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Short-term modulation of distal tubule fluid nitric oxide in vivo by loop NaCl reabsorption

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Short-term modulation of distal tubule fluid nitric oxide in vivo by loop NaCl reabsorption.

Background. Intrarenal nitric oxide (NO) production and signaling effects are influenced by NaCl loading. To gain further insight into NO mechanisms we determined whether rat distal tubular fluid (DTF) [NO] and collected NO may acutely change when NaCl loop delivery is altered.

Methods. An NO microelectrode was used to measure real-time DTF [NO] and DT-collected NO. With proximal flow blocked (open system), 150 mmol/L NaCl, with and without 10^{-4} mol/L furosemide was perfused with measurement of loop [Cl] reabsorption. Using a closed system, DTF [NO] was also determined using several different loop perfusates.

Results. In the open system, perfusion with 40 nL/min of 150 mmol/L NaCl to which 10^{-4} mol/L furosemide was added, DT [NO] and DT-collected NO was approximately twice that measured with perfusion of 150 mmol/L NaCl alone, while loop Cl reabsorption decreased by half. In the closed system, perfusion at 10 nL/min of 150 mmol/L NaCl + furosemide 10^{-4} mol/L also induced a significant rise in DTF [NO] and collected NO. Perfusion of 10^{-3} mol/L S-methyl-L-thiocitrulline (SMTC) with 150 mmol/L NaCl, induces a significant drop in DT [NO], but without a significant increase in collected NO. Furthermore, with addition of 10^{-3} mol/L SMTC to the 150 mmol/L NaCl + 10^{-4} furosemide perfusate, the rise in DT [NO] was prevented. Analysis of covariance showed that flow changes within, or between all groups, had no significant additional effect.

Conclusion. In both open and closed loop perfusion systems, 10^{-4} mol/L furosemide inhibition of NaCl transport stimulates net loop NO emission independent of flow; 10^{-3} mol/L SMTC + 150 mmol/L NaCl reduces DT [NO], but not DT-collected NO. Short-term net NO emission from the entire loop, as collected in distal tubule fluid, increases with inhibition of loop NaCl transport.

In the loop of Henle, NO is believed to alter solute transport and influence the tubuloglomerular feedback (TGF) response [1–5]. In the isolated rabbit cTAL, with attached glomerulus, it has been recently reported that macula densa (MD) cell NO synthesis increases with increased NaCl perfusion [6]. Also, in short-term in vitro experiments, NO has been shown to be synthesized by the TAL and inhibits TAL NaCl transport [7]. Moreover, there is evidence that NO derived from TAL cells may diffuse to the MD site and further mitigate TGF afferent arteriolar constriction [8]. However, no in vivo data are available regarding the effects of NaCl loads or transport, on NO synthesis by the entire loop, which includes the approximately 3 mm TAL segment in the rat [9]. The TAL segment, comprising perhaps 800 cells[10]—about 20 times the number of MD cells [5]—could be a significant source of net loop NO emission, unless rapidly degraded in tubular fluid, or downregulated by a high NaCl load.

Accordingly, we reasoned if net loop NO emission is released in significant quantities, it might also be detected in distal tubular fluid. Using an NO specific electrode we recently described [11], real-time distal tubular fluid [NO] [DTF (NO)] was measured using the classic open system, where endogenous proximal flow was blocked, and 150 mmol/L NaCl was perfused at 10 and 40 nL/min, with and without 10^{-4} mol/L furosemide. With similar changes in DT flow, with 40 nL/min perfusion of 150 mmol/L NaCl to which furosemide 10^{-4} mol/L was added, DT [NO] and net NO emission was approximately twice that measured with perfusion of 150 mmol/L NaCl alone, while loop Cl reabsorption decreased by half. In closed loop perfusions, 150 mmol/L NaCl was perfused at 10 nL/min, added to the endogenous flow with and without 10^{-4} mol/L furosemide. The 10^{-4} mol/L furosemide perfusate resulted in significantly higher DTF [NO] and collected NO with a similar modest increase in DT flows.

The results indicate that short-term net NO emission from the entire loop, as collected in distal tubule fluid, increases with inhibition of loop NaCl transport.

METHODS

Adult male Sprague-Dawley rats from Charles River, Canada (St. Constant, Quebec, Canada) weighing

[1]Statistical analyses were performed by James Jaffey.

Key words: nitric oxide, loop of Henle NaCl transport, distal tubule.

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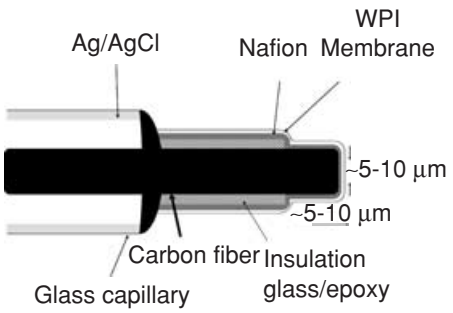

Fig. 1. Modified World Precision Instruments (WPI) integrated amperometric nitric oxide (NO)- sensing electrode. The basic features of the electrode structure are shown, as previously described [11]. See also **Methods**. Reproduced with permission.

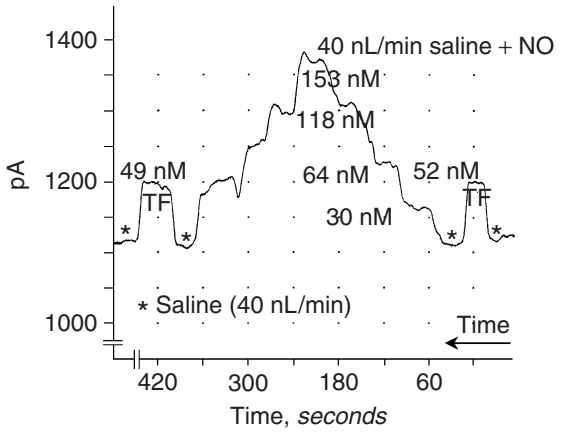

Fig. 2. Intratubular NO measurements from a single proximal tubule with perfusion of saline and/or saline equilibrated with NO. Perfusion of 40 nL/min saline close to the electrode tip reduced the pA current to values similar to that obtained from surface fluid. Maintaining a constant 40 nL/min flow, stepwise increments of the saline + NO and NO-free saline solutions resulted in expected [NO] changes [11]. See also **Methods**. Reproduced with permission.

between 250 and 350 g were fed a standard laboratory rat chow (diet 5012; Ralston Purina of Canada, Woodstock, Ontario, Canada) and had free access to tap water. Animals were anesthetized with 100 mg/kg thiobutabarbital sodium (Inactin; Research Biochemical International, Natick, MA, USA) and prepared for microperfusion, as described previously [12].

Our [NO] measurement technique has also been previously described [11], and is briefly reviewed here. The structure of the electrode, shown in Figure 1, is a specially modified [World Precision Instruments (WPI P/N ISO-NOP007)] design for our in vivo use. Glass insulation is used to preclude NO reactivity outside the tubule; the reactive membrane length is ~5–15 µm, and the tip diameter usually about 5–7 µm. We monitor electrodes for NO specificity by testing against nitrite and ascorbic acid. After calibration in vitro, using a S-nitroso-N-acetylpenicillamine (SNAP)-copper sulfate solution, a zero point is needed between repeated punctures. We have chosen to use saline on the surface of the rat kidney to be that point. Thus, after an "extratubular" zero is determined, the puncture is made along the "prepuncture" track, and the picoamperes (pA) response is taken from that "zero." We have also considered the possibility that NO leaking from the tubule may influence the zero point, but we have determined that is not a significant concern; samples of surface fluid were pipetted into the copper sulfate solution without any significant response, even after many tubular segments were punctured. We presumed that if the pA signal was solely due to intratubular NO, the signal should be abolished by placing a high-flow saline perfusion pipette close to the sensing electrode. Indeed, 40 nL/min saline diminished the pA current in a repeated and reproducible fashion (Fig. 2) to values similar to that obtained from surface fluid. Assuming that the aqueous intratubular milieu is not materially different from the in vitro calibration conditions, the electrode should respond in an expected manner, within the tiny tubule, when different concentrations of NO were perfused upstream, using two perfusion solutions (with and without NO) with separate flows ranging from zero to 40 nL/min, with the total delivery always 40 nL/min. In fact, at constant 40 nL/min flow, we showed first an intratubular zero point, and then appropriate step-wise increment and decrement of responses when an NO containing saline solution was mixed with an NO free solution, without altering flow, as shown in Figure 2. Finally, Figure 3 shows an actual recording—with compressed time axis—from Group 2, as described below.

Experimental groups

The following solutions were perfused through the loop, after blockade of endogenous proximal flow (open system): Group 1 (5 rats, 6 tubules), 150 mmol/L NaCl perfused at 10 and 40 nL/min with quantitative collections for flow, and analysis of Cl concentrations, so that collected NO amounts could be calculated, as well as absolute net Cl reabsorption, $J_{Cl.}$. Group 2 (6 rats, 10 tubules), as for Group 1, with 10^{-4} mol/L furosemide added to the 150 mmol/L NaCl perfusate.

In 6 additional groups of normal rats, loops of Henle were perfused with different solutions from the late proximal site at 10 nL/min, without blocking normal flow (closed system), not disrupting proximal tubule function [13], and attempting to minimally alter loop flow; Group 3 (5 rats, 10 tubules), 150 mmol/L NaCl; Group 4 (6 rats, 7 tubules), 150 mmol/L NaCl + 10^{-4} mol/L furosemide; Group 5 (5 rats, 8 tubules), 150 mmol/L NaCl + 10^{-3} mol/L SMTC.

In two additional groups, to permit evaluation of higher NaCl reabsorption and higher flows also in the closed system, perfusions were undertaken with 450 mmol/L NaCl,

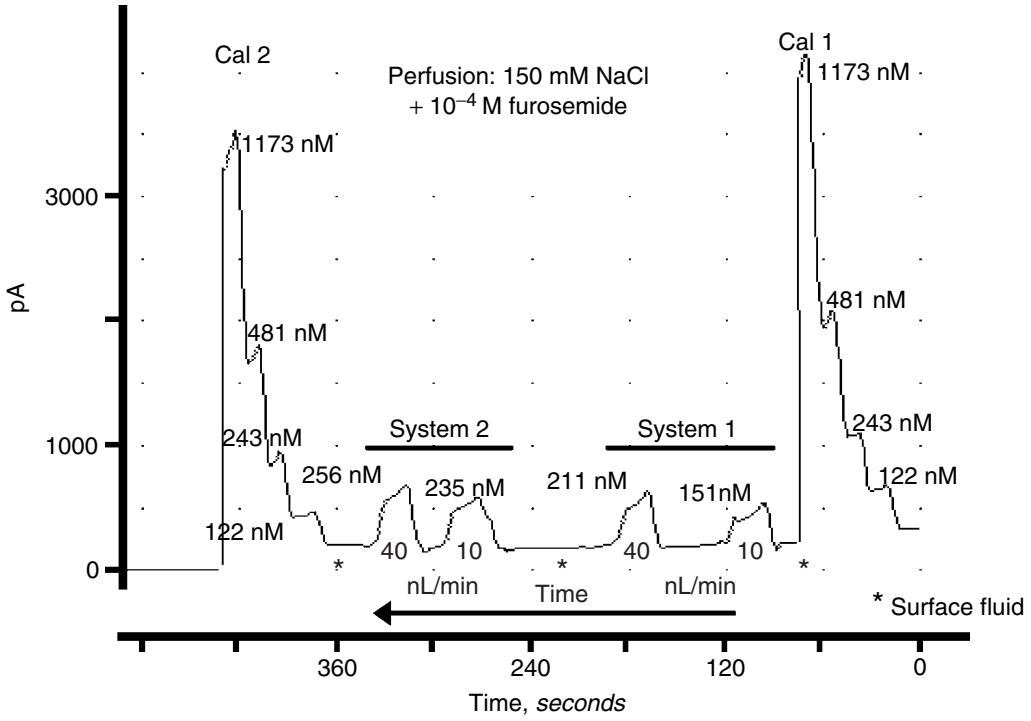

Fig. 3. Intratubular real-time NO recordings from Group 2. Responses, pA and nmol/L during loop perfusion of two tubules from Group 2, with perfusion of 150 mmol/L NaCl + 10^{-4} mol/L furosemide at 10 and 40 nL/min. Reading from left to right, DT [NO] is determined between SNAP-copper sulfate calibrations.

Group 6 (4 rats, 10 tubules), and 900 mmol/L mannitol, Group 7 (4 rats, 7 tubules). Finally, in Group 8 (5 rats, 8 tubules), the effects of combined furosemide and SMTC added to 150 mmol/L NaCl were measured.

In these studies, the direct measurement of [NO] in real time requires a separate puncture into a saline superfused kidney surface; oil impedes the NO electrode sensitivity and saline is also needed for a "zero" point. Thus, in contrast to the usual micropuncture procedure, where tubular fluid is quantitatively collected for volume flows and chloride analyses, separate punctures of the same distal tubule were needed, with oil on the kidney surface. This approach was taken with Groups 1, 2, 6, 7, and 8, while the other volume collections were made under identical experimental conditions, in different tubules, in separate oil superfused kidneys, but without DT [NO] measured.

Statistical analyses

Paired and unpaired *t* testing was carried out in all groups as appropriate (see **Results** and Tables 1 and 2). However, to examine the possible independent effect of flows, both within and between groups, an analysis of covariance (ANCOVA) was carried out using individual flow data from individual tubules, along with the group effects. The SAS (SAS Institute, Cary, NC, USA) procedure general linear model (GLM) was used for this analysis. Finally, for Groups 1 and 2, Pearson correlation coefficients were determined for several paired variables: DT [NO], DT-collected NO, DT [Cl], DT Cl delivery, and J_{Cl}.

RESULTS

Figure 4 and Table 1 show results from Groups 1 and 2, where at both 10 and 40 nL/min perfusion, furosemide inhibition significantly increased DT-collected NO, while J_{Cl} is significantly reduced. The DT [NO] at 40 nL/min is also significantly higher with furosemide inhibition, but does not quite reach significance at 10 nL/min. Indeed, the effects of furosemide, as well as flows, and J_{Cl} very closely approximate the data of Wright et al [14] obtained 30 years ago. When single-tubule values were pooled from Groups 1 and 2, to which 20 nL/min additional perfusion data were added (not reported elsewhere, see above) the DT-collected NO was highly correlated with DT Cl delivery, $P < 0.0001$.

Table 2 shows data from Groups 3–5. In Groups 3 and 4, DT-collected NO is significantly higher, $P < 0.05$ or less, compared to free-flow control during closed perfusion with 150 mmol/L NaCl or with furosemide addition. Importantly, the increase in collected NO with furosemide addition (Group 4) is associated with a significant increase in DT [NO] versus Group 4 control, $P < 0.05$, despite a significant increase in DT flow. With further addition of 10^{-3} mol/L SMTC to the furosemide/

Table 1. Perfusion of 10 and 40 nL/min of 150 mmol/L NaCl with and without 10^{-4} mol/L furosemide

	Perfusion solution			
	Group 1 150 mmol/L NaCl		**Group 2** 150 mmol/L NaCl + 10^{-4} mol/L furosemide	
Rats/tubules	5/6		6/10	
Perfusion rate *nL/min*	10	40	10	40
Flow *nL/min*	5.2 ± 0.8	25.4 ± 2.5[a]	6.4 ± 0.5	29.2 ± 2.5[b]
[NO] *nmol/L*	161 ± 33	116 ± 25	219 ± 28	214 ± 32[c]
Collected NO *fmol·min^{-1}*	778 ± 119	2749 ± 549[a]	1364 ± 249	6073 ± 940[b,c]
[Cl] *mmol/L*	51 ± 5	135 ± 6[a]	159 ± 7[d]	166 ± 4[c]
J_{Cl} *pmol·min^{-1}*	1277 ± 36	2673 ± 335[a]	480 ± 71[d]	1321 ± 429[b,c]

Values are mean ± SEM.
[a] $P < 0.05$ vs. 10 nL/min (Group 1), by paired *t* test.
[b] $P < 0.05$ vs. 10 nL/min (Group 2), by paired *t* test.
[c] $P < 0.05$ vs. 40 nL/min (Group 1), by unpaired *t* test.
[d] $P < 0.05$ vs. 10 nL/min (Group 1), by unpaired *t* test.

Table 2. Perfusion at 10 nL/min added to endogenous late proximal fluid

Group	Perfusion solution		Rats/tubules	DT V nL/min	DT [NO] nmol/L	Collected NO fmol·min^{-1}
3	150 mmol/L NaCl	Control	5/10	7.5 ± 1.0	198 ± 16	1484 ± 120
		Perfusion	5/10	12.1 ± 2.7	196 ± 28	2369 ± 334[a]
4	150 mmol/L NaCl	Control	6/7	7.5 ± 0.8	169 ± 26	1266 ± 197
	+ 10^{-4} mol/L furosemide	Perfusion	6/7	12.8 ± 1.1[a]	245 ± 31[a]	3136 ± 395[a,b]
5	150 mmol/L NaCl	Control	5/10	5.5 ± 1.3	117 ± 14	644 ± 77
	+ 10^{-3} mol/L SMTC	Perfusion	5/10	11.3 ± 1.9[a]	78 ± 11[a]	884 ± 126

Values are mean ± SEM.
[a] $P < 0.05$ vs. control, by paired *t* test.
[b] $P < 0.05$ vs. Group 3, control, by unpaired *t* test.

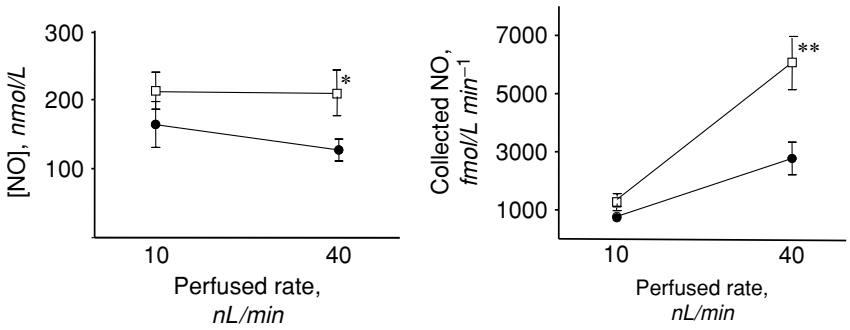

Fig. 4. Distal tubule fluid [NO] and collected NO during 10 and 40 nL/min loop perfusions (open system). Results shown are with 150 mmol/L NaCl (closed circles) or 150 mmol/L NaCl + 10^{-4} mol/L furosemide (open squares). During furosemide inhibition DTF [NO] is significantly higher at 40 nL/min. Left panel, $^{*}P < 0.05$. Collected NO with furosemide inhibition (40 nL/min perfusion) is significantly ($^{**}P < 0.01$) higher than the 10 nL/min perfusions from both groups, and is also significantly different ($P < 0.05$) than the 40 nL/min Group 1 response. See also Table 1 and **Results**.

150 mmol/L NaCl solution (Group 8, not shown elsewhere), the rise in DT[NO] was prevented: 175 ± 23 versus 180 ± 23 nmol/L. Table 2 and Figure 5 also show that in Group 5, addition of 10^{-3} mol/L SMTC to 150 mmol/L NaCl induces a significant drop in DT [NO], but without a significant increase in collected NO.

In Group 6, 450 mmol/L NaCl perfusion, DT [NO] during the control period was 142 ± 26 nmol/L, which was significantly reduced to 85 ± 22 nmol/L during perfusion, $P < 0.01$ by paired *t* testing. In Group 7 (900 mmol/L mannitol) the mean DT [NO] perfusion value of 138 ± 37 nmol/L was higher than the control period value of 109 ± 22 nmol/L, but did not reach statistical significance. In contrast, DT-collected NO values increased significantly for Group 6 during control and perfusion periods: 694 ± 129 versus 1688 ± 445 fmol·min^{-1}, $P < 0.02$. Corresponding Group 7 values were 697 ± 164 and 3769 ± 1073 fmol·min^{-1}, with both perfusion values significantly different from controls, $P < 0.01$. Mannitol perfusion in Group 7 was also associated with a fourfold increase in flow, 6.8 ± 1.2 vs. 28.5 ± 3.1 nL/min, with the perfusion flow rate being ~10 nL higher than that measured in Group 6, but nevertheless, the DT [NO] did not decrease. Measurement of DT [Cl] in both Groups 6 and 7 permitted rough estimates of chloride reabsorption (late proximal Cl entry continues in these closed perfusions) and indicate, as expected, NaCl reabsorption is enhanced with 450 NaCl, and is precipitously reduced with 900 mmol/L mannitol.

Analysis of possible flow effects

As noted in **Methods**, flow effects on [NO] and DT-collected NO were examined by ANCOVA in all single tubules both within and between groups. Thus, even though mean flows in Groups 1 and 2 were almost identical (open system) at 10 and 40 nL/min, there was no significant within group effect, even with the addition

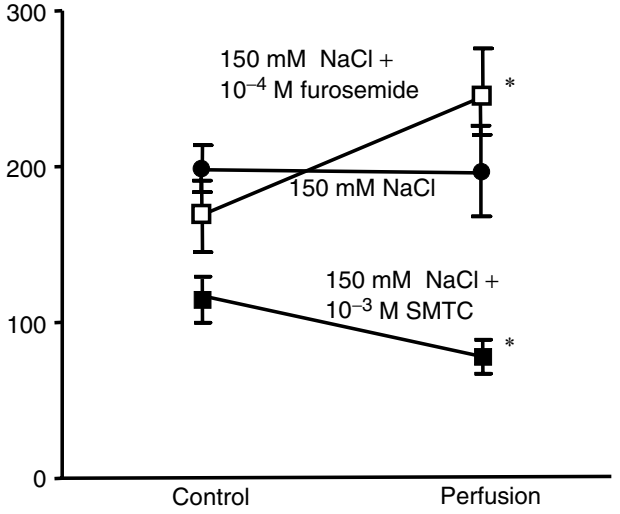

Fig. 5. Distal tubule fluid [NO] during 10 nL/min loop perfusion (closed system) in Groups 3–5. Group 3, 150 mmol/L NaCl (closed circles); Group 4, 150 mmol/L NaCl + 10^{-4} mol/L furosemide (open squares); Group 5 (closed squares) 150 mmol/L NaCl + 10^{-3} mol/L SMTC. $^*P < 0.05$ by paired *t* testing of perfusion vs. control values for individual tubules. See also Table 2 and **Results**.

of tubules perfused at 20 nL/min, not shown elsewhere in this report. Similarly, in the closed perfusion studies, with Group 3 (150 mmol/L NaCl), Group 4 (150 mmol/L NaCl +10^{-4} mol/L furosemide), and Group 5 (150 NaCl mmol/L+ 10^{-3} mol/L SMTC), no significant flow effects were present. All significant changes in [NO] and collected NO are attributable to the perfusate used. Most important, even with Group 6 (450 NaCl mmol/L), and Group 7 (900 mmol/L mannitol), despite an approximately 10 nL/min increase in flow, analysis of within group and between group, single-tubule responses clearly reveal that flow, per se, did not significantly influence NO responses independent of the perfusate intervention. Although it might be expected that in Groups 3–5 there are no significant flow effects, where flow does not increase by more than about 5 nL/min, it might seem more likely that high flows would have an effect. But even though 900 mmol/L mannitol perfusion may have delivered more NO to the DT site by convective flow, this effect was not significant. In short, no significant changes in DT [NO] and collected rates of NO are attributable to changes in flow; rather, the significant changes result from the composition of the perfusates used (Group effects).

DISCUSSION

In the present studies we directly measured nitric oxide concentrations [NO] in distal tubular fluid (DTF) during perfusion of solutions that altered loop NaCl reabsorption. We hypothesized that with the use of real-time NO specific electrodes, DTF [NO] should change perceptibly if loop net NO emission is altered by a substantial portion of epithelial cells making up the entire loop, or, if very few cells were involved, the local changes in NO emission would have to be profound.

Using the classic open loop perfusion system [14], which prevents both proximal tubular fluid NaCl and NO from entering the loop, we perfused 150 mmol/L NaCl with and without 10^{-4} mol/L furosemide; at both 10 and 40 nL/min perfusion, with DT flow unchanged, furosemide inhibition significantly increased DT-collected NO, while significantly suppressing J_{Cl}. Using the closed loop perfusion system, considered by some to be more physiologic because endogenous flow is intact and has provided unexpected TGF responses at low flows [13], we perfused the loop with 150 mmol/L NaCl, and evaluated the effects of 10^{-4} mol/L furosemide. Again, furosemide elicited significant increases in both DT [NO] and DT-collected NO, with the identical modest increase in DT flow. Although perfusion of the specific nNOS inhibitor SMTC reduced DT [NO] when compared to free-flow controls, and prevented the furosemide-induced rise in DT [NO], it did not reduce the collected NO at the DT site. To assess higher NaCl reabsorption and higher flow rates, perfusions were undertaken with 450 mmol/L NaCl and 900 mmol/L mannitol. Similar to the furosemide effect, mannitol elicited a fivefold increase in collected NO.

Finally, in all protocols we examined between and within group flow effects. ANCOVA of all individual tubules within and between groups indicates the significant changes in DT [NO] and collected rates of NO are attributable not to changes in flow, but rather to the composition of the perfusates used.

How far along the nephron may changes in tubular fluid [NO] be detected?

It is clear that along the nephron, as flow varies and water is abstracted, tubular fluid NO concentrations must be the result of a dynamic equilibration process whereby NO freely diffuses into the lumen from the interstitial sink of NO, as well as being influenced by multidirectional diffusion of NO from those apical cells which generate NO. Changes in DTF [NO] as a result of loop effects might be readily detectable if NO is stable in tubular fluid over 1 to 3 minutes, and if the change in net NO entry into the loop tubular fluid is brisk—for example, if the approximate 800 cells [10] of the cortical TAL added nitric oxide gas to the tubule fluid. Assuming that tubular fluid, free of heme, corresponds to a simple aqueous solution, it has been calculated that nmol/L NO concentrations have an unexpectedly long half-life—from 100 to 500 seconds [15, 16]. Using an NO electrode design similar to ours (from the same manufacturer), Hakim et al [17] showed that a 1200 nmol/L NO concentration in aqueous solution with a pO_2 of 40 mm Hg, has a half-life of 7 minutes. Moreover, very recent studies involving direct measurement and infusion of NO equilibrated saline into the human brachial artery show, remarkably, that intact NO can travel significant distances along the vascular bed [18]. Therefore,

based on these considerations, we believe there is no evidence to dismiss the possibility that alterations in net emission rates of NO from the entire loop may elicit 50 to 200 nmol/L NO concentration changes in distal tubule fluid.

Interpretation of results

We stress the following comments are speculative—we have provided no measurements of any aspects of cellular NO emission by loop segments or the TGF response. It is clear that 10^{-4} mol/L furosemide, when perfused with isotonic NaCl at high flow in the open system, or at 10 nL/min in the closed system, results in significant increases in both DT [NO] and collected NO, either when flows are unchanged (Group 1), or with modest increases in flow (Group 4). Thus, reduced NaCl loop transport, as indicated by these observations alone, is associated with increased DT [NO] and net NO emission from the entire loop. In the open system, the observation that the increase in DT [NO] is not significant at 10 nL/min 150 mmol/L NaCl perfusion is perhaps consistent with past observations (using the closed system), that at low flow, NOS inhibitors have minimal or no effect on the TGF response [13].

It is not clear that the significant fall in DT [NO] associated with SMTC perfusion, or its effect to prevent the furosemide-induced increase in DT [NO] (Group 8), necessarily conflicts with the report of Liu et al, showing that the NO-sensitive fluorophore 4,5-diaminofluorescin (DAF-2) detected increased MD intracellular NO formation when NaCl delivery is acutely increased [6]. It is also uncertain that MD cell NO content need necessarily parallel DT NO measurements 3 to 500 μm downstream [5]. In fact, NO collected at the DT site, likely a reflection of net loop NO emission, is not increased with SMTC perfusion. Although it is very likely NO concentration changes are important at local signaling/transport sites, our data do not suggest that net loop NO synthesis or emission are changed by SMTC. In any case, we may speculate that with high NaCl loads, it is still possible for the MD cells to increase NO synthesis, while a much greater NO emission into tubular fluid, from the entire loop, may fall. We note that TAL cells, likely 20 times the number of MD cells [10], also synthesize significant quantities of eNOS-derived NO to diffuse to the MD site [6]. Indeed, it is possible that this TAL NO emission may be downregulated by high NaCl delivery, but there are no data yet available to support this conjecture.

A final speculation is derived from the view that NO likely inhibits collecting duct sodium transport [19]. The demonstration of higher rates of NO entry into the distal nephron under conditions of decreased loop NaCl transport might be considered to act as "feed-forward" signal, which would support continued reduction of downstream NaCl transport.

CONCLUSION

Our results indicate that short-term net NO emission from the entire loop, as collected in distal tubule fluid, increases with inhibition of loop NaCl transport.

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