE. NHE-mediated Na entry causes an increase in intracellular Na, just as NKCC2 would have if it was not inhibited, and the increase in intracellular Na initiates TGF just as it would have if NKCC2 had mediated its entry. Thus, the vasoconstriction caused by increasing luminal NaCl that is mediated by NHE can only be seen when NKCC2 is inhibited and it can operate in a mode that is the opposite of what happens when NKCC2 is not blocked by furosemide.

Fig. 3. A: effect of simultaneously inhibiting NKCC2 and NHE on CTGF response. Left: in the presence of furosemide (○), adding NHE blocker DMA (●) caused $P_{SF}$ to increase in response to increasing nephron perfusion, suggesting that when NKCC2 and NHE are both blocked, the afferent arteriole (Af-Art) vasodilator effect induced by CTGF can be revealed with increasing nephron perfusion. Right: maximum $P_{SF}$ responses in the furosemide and furosemide+DMA curves. **P < 0.01, ***P < 0.001. B: effect of inhibiting CTGF with ENaC blocker benzamil on PSF when both NKCC2 and NHE are blocked. Left: in the presence of furosemide and DMA (○), increasing the nephron perfusion caused an increase in $P_{SF}$; addition of benzamil to the perfusate blocked this effect (●). Right: maximum $P_{SF}$ responses in the furosemide+DMA curve and furosemide+DMA+benzamil curve. Benzamil prevented the Af-Art vasodilation observed when both NKCC2 and NHE are blocked, indicating that CTGF causes Af-Art vasodilation that can be revealed when both NKCC2 and NHE are completely blocked. *P < 0.05, ***P < 0.001.

Fig. 4. Effects of the Na-Cl cotransporter (NCC) blocker hydrochlorothiazide (HCTZ) on $P_{SF}$ when NKCC2 and CTGF are inhibited. Left: in the presence of furosemide and benzamil (○), adding the NCC inhibitor HCTZ (●) did not affect $P_{SF}$, suggesting that the distal convoluted tubule does not participate in the regulation of Af-Art tone. Right: maximum $P_{SF}$ responses in furosemide+benzamil and furosemide+benzamil+HCTZ curves.
cells of the CT abundantly express NHE in the luminal membrane (5), it is possible that CT intercalated cells mediate this constrictor phenomenon.

We also perfused the nephron with HCTZ, to test whether HCTZ-sensitive NCC plays a role in the regulation of Af-Art constriction, but we found no effect. Recently, another Na transporter has been described in the nephron, the Na-driven Cl/HCO$_3$ exchanger (NDCEBE), which can mediate electroneutral Na reabsorption by acting jointly with pendrin (10). Since this transport is also sensitive to HCTZ, our findings also ruled out the role for NDCEBE in controlling Af-Art tone. Since this is the first report on NHE-induced Af-Art constrictor mechanism, many questions still remain to be answered, in particular defining the exact segment of the distal nephron that initiates this novel mechanism.

Given our finding that both NKCC2 and NHE mediate a decrease in P$_{SF}$, we tested whether we could measure a CTGF-dependent increase in P$_{SF}$. To do this, we studied the effect of furosemide and DMA on changes in P$_{SF}$ induced by increasing tubular perfusion. Here, we report for the first time that in vivo CTGF increased P$_{SF}$ in absolute terms when NHE and NKCC2 were inhibited. This increase in P$_{SF}$ could be due to either dilation of the Af-Art or constriction of Ef-Art during the CTGF. However, CTGF is initiated by the ENaC in the CT that has contact with the Af-Art but not with the Ef-Art, thus it is unlikely that the increase in P$_{SF}$ is mediated by Ef-Art constriction. These data support the hypothesis that when NKCC2 and CTGF are blocked, activation of NHE in the nephron constricts the Af-Art and antagonizes CTGF in response to the increase in tubular perfusion. Furthermore, it also supports the hypothesis that when in vivo both NKCC2 and NHE are blocked, CTGF causes an increase in P$_{SF}$ or Af-Art dilation.

During the treatment with furosemide and benzamil, the NHE-induced decrease in P$_{SF}$ may participate in nephron autoregulation of glomerular filtration. It is also possible that it participates in antagonizing CTGF. An increase in CTGF may explain the higher glomerular pressure and renal damage in salt-sensitive hypertensive individuals (22), such as African-Americans, the elderly, and the diabetic. ENaC-blocking drugs (potassium-sparing diuretics), by blocking CTGF and decreasing glomerular perfusion pressure, could be useful in preventing hypertensive nephrosclerosis. Also, CTGF is a novel regulatory mechanism of the renal microcirculation that may explain the Af-Art dilatation and increased GFR observed during high-salt intake, perhaps by antagonizing or resetting TGF. During high-salt intake, O$_2$ consumption by the nephron is higher because of increased Na$^+$ reabsorption; thus, CTGF could help protect the kidney from ischemia by increasing renal blood flow. On the other hand, CTGF may be detrimental in certain situations, such as in diabetes with osmotic diuresis, where Af-Art dilation might increase intraglomerular pressure and glomerular damage.

In summary, our studies provide the first evidence for the existence of a constrictor phenomenon initiated in the nephron that controls Af-Art tone and that is apparent when both NKCC2 and CTGF are inhibited. We showed that inhibiting both NKCC2 and CTGF causes constriction of the Af-Art in response to increases in nephron tubular flow rate and that is mediated by NHE, rather than by NCC. We also showed that when NHE and NKCC2 are both blocked, CTGF causes in vivo vasodilatation of the Af-Art, seen as an absolute increase in P$_{SF}$.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


