

Effect of blockade of nitric oxide synthesis on the renin secretory response to frusemide in conscious rabbits

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(Received 6 June 1994/23 January 1995; accepted 14 February 1995)

1. The enzyme nitric oxide synthase is present in the macula densa and may participate in the control of renin secretion by the adjacent juxtaglomerular cells. In the present study, we investigated the effect of inhibiting nitric oxide synthase on the renin secretory response to frusemide, which stimulates renin secretion by blocking $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transport in the macula densa.

2. Injection of frusemide in 12 conscious rabbits elicited a transient increase in mean arterial pressure from 84 ± 2 to 92 ± 3 mmHg at 5 min ($P < 0.01$) and a sustained increase in heart rate from 246 ± 6 to 281 ± 10 beats/min at 45 min ($P < 0.01$). Plasma renin activity increased from 8.0 ± 1.2 to 14.3 ± 1.8 , 12.4 ± 1.6 and 11.6 ± 1.5 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ at 15, 30 and 45 min respectively ($P < 0.01$). There were no changes in plasma sodium and potassium concentrations or osmolality.

3. Inhibition of nitric oxide synthase with N^G -nitro-L-arginine methyl ester increased mean arterial pressure by 9 mmHg, decreased heart rate and plasma renin activity, and markedly suppressed the renin response to frusemide (from 4.6 ± 0.7 to 7.6 ± 1.7 , 4.7 ± 1.0 and 4.6 ± 0.7 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ at 15, 30 and 45 min respectively). By contrast, infusion of an equipressor dose of phenylephrine did not suppress the renin response to frusemide.

4. Histochemical studies with the NADPH diaphorase technique confirmed the presence of nitric oxide synthase in the macula densa, and suggested that enzyme activity is increased by treatment with frusemide.

5. These results are consistent with a role for the L-arginine–nitric oxide pathway in the modulation of renin secretion by the macula densa.

INTRODUCTION

Anatomical and functional studies have established that the macula densa plays an important role in the regulation of renin secretion [1–5]. Specifically, there is evidence that the macula densa functions as a sodium/chloride sensor by a mechanism involving an $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transporter in

the luminal membrane of the macula densa cells [5–7]. Alterations in $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transport result in inverse changes in renin secretion by the adjacent juxtaglomerular cells [8].

One of the least understood aspects of this macula densa control of renin secretion is how the cells of the macula densa communicate with the juxtaglomerular cells. Several mediators have been proposed, including adenosine and prostacyclin [5, 9, 10]. Nitric oxide may also play a role. This proposal is based primarily on the findings that the macula densa contains a high concentration of the enzyme nitric oxide synthase [11–13] and that arginine analogues which inhibit nitric oxide synthase alter the rate of renin secretion [14–19].

The aim of the present investigation was to test the hypothesis that the L-arginine–nitric oxide pathway participates in the control of renin secretion by the macula densa. Experiments were performed in conscious rabbits to determine if inhibition of nitric oxide synthesis alters the renin secretory response to frusemide, a diuretic that stimulates renin secretion by, at least in part, inhibiting $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transport in the macula densa [20, 21]. In addition, the NADPH diaphorase histochemical technique was used to determine if acute administration of frusemide alters nitric oxide synthase activity in the macula densa.

METHODS

The experiments were performed on male New Zealand White rabbits weighing 2.5–3.5 kg (Nitabell Rabbitry, Hayward, CA, U.S.A.). The rabbits were housed in separate cages in a 12 h light-cycled and temperature-controlled room. They were fed a commercial diet (Purina rabbit chow, Ralston-Purina, St. Louis, MO, U.S.A.) and were provided with water *ad libitum*. Surgical and experimental procedures were approved by the University of California, San Francisco Committee on Animal Research.

Surgical preparation

Surgical procedures were performed under aseptic

Key words: frusemide, nitric oxide, N^G -nitro-L-arginine methyl ester, macula densa, renin release.

Abbreviations: HR, heart rate; L-NAME, N^G -nitro-L-arginine methyl ester; MAP, mean arterial pressure; PRA, plasma renin activity.

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NAC 2-779

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conditions during anaesthesia with intramuscular ketamine (35–50 mg/kg; Parke-Davis, Morris Plains, NJ, U.S.A.) and xylazine (5–10 mg/kg; Lloyd Laboratories, Shenandoah, IA, U.S.A.). A catheter consisting of 10 cm of medical-grade Silastic (0.76 mm inner diameter, 1.65 mm outer diameter; Dow-Corning Corp., Midland, MI, U.S.A.) connected to PE60 tubing was inserted into a femoral artery and advanced into the aorta to a point distal to the kidneys. Two Tygon catheters (0.76 mm inner diameter, 1.27 mm outer diameter) were inserted into a jugular vein and positioned near the heart. The three catheters were led subcutaneously to a point between the scapulae, where they emerged through a small skin incision, and were protected in a pocket of a nylon mesh jacket. The rabbits were allowed to recover for at least 3 days after surgery, during which time they were treated with intravenous ampicillin (10 mg/day; Sigma, Chemical Co., St Louis, MO, U.S.A.). The catheters were flushed with sterile heparinized isotonic saline (100 units/ml) at least every other day. During the recovery period, the rabbits were brought to the laboratory and accustomed to the experimental environment.

Experimental protocols

On the day of an experiment, a rabbit was brought to the laboratory, where it rested comfortably in a partly covered cage. Arterial blood pressure and heart rate were continuously monitored using a pressure transducer (Cobe Laboratories, Lakewood, CO, U.S.A.) and a custom-built cardiovascular analyser. The cardiovascular data were simultaneously recorded on a Grass polygraph, and digitized, stored and analysed using a PDP 11/23+computer (Digital Equipment Corporation, Maynard, MA, U.S.A.). Blood samples (volume = 1.2 ml) for analysis were collected from the femoral arterial catheter and replaced with an equal volume of sterile isotonic NaCl. We have previously observed that the volume of blood removed during the course of these experiments does not change the haematocrit. Experiments were begun when blood pressure and heart rate had remained stable at their basal values for at least 15 min. The experiments were performed according to the following protocols.

Effect of inhibition of nitric oxide synthesis on the renin and cardiovascular responses to frusemide. In 12 rabbits, blood pressure and heart rate were monitored during a 15-min control period, at the end of which a blood sample was collected. An intravenous infusion of 0.9% saline at 0.02 ml/min was then started and continued for the duration of the experiment. Fifteen minutes after the start of the saline infusion, another blood sample was collected. Frusemide (American Regent Laboratories, Shirley, NY, U.S.A.) was then injected intravenously in a single dose of 2.0 mg/kg. Blood samples were collected 15, 30, and 45 min after frusemide administration. This procedure was repeated in the same

rabbits on the following day, but the saline infusion was replaced with an intravenous infusion of the nitric oxide synthase inhibitor *N*^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma) [22, 23] at 0.5 mg min⁻¹ kg⁻¹. On the third day, the effect of frusemide in the presence of isotonic saline was tested again.

Effect of phenylephrine on the renin and cardiovascular responses to frusemide. The aim of this experiment was to investigate the possibility that L-NAME might alter the renin response to frusemide by increasing blood pressure. In nine rabbits, the effects of frusemide were tested as described in the protocol above, but the L-NAME infusion on day 2 was replaced with an intravenous infusion of phenylephrine (Neo-Synephrine, Winthrop, NY, U.S.A.). The dose of phenylephrine was adjusted between 0.5 and 2.0 µg min⁻¹ kg⁻¹ in order to produce the same increase in blood pressure as that produced by L-NAME.

NADPH diaphorase histochemistry. Rabbits received an intravenous injection of isotonic saline (2 ml, *n* = 3) or frusemide (10 mg/kg, *n* = 3). Thirty minutes later the rabbits were anaesthetized with sodium pentobarbital (30 mg/kg) and the kidneys rapidly removed. Blocks of renal cortex were fixed for 4 h in phosphate buffer (pH 8.0) containing 2% formaldehyde and 0.002% picric acid, immersed in 15% sucrose for 24 h and frozen. Tissue sections of 7 µm were cut in a cryostat, placed on charged glass slides, and dried. The sections were incubated at 37°C for 60–90 min in phosphate buffer (pH 8.0) containing NADPH (1 mg/ml), nitroblue tetrazolium (0.1 mg/ml) and 0.2% Triton X-100. The sections were then rinsed, mounted in glycerol and examined by light microscopy.

Analytical methods

Plasma renin activity (PRA) was measured using a radioimmunoassay for angiotensin I and expressed as pmol of angiotensin I generated per ml of plasma during a 2-h incubation at 37°C and pH 6.5 (pmol 2 h⁻¹ ml⁻¹) [24]. Plasma sodium and potassium concentrations were measured by flame photometry and plasma osmolality by freezing point depression.

Statistical analysis

Results are expressed as the means ± SEM. Data were analysed using analysis of variance for repeated measures followed by the Neuman-Keuls or Dunnett test [25]. Changes were considered to be statistically significant when *P* < 0.05.

RESULTS

Effect of inhibition of nitric oxide synthesis on the renin and cardiovascular responses to frusemide

The effects of frusemide and L-NAME on blood pressure, heart rate and plasma renin activity are

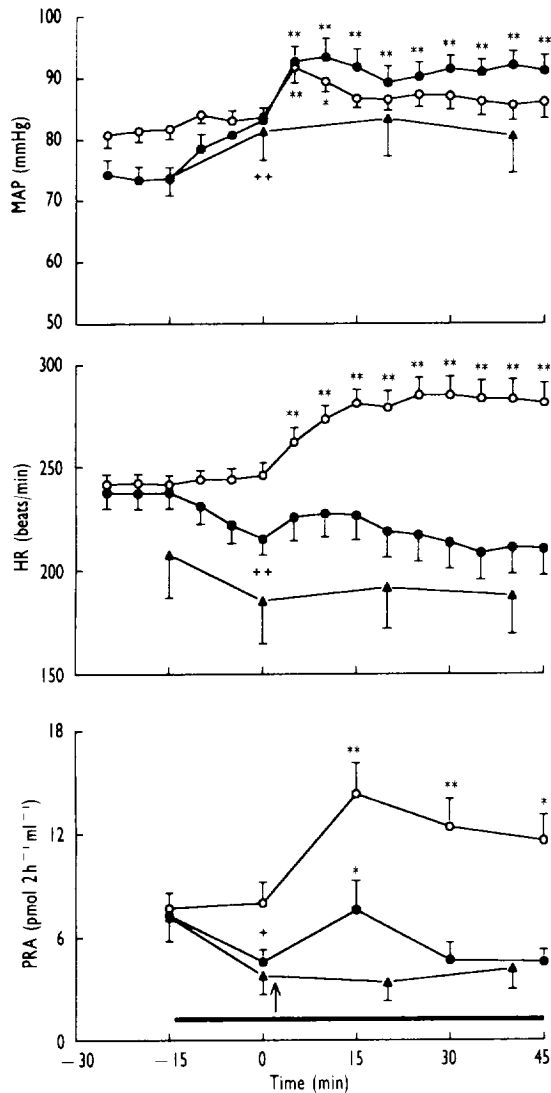


Fig. 1. Effects of frusemide on mean arterial pressure (MAP), heart rate (HR) and plasma renin activity (PRA) during infusion of saline vehicle or L-NAME. Infusion of L-NAME or saline was started immediately after completion of control measurements at -15 min and continued throughout the experiment as indicated by the horizontal bar. Frusemide was injected at 0 min as indicated by the arrow. Values represent the means \pm SEM of observations made in 12 rabbits. Previously reported data [14] showing the effects of L-NAME without frusemide are also shown. * $P < 0.05$, ** $P < 0.01$ compared with -15 min value. * $P < 0.05$, ** $P < 0.01$ compared with 0 min value. \circ , Saline/frusemide; \bullet , L-NAME/frusemide; \blacktriangle , L-NAME/saline.

summarized in Fig. 1. Injection of frusemide elicited a transient increase in mean arterial pressure (MAP) from 84 ± 2 to 92 ± 3 mmHg at 5 min ($P < 0.01$), and increased heart rate (HR) from 246 ± 6 to 281 ± 10 beats/min at 45 min ($P < 0.01$). PRA increased from 8.0 ± 1.2 to 14.3 ± 1.8 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ 15 min after the injection and remained elevated for the next 30 min. Administration of frusemide did not change plasma sodium (142 ± 0.4 to 143 ± 0.4 mmol/l) or potassium (4.2 ± 0.1 to 4.1 ± 0.1

mmol/l) concentrations, or osmolality (289 ± 1 to 288 ± 1 mosmol/kg H_2O). On the following day, infusion of L-NAME increased MAP from 74 ± 2 to 83 ± 2 mmHg ($P < 0.01$), decreased HR from 238 ± 8 to 215 ± 8 beats/min ($P < 0.01$) and decreased PRA from 7.3 ± 0.9 to 4.6 ± 0.7 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ after 15 min ($P < 0.01$). L-NAME markedly reduced the renin response to subsequent administration of frusemide. Thus, while frusemide alone increased PRA by 6.3, 4.4 and 3.6 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ at 15, 30 and 45 min respectively, frusemide in the presence of L-NAME increased it by only 3.0, 0.1 and 0 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$. Treatment with L-NAME also prolonged the pressor response to frusemide and inhibited the HR response.

Previously reported data [14] showing the effects of L-NAME without frusemide are also shown in Fig. 1. It can be seen that the maximum cardiovascular and renin responses to this dose of L-NAME occur during the first 15 min of the L-NAME infusion.

Administration of frusemide on the third day produced changes in MAP (83.4 ± 4.9 to 88.5 ± 3.9 mmHg at 5 min), HR (198 ± 6 to 257 ± 11 beats/min at 45 min) and PRA (5.6 ± 1.2 to 12.4 ± 3.8 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ at 15 min) that were not significantly different from those on the first day.

Effect of phenylephrine on the renin and cardiovascular responses to frusemide

The effect of phenylephrine on the renin and cardiovascular responses to frusemide are summarized in Fig. 2. Frusemide alone increased PRA from 4.9 ± 1.0 to 9.3 ± 1.3 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ at 15 min ($P < 0.01$). Infusion of phenylephrine increased MAP from 73 ± 3 to 82 ± 4 mmHg ($P < 0.01$) (an increase identical to that produced by L-NAME) and decreased PRA from 5.1 ± 0.7 to 2.6 ± 0.5 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ ($P < 0.05$). However, in contrast to L-NAME, phenylephrine did not reduce the subsequent PRA response to frusemide (to 7.3 ± 1.9 pmol $2\text{ h}^{-1}\text{ ml}^{-1}$ at 15 min, $P < 0.05$), although the response was more variable in the presence of phenylephrine. The MAP and HR responses to frusemide were also unaltered by phenylephrine.

NADPH diaphorase histochemistry

NADPH diaphorase staining was observed in macula densas of kidneys from control rabbits and from rabbits treated with frusemide. Representative sections of renal cortex from two control and two frusemide-treated rabbits are shown in Fig. 3. Staining of the macula densa was generally more intense in frusemide-treated rabbits than in control rabbits. However, quantitation of the staining intensity is required before firm conclusions can be made concerning the effect of frusemide.

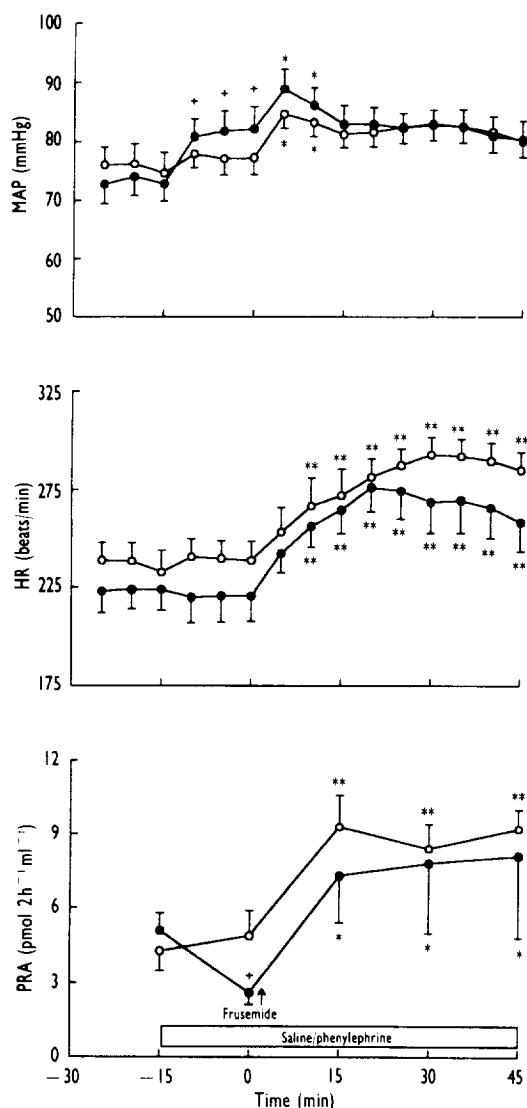


Fig. 2. Effects of frusemide on mean arterial pressure (MAP), heart rate (HR) and plasma renin activity (PRA) during infusion of saline vehicle (○) or phenylephrine (●). Infusion of phenylephrine or saline was started immediately after completion of control measurements at -15 min and continued throughout the experiment. Frusemide was injected at 0 min. Values represent the means \pm SEM of observations made in nine rabbits. * $P < 0.05$ compared with -15 min value. ** $P < 0.05$, ** $P < 0.01$ compared with 0 min value.

DISCUSSION

The aim of this investigation was to determine if nitric oxide participates in macula densa-mediated control of renin secretion. In order to do this, it was necessary to identify a stimulus to renin secretion that operates specifically through the macula densa mechanism. This is difficult because many stimuli which are believed to activate the macula densa mechanism, e.g. sodium deficiency, haemorrhage and decreased renal perfusion pressure, also activate the renal baroreceptor and/or extrarenal mechanisms controlling renin secretion [1, 2, 4].

One exception is administration of frusemide. Although this diuretic has multiple and complex effects on renin secretion when administered chronically [2], it appears to stimulate renin secretion by a direct action on the macula densa when administered acutely [21, 26, 27, 30]. Thus, in the present experiments, the maximum renin response to frusemide occurred at 15 min, at which time volume depletion would have been insignificant. Other investigators have reported that frusemide increases renin secretion when urinary losses are prevented [26, 27]. Blood pressure increased transiently following frusemide administration, and this would be expected to decrease, rather than increase, renin secretion [1, 2]. Plasma sodium and potassium concentrations and osmolality did not change. Frusemide has been reported to increase renal sympathetic nerve activity in rats [28] and plasma noradrenaline concentration in dogs [29], but the renin secretory response to acute administration of frusemide is not blocked by β -adrenoceptor blockade [27, 30], suggesting that it is not mediated by the sympathetic nervous system. Moreover, in preliminary experiments we observed that frusemide does not increase renal sympathetic nerve activity in conscious rabbits (K. Kumagai and I. A. Reid, unpublished work). Finally, frusemide stimulates renin secretion by microdissected afferent arterioles with the macula densa attached, but not from microdissected afferent arterioles alone [21]. Thus, the stimulation of renin secretion by frusemide in the present study was probably mediated by the macula densa. Recent evidence indicates that this stimulation results from inhibition of $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ co-transport in the macula densa [5-8, 20].

It has been reported that the macula densa contains a high level of nitric oxide synthase as detected by immunocytochemistry and NADPH diaphorase histochemistry [11-13], and this was confirmed in the present study. Furthermore, it has been suggested that nitric oxide originating in the macula densa participates in the control of renin secretion [11, 12]; indeed, it may be an important chemical mediator between the macula densa and the juxtaglomerular cells. In order to test this possibility, we investigated the effect of inhibiting nitric oxide synthase on the renin response to frusemide. L-NAME decreased resting PRA and markedly suppressed the renin response to frusemide. Before L-NAME, there were significant increases in PRA 15, 30 and 45 min after frusemide; after L-NAME, there was only a small, transient increase at 15 min.

It is important to note that L-NAME was administered systemically in the present study so that blockade of nitric oxide synthase was not limited to the kidney. Thus, the possibility must be considered that the inhibitory effect of L-NAME on the renin response to frusemide was a consequence of inhibition of extrarenal nitric oxide synthase, in particular

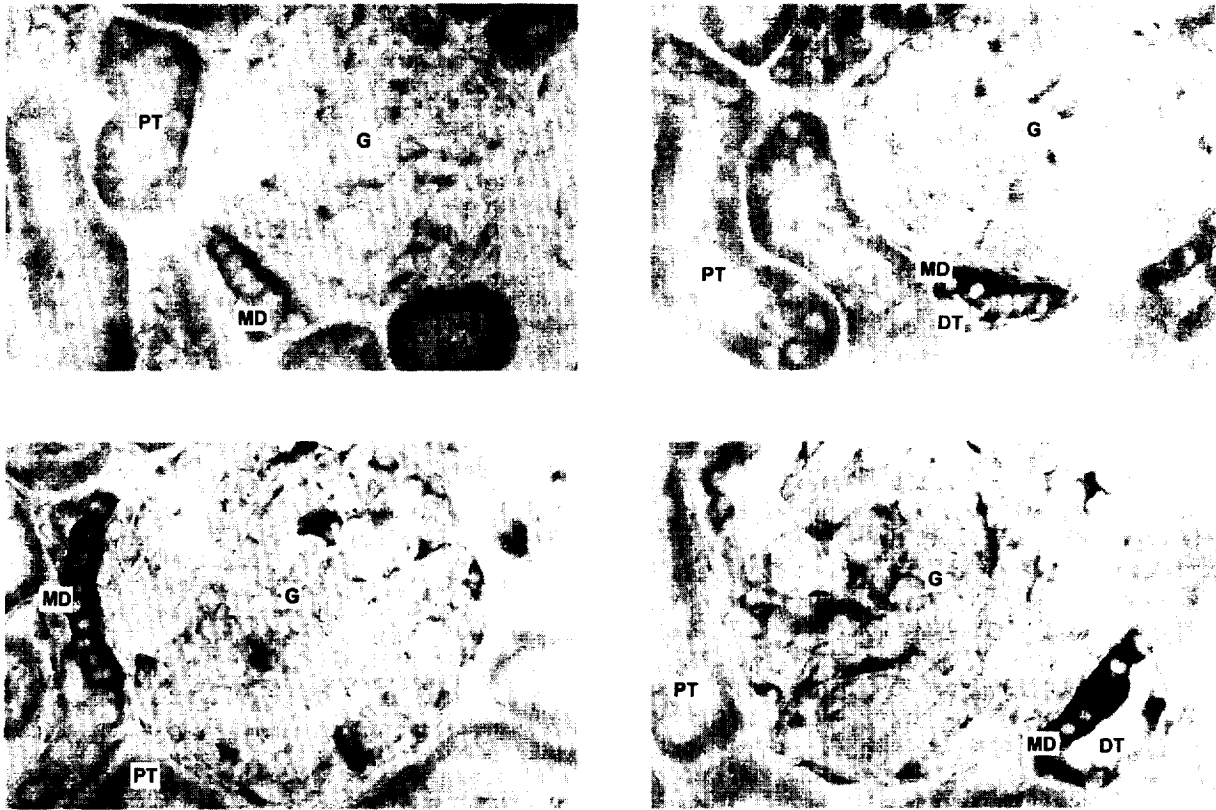


Fig. 3. NADPH histochemistry of sections of kidneys from two control rabbits (top) and two frusemide-treated rabbits (bottom). G, glomerulus; MD, macula densa; PT, proximal tubule; DT, distal tubule.

the increase in blood pressure. However, infusion of an equipressor dose of phenylephrine did not inhibit the renin response to frusemide, and it therefore seems reasonable to suggest that L-NAME inhibits the renin response to frusemide by blocking nitric oxide synthesis within the kidney. Nevertheless, it is difficult to rule out the systemic effects of blockade of nitric oxide synthesis, and additional studies with intrarenal infusion of L-NAME are needed to test this proposal more directly.

How could nitric oxide translate changes in macula densa $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ transport into changes in renin secretion? Specifically, does $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ transport affect macula densa nitric oxide synthase activity, and what effect does nitric oxide have on renin secretion? At present, there is no clear answer to either question, but the following is presented as a working hypothesis. First, it is proposed that nitric oxide originating in the macula densa diffuses into the adjacent juxtaglomerular cells. Here it could increase cyclic GMP concentration, which in turn could stimulate renin secretion, possibly by decreasing the intracellular concentration of calcium [4, 31]. In support of this proposal are reports that blockade of nitric oxide synthase decreases renal cyclic GMP levels [32] and inhibits renin secretion *in vitro* and *in vivo* [14–19]. Second, it is proposed that $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transport by the macula densa decreases calcium concentration in cells of

the macula densa and, as a result, decreases nitric oxide synthase activity and nitric oxide formation. Blocking $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transport with frusemide would increase nitric oxide synthase activity (as the results of the present histochemical study suggest), resulting in an increase in renin secretion which would be prevented by L-NAME (as was observed in the present study). According to this scheme, it would be predicted that the renin response to a reduction in the delivery of sodium chloride to the macula densa would also be blocked by inhibition of nitric oxide synthase. Recent studies by He et al. [33] using a perfused juxtaglomerular apparatus preparation have shown this to be the case.

Several key questions concerning this hypothesis remain to be resolved. In particular, there is, to our knowledge, no direct evidence that $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transport by the macula densa inhibits nitric oxide synthase activity. However, nitric oxide synthase requires calcium for enzyme activity [34, 35], and it has been reported that perfusion of the macula densa with a low concentration of sodium chloride increases cytosolic free calcium concentration in macula densa cells [36]. Thus, it would be anticipated that $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ co-transport by the macula densa would decrease calcium concentration and suppress nitric oxide synthase activity. Although negative results have been obtained [36],

frusemide has been reported to increase calcium levels in the kidney [37], and this would be expected to increase nitric oxide synthase activity. Our observation that frusemide increases NADPH diaphorase staining in the macula densa is consistent with such an action. Another problem is that, although some studies indicate that cyclic GMP stimulates renin secretion, others suggest that it inhibits or has no effect [4, 31, 38–42].

It is important to acknowledge that, although our results are consistent with a role for nitric oxide in the macula densa control of renin secretion, they by no means prove it. It is possible, for example, that the action of L-NAME in suppressing the renin secretory response to frusemide results from blockade of nitric oxide synthesis in cells other than those of the macula densa, such as mesangial [43], endothelial or other cells. A systemic effect of L-NAME also cannot be ruled out, although the present results suggest that changes in blood pressure are not responsible. Finally, even if the nitric oxide does originate in the macula densa, it may play a more generalized permissive or modulatory role rather than a mediator role. Nevertheless, the fact that there is abundant nitric oxide synthase in the macula densa together with our observation that the activity of this enzyme is apparently increased by frusemide warrants additional investigation of the role of the macula densa L-arginine–nitric oxide pathway in the regulation of renin secretion.

As has been observed by other investigators [29], frusemide elicited a transient increase in blood pressure and a prolonged increase in heart rate. Neither response appears to be due to the accompanying increase in renin secretion [29] but, beyond this, the mechanisms underlying these responses do not appear to have been identified. It was therefore of interest that, in the present study, blockade of nitric oxide synthesis inhibited the heart rate (but not the pressor) response to frusemide. This finding, together with evidence that implicates nitric oxide in the control of heart rate [44–47], suggests that nitric oxide participates in the heart rate response to frusemide.

ACKNOWLEDGMENTS

This research was supported by NIH Grant HL-29714 and NASA Grant NAG 2-779. We thank Dina San Juan for measuring plasma renin activity, and Laura Nunes for performing the NADPH diaphorase histochemistry.

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