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Nitric oxide synthase in macula densa regulates glomerular capillary pressure

(kidney/tubuloglomerular feedback response/glomerular filtration rate/afferent arteriole)

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ABSTRACT **Tubular-fluid reabsorption by specialized cells of the nephron at the junction of the ascending limb of the loop of Henle and the distal convoluted tubule, termed the macula densa, releases compounds causing vasoconstriction of the adjacent afferent arteriole. Activation of this tubuloglomerular feedback response reduces glomerular capillary pressure of the nephron and, hence, the glomerular filtration rate. The tubuloglomerular feedback response functions in a negative-feedback mode to relate glomerular capillary pressure to tubular-fluid delivery and reabsorption. This system has been implicated in renal autoregulation, renin release, and long-term body fluid and blood-pressure homeostasis. Here we report that arginine-derived nitric oxide, generated in the macula densa, is an additional intercellular signaling molecule that is released during tubular-fluid reabsorption and counters the vasoconstriction of the afferent arteriole. Antibody to rat cerebellar constitutive nitric oxide synthase stained rat macula densa cells specifically. Microperfusion of the macula densa segment of single nephrons with N^{ω}-methyl-L-arginine (an inhibitor of nitric oxide synthase) or with pyocyanin (a lipid-soluble inhibitor of endothelium-derived relaxation factor) showed that generation of nitric oxide can vasodilate the afferent arteriole and increase glomerular capillary pressure; this effect was blocked by drugs that prevent tubular-fluid reabsorption. We conclude that nitric oxide synthase in macula densa cells is activated by tubular-fluid reabsorption and mediates a vasodilating component to the tubuloglomerular feedback response. These findings imply a role for arginine-derived nitric oxide in body fluid-volume and blood-pressure homeostasis, in addition to its established roles in modulation of vascular tone by the endothelium and in neurotransmission.**

Goormaghtigh (1) suggested that the macula densa is the sensor for a stimulus from tubular fluid that is conveyed to the glomerulus. Subsequently, Thurau and Schnermann (2) identified that the stimulus was the delivery and reabsorption of NaCl by this segment. This tubuloglomerular feedback response functions as a negative-feedback control mechanism, whereby glomerular filtration of NaCl, with delivery to and reabsorption by the macula densa, induces release of mediator(s) that cause afferent-arteriolar vasoconstriction and a reduction in glomerular capillary pressure and glomerular filtration rate (2). Although the signaling mechanisms or molecules inducing afferent-arteriolar vasoconstriction have not been clearly defined, the response is promoted by adenosine acting on adenosine type 1 receptors (3), angiotensin II (4), and thromboxane A_2 (5).

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Previous studies have established that L-arginine-derived nitric oxide (NO) is produced by several cells within the kidney, including isolated glomerular mesangial (6) and endothelial cells (7), and a renal epithelial cell line (8), but its integrative role in the control of renal function is not yet clear (9). In the vessel wall, the endothelium can mediate vasodilator responses to agents such as acetylcholine (10) and can blunt the actions of certain vasoconstrictors (11). In the isolated or intact kidney, the L-arginine–NO pathway determines basal vascular resistance and mediates vasodilator responses to acetylcholine (12). We investigated the hypothesis that L-arginine-derived NO offsets the action of those mediators that cause vasoconstriction of the afferent arteriole upon activation of NaCl reabsorption by the macula densa segment of the nephron. This hypothesis implies that NO mediates a vasodilator arm of the tubuloglomerular feedback response.

MATERIALS AND METHODS

NO synthase was located immunohistochemically in the rat kidney with a polyclonal rabbit antibody (13) to rat cerebellar constitutive NO synthase (14) that does not crossreact to macrophage-inducible or endothelial-constitutive NO synthase (15). Furthermore, the NADPH-diaphorase activity (nitro blue tetrazolium formazan formation) of NO synthase was used to verify the site(s) of NO synthase with a method that does not depend upon immunoreactivity. Kidneys were excised, frozen in hexane/dry ice, and mounted on a microtome chuck. Eight-micrometer-thick sections were cut by using a cryomicrotome (Reichert–Jung Frigocut 2800), thaw-mounted onto microscope slides, fixed by immersion in acetone at 4°C for 5 min, and air-dried. Slides were stored at 4°C until used. For histochemical staining of NO synthase, slides were preincubated in phosphate-buffered saline for 5 min and then with mono-specific polyclonal rabbit antiserum against NO synthase from rat cerebellum (6763-5), which was diluted 1:100 in phosphate-buffered saline/1% bovine serum albumin and applied for 30 min at 37°C. Slides were then washed twice in phosphate-buffered saline for 5 min and in Tris·HCl buffer, pH 7.6 for 10 min. A peroxidase-conjugated second antibody, goat anti-rabbit IgG, was added for 10 min, and slides were again washed in phosphate-buffered saline. The peroxidase label was developed by using diaminobenzidine dissolved in imidazole buffer, pH 7.6 for 6–10 min,

Abbreviations: PSF, proximal stop-flow pressure; L-NMA, N^{ω}-methyl-L-arginine; D-NMA, N^{ω}-methyl-D-arginine.

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washing in Tris·HCl buffer, and dehydration. For histochemical staining of NADPH diaphorase (data not shown), slides were immersed for 20–30 min at 37°C in 50 mM Tris·HCl, pH 8.0/1 mM NADPH/0.5 mM nitro blue tetrazolium/0.2% Triton X-100. The slides were washed briefly in phosphate-buffered saline, counterstained with eosin, and dehydrated with a graded series of ethyl alcohols. Slides from both staining procedures were mounted by using Permount and no. 1-1/2 glass coverslips (14).

The function of macula densa NO synthase in the control of glomerular capillary pressure was investigated by using micropuncture and microperfusion studies in single outer cortical nephrons (5). Male Sprague–Dawley rats (body weight, 175–250 g) were anesthetized with Inactin (100 mg·kg^{-1}; Byk-Guiden Pharmazeutika). For orthograde perfusion of the loop of Henle, a microperfusion pipette (4–6 μm o.d.) containing artificial tubular fluid and driven by a nanoliter perfusion pump (model A1400; World Precision Instruments, Sarasota, FL) was inserted into an end-proximal surface tubule. An immobile wax block was inserted into the nephron proximal to the perfusion site. To assess changes in glomerular capillary pressure, a pressure-measuring ultramicropipette (1–2 μm o.d.) was inserted into the nephron proximal to the wax block to measure proximal stop-flow pressure (PSF). This pipette was filled with 2 M NaCl solution and connected to a servo-null pressure-recording device (model 4A, Instrumentation for Physiology and Medicine, La Jolla, CA). PSF is determined by the net filtering pressure at the glomerulus. Because the plasma oncotic pressure remains stable during short-term studies, changes in PSF provide a dynamic measure of glomerular capillary pressure.

The first series of functional studies assessed the regulation of the glomerular capillary pressure by the macula densa. Glomerular capillary pressure was measured while macula densa function was intact during perfusion of the loop of Henle with artificial tubular fluid; these values were contrasted with measurements made while macula densa function was prevented in the absence of perfusion of this segment. The role of L-arginine-derived NO was assessed by infusion of N^{ω}-methyl-L-arginine (L-NMA, an inhibitor of NO synthase; 10^{-4} M) into the efferent arteriole. This arteriole supplies the peritubular capillaries and thereby provides a route for drug delivery into the cortical interstitium surrounding the test nephron and its macula densa segment. Perfusion with vehicle does not alter the glomerular capillary pressure (16, 17).

The second series of studies assessed the role of NO in the macula densa–afferent arteriolar signaling pathway by measuring changes in glomerular capillary pressure produced by adding drugs to artificial tubular fluid perfused orthogradely into the loop of Henle supplying the macula densa segment. The drugs included sodium nitroprusside (an NO-releasing compound; 10^{-6} M); L-NMA (an inhibitor of NO synthase; 10^{-6}–10^{-3} M); N^{ω}-methyl-D-arginine (D-NMA, the pharmacologically inert enantiomer of L-NMA; 10^{-6}–10^{-3} M); L-arginine (substrate for NO synthase; 5×10^{-4} M); pyocyanin, [a lipid-soluble inhibitor of endothelium-derived relaxation factor (18); 10^{-6}–10^{-3} M]; furosemide (10^{-4} M) or mannitol (300 mM), which are two diuretics that inhibit NaCl reabsorption by the macula densa segment of the nephron (2, 19) or vehicle.

For the third series of studies, the effects of the macula densa NO signaling pathways were tested by adding L-NMA (10^{-5} M) to artificial tubular fluid perfused retrogradely directly into the macula densa segment by a micropipette inserted into the early distal tubule upstream from a wax block. The nephron was vented downstream from the proximal wax block (16).

RESULTS

NO synthase immunoreactivity was detected in the macula densa of the kidney—i.e., in the tubular epithelial cells adjacent to the afferent arteriole of cortical nephrons (Fig. 1) but not in other glomerular or tubular cell types. As shown for other neural (14) and nonneural (13) cells, NADPH-diaphorase histochemical activity colocalized with NO synthase immunoreactivity (data not shown). When long nitro blue tetrazolium incubation times were used, several epithelial cells of collecting tubules stained weakly positive for NADPH diaphorase. This finding of a specific location of the type I NO synthase (constitutive, brain-type) within macula densa cells should provide a useful tool for identifying these specialized cells for isolation and clonal cell culture.

For the first series of functional studies, compared with vehicle (n = 10), perfusion of L-NMA (n = 12) in artificial plasma into the efferent arteriole supplying the cortical interstitium reduced PSF modestly, but significantly ($P < 0.01$), by 1.12 ± 0.34 mmHg (1 mmHg = 133 Pa) (mean ± SEM) in the absence of perfusion of the loop of Henle (i.e., macula densa reabsorption prevented). However, during perfusion of the loop of Henle with artificial tubular fluid at 40 nl·min^{-1} (macula densa reabsorption intact), perfusion of L-NMA into the efferent arteriole led to a significantly ($P < 0.02$) greater fall in PSF, which averaged 3.50 ± 0.92 mmHg.

For the second series of functional studies, single nephrons were perfused orthogradely from the late proximal tubule. When added to this perfusion system, both L-NMA and pyocyanin caused dose-dependent reductions in glomerular capillary pressure. This effect was not seen with D-NMA, the inactive D-enantiomer of L-NMA (Fig. 2). Perfusion of eight nephrons with L-arginine (5×10^{-4} M) did not alter PSF when administered alone (vehicle, 25.5 ± 0.5 mmHg vs. L-arginine, 26.0 ± 0.4 mmHg; not significant). However, the reduction in PSF produced by perfusion of the loop of Henle with L-NMA (10^{-4} M) was blunted ($P < 0.001$) when coperfused with a 5-fold molar excess of L-arginine (L-NMA alone, −3.9 ± 0.6 mmHg vs. L-NMA plus L-arginine, −0.2 ± 0.4 mmHg). In rats not receiving drugs (time controls) there are no changes in PSF over a 90-min period of observations (n = 24) (5, 16).

Pyocyanin was found to inhibit the endothelium-dependent vasodilating action of bradykinin in isolated vascular rings;

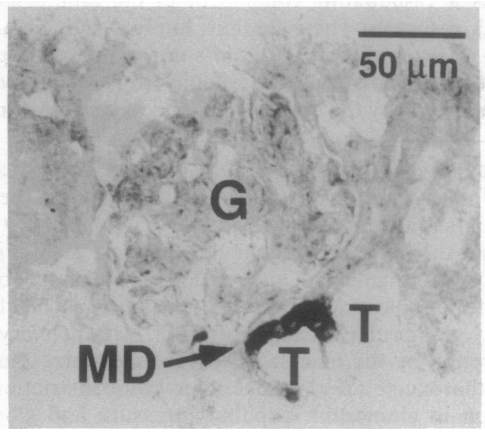

FIG. 1. Immunohistochemical localization of NO synthase in macula densa of rat kidney. Note the section through the glomerular capillary tuft and the adjacent tubular structures that show dense localization of NO synthase within the cells of the macula densa. In contrast, the remaining cells of the tubule (thick ascending limb cells), other tubule cells within the cortex, and glomerular cells including the endothelium were not recognized. T, tubule; G, glomerulus; MD, macula densa. An identical localization was observed for NADPH-diaphorase histochemical staining.

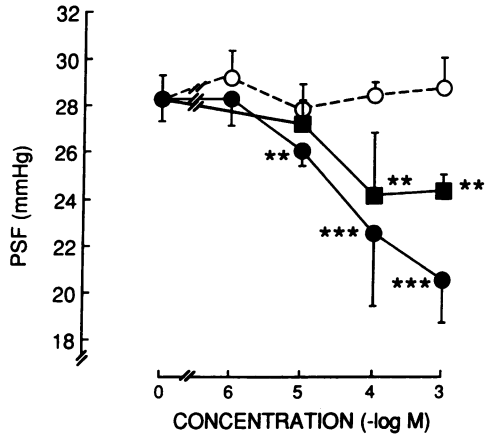

FIG. 2. Concentration–response curves for the effects of L-NMA (●), D-NMA (○), and pyocyanin (■) on PSF (an index of glomerular capillary pressure). Each point represents mean ± SEM values obtained from 8 to 12 individual nephrons. Note the dose-dependent reductions in glomerular capillary pressure elicited by both L-NMA and pyocyanin but the lack of effect of D-NMA. **, $P < 0.01$; ***, $P < 0.005$ (compared to perfusion with vehicle).

this action has been ascribed to scavenging of NO (18) but could also involve intracellular substrate cycling and the generation of superoxide (with a consequent inactivation of NO) or a direct interaction with the active site of guanylate cyclase (compare with methylene blue; 20). The hypothesis that L-NMA inhibits endogenous NO synthase specifically, whereas pyocyanin inhibits the actions of NO derived from endogenous or exogenous sources, was tested in two further sets of nephrons (Fig. 3 *A* and *B*). The glomerular capillary pressure was increased in all nephrons during loop perfusion with sodium nitroprusside (an NO-releasing compound), whereas, as in the previous series, this pressure was reduced consistently by both L-NMA and pyocyanin. Pyocyanin, but not L-NMA, abolished the increase in glomerular capillary pressure elicited by sodium nitroprusside. Collectively, these results imply that, in this *in vivo* nephron system, L-NMA is a potent, stereospecific, and reversible inhibitor of endogenous NO synthase, whereas pyocyanin scavenges or inactivates NO once it is formed and that the local formation of NO within the nephron regulates the glomerular capillary pressure.

The hypothesis that NO is a mediator of tubuloglomerular feedback requires evidence not only that NO can be produced within macula densa cells but also that it relates glomerular capillary pressure to macula densa NaCl reabsorption. Therefore, further nephron perfusion studies were undertaken with furosemide or mannitol added to the luminal perfusate to block macula densa cell reabsorption (2, 19). Luminal perfusion with L-NMA reduced the glomerular capillary pressure uniformly (Fig. 3*C*). As anticipated (19, 21), perfusion with furosemide increased the glomerular capillary pressure, which implies that reabsorption by macula densa cells increases the vasoconstrictor tone of the afferent arteriole. However, in the presence of luminal furosemide, coperfusion with L-NMA no longer reduced the glomerular capillary pressure; indeed, a small increase was apparent. These data indicate (*i*) that furosemide, which blocks macula densa cell function, blocks the primary vasoconstrictor pathway that reduces glomerular capillary pressure, and (*ii*) that the NO-mediated vasodilator pathway, which physiologically counteracts this vasoconstrictor tone, depends upon an intact macula densa reabsorptive process. This conclusion was strengthened by the finding that luminal perfusion with mannitol, an osmotic diuretic, also prevented any change in PSF during loop perfusion with L-NMA (change in PSF with

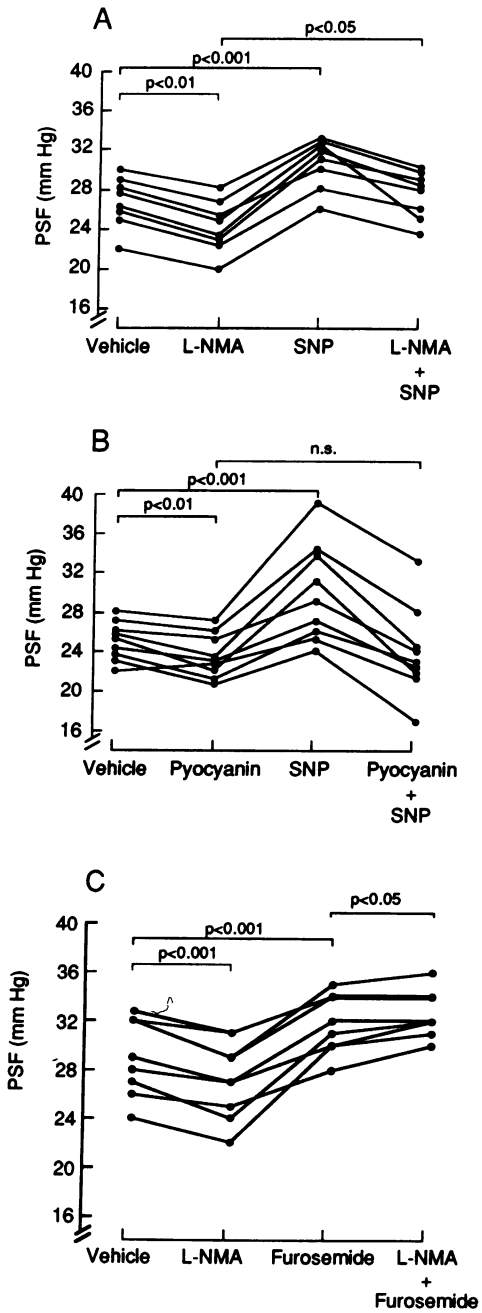

FIG. 3. Effects of L-NMA (10^{-5} M), pyocyanin (10^{-4} M), sodium nitroprusside (SNP; 10^{-6} M), and furosemide (10^{-4} M) on PSF. Individual values of PSF were measured during orthograde perfusion of the loop of Henle with artificial tubular fluid at 40 nl·min^{-1} containing the indicated agents. Note that in all nephrons, L-NMA (*A*) and pyocyanin (*B*) decreased glomerular capillary pressure, whereas sodium nitroprusside increased it. Moreover, the increase in PSF elicited by nitroprusside was blocked by pyocyanin but was unaffected by L-NMA. (*C*) Demonstration that the decrease in PSF elicited by L-NMA was reversed by coperfusion with furosemide (10^{-4} M), which blocks macula densa cell Na/K/2 Cl cotransport (21). n.s., Not significant.

L-NMA plus mannitol, −0.2 ± 0.2 mmHg; $n = 7$; not significant).

The functional significance of NO synthase in macula densa cells was shown further in the third series of experiments in which the glomerular capillary pressure was assessed in response to retrograde perfusions with L-NMA or its vehicle from the early distal tubule directly into the macula densa segment. Compared with vehicle, perfusion of the

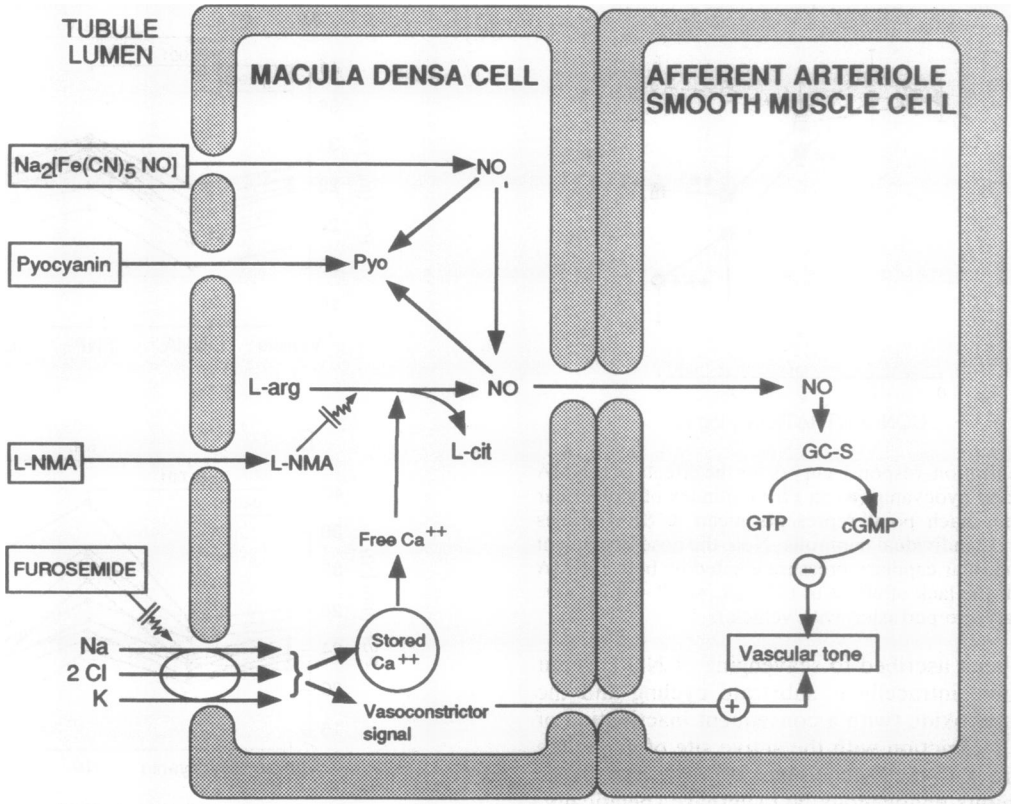

FIG. 4. Proposed role of macula densa NO synthase in the regulation of renal afferent arteriolar smooth muscle tone based on the actions of drugs used in the present study. Luminal perfusion with NaCl-containing tubular fluid results in macula densa cell Na/K/2 Cl uptake via a furosemide or mannitol-sensitive transport process. This uptake triggers both the generation of the agent(s) that mediate the tubuloglomerular feedback-induced vasoconstriction of the afferent arteriole and is also reported (19) to increase intracellular calcium concentration, which, in turn, could activate NO synthase. NO diffuses from the macula densa into the adjacent afferent arteriolar smooth muscle cells, where it activates soluble guanylyl cyclase (GC-S), leading to formation of cGMP from guanosine triphosphate (GTP). This reaction counters the action of the primary vasoconstrictor mediator(s) of the tubuloglomerular feedback. Nitroprusside releases NO. Pyocyanin inactivates NO derived from nitroprusside or NO synthase, whereas L-NMA specifically blocks the synthesis of L-arginine-derived NO. L-cit, L-citrulline.

macula densa with L-NMA (10^{-5} M) consistently reduced PSF by 2.7 ± 0.8 mmHg (n = 7).

DISCUSSION

Reabsorption of tubular fluid by the specialized tubular cells of the macula densa initiates the tubuloglomerular feedback response. This is the focal point for integrating tubular and vascular functions in the kidney and, therefore, mediators of this response may have important physiologic actions in the kidneys (1–5). Tubuloglomerular feedback has been implicated in renal autoregulation (22), the response of the kidney to vasoactive hormones (4, 5, 16, 17), the control of body sodium homeostasis (23), and the development of genetic hypertension (23, 24). Low doses of drugs that block NO synthase have little effect on renal autoregulation at reduced perfusion pressures when macula densa NaCl delivery and function are restricted (25) but reduce renal blood flow at increased renal perfusion pressure (26). These results are consistent with release of NO from the macula densa leading to afferent arteriole vasodilation during high renal-perfusion pressure that increases glomerular filtration rate and macula densa NaCl delivery and reabsorption. In this study, results from two protocols concurred in demonstrating that the vasorelaxation of the afferent arteriole by NO release was more pronounced when the macula densa tubular fluid delivery and reabsorption were intact. (*i*) Reduction in glomerular capillary pressure produced by local infusion of L-NMA into the cortical interstitium via the efferent arteriole was 3-fold greater during perfusion of the macula densa segment with tubular fluid. (*ii*) Reduction in glomerular capillary pressure produced by perfusion of the loop of Henle and macula densa segment with L-NMA was prevented or reversed by coperfusion with mannitol or furosemide, which blocks reabsorption of solutes by the macula densa (Fig. 3*C*).

The findings that NO synthase is located in macula densa cells, where its activity is regulated by tubular fluid reabsorption, and that the NO formed mediates vasorelaxation of the afferent arteriole imply that NO synthase, its target enzyme soluble guanylyl cyclase, and cGMP represent a previously unrecognized vasodilator component of the tubuloglomerular feedback response (Fig. 4). Macula densa cells contain an apical Na/K/2 Cl furosemide-sensitive cotransport process, which when activated releases stored Ca^{2+} from within the cells (19). Therefore, an increase in intracellular calcium may link reabsorption of tubular fluid to activation of the Ca^{2+}-dependent constitutive NO synthase in macula densa cells. An increase in macula densa cell transport also regulates renin release from the adjacent afferent arteriole (27). Therefore, the macula densa may also participate in the observed regulation of renin secretion by NO (28). Finally, alterations in this intrarenal NO pathway may contribute to hypertension. Rats with genetic, salt-sensitive hypertension fail to adjust renal hemodynamics appropriately to changes in salt intake (29) and have a blunted renal and systemic vascular response to inhibition of NO synthase (30). Our findings imply a previously unrecognized role for arginine-derived NO in body fluid-volume and blood-pressure homeostasis over and above its important established functions as a modulator of vascular tone and neurotransmission.

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