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Crosstalk between the connecting tubule and the afferent arteriole regulates renal microcirculation

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The renal afferent arterioles (Af-Arts) account for most of the renal vascular resistance, which is controlled similar to other arterioles and by tubuloglomerular feedback (TGF). The latter signal is generated by sensing sodium chloride concentrations in the macula densa; this in turn results in a signal which acts through the extraglomerular mesangium leading to constriction of the Af-Art. In the outer renal cortex, the connecting tubule (CNT) returns to the glomerular hilus and contacts the Af-Art suggesting that crosstalk may exist here as well. To investigate this, we simultaneously perfused a microdissected Af-Art and adherent CNT. Increasing the sodium chloride concentration perfusing the CNT significantly dilated preconstricted Af-Arts. We called this crosstalk 'connecting tubule glomerular feedback' (CTGF) to differentiate it from TGF. We tested whether entry of Na^+ and/or Cl^- into the CNT is required to induce CTGF by replacing Na^+ with choline$^+$. Increasing choline chloride concentration did not dilate the Af-Art. To test whether epithelial Na channels (ENaCs) mediate CTGF, we blocked ENaC with amiloride and found that the dilatation induced by CTGF was completely blocked. Inhibiting sodium chloride cotransporters with hydrochlorothiazide failed to prevent Af-Art dilatation. Finally, we tested whether nitric oxide released by the CNT mediates CTGF by the addition of a non-selective nitric oxide synthase inhibitor to the CNT. This potentiated CTGF rather than blocking it. We suggest that crosstalk exists between CNTs and attached Af-Arts, which is initiated by sodium reabsorption through amiloride-sensitive channels and this can contribute to the regulation of renal blood flow and glomerular filtration.

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The renal afferent arterioles (Af-Arts) account for most renal vascular resistance; they control glomerular filtration rate (GFR) and peritubular pressure, and thus renal function. In addition, Af-Art and efferent arteriole resistance control intraglomerular pressure, which is important not only for filtration pressure but also the development of glomerulosclerosis in hypertension and diabetes. Af-Art resistance is regulated by factors similar to other arterioles, and in addition is controlled in part by tubuloglomerular feedback (TGF). TGF operates via the macula densa: when concentrations of sodium and chloride in the macula densa are increased, a signal is transmitted through the extraglomerular mesangium that constricts the Af-Art.[1] In humans and other mammals, there is a transitional region of the nephron between the distal convoluted tubule and the cortical collecting duct, called the connecting tubule (CNT). This segment of the nephron plays a significant role in the regulation of Na^+ absorption and K^+ secretion.[2] Na^+ transport in the CNT can be described as a two-step process: Na^+ enters the apical membrane of the cell via the epithelial sodium channel (ENaC), whereas Na^+-K^+-ATPase in the basolateral membrane is responsible for Na^+ exit out of the cell. The apical and basolateral membrane also contains Na^+/H^+ exchangers; however, they play only a small role in Na^+ transport. There is some controversy as to whether the CNT has thiazide-sensitive Na^+/Cl^- cotransporter. K^+ secretion occurs via the inwardly rectifying potassium channel (renal outer-medullary potassium channel) (for a review of Na^+ and K^+ transport, see Reilly and Ellison[3]). We and others have shown that in the superficial nephrons of the renal cortex, the CNT returns to the vascular pole of the glomerulus and accompanies the Af-Art for varying distances.[4–6] This morphology is compatible with the existence of a feedback mechanism between the CNT and the Af-Art. However, there is no direct evidence to date demonstrating crosstalk between the CNT and the Af-Art, and thus the physiological significance of this anatomical relationship is not known. We hypothesized that the CNT participates in the regulation of Af-Art resistance. In order to test this hypothesis, we developed an *in vitro* technique that consists of simultaneous perfusion of a microdissected rabbit Af-Art and adherent CNT, thereby avoiding the confounding influence of the multiple systemic factors that regulate the

renal microcirculation. Using this preparation, we found that increasing NaCl in the perfusate of the CNT caused strong dilatation of the preconstricted Af-Art. We called this crosstalk 'connecting tubule glomerular feedback (CTGF)' to differentiate it from TGF, which is the crosstalk between the macula densa and the Af-Art. We also found that CTGF did not occur when NaCl was replaced with choline chloride, or when the ENaC was inhibited with amiloride. We believe this is a novel mechanism of regulation of the Af-Art.

RESULTS

We first perfused the Af-Art at 60 mm Hg while perfusing the CNT with either 10 or 80 mM NaCl. We found that in non-constricted Af-Arts, increasing luminal NaCl in the CNT caused modest dilatation (from 18.3 ± 1.1 to $21.2 \pm 1.3\,\mu$m; $n = 3$). As isolated arterioles have little or no tone, we tested whether preconstricting the Af-Art with norepinephrine ($2–5 \times 10^{-7}$ M) would potentiate vasodilatation. When the CNT was perfused with 10 mM NaCl, adding norepinephrine to the bath decreased Af-Art diameter from 18.4 ± 0.7 to $10.1 \pm 1.3\,\mu$m. When the solution was changed to 80 mM NaCl, diameter increased to $17.3 \pm 1.6\,\mu$m ($n = 6$; $P < 0.05$). When the solution was switched back to 10 mM NaCl, diameter returned to preconstricted levels ($10.8 \pm 1.4\,\mu$m). When the CNT perfusate was again changed to 80 mM NaCl, diameter increased to $17.0 \pm 1.0\,\mu$m (Figure 1). These data indicate that there is crosstalk between the Af-Art and the CNT which is initiated by an increase in luminal NaCl in the CNT and that the response is stable over time.

We next tested whether Na^+ or Cl^- is required to induce Af-Art dilatation by replacing NaCl with choline chloride. When choline chloride in CNT was increased from 10 to 80 mM, preconstricted Af-Art diameter did not change significantly (from 10.5 ± 1.3 to $10.8 \pm 1.5\,\mu$m; $n = 5$). However, when a second CTGF was performed in the same preparation and choline chloride was switched to NaCl, preconstricted Af-Arts dilated from 10.7 ± 1.1 to $16.9 \pm 0.6\,\mu$m, $P < 0.05$ (Figure 2). These data indicate that Na^+ rather than Cl^- initiates CTGF in the CNT.

To test whether Na^+ transport is required for CTGF and which Na^+ transporter is involved, we tested the effects of amiloride (which blocks ENaC) and hydrochlorothiazide (which blocks Na^+/Cl^- cotransport). During the control CTGF, preconstricted Af-Art diameter increased from 13.2 ± 1.5 to $17.8 \pm 1.1\,\mu$m ($n = 7$; $P < 0.05$). When we blocked ENaC by adding 10^{-6} M amiloride to the CNT perfusate together with 10 mM NaCl, preconstricted Af-Art diameter did not change and the dilatation induced by high NaCl was blocked, as diameter remained unchanged (from 12.4 ± 1.3 to $12.2 \pm 1.4\,\mu$m; $P =$ NS) (Figure 3). In contrast, when we blocked the Na^+/Cl^- cotransporter by adding 10^{-3} M hydrochlorothiazide to the CNT perfusate, 80 mM NaCl dilated preconstricted Af-Arts from 11.4 ± 1.3 to $15.5 \pm 1.5\,\mu$m ($n = 6$; $P < 0.05$) (Figure 4). These data indicate that Na^+ reabsorption by the ENaC initiates CTGF.

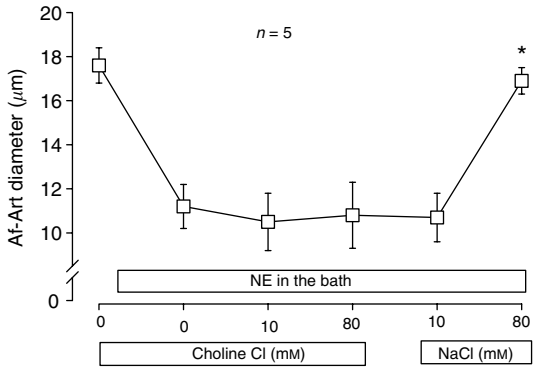

Figure 2 | Effect of perfusing the CNT with various concentrations of choline chloride on preconstricted Af-Arts. Unlike high NaCl, increasing choline chloride concentration did not dilate preconstricted Af-Arts. Switching to high NaCl caused preconstricted Af-Arts to become dilated, confirming that Na^+ rather than Cl^- initiates the CTGF response ($n = 5$; $^*P < 0.05$, high vs low NaCl).

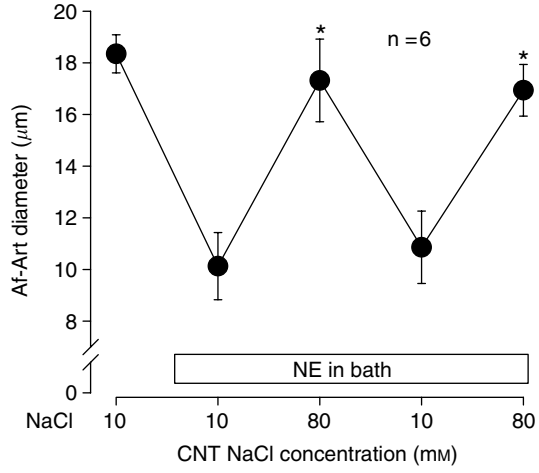

Figure 1 | Effect of perfusing the CNT with a low or high concentration of NaCl on preconstricted Af-Arts. The time control demonstrated that CTGF responses are reproducible ($n = 6$; $^*P < 0.05$, high vs low NaCl).

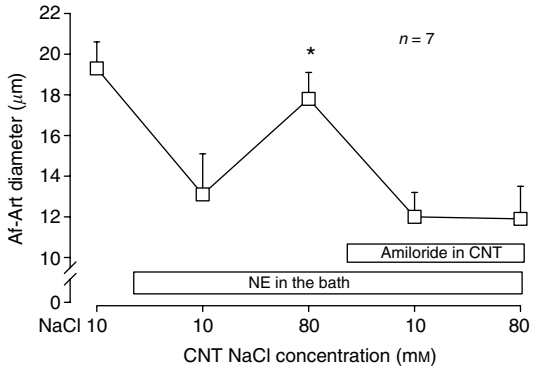

Figure 3 | Effect of adding amiloride to the CNT lumen (thereby blocking ENaC), on Af-Art dilatation induced by high NaCl in the CNT. Amiloride (10^{-6} M) blocked the preconstricted Af-Art dilation induced by high NaCl ($n = 7$; $^*P < 0.05$, high vs low NaCl).

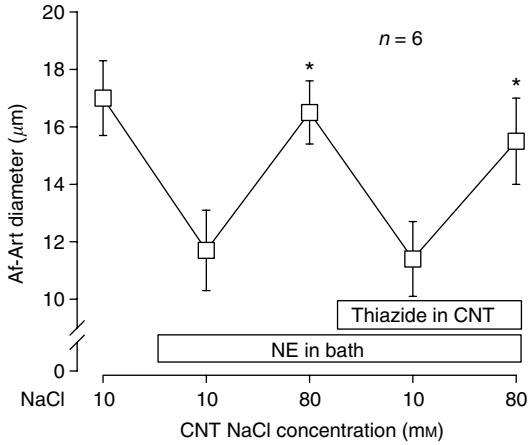

Figure 4 | Effect of adding hydrochlorothiazide to the CNT lumen (thereby blocking Na^+/Cl^- cotransport) on Af-Art dilatation induced by high NaCl in the CNT. Hydrochlorothiazide did not block the preconstricted Af-Art dilation induced by high NaCl ($n=6$; $^*P<0.05$, high vs low NaCl).

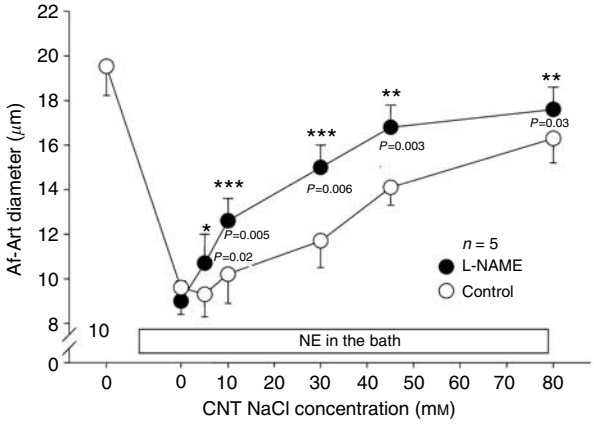

Figure 5 | Dose–response curve for increasing NaCl concentrations in the CNT lumen. Open circles show that increasing NaCl concentration dilated preconstricted Af-Arts in a dose-dependent manner. Closed circles show a second dose–response curve in the same preparation but with L-NAME in the CNT perfusate. $^{\#}P<0.05$, 0 NaCl vs various NaCl concentrations); $^*P<0.05$, without vs with L-NAME in the CNT; $n=5$.

As the amount of Na^+ that reaches the CNT is not well established, we obtained a dose–response curve for increasing NaCl concentrations (5, 10, 30, 45, and 80 mM NaCl) in the CNT perfusate. As shown in Figure 5, increasing NaCl in the CNT lumen dilated preconstricted Af-Arts in a dose-dependent manner; diameter increased from 9.6 ± 1.2 to 10.2 ± 1.3, 11.7 ± 1.2, 14.1 ± 0.8, and 16.3 ± 1.1 μm at 10, 30, 45, and 80 mM NaCl, respectively. These data indicate that CTGF response is related to the amount of Na^+ in the CNT.

To determine whether the Af-Art dilatation is caused by nitric oxide (NO) released from the CNT, we repeated the dose–response curve with N^G-nitro-L-arginine methyl ester (L-NAME;10^{-4} M), a NO synthase inhibitor, present in the CNT perfusate. When L-NAME was added to the lumen to inhibit NO production by the CNT, increasing luminal NaCl caused greater dilatation of the Af-Art; diameter increased from 9.0 ± 0.9 to 10.7 ± 1.3, 12.6 ± 1.0, 15.0 ± 1.0, 16.8 ± 1.0, and 17.6 ± 1.0 μm at 5, 10, 30, 45, and 80 mM NaCl, respectively ($n=5$; $P<0.05$, with vs without L-NAME) (Figure 5). These data indicate that NO released from the CNT is not the mediator of the vasodilatation. As inhibition of NO synthesis potentiates the response to NaCl, this suggests that NO produced by the CNT negatively modulates CTGF, perhaps by inhibiting Na^+ absorption.

DISCUSSION

We tested the hypothesis that there is crosstalk between the CNT and the Af-Art. We found that increasing luminal NaCl concentrations in the CNT caused dilatation of the Af-Art. In the absence of preconstriction, increasing NaCl in the CNT lumen caused slight dilatation of the Af-Art. Because arteries and arterioles devoid of tone respond poorly to vasodilators, we investigated whether preconstricting the Af-Art with norepinephrine would potentiate CTGF. We found that in the preconstricted Af-Art, increasing luminal NaCl in the CNT almost completely reversed its diameter to the non-constricted level. Af-Art dilatation appeared to be initiated by Na^+ absorption in the CNT, as when we replaced NaCl with choline chloride in the CNT perfusate we were not able to induce a CTGF response. In addition, this crosstalk appeared to be initiated by Na^+ reabsorption via ENaC, as amiloride blocked the Af-Art dilatation induced by high Na^+ in the CNT, whereas hydrochlorothiazide did not. Moreover, our results suggest that NO produced by the CNT does not mediate Af-Art dilatation but rather blunts it, probably by reducing Na^+ transport in the CNT. We called the crosstalk between the CNT and the Af-Art 'connecting tubule glomerular feedback' or CTGF to differentiate it from tubuloglomerular feedback or TGF, which is the crosstalk between the macula densa and the Af-Art. There are similarities and differences between TGF and CTGF. TGF operates via the macula densa, whereas CTGF operates via the CNT. Both sense changes in Na^+ concentration; however, Na^+ enters into the macula densa cells via the $Na^+K^+2Cl^-$ cotransporter whereas in the CNT it enters via the ENaC. Both TGF and CTGF are potentiated by inhibition of NO synthesis in the tubule; however, TGF causes constriction of the Af-Art whereas CTGF causes dilatation, so that while TGF decreases, CTGF probably increases renal blood flow and GFR.

In humans and all other mammals studied to date, there is a transitional region of varying length between the distal convoluted tubule and the cortical collecting duct, called the connecting tubule or CNT. The CNT consists of three specific cell types: CNT and intercalated cells type A (light) and type B (dark).[3] The identification of the CNT during the microdissection was based on previous studies by us and others, indicating that the nephron segment which makes contact with the Af-Art of the parent glomerulus is

the CNT. This anatomical relation has been found in rats, rabbits, and humans[4–7] (for a review see Reilly and Ellison[3]). In an immunocytochemical localization of renin (Af-Art) and kallikrein (CNT) in the rat renal cortex, we found that the Af-Art from the superficial glomeruli come within 3 μm of the CNT 90% of the time, whereas this occurred 87 and 73% of the time in the midcortical and juxtamedullary glomeruli.[4] During microdissection the CNT is easily differentiated from the distal convolute tubule (DCT) (for a review see Morel *et al.*[8]). The DCT is bright, thin, and more transparent than the CNT, which is larger and has a granular appearance. The CNT was once called granular DCT and granular cortical collecting tubule; however, in 1979 Imai[9] proposed the term CNT because the morphological and functional characteristics of the two segments were identical.[8,9–11] This anatomical relationship between the Af-Art and the CNT[5] is compatible with the existence of a feedback mechanism between the CNT and the Af-Art and our study clearly demonstrated that this is the case. Morsing *et al.*[12] performed *in vivo* micropuncture studies of TGF with and without interruption of distal flow and found that interruption of flow caused a greater decrease in the proximal tubule maximum stop-flow pressure, suggesting greater constriction of the Af-Art, as stop-flow pressures in the proximal tubule correlate inversely with Af-Art resistance. Morsing's findings and ours are compatible with the existence of CTGF *in vivo*. Furthermore, there is another anatomical relationship, as the CNT in the midcortical and juxtamedullary nephrons frequently forms branching structures (termed arcades) that ascend through the cortical labyrinth and run close to the interlobular artery.[3] Thus, it could be that CTGF is not limited to regulation of the Af-Art but the mechanism is more general and also regulates interlobular arterial resistance.

Our data suggest that CTGF is initiated by changes in Na^+ concentration, as after we replaced Na^+ with choline chloride CTGF no longer occurred. We next questioned which Na^+ transporter is involved. There are several lines of evidence for Na^+ channel-mediated transport in the CNT. Data suggest that Na^+ is reabsorbed in this segment by an amiloride-sensitive electrogenic process.[3,13] Frindt and Palmer.[13] measured amiloride-sensitive Na^+ channel activity in the rat CNT using cell-attached patch-clamp and whole-cell clamp approaches and confirmed that channel activity in this segment is qualitatively similar to, but quantitatively greater than, that in the cortical collecting tubule. Our results suggested that Na^+ entry is caused by ENaC, as amiloride blocks CTGF. In contrast, hydrochlorothiazide, which blocks the Na^+/Cl^- cotransporter, did not alter CTGF, suggesting that the Na^+/Cl^- cotransporter does not participate in CTGF. This is consistent with a previous report that the CNT does not express the apical thiazide-sensitive NaCl cotransporter.[3] Based on these findings, we concluded that increases in CNT Na^+ reabsorption via ENaC rather than Na^+/Cl^- cotransport dilate the preconstricted Af-Art.

The Na^+ concentration in the lumen of the CNT is not precisely known, as given its anatomical location it cannot be examined by micropuncture. However, under normal circumstances luminal NaCl concentration at the macula densa ranges from 25 to 52 mM.[14] Given the rate at which the distal convoluted tubule absorbs NaCl and its length, one can estimate that the NaCl concentration entering the CNT ranges from 12 to 26 mM. As the CNT reabsorbs both Na^+ and water, which are regulated independently, the NaCl concentration leaving the CNT could vary over a wider range and may be as low as 5–10 mM or as high as 50–60 mM. We obtained a dose–response curve, changing NaCl in the CNT perfusate from 0 to 5, 10, 30, 45, and 80 mM, which showed that the degree of Af-Art dilatation was directly related to the amount of NaCl perfused into the CNT and that 30 mM NaCl already produces statistically significant dilatation. Thus, the concentrations of NaCl that cause the Af-Art to dilate are in a range that one can expect to encounter in the CNT. Furthermore, when nitric oxide synthase in the CNT was inhibited, the dose–response curve was shifted to the left and even NaCl concentrations as low as 5 mM caused statistically significant dilatation. In the kidney, NO causes natriuresis and diuresis. The mechanism involved includes increases in GFR, renal blood flow, and inhibition of salt and water reabsorption along the nephron.[15–17] *In vivo* and *in vitro* studies indicate that inhibition of NO synthesis in the macula densa potentiates TGF.[18–20] We have provided evidence that NO acts in the macula densa itself rather than by diffusing to the Af-Art[19] and have reported that NO inhibits transport in the microperfused cortical collecting duct as well as the thick ascending limb.[21,22] In cultured cortical collecting duct cells, NO acting via cyclic guanosine monophosphate inhibits Na^+ transport by affecting apical membrane channels.[23] Frindt and Palmer[13] confirmed that in the rat CNT Na^+ channel activity is qualitatively similar to that in the cortical collecting tubule. Although NO has been studied extensively in the kidney,[24–26] its role in the CNT is unknown. We proposed that NO produced by the CNT acts in an autocrine manner in the CNT cells to block Na^+ transport by ENaC, thus inhibiting CTGF. Indeed, our data demonstrated that inhibiting NO synthesis in the CNT with L-NAME augmented CTGF, causing greater Af-Art dilatation. Which nitric oxide synthase isoforms are responsible for the release of NO by the CNT, as well as the second-messenger cascades that mediate inhibition of transport, need to be investigated further. It is unlikely that NO diffusing from the CNT to the Af-Art acts in a paracrine manner to dilate the Af-Art, since when we inhibited nitric oxide synthase in the CNT lumen using L-NAME we found that CTGF was potentiated.

Although the signal sent from the CNT to the Af-Art is unknown, there are several likely candidates, because the CNT expresses the enzymes responsible for generating many paracrine and endocrine factors that are direct or indirect vasodilators. The CNT synthesizes kallikrein and eicosanoids that can cause vasodilatation, and also synthesizes renin which is a vasoconstrictor (for a review, see Meneton *et al.*[2]).

Like all cells, the CNT also has the capacity to generate ATP, adenosine, and other purinergic agonists. Many of these autacoids could not only mediate but also regulate CTGF; however, at present the mechanism by which CTGF dilatates the Af-Art is not known. An important consideration is that CTGF caused Af-Art dilatation, whereas macula densa TGF caused constriction; thus, different mediators have to be involved. We have provided evidence that the mediator of TGF is adenosine, which constricts the Af-Art via the A_1 receptor, whereas the effector of CTGF remains to be investigated.[27,28]

CTGF is a novel regulatory mechanism of the renal microcirculation that may result in the dilation of the Af-Art and increase in GFR observed during high salt intake, perhaps by antagonizing or resetting TGF. Also, as during high salt intake there is an increased O_2 consumption by the nephron because of increased sodium reabsorption, it could be that CTGF helps protect the kidney from renal ischemia by increasing renal blood flow. On the other hand, in diabetes with osmotic diuresis and in salt-sensitive hypertension during high sodium intake, CTGF may cause an increase in intraglomerular pressure and renal damage by dilating the Af-Art. From a homeostatic point of view, TGF is a positive feedback that during salt loading will sense increases in Na^+ at the end of the ascending loop of Henle causing increase in the vascular resistance and a decrease in GFR, thus favoring Na^+ retention. On the other hand, CTGF from the homeostatic point of view is a negative feedback that senses Na^+ in the CNT and causes an increase in renal blood flow and GFR, thus favoring Na^+ excretion and a rapid return of the body to Na^+ balance. The CNT is at the end of the DCT, these two nephron segments are aldosterone-sensitive and they have a pivotal role in the regulation of Na and K excretion.[2]

In conclusion, our studies provide direct evidence of crosstalk between the CNT and Af-Art. It is initiated by increasing NaCl concentration, which stimulates Na^+ transport via epithelial ENaC in the CNT and dilates the Af-Art. NO produced by CNT inhibits CTGF, probably by blocking Na^+ transport. This novel regulatory mechanism of the renal microcirculation may participate in the vasodilatation observed during high salt intake, perhaps by antagonizing TGF.

MATERIALS AND METHODS

Rabbits were fed standard chow (Ralston Purina, St Louis, MO, USA) and tap water *ad libitum* and anesthetized with intraperitoneal ketamine (60 mg/kg). We used rabbits as in this species the CNT is well demarcated and microdissection of the CNT and attached Af-Art is easier than in rats or mice.[29] To isolate and microperfuse the Af-Art and CNT, we used methods similar to those described previously.[18,19,30–32] The kidneys were sliced along the corticomedullary axis and slices placed in ice-cold minimum essential medium (Gibco Laboratories, Grand Island, NY, USA) containing 5% bovine serum albumin (Sigma, St Louis, MO, USA). Isolation of the Af-Art and CNT: a single superficial Af-Art with its glomerulus intact was dissected from each rabbit together with the adherent CNT with fine

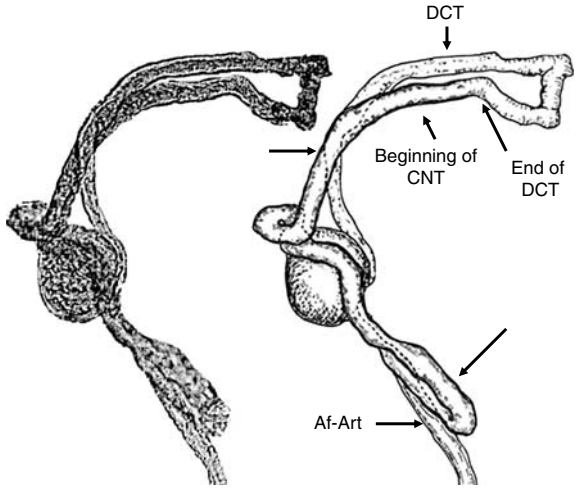

Figure 6 | Left: photography showing a microdissected rabbit distal nephron, glomerulus, and Af-Art. Right: a diagram of the photography in the left, identifying the DCT, the CNT, and the Af-Art. Note the transition form DCT to the CNT and its return to the Af-Art. This segment of the nephron can easily be located by microdissection. The DCT is bright, thin, and more transparent than the CNT which is larger and has a granular appearance (for a review see Morel *et al.*[8]).

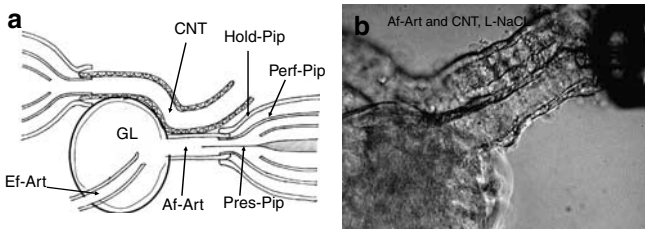

Figure 7 | Images of simultaneous perfusion of a microdissected rabbit Af-Art and attached CNT. (**a**) schematic representation of the perfusion system. (**b**) Picture of the simultaneous perfusion of a microdissected rabbit Af-Art and attached CNT. CNT = connecting tubule; Af-Art = afferent arteriole; Ef-Art = efferent arteriole; GL = glomerulus; Hold-Pip = holding pipette; Perf-Pip = perfusion pipette; Pres-Pip = pressure pipette.

forceps. For this, we separated the proximal tubule and identified the macula densa attached to the glomerular vascular pole as the beginning of the DCT.[30] We follow the DCT which is bright and transparent and it becomes the CNT, which is of granular appearance and larger diameter than the DCT. The CNT returns toward the same glomerular vascular pole and runs along the Af-Art and attached to it (Figures 6 and 7). Using a micropipette, the microdissected complex is transferred to a temperature-regulated perfusion chamber mounted on an inverted microscope with Hoffmann modulation. Both the Af-Art and CNT were cannulated with an array of glass pipettes as described previously.[30] The methods of cannulating and perfusing the tubular segment are similar to those originally described by Burg.[33] Figure 7 illustrates microperfusion of an Af-Art with CNT intact. The Af-Art was perfused with minimum essential medium oxygenated with room air and containing 5% bovine serum albumin. Intraluminal pressure was measured by Landis' technique,[34] and maintained at 60 mm Hg. The CNT was perfused with solutions having varying NaCl

concentrations. The basic solution contained (in mM): 4 $KHCO_3$, 10 HEPES, 0.5 Na acetate, 0.5 Na lactate, 0.5 K_2HPO_4, 1.2 $MgSO_4$, 1 $CaCO_3$, and 5.5 glucose, adding 1 M NaCl to achieve the desired final NaCl concentration. The driving force to maintain the tubular perfusion rate was provided by hydrostatic pressure causing a tubular flow of approximately 15 nl. The bath was superfused with minimum essential medium containing 0.15% bovine serum albumin at a rate of 1 ml/min.

Microdissection and cannulation of the Af-Art and tubular segment were completed within 90 min at 8°C after which the temperature was gradually raised to 37°C. Once it was stable, a 30-min equilibration period was allowed before taking any measurements. Images of the Af-Art were displayed at magnifications up to $\times 1980$. As our preliminary studies showed that increasing NaCl in the CNT perfusate causes modest dilatation of the Af-Art, and as isolated arteries have little or no tone, the studies were performed in preconstricted Af-Arts. Af-Art diameter was measured in the region of the maximal response to norepinephrine at three sites separated by 3–5 μm and expressed as the average of these three measurements. Diameter was recorded at 5 s intervals with a video camera and measured with a computer equipped with a Metavue image analysis system.

Statistics

Values are expressed as mean $\pm$ s.e.m. A paired *t*-test was used to examine whether the diameter at a given concentration was different from control. Analysis of variance was used to examine whether dose–response curves differed between groups, and a two-sample *t*-test was used to examine whether the changes in diameter at a given concentration differed between groups. $P<0.05$ was considered significant, using Bonferroni's correction for multiple comparisons.

ACKNOWLEDGMENTS

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REFERENCES

1. Navar LG, Inscho EW, Majid DSA *et al.* Paracrine regulation of the renal microcirculation. *Physiol Rev* 1996; **76**: 425–536.
2. Meneton P, Loffing J, Warnock DG. Sodium and potassium handling by the aldosterone-sensitive distal nephron: the pivotal role of the distal and connecting tubule. *Am J Physiol Renal Physiol* 2004; **287**: F593–F601.
3. Reilly RF, Ellison DH. Mammalian distal tubule: physiology, pathophysiology, and molecular anatomy. *Physiol Rev* 2000; **80**: 277–313.
4. Barajas L, Powers K, Carretero O *et al.* Immunocytochemical localization of renin and kallikrein in the rat renal cortex. *Kidney Int* 1986; **29**: 965–970.
5. Dørup J, Morsing P, Rasch R. Tubule–tubule and tubule–arteriole contacts in rat kidney distal nephrons. A morphologic study based on computer-assisted three-dimensional reconstructions. *Lab Invest* 1992; **67**: 761–769.
6. Faarup P. On the morphology of the juxtaglomerular apparatus. *Acta Anat* 1965; **60**: 20–38.
7. Vio CP, Figueroa CD, Caorsi I. Anatomical relationship between kallikrein-containing tubules and the juxtaglomerular apparatus in the human kidney. *Am J Hypertens* 1988; **1**: 269–271.
8. Morel F, Chabardes D, Imbert M. *Methods in Pharmacology: Renal Pharmacology*. 4B edn. Plenum: New York, 1976, pp 297–323.
9. Imai M. The connecting tubule: a functional subdivision of the rabbit distal nephron segments. *Kidney Int* 1979; **15**: 346–356.
10. Imai M, Nakamura R. Function of distal convoluted and connecting tubules studied by isolated nephron fragments. *Kidney Int* 1982; **22**: 465–472.
11. Kaissling B, Kriz W. Structural analysis of the rabbit kidney. *Adv Anat Embryol Cell Biol* 1979; **56**: 1–123.
12. Morsing P, Velazquez H, Ellison D *et al.* Resetting of tubuloglomerular feedback by interrupting early distal flow. *Acta Physiol Scand* 1993; **148**: 63–68.
13. Frindt G, Palmer LG. Na channels in the rat connecting tubule. *Am J Physiol Renal Physiol* 2004; **286**: F669–F674.
14. Holstein-Rathlou NH, Marsh DJ. Oscillations of tubular pressure, flow, and distal chloride concentration in rats. *Am J Physiol* 1989; **256**: F1007–F1014.
15. Deng X, Welch WJ, Wilcox CS. Renal vasodilatation with L-arginine. Effects of dietary salt. *Hypertension* 1995; **26**: 256–262.
16. Majid DS, Williams A, Kadowitz PJ *et al.* Renal responses to intra-arterial administration of nitric oxide donor in dogs. *Hypertension* 1993; **22**: 535–541.
17. Wilcox CS, Deng X, Welch WJ. NO generation and action during changes in salt intake: roles of nNOS and macula densa. *Am J Physiol* 1998; **274**: R1588–R1593.
18. Ito S, Ren Y. Evidence for the role of nitric oxide in macula densa control of glomerular hemodynamics. *J Clin Invest* 1993; **92**: 1093–1098.
19. Ren Y, Garvin JL, Carretero OA. Role of macula densa nitric oxide and cGMP in the regulation of tubuloglomerular feedback. *Kidney Int* 2000; **58**: 2053–2060.
20. Welch WJ, Wilcox CS. Tubuloglomerular feedback and macula densa-derived NO,. In: Goligorsky MS, Gross SS (eds). *Nitric Oxide and the Kidney. Physiology and Pathophysiology*. chap. 11 Chapman & Hall: New York, 1997, pp 216–232.
21. Plato CF, Stoos BA, Wang D *et al.* Endogenous nitric oxide inhibits chloride transport in the thick ascending limb. *Am J Physiol* 1999; **276**: F159–F163.
22. Stoos BA, Garcia NH, Garvin JL. Nitric oxide inhibits sodium reabsorption in the isolated perfused cortical collecting duct. *J Am Soc Nephrol* 1995; **6**: 89–94.
23. Stoos BA, Carretero OA, Garvin JL. Endothelial-derived nitric oxide inhibits sodium transport by affecting apical membrane channels in cultured collecting duct cells. *J Am Soc Nephrol* 1994; **4**: 1855–1860.
24. Ito S. Nitric oxide in the kidney. *Curr Opin Nephrol Hypertens* 1995; **4**: 23–30.
25. Lahera V, Navarro-Cid J, Cachofeiro V *et al.* Nitric oxide, the kidney, and hypertension. *Am J Hypertens* 1997; **10**: 129–140.
26. Raij L, Baylis C. Glomerular actions of nitric oxide. *Kidney Int* 1995; **48**: 20–32.
27. Ren Y, Arima S, Carretero OA *et al.* Possible role of adenosine in macula densa control of glomerular hemodynamics. *Kidney Int* 2002; **61**: 169–176.
28. Ren Y, Garvin JL, Liu R *et al.* Role of macula densa adenosine triphosphate (ATP) in tubuloglomerular feedback. *Kidney Int* 2004; **66**: 1479–1485.
29. Kaissling B, Kriz W. Structural analysis of the rabbit kidney. *Adv Anat Embryol Cell Biol* 1979; **56**: 1–123.
30. Ito S, Carretero OA. An *in vitro* approach to the study of macula densa-mediated glomerular hemodynamics. *Kidney Int* 1990; **38**: 1206–1210.
31. Ren Y, Carretero OA, Ito S. Influence of NaCl concentration at the macula densa on angiotensin II-induced constriction of the afferent arteriole. *Hypertension* 1996; **27**: 649–652.
32. Wang H, Carretero OA, Garvin JL. Nitric oxide produced by THAL nitric oxide synthase inhibits TGF. *Hypertension* 2002; **39**: 662–666.
33. Burg MB. Perfusion of isolated renal tubules. *Yale J Biol Med* 1972; **45**: 321–326.
34. Osgood RW, Patton M, Henley MJ *et al. In vitro* perfusion of the isolated dog glomerulus. *Am J Physiol* 1983; **244**: F349–F354.