Permissive role of nitric oxide in macula densa control of renin secretion

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Increases in plasma renin concentration (PRC) in response to a 3-day infusion of bumetanide (50 mg·kg−1·day−1) or an acute injection of furosemide (50 mg/kg ip) were not markedly altered in nNOS−/− mice. Responses to furosemide were also maintained in eNOS−/− mice, but the administration of L-nitro-l-arginine methyl ester (L-NAME) markedly attenuated the PRC response to furosemide in these mice. In the isolated kidney preparation, bumetanide caused similar relative increases in renin secretion in kidneys of wild-type, nNOS−/−, and eNOS−/− mice. Bumetanide only marginally increased renin secretion in L-NAME-treated kidneys, but the bumetanide effect was normalized by 7-nitroindazole (7-NI) treatment. Basal PRC was significantly reduced in male nNOS−/− mice compared with nNOS+/+ (189 ± 28 vs. 355 ± 57 ng ANG I·ml−1·h−1; P = 0.017). There was no significant difference in PRC between eNOS+/+ and eNOS−/− mice. Basal renin secretion rates in perfused kidneys isolated from nNOS−/− or eNOS−/− mice were markedly reduced compared with wild-type controls. Our data suggest that NO generated by macula densa nNOS does not play a specific mediator role in macula densa-dependent renin secretion. However, NO independent of its exact source permits the macula densa pathway of renin secretion to function normally.

The macula densa (MD) cells located at the terminal end of the loop of Henle are believed to play an important role in both the regulation of preglomerular resistance and the control of renin secretion. Commensurate with their distinct function as sensor cells in these local regulatory pathways, MD cells have a number of unique characteristics that distinguish them from neighboring cells of the cortical thick ascending limb. One of the more striking of these discriminating features is the expression of substantial amounts of neuronal nitric oxide synthase (nNOS) and the ability to generate nitric oxide (NO) (31, 53).

A considerable body of experimental evidence indicates that the expression of nNOS in the MD and the expression and secretion of renin by juxtaglomerular apparatus (JGA) are largely excluded, general NOS inhibition will reduce NO generation by all NOS isoforms. These studies therefore are not able to discriminate between a regulatory role of NO generated by MD nNOS and a permissive role of NO generated by other NOSs.
or endothelial NOS (eNOS) alter the response of renin secretion to chronic or acute administration of a loop diuretic as assessed by measurements of plasma renin in vivo or renin secretion rates in the isolated kidney? Second, is the loop diuretic-stimulated renin secretion in the absence of either eNOS or nNOS affected by superimposed NOS blockade with Nω-nitro-l-arginine methyl ester (l-NAME), and can this response be restored by exogenous NO? Third, does blockade of β-adrenergic input affect the loop diuretic-induced response of renin release. Fourth, does the absence of either nNOS or eNOS alter baseline renin secretion? Our results from studies both in vivo and in the isolated, perfused kidney suggest that loop diuretics induce similar relative increases in renin secretion in the absence of either nNOS or eNOS but not when all NOS activity is inhibited. Inhibition of β-adrenergic receptors reduced basal plasma renin concentration (PRC) but did not alter the efficacy of loop diuretics to increase PRC. The administration of exogenous NO restored sensitivity of renin secretion to loop diuretics in l-NAME-treated, isolated, perfused kidneys. We believe that our data are most consistent with the conclusion that NO specifically derived from nNOS is not required for renin release.

Studies in Isolated, Perfused Mouse Kidney

Mice were anesthetized with a combination of 100 mg/kg 5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid (Trapanal; Byk Gulden) and 80 mg/kg ketamine-HCl (Curamed). After a midline incision, ligations were placed around the abdominal aorta proximal and distal to the right renal artery, around the mesenteric artery and the vena cava inferior. Subsequently, the aorta was clamped distal to the right renal artery so that the perfusion of the right kidney was not disturbed during the following injection of the perfusion cannula into the aorta distal to the clamp. After ligation of the mesenteric artery, a metal perfusion cannula (outer diameter 0.8 mm) was inserted into the abdominal aorta and placed close to the aortic clamp distal to the origin of the right renal artery. After removal of the aortic clamp, the cannula was advanced to the origin of the right renal artery and fixed in this position. The aorta was ligated proximal to the right renal artery, and perfusion was started in situ with an initial flow rate of 1 ml/min. Using this technique, a significant ischemic period of the right kidney was avoided. The perfused kidney was then excised, placed in a thermostated moistening chamber, and perfused at a constant pressure of 100 mmHg was established. To this end, perfusion pressure was monitored within the perfusion cannula (ISOTEC Pressure Transducer, Hugo Sachs Elektronik), and the pressure signal was used for feedback control (SCP 704, Hugo Sachs Elektronik) of a peristaltic pump.

Finally, the renal vein was cannulated with a polypropylene catheter (1.5-mm outer diameter), and the vena cava inferior was ligated. The venous effluent was drained outside the moistening chamber and collected for determination of renin activity and venous blood flow measurement.

The basic perfusion medium, taken from a thermostated (37°C) reservoir of 200-ml volume, consisted of a modified Krebs-Henseleit solution containing (in mM): all physiological amino acids in con-
centrations between 0.2 and 2.0 mM, 8.7 glucose, 0.3 pyruvate, 2.0 t-lactate, 1.0 α-ketoglutarate, 1.0 t-maleate, and 6.0 urea. The perfusate was supplemented with 6 g/l00 ml bovine serum albumin, 1 mU/100 ml vasopressin lysine, and with freshly washed human red blood cells (10% hematocrit). Ampicillin (3 mg/100 ml) and fluocxacillin (3 mg/100 ml) were added to inhibit possible bacterial growth in the medium. To improve the functional preservation of the preparation, the perfusate was continuously dialyzed against a 10-fold volume of the same composition, but lacking erythrocytes and albumin. For oxygenation of the perfusion medium, the dialyzer was gassed with a mixture of 94% O2-6% CO2. Perfusion flow rates were obtained from the revolutions of the peristaltic pump, which was calibrated before and after each experiment. Renal flow rate and perfusion pressure were continuously monitored by a potentiometric recorder. After constant perfusion pressure was established, perfusate flow rates usually stabilized within 15 min. Stock solutions of the drugs to be tested were added to the dialysate.

For determination of perfusate renin activity, venous effluent was collected over a period of 1 min at intervals of 4 min. Samples were centrifuged at 1,500 g for 15 min, and the supernatants were stored at −20°C until assayed for renin activity. For this purpose, perfusate samples were incubated for 1.5 h at 37°C and with plasma from bilaterally nephrectomized male rats as a renin substrate. The generated ANG I (ng·ml⁻¹·h⁻¹) was determined by radioimmunoassay (Byk & Diasonor Diagnostics). Renin secretion rates were calculated as the product of the venous renin activity and the venous flow rate (ml⁻¹·min⁻¹·g kidney wt⁻¹).

Isolation of JG cells and primary cell culture. Mouse JG cells were isolated as previously described (9). Briefly, cells from 2 mouse kidney homogenates were separated by Percoll gradient centrifugation (n = 4 preparations each), and the JG cell-enriched fraction was seeded in 96-well plates. Cells were kept at 37°C in a 5% CO2 atmosphere using DMEM supplemented with 5% FCS, L-glutamine, Na-pyruvate, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). After 20 h of primary culture, the culture medium was removed and the cultures were washed once with 100 µl RPMI-1640 medium containing 2% FCS. Then, 100 µl of fresh and prewarmed culture medium containing the chemicals to be tested (forskolin, 10 µM; IBMX, 100 µM) were added and incubation was continued for 20 h. To normalize for possible differences between different preparations, renin secretion rates were calculated as fractional release of total renin [i.e., renin activity released to the medium (renin activity released to the medium + renin activity remaining in the cells)].

Statistics

Statistical comparisons were made by paired t-test when the effect of a single intervention was tested in the same animal (basal vs. furosemide or l-NAME) and by unpaired t-test when two values between different animals were compared. Comparisons between more than two groups were done by ANOVA with repeated measures and Bonferroni correction for post hoc analysis (basal-propranolol-propranolol plus furosemide or basal-furosemide-furosemide+l-NAME).

RESULTS

Effect of Chronic Bumetanide

As shown in Fig. 1, a 3-day infusion of bumetanide (50 mg·kg⁻¹·day⁻¹) caused significant increases of PRC to 2,601 ± 506 in nNOS+/+ (n = 6; P < 0.01) and to 1,003 ± 274 ng ANG I·ml⁻¹·h⁻¹ in nNOS−/− mice (n = 6; P < 0.05). Relative increases in PRC over the overall baseline averages were comparable between genotypes (7.3-fold in nNOS+/+ vs. 5.3-fold in nNOS−/− mice).

Effect of Acute Furosemide

Because exposure of mice to a 3-day infusion of a loop diuretic may affect renin secretion through other mechanisms than the MD including changes in renin gene expression, a second series of experiments was performed in which one single injection of furosemide (50 mg/kg ip) was given, and tail blood was collected 45 min later. Administration of furosemide increased PRC from 289 ± 78 to 1,634 ± 383 ng ANG I·ml⁻¹·h⁻¹ in nNOS+/+ and from 228.3 ± 33 to 1,356 ± 166 ng ANG I·ml⁻¹·h⁻¹ in nNOS−/− mice (paired t-test: P < 0.01 for both genotypes; n = 6) (Fig. 2). Relative increases in plasma renin levels after furosemide were comparable between nNOS+/+ and nNOS−/− mice (8.2 ± 2.9- vs. 6.3 ± 0.7-fold; P = 0.53). Thus a selective lack of nNOS does not drastically affect the ability of furosemide to increase plasma renin and presumably renin secretion.

As shown in Fig. 3, furosemide increased PRC from 123 ± 18 to 2,322 ± 343 ng ANG I·ml⁻¹·h⁻¹ in eNOS+/- (ANOVA: P < 0.01) and from 228.3 ± 33 to 1,356 ± 166 ng ANG I·ml⁻¹·h⁻¹ in nNOS−/− mice (paired t-test: P = 0.53). Thus a selective lack of nNOS does not drastically affect the ability of furosemide to increase plasma renin and presumably renin secretion.

Effect of l-NAME

As shown in Fig. 2, l-NAME increased PRC from 123 ± 18 to 1,366 ± 333 ng ANG I·ml⁻¹·h⁻¹ in eNOS+/- (ANOVA: P < 0.01) and from 228.3 ± 33 to 1,356 ± 166 ng ANG I·ml⁻¹·h⁻¹ in nNOS−/− mice (paired t-test: P = 0.53). Thus a selective lack of nNOS does not drastically affect the ability of furosemide to increase plasma renin and presumably renin secretion.

Fig. 1. Individual determinations of plasma renin concentration (PRC) in 6 neuronal nitric oxide synthase (nNOS)+/+ (open symbols) and 6 nNOS−/− mice (filled symbols) before (basal) and after a 3-day minipump infusion of bumetanide (50 mg·kg⁻¹·day⁻¹). Horizontal lines indicate means. *P < 0.05; **P < 0.01 vs. respective baseline values (paired t-test).

Fig. 2. Individual determinations of PRC in 6 nNOS+/+ and 6 nNOS−/− mice before (basal) and 45 min after a single injection of furosemide (50 mg/kg ip). Horizontal lines indicate means. *P < 0.01 vs. respective baseline values (paired t-test).
ANG I ml⁻¹h⁻¹ in eNOS⁻/⁻ mice (ANOVA: P < 0.05; n = 5). Furosemide-stimulated values were significantly higher in eNOS⁺/+ than in eNOS⁻/⁻ mice (ANOVA: P < 0.001), a result that was mainly due to an unusually strong response in this group of wild-type mice (relative increase 19.6 ± 2- vs. 8.7 ± 1.5-fold in eNOS⁻/⁻; P = 0.003).

Effect of Furosemide During l-NAME Inhibition of NOS

Using the same groups of eNOS⁺/+ and eNOS⁻/⁻ mice, we tested whether nonspecific blockade of NOS activity with l-NAME affects furosemide-induced renin secretion. Results from these studies are included in Fig. 3. After l-NAME, the effect of furosemide was found to be markedly blunted in both genotypes. While, as indicated above, furosemide stimulated PRC of eNOS⁺/+ mice 19.6-fold without l-NAME, NOS inhibition reduced the stimulation to 3.3-fold. In eNOS⁻/⁻ mice, l-NAME reduced the stimulation of PRC from 8.6- to 3.2-fold (Fig. 3). As tested by ANOVA, PRC values under basal conditions and after the administration of furosemide combined with l-NAME were not significantly different between genotypes (P > 0.05).

Effect of l-NAME

To compare the effects of isolated NOS isoform deficiencies with general NOS blockade, we determined the influence of l-NAME on PRC in nNOS and eNOS wild-type and knockout mice (Fig. 4). l-NAME decreased PRC from 312 ± 34 to 34 ± 7 ng ANG I ml⁻¹h⁻¹ in nNOS⁺/+ (paired t-test: P = 0.005) and from 331 ± 50 to 52 ± 15 in nNOS⁻/⁻ mice (paired t-test: P = 0.004). In eNOS⁺/+ and eNOS⁻/⁻ mice, l-NAME reduced basal PRC from 408 ± 82 to 39 ± 4.5 ng ANG I ml⁻¹h⁻¹(paired t-test: P = 0.002) and from 240 ± 33 to 130 ± 26 ng ANG I ml⁻¹h⁻¹, respectively (paired t-test: P = 0.047; Fig. 4).

Thus l-NAME reduced PRC by a factor of ~10 in wild-type mice, whereas the relative reduction of PRC by l-NAME was 7.5 ± 1.4 in nNOS⁻/⁻ (P = 0.3 compared with wild-type) and only 2.2 ± 0.6 in eNOS⁻/⁻ mice (P = 0.007 compared with wild-type).

Effect of Furosemide During β-Adrenergic Blockade

To minimize the possibility that β-adrenergic activation contributes to the action of loop diuretics, perhaps by the adrenergic stimulation resulting from volume depletion, we examined the effect of acute administration of furosemide during β-adrenergic blockade. Propranolol reduced PRC from 270 ± 84 to 35.6 ± 10.6 ng ANG I ml⁻¹h⁻¹ in wild-type mice (n = 5) and from 198 ± 53 to 77.6 ± 18 ng ANG I ml⁻¹h⁻¹ in nNOS⁻/⁻ mice (n = 4), changes that did not reach the 5% significance level (ANOVA; P > 0.05; Fig. 5). Furosemide administered to mice pretreated with propranolol caused significant increases in PRC, to 443.4 ± 110 in nNOS⁺/+ and to 344.1 ± 49.6 ng ANG I ml⁻¹h⁻¹ in nNOS⁻/⁻ mice. As tested by ANOVA, these values were not significantly different compared with basal (P > 0.05 for both genotypes), but they were significantly higher compared with propranolol alone (P < 0.05 for nNOS⁺/+ and P < 0.01 for nNOS⁻/⁻). Thus renin secretion in response to the loop diuretic was unaffected by the β-receptor antagonist in both nNOS⁺/+ and −/− mice, supporting the assumption that furosemide administration is a suitable test of the MD pathway of renin release.

Baseline Plasma Renin in nNOS- or eNOS-Deficient Mice

Baseline PRC values in male nNOS⁺/+ mice averaged 355 ± 57 ng ANG I ml⁻¹h⁻¹ (n = 24) compared with 189 ± 28 ng ANG I ml⁻¹h⁻¹ for nNOS⁻/⁻ mice (unpaired t-test: P = 0.017; n = 21). PRC values averaged 123 ± 18 ng ANG I ml⁻¹h⁻¹ in eNOS⁺/+ (n = 9) and 137 ± 13 ng ANG I ml⁻¹h⁻¹ in eNOS⁻/⁻ (n = 11).
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Effect of Loop Diuretics on Renin Secretion in Isolated, Perfused Kidney

Basal renin secretion rates in isolated, perfused kidneys from nNOS−/− and eNOS−/− mice were as low as 10 and 30% of the values found in kidneys from nNOS+/+ and eNOS+/+ mice, respectively (Fig. 6). The implication that NO deficiency is responsible for this reduction in renin secretion is supported by the finding that acute pharmacological inhibition of all endogenous NOS with L-NAME (1 mM) significantly reduced basal renin secretion rates in wild-type controls to ~10% of control levels (P < 0.05; Fig. 7). Thus in the perfused kidney, NO, whether derived from nNOS or eNOS, appears to be a potent stimulatory factor of basal renin secretion.

Addition of bumetanide (100 μM) to the perfusate caused an ~2.5-fold increase in renin secretion in wild-type mice (Fig. 6). In kidneys from both nNOS−/− and eNOS−/− mice, bumetanide caused an increase in renin secretion that was comparable to their respective wild types in relative terms, although the absolute magnitude of the increase in renin release was reduced because of the much lower basal levels (see Fig. 6, inset). Bumetanide also caused an increase in perfusion flow, from 6 ± 0.02 to 6.5 ± 0.06 ml/min•g kidney wt−1 in wild-type (P < 0.001), from 6.5 ± 0.01 to 6.8 ± 0.05 ml/min•g kidney wt−1 in nNOS−/− (not significant), and from 2 ± 0.02 to 2.3 ± 0.03 ml/min•g kidney wt−1 in eNOS−/− (P < 0.05), indicating both an intact tubuloglomerular feedback and an increased vascular resistance in kidneys derived from eNOS−/− mice. A causal role of NO in the stimulation of renin secretion by bumetanide is suggested by the observation that the administration of L-NAME in the continuous presence of bumetanide returned renin secretion to basal levels in wild-type as well as nNOS−/− and eNOS−/− mice (Fig. 6). A striking dependency of the renin-stimulatory effect of bumetanide on an intact NOS system was found in additional experiments in wild-type mice in which the addition of bumetanide to the perfusate of kidneys only marginally increased renin secretion in kidneys in which NOS activity had been blocked by prior administration of L-NAME (Fig. 8).

To further examine whether NO synthesis is required for bumetanide to stimulate renin secretion, endogenous NO generation was blocked by L-NAME, and exogenous NO was added in the form of S-nitroso-N-acetyl-penicillamine (SNAP) in a concentration just capable of restoring basal renin secretion to control levels (Fig. 7). Under these conditions of “clamped” NO concentrations and with endogenous NO activity inhibited by L-NAME, bumetanide was still capable of causing a 2.5-fold increase in renin secretion, an effect similar in magnitude to that seen in untreated kidneys (Fig. 6).

Renin Secretion From Isolated JG cells in Primary Culture

Experiments were performed in isolated JG cells to examine the possibility of an inherently altered renin secretion in nNOS−/− compared with wild-type mice (Fig. 9). Baseline renin secretion was found to be similar in JG cells isolated from kidneys of nNOS+/+ and nNOS−/− mice (n = 4 preparations each). Direct stimulation of adenylate cyclase by forskolin (10 μM) doubled renin secretion in JG cells from

![Image](http://ajprenal.org/old/ajprenal/v286n5/i1294i1298f06.png)

Fig. 6. Effect of bumetanide (100 μM) and superimposed inhibition of NOS activity with L-NAME (1 mM) on renin secretion rates in isolated, perfused kidneys of C57BL/6 wild-type (WT), nNOS−/−, and eNOS−/− mice. Inset: relative changes in renin secretion rates as %baseline (n = 4 each).
both strains (ANOVA: \( P < 0.05 \)). When tested by ANOVA with repeated measures, the tendency of renin secretion to increase during inhibition of cAMP-degrading phosphodiesterase activity (IBMX, 100 \( \mu \text{M} \)) did not reach the 5% significance level in JG cells from either nNOS\( ^{+/+} \) or nNOS\( ^{-/-} \) mice. The combination of forskolin and IBMX increased renin secretion (\( P < 0.05 \) vs. control); again, no differences were detected between the two genotypes. Thus the intrinsic ability of JG cells to respond to an increase in cytosolic cAMP does not seem to be different between nNOS\( ^{+/+} \) and nNOS\( ^{-/-} \) mice.

**DISCUSSION**

The principal purpose of this study was to further investigate the role of NO in MD-dependent renin secretion. This question has been of considerable interest because of the striking observation that nNOS, one of the NO-generating enzymes, is expressed at high levels in MD cells and that its level of expression varies in parallel to that of renin in a number of experimental conditions (4, 32, 43, 46, 50). For two major reasons, the question of a specific role of nNOS in MD-dependent renin release has been difficult to address experimentally. Interference with MD nNOS has required the application of inhibitors with selectivity for nNOS, but the efficacy and specificity of these agents are hard to assess in vivo. Second, the technical difficulties in studying the MD pathway of renin secretion are formidable because of numerous other pathways impinging on renin-secreting cells. In addition, it is not always easy to alter the composition of fluid at the MD in an exactly predictable fashion. As a consequence, a specific role of NO generated by nNOS by MD cells in MD-dependent renin secretion has remained unclear. The primary aim of these studies was to further pursue this question in mice with genetic nNOS deficiency both in vivo and in the isolated, perfused kidney as an alternative approach to eliminate nNOS function, using the administration of loop diuretics as a method to alter MD-dependent renin secretion selectively.

Overall, our observations provide evidence that MD nNOS is not required for the activation of the renin-angiotensin system by loop diuretics. First, the stimulation of renin secretion as deduced from the change of PRC caused by prolonged...
or acute administration of loop diuretics was essentially normal in nNOS-deficient mice. Second, in perfused kidneys isolated from nNOS knockout mice the relative change of renin secretion in response to an acute administration of bumetanide was well maintained, although basal renin secretion was markedly reduced. Complete absence of nNOS protein expression in MD cells has been documented previously in nNOS−/− mice (49, 51).

Thus the relevance of our observations for MD-dependent renin release rests on the strength of the evidence that loop diuretics exert their effect on renin secretion through the MD mechanism. MD dependence of the loop diuretic response of renin secretion was first suggested by Vander and Carlson (52); this proposal was later supported by studies in isolated tubule preparations in which inhibition of renin secretion by high NaCl concentrations was blocked by loop diuretics (15, 19, 29). A predictable complication in vivo is a superimposed decrease in pressure at the site of the baroreceptor, because it typically seen with loop diuretics may cause an increase, not a decrease in pressure. While some studies suggest an acute and probably direct effect of loop diuretics on renin secretion from wild-type or nNOS knockout mice, although it tended to reduce baseline renin concentrations as demonstrated previously (22). Lack of a marked contribution of sympathetic activation to the renin secretory response of loop diuretics is also supported by the maintained effect of bumetanide in the isolated, perfused kidney, where regulated sympathetic input is absent and where renal perfusion pressure is externally controlled. We also consider it unlikely that the renal baroreceptor contributes to a major extent to the loop diuretic-induced stimulation of renin secretion. While some studies suggest an acute and probably direct relaxing effect of loop diuretics on venous tone, mean arterial blood pressure does not appear to be systematically altered in the short term before major salt losses have occurred (1, 21, 33, 37). Furthermore, if the renal baroreceptor is located in the JGA region of the afferent arteriole, the renal vasodilatation typically seen with loop diuretics may cause an increase, not a decrease in pressure at the site of the baroreceptor, because it is well established that these agents cause an increase in glomerular capillary pressure (5, 23, 27). In addition, our studies in the isolated kidney show that the increase in perfusion flow rate caused by bumetanide is only between 5 and 10%, a change that is unlikely to be primarily responsible for the marked increase in renin release. Finally, even though not particularly likely in view of the similar responses to loop diuretics, we have addressed the possibility that JG cells from nNOS−/− mice may differ in their response to the major stimuli of renin secretion from wild-type JG cells.

However, isolated JG cells in primary culture from nNOS−/− and nNOS+/+ mice showed comparable responses of renin secretion to forskolin and the phosphodiesterase inhibitor IBMX. Thus the results from these studies indicate that NO specifically generated by MD nNOS is not a mandatory component in MD control of renin secretion. A causal role for NO generation by nNOS in the MD in the stimulation of renin secretion by low NaCl would also have been difficult to reconcile with the recent finding of an increase in MD NO during perfusion of the loop of Henle with high NaCl (28).

Our observations and conclusions are in conflict with the previous finding that the acute administration of the nNOS inhibitor 7-NI prevented the rise in renin secretion caused by furosemide in rats (1). The reason for this discrepancy is unclear, but the effect of 7-NI seems to be different from genetic ablation of nNOS in other respects. For example, 7-NI given for 4 wk has been found to elevate blood pressure, an effect not seen in nNOS−/− mice (34). Indirect evidence that 7-NI may inhibit eNOS in vivo has been presented in rats previously (54). Thus the in vivo selectivity of 7-NI for nNOS that results from limited uptake into endothelial cells compared with neurons may not be absolute (30). In addition, it is unclear whether uptake into MD cells can be a factor modifying the effect of 7-NI. Finally, it is entirely possible that the role of MD-generated NO in renin secretion differs between rats and mice given that the renin-angiotensin system in mice has species-specific characteristics. 7-NI has also been shown to reduce the stimulatory effect of a low-salt diet on renin secretion, another intervention that may include an MD-dependent component (2). However, in another study, a similar administration of 7-NI for several days stimulated renin secretion above the level caused by a low-salt diet alone (34). Furthermore, 7-NI did not alter the increase in renal renin content caused by a low-salt diet (14). Because the administration of a low-salt diet is a complex intervention that does not specifically test MD mediation of renin secretion, conclusions drawn from these studies related to MD-mediated renin secretion are unreliable.

In the absence of a demonstrable effect of nNOS deficiency on loop diuretic-stimulated renin secretion, we have extended our studies to an examination of a possible role of eNOS, another potential source of NO in the JGA. Our data show that the renin secretory response to furosemide was maintained, although perhaps somewhat blunted compared with wild-type mice of the same strain. Furthermore, the effect of bumetanide on renin secretion of kidneys isolated from eNOS−/− was comparable in relative terms to that seen in wild-type or nNOS−/− mice. Thus selective deficiencies in either eNOS or nNOS do not cause marked alterations in the response of renin release to loop diuretics, suggesting that MD-dependent renin secretion is not dependent on NOS isoform-specific NO formation. We did not investigate the effect of loop diuretics in inducible NOS (iNOS)−/− mice.

However, in view of the results for both nNOS- and eNOS-deficient mice and considering the low expression of iNOS in the kidney, we assume that it is highly unlikely that renin secretion in response to loop diuretics would be affected by specific iNOS deficiency. Before concluding that NO is irrelevant for MD control of renin secretion, we considered the possibility that MD control of renin secretion, while not being mediated by NO, requires some background level of NO that in the nNOS- or eNOS-deficient mice is furnished by the intact NOS isoform. In the absence of the availability of nNOS/eNOS double knockout mice, we attempted to study this possibility by using L-NAME as an inhibitor of all NOS enzymes. If NO is required for MD control of renin secretion, one would expect reduced efficacy of loop diuretics to cause renin release during...
general NOS inhibition. The strongest evidence in support of this notion comes from our observation in the isolated, perfused kidney that L-NAME virtually abolished the effect of bumetanide on renin secretion (Figs. 6 and 8). Because perfusion pressure is servo-controlled in this preparation, this effect cannot be the result of the change in pressure that accompanies L-NAME administration in vivo. One may argue that even in the absence of changes in renal perfusion pressure the effect of L-NAME may be indirectly mediated through its intrarenal hemodynamic effects. However, the predicted L-NAME-induced increase in preglenomeral resistance in combination with a constant perfusion pressure would be expected to lead to an actual decrease in pressure at the vascular pole of the glomerulus, the likely site of the renal baroreceptor, causing an increase rather than a decrease in renin secretion. Thus we conclude that the strong attenuation of loop diuretic-stimulated renin release by L-NAME in isolated, perfused kidneys from wild-type, nNOS, and eNOS knockout mice is probably due to a direct effect of NO removal from the JG cell environment. Inhibition of basal and loop diuretic-induced renin secretion by L-NAME has previously also been demonstrated in the isolated, perfused rat kidney (11, 25). Furthermore, NOS inhibition abolished the increase in renin release caused by furosemide pretreatment in dissected rat renal microvessels (6). Perhaps the most direct previous evidence for a role of NO in MD-dependent renin release comes from experiments in the isolated, perfused JGA preparation in which a nonspecific NOS blocker prevented the increase in renin secretion in response to a reduction of luminal NaCl concentration (16). Our in vivo support for a permissive role of NO in MD control of renin secretion is somewhat less convincing. Nevertheless, L-NAME markedly attenuated the increase in PRC caused by furosemide in intact wild-type mice (Fig. 3). It is possible that suppression of renin is due, in part, to the increase in arterial pressure after L-NAME administration, a possibility that is supported by the smaller suppressing effect of L-NAME in eNOS−/− mice in which blood pressure is not elevated by L-NAME (18, 24). Previous studies addressing the importance of blood pressure control during L-NAME treatment have been inconclusive. While in one study L-NAME reduced plasma renin whether renal perfusion pressure was allowed to increase or kept constant, another group reported that the decrease in plasma renin with L-NAME converted to an increase when perfusion pressure was controlled (20, 45). The causes for this discrepancy are unclear, but it would appear that other consequences of L-NAME administration can override the impact of renal perfusion pressure as such. We believe that the bulk of the present evidence suggests that NO generation is required for renin secretion to respond to the MD input but that MD nNOS is not an exclusive source for this NO.

We believe that our data are most consistent with the notion that exposure of JG cells to NO is necessary for the MD-derived mediator to regulate renin secretion efficiently, i.e., that NO is a permissive factor in this pathway. The most direct evidence for this is the observation shown in Fig. 7. When endogenous NO generation was blocked, exogenous NO supplied in sufficient amounts to restore basal renin secretion was found to reestablish the renin-stimulatory effect of bumetanide. Because under these circumstances any regulatory change in endogenous NO production is unlikely because of the presence of L-NAME, it appears that exposure of JG cells to a constant amount of NO permits the expression of the stimulatory effect of bumetanide. While the administration of SNAP is expected to increase renal perfusate flow, one would expect that at constant perfusion pressure the reduction in renal vascular resistance is likely to increase pressure in the afferent arteriole, the likely site of the renal baroreceptor. Because this would be predicted to inhibit renin secretion, the stimulatory effect of SNAP is probably not an indirect consequence of its hemodynamic actions. The mechanism of this permissive action of NO remains to be determined, but the following explanation may be worth pursuing. Given that cAMP is the main stimulus for renin secretion and expression, it may be reasonable to assume that the mediator of MD-dependent renin secretion acts through the cAMP/PKA pathway (13). Levels of cAMP in JG cells are primarily determined by the activity of adenylate cyclase, but there is good evidence that the activity of the cAMP-degrading phosphodiesterase III (PDE III) is another determinant of cAMP levels (10, 25, 26). Inhibition of PDE III activity by cGMP establishes a link between levels of NO and cAMP degradation with the result that cellular cAMP levels should vary in direct proportion to NO. Thus by setting basal cAMP, NO may be a determinant of basal renin secretion (7, 8, 25, 38). Furthermore, the absolute magnitude of the response to a cAMP-stimulating input, like that originating in MD cells, would depend on the rate of cAMP build-up, and thereby on the degree of PDE III inhibition by NO-dependent cGMP (7).

Although studies of the effect of specific NOS deficiencies on basal renin secretion were not a primary goal of the present experiments, it is noteworthy that basal renin release in nNOS- and eNOS-deficient mice appears to be widely different when assessed in vivo compared with the isolated, perfused kidney. Mice without nNOS were found to have a reduction in basal plasma renin to ~50% of the levels found in wild-type mice, a difference that was significant for all observations taken together, but one that did not reach significance levels for smaller subgroups due to substantial individual data scatter.

Basal levels of plasma renin were unchanged in eNOS knockout mice, results that confirm earlier reports of unaltered or even increased levels of plasma renin in eNOS-deficient mice (3, 44). While the effects of selective nNOS or eNOS deficiencies on basal renin release appear to be marginal or absent in vivo, our data indicate that the deficiency of both nNOS and eNOS is associated with drastically reduced rates of basal renin secretion in the isolated, perfused kidney preparation. The reasons for the marked dependence of renin release on an intact NO generation in the isolated kidney are unclear. One possibility may be that the absence of sympathetic input in the isolated kidney may lower cAMP formation in JG cells and that inhibition of cAMP degradation becomes critical in maintaining cAMP levels. This would increase the relative importance of PDE III inhibition and may make renin secretion more NO dependent (26). Obviously, this is a speculation that needs to be corroborated by studies directly aimed at this issue.

In summary, MD-dependent renin secretion as tested by the renin secretory response to loop diuretics in vivo or in the isolated, perfused mouse kidney does not require intact nNOS activity in MD cells or eNOS activity in endothelial cells. However, the marked reduction of the renin-stimulatory efficacy of loop diuretics by nonspecific NOS blockade indicates...
that NO generated by total NOS activity is necessary for MD control of renin secretion to operate efficiently. Overall, our data suggest that NO is a permissive rather than a mediating factor in the MD control of renin release.

**REFERENCES**


