Local inhibition of nitric oxide temporarily stimulates aldosterone secretion in conscious sheep in vivo

RENATO SALEMI,1 JOHN G. MCDougALL,1 KENNETH J. HARDY,2 AND E. MARELYN WINTOUR1
1Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria 3052; and 2Department of Surgery, Austin and Repatriation Medical Centre, Heidelberg, Victoria 3084, Australia

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IT HAS BEEN ESTABLISHED that aldosterone is the major mineralocorticoid. Nevertheless, the control of its secretion is far from being fully elucidated. Five important acute physiological modulators have been established in all species studied to date. In order of importance under physiological conditions, these are 1) the renin-angiotensin (RAS), and in particular angiotensin II (ANG II), 2) plasma K+ concentration, 3) atrial natriuretic peptide, 4) ACTH, and, to a lesser extent, 5) plasma sodium (Na+) concentration. In addition, there also exist mechanisms for the chronic, long-term regulation of aldosterone secretion. Dietary Na+ restriction and active Na+ depletion are potent stimuli to aldosterone production in vivo. Finally, the K+ status is also an important contributor to chronic aldosterone control. The regulation of aldosterone secretion has been reviewed by Muller (17).

The search continues for other physiological factors that may have a role as either inhibitors or stimuli to aldosterone, and one particular candidate has received much attention in recent times. The recent discovery of nitric oxide synthase (NOS) in rat and bovine adrenal glands has paved the way for the possible inclusion of NO in the complex myriad of factors responsible for the modulation of aldosterone secretion.

The expression of a constitutive NOS enzyme was first shown in adrenal cytosol (23), and subsequently neuronal NOS (nNOS) was identified immunohistochemically in the nerve fibers and ganglion cells within the medulla and scattered throughout the cortex (4). More recent studies have identified nNOS-immunoreactive nerve fibers around blood vessels and cells in the zona glomerulosa (ZG), with varicosities contacting the cytolemma of both vascular smooth muscle and endocrine cells (27). Hence, NOS-immunoreactive nerve fibers may exert influences through direct contact with medullary and cortical endocrine cells, in addition to vasodilating activity in the blood vessels. The only evidence of the presence of NOS in the ZG cell itself comes by way of recent immunoblotting and immunocytochemical experiments. These studies have indicated the presence of endothelial NOS (eNOS) in the ZG of rat adrenal tissue (19).

A variety of NO-related effects on aldosterone secretion have been shown in vitro, where NO has proven to be both stimulatory (18) and inhibitory (12, 19). In the most recent studies in which the eNOS gene was expressed in adrenal ZG cells, it was found that endogenous NO inhibits aldosterone synthesis (13). The differences in the effects of NO on adrenal gland function may reflect differences in tissue preparations used. Although fewer in vivo studies on the effects of NO on aldosterone secretion have been performed, similar contradictory findings have been observed (25, 29).

The impact of systemic NO inhibition on aldosterone synthesis is difficult to analyze in vivo. The chronic
blockage of NO with $N^G$-nitro-lys-arginine methyl ester (L-NAME) has been shown to increase blood pressure (7). It is well established that altering blood pressure may cause changes in the RAS, which in turn may cause secondary changes in secretion of adrenocortical steroids (20). An increase in plasma ACTH concentrations induced by L-NAME administration in vivo has also been described (8). It is difficult to ascertain in vivo whether aldosterone responses to chronic NO inhibition are a direct effect on steroid secretion or primarily a homeostatic response to the changes in arterial pressure and secondary effects resulting from changes in the RAS. Thus, to determine the effects of NO on aldosterone synthesis in vivo, it would be necessary to isolate the adrenal gland from reflex responses to changes in other systems that influence the adrenal gland or to selectively administer the NOS inhibitor to the adrenal gland. The cervical adrenal autotransplant preparation affords us the opportunity to do both.

To our knowledge, there has been no investigation to date on the direct effect of L-NAME administration on the intact adrenal gland in conscious subjects in an in vivo situation in which the integrity of the adrenal gland is maintained and it is isolated from interfering factors in the circulation and from systemic hemodynamic changes. Experiments utilizing this methodology must be done to properly gauge the realistic role of NO in the control of aldosterone secretion in the “whole animal sense,” and to give the results some physiological relevance, which is lacking in studies in isolated cells. Hence, the following series of experiments has been designed to assess the direct effects of NO on basal aldosterone secretion in the autotransplanted sheep adrenal gland. This will give us some indication of whether NO may be the long sought-after “sodium status factor,” a factor whose identity is yet to be established but which is thought to be responsible for the hypersecretion of aldosterone during established Na$^+$ depletion, independent of circulating ANG II levels. An additional aim of the study was to determine the effect, if any, of NO on cortisol secretion. This was done in an effort to establish the specificity of effect of NO blockage on the adrenal gland.

**MATERIALS AND METHODS**

*Animals*

Adult crossbreed Merino ewes were used in all studies. All animals were oophorectomized and had one adrenal gland completely removed to eliminate the effects of these organs on the function of the remaining adrenal gland. Also at this same time, cervical carotid/jugular loops were created on both sides of the animals. After a brief recovery period, all animals had a cervical adrenal autotransplant prepared ≥6 mo before experimentation to allow for recovery from surgery (9). After this 6-mo recovery period, the autotransplanted adrenal gland functions normally, as it has an adequate blood supply and it has been shown that the adrenal medulla undergoes varying degrees of denervation and that this state of denervation varies from animal to animal (9). However, complete loss of innervation is seldom seen. Sheep were fed a diet of oaten chaff (800 g/day) containing 80–120 mmol/kg Na$^+$ and 270–380 mmol/kg K$^+$, and water was offered ad libitum. All experiments were approved by the Animal Experimentation Ethics Committee of the Howard Florey Institute.

**General Methods**

Infusions into the adrenal arterial supply were via the carotid/adrenal artery by a needle and cannula (Critchley Electrical, Silverwater, NSW, Australia) attached to a slow infusion pump (C. F. Palmer, London, UK). Circulation of the infused agents was limited to the local vasculature of the adrenal transplant preparation by inflation of pneumatic cuffs (to 300 mmHg) around the exteriorized left jugular vein/carotid artery cranial to the adrenal and by application of finger pressure to the exteriorized jugular/adrenal vein distal to the adrenal. Cannulation of the jugular/adrenal vein (Portex Limited, Hythe, Kent, UK) permitted collection of adrenal venous blood. Figure 1 depicts the cervical adrenal autotransplant preparation during experimentation.

**Plasma Analysis**

Adrenal venous blood was collected into tubes containing heparin during control and test infusions. Blood flow (BF) was calculated from the volume of adrenal vein blood collected for each timed bleed and was expressed in milliliters per minute. Samples were centrifuged for 5 min at 12,000 rpm (Biofuge A, Heraeus Septatech, Osterode, Germany), and hematocrit was read from a standard scale (Heraeus Septatech). Adrenal venous plasma was obtained by centrif-
doses for 30 min each dose. L-NAME was infused at rates of 1.3, 13, and 130 µg/h, which approximates to 0.2, 2, and 22 nmol/l in the adrenal blood flow. The rates of infusion assume adrenal BF of the order of 1 l/h. Adrenal venous blood samples were taken every 15 min throughout the entire study and analyzed for aldosterone, plasma [Na], and plasma [K]. BF was also measured every 15 min.

**Experiment 2: effect of sustained infusion of L-NAME on basal ASR.** This experiment was composed of two different treatments. **Treatment 1** was a control experiment in which saline was infused continuously for 4 h. **Treatment 2** involved a 30-min control period, after which L-NAME was infused directly into the adrenal arterial supply at the effective dose of 130 µg/h. This infusion was sustained for 4 or 8 h. For both treatments, adrenal venous blood samples were taken every 30 min throughout the entire infusion periods. These blood samples were analyzed for ASR, BF, plasma [Na], plasma [K], and FSR.

**Experiment 3: effect of systemic L-NAME infusion on basal ASR.** This study was performed to determine whether L-NAME has any effect on ASR via systemic cardiovascular actions or via an activation of the RAS or ACTH release from the pituitary gland. The same dose of L-NAME used in the direct adrenal experiments employed thus far was used for this investigation. L-NAME was infused systematically via the right jugular vein at a dose of 130 µg/h. This infusion was maintained for 4 h. It was preceded by a 30-min control infusion period. Bleeds were taken immediately after the control period to establish a baseline level of secretion, and also at 30, 60, 120, 180, and 240 min of systemic L-NAME infusion. Bleeds were analyzed for the usual parameters.

**Statistical Analysis**

Results are expressed as means ± SE. One-way ANOVA with repeated measures was used to determine differences within the varied treatments of the study. Significance was assumed when $P < 0.05$. Where significant differences were obtained by ANOVA, an all pairwise Bonferroni $t$-test modified for multiple comparisons was applied. Changes were considered significant at $P < 0.05$.

**RESULTS**

ASR was observed to increase significantly only at the highest dose of L-NAME employed. ASR rose from a control level of 67.0 ± 2.7 pmol/min at 0 min to 207.4 ± 17.0 pmol/min at 90 min ($P < 0.001$). ASR at 75 min was increased significantly to 182.3 ± 27.7 pmol/min ($P < 0.01$). The level of aldosterone stimulation achieved with this dose of L-NAME is approximately threefold after only a 30-min infusion (Fig. 2;
The normal, resting range of basal aldosterone secretion is between 40 and 80 pmol/min; thus, clearly, L-NAME is having a stimulatory effect. It was the dose of L-NAME (130 μg/h) used at these two time points that was chosen and used in all subsequent studies. No significant changes were observed in the other parameters measured, although BF was seen to fall consistently throughout (17.3 ± 1.4 ml/min at 0 min vs. 13.2 ± 0.8 ml/min at 90 min). This fall, although not significant, suggests that L-NAME has a reducing effect on BF. The effect may become significant with a longer infusion period.

In experiment 2, the control treatment shows that the NS vehicle has no significant effect on basal ASR, with all values falling within the normal range for the duration of the 4-h NS infusion. Similarly, no changes were observed for BF, plasma [Na] (data not shown), plasma [K] (data not shown), and FSR. Conversely, ASR increased steadily throughout the entire 4-h infusion of L-NAME. Aldosterone output rose significantly from 38.6 ± 4.0 pmol/min before infusion (0 min) to its maximum of 221.0 ± 12.4 pmol/min at 240 min (P < 0.001). This represents a fivefold stimulation of aldosterone secretion. The effect became significant as early as 150 min (95.3 ± 9.4 pmol/min; P < 0.01). BF was also seen to change significantly in this study. This parameter was seen to decrease from 17.0 ± 0.5 ml/min at 0 min to 10.5 ± 1.1 ml/min at 210 min and continued to decrease until the termination of L-NAME infusion. No changes in plasma [Na] or plasma [K] were observed (data not shown). Similarly, the 4-h infusion of L-NAME had no effect on basal FSR (Fig. 3; n = 5 for control; n = 6 for L-NAME for 4 h).

When L-NAME was infused for 8 h, it significantly stimulated basal aldosterone secretion from 72.8 ± 13.6 pmol/min at 0 min to 179.0 ± 31.5 pmol/min at 240 min (P < 0.001), a 2.5-fold stimulation. Beyond this time point, ASR was seen to fall dramatically and significantly. The stimulation of aldosterone was reduced, with ASR dropping to a basal value of 42.4 ± 6.8 pmol/min at 300 min (P < 0.001), a complete abolition of aldosterone secretion stimulated by L-NAME. This reduction of secretion was sustained for the remaining period of L-NAME infusion, ~3 h. The effect of L-NAME on adrenal BF in this experiment was similar to the effect observed in the earlier study. L-NAME again significantly reduced BF from 15.1 ± 0.9 ml/min at 0 min to 9.8 ± 1.0 ml/min at 240 min (P < 0.01), and this reduction of BF was maintained at this magnitude until the end of infusion. No significant changes in plasma [Na] or plasma [K] were observed (Fig. 4; n = 6). Despite no apparent effect of L-NAME on FSR over 4 h, cortisol secretion was greatly reduced by the 8-h time point of L-NAME infusion, to levels below control. FSR was observed to fall from 52.8 ± 5.1 nmol/min at 240 min to 19.5 ± 2.2 nmol/min at 480 min (P < 0.05; data not shown).

Experiment 3 results are shown in Fig. 5 (n = 4). No effect of systemic intravenous administration of L-NAME was observed on ASR, with all values falling within the normal range, and similarly for BF and plasma [Na]. At 4 h of systemic L-NAME infusion, plasma [K] rose significantly from 4.0 ± 0.1 mmol/l at 0 min to 4.5 ± 0.1 mmol/l (P < 0.05).

**DISCUSSION**

It seems evident from these studies that the blockade of NO production is having a small stimulatory effect on aldosterone secretion. This stimulation by L-NAME (5-fold) is only minor compared with the much bigger aldosterone responses that are attributed to the known major agonists of aldosterone secretion, such as ANG II, ACTH, and K+ (10-fold) (2, 3). The stimulation of aldosterone output achieves significance at 2.5–4 h of L-NAME infusion. The observed effects of L-NAME on basal aldosterone secretion imply that endogenous NO levels in the adrenal gland provide a constant inhibition or tonic restraint on aldosterone production. The lack of effect of L-NAME on cortisol secretion over 4 h suggests that NO has no role to play in the zona fasciculata (ZF) and that its influence on adrenal gland steroidogenesis is limited specifically to the ZG.

The decrease in BF through the adrenal gland observed with L-NAME is consistent with the finding that
NO acts to enhance BF in this organ (5). This effect becomes significant at ~3.5 h of L-NAME infusion, which correlates well with the 2.5–4 h required for aldosterone secretion to be enhanced. Therefore, L-NAME is having a dual effect on the adrenal gland at this time point, both on BF and on ZG cell function.

When NO production is blocked for a lengthy period, the heightened aldosterone response is ultimately abolished despite continued delivery of L-NAME to the adrenal. A complete abolition of the response is seen, with ASR falling to lower than control levels, after 5 h of L-NAME infusion. This suggests that L-NAME behaves in a manner similar to ACTH (15), with stimulation of aldosterone by short-term exposure but with a complete reversal of effect with longer-term exposure. ACTH reversal requires 24–48 h, and possible cellular and morphological mechanisms for this effect have been suggested. It is difficult to think of a mechanism to explain the reversal of stimulation seen with L-NAME in such a short time frame. This may come in the form of a contribution from another endogenous aldosterone inhibitor, perhaps ANP or adrenomedullin, both of which are located within the adrenal gland, or the recruitment of other NOS isoforms, such as inducible NOS or iNOS, from areas of the adrenal not normally involved in steroidogenic control, such as the vascular endothelium or endothelial cells located in the medulla (1). The above argument is purely speculative, and no evidence exists to suggest that such an aldosterone control mechanism occurs. The fact that adrenal BF remains lowered throughout the entire infusion period argues against the renewed generation of NO within the adrenal gland. Because of the limited scope

Fig. 4. Experiment 2: effect of sustained L-NAME infusion (8 h) on ASR (A), BF (B), plasma [Na] (C), and plasma [K] (D). Control infusion was 0.15 mol/l NS for 30 min. L-NAME was given at a dose of 130 mg/l of adrenal BF. Normal range represents expected basal secretion of aldosterone in normal conscious resting animal. Values are means ± SE (n = 6). †††P < 0.001 vs. 240 min; **P < 0.01 and ***P < 0.001 vs. 0 min.

Fig. 5. Experiment 3: effect of systemic L-NAME infusion on ASR (A), BF (B), plasma [Na] (C), and plasma [K] (D). Control infusion was 0.15 mol/l NS for 30 min. L-NAME was given at a dose of 130 µg/h. Normal range represents expected basal secretion of aldosterone in normal conscious resting animal. Values are means ± SE (n = 4). *P < 0.05 vs. 0 min.
of these experiments, confident conclusions cannot be drawn regarding the role of NO in adrenal steroidogenesis. A more comprehensive assessment of the NO system in this preparation, such as investigating the effects of an analog of NO or of acetylcholine, would go a long way in elucidating the effect of NO on the adrenal gland.

A similar reduction in cortisol secretion was observed after 8 h of L-NAME infusion. Although the magnitude of the reduction in FSR is significant, this observed effect on cortisol is more gradual than the sudden and dramatic abolition seen with aldosterone secretion. This information suggests that the drop in FSR is likely due to normal diurnal variation in cortisol levels, in which plasma levels of this hormone are reduced later in the day. The diurnal variation and the fact that the FSR value at 8 h of L-NAME infusion is, nevertheless, high enough to indicate that ACTH is contributing to the observed FSR are quite telling observations. They illustrate that the adrenal gland, or the ZF at the very least, remains competent after prolonged exposure to l-NAME, and it is unlikely that l-NAME is having a general toxic effect on the adrenal gland. However, studies to determine the aldosterone response to a known secretagogue after 5 h of L-NAME infusion would be the only way to ascertain beyond doubt whether prolonged exposure to l-NAME renders the ZG incompetent. Such studies in the future would indeed be worthwhile.

The fact that a systemic infusion of l-NAME failed to affect ASR indicates that this preparation is not being influenced substantially by cardiovascular and/or circulating factors. Despite this, there was a rise in plasma [K+] observed in this experiment. It is important to keep in mind that these are relatively long-term experiments, and [K+] may be affected by other unrelated factors throughout the day. Also, it is impossible to keep this adrenal preparation completely isolated from the systemic circulation throughout the entire experiment, particularly for longer-term manipulations, and some systemic interference may be possible, although this interference would be minimal.

Confusion surrounds the potential mechanism by which NO may control aldosterone secretion. NO may exert direct effects on guanylate cyclase, activating it to generate cGMP. The inhibitory cGMP-mediated effects of ANP on aldosterone synthesis have been well characterized and are thought to involve ANP binding to the particulate form of guanylate cyclase (16, 22). However, the presence of the soluble form of guanylate cyclase in the adrenal gland, which is the preferred target for NO, has not been shown conclusively. The NO-mediated inhibition of aldosterone synthesis is therefore likely to involve mechanisms other than the activation of cGMP. Another mechanism of inhibition appears to occur via direct interaction of NO with cytochrome P-450 enzymes of the steroidogenic pathway (21). Studies of the biochemical interactions between NO and various cytochrome P-450 enzymes have clearly demonstrated the ability of NO to rapidly bind the heme portion of the cytochrome P-450 enzyme (24, 31). This binding process inactivates the enzyme by preventing binding to oxygen (30). Wink et al. (31) and Hanke et al. (12) reported that cytochrome P-450 steriodogenic enzymes involved in aldosterone production can be directly inhibited by NO. Furthermore, NO has been shown to nitrosylate cellular proteins, and this nitrosylation of proteins may serve as a signaling mechanism much like protein phosphorylation (26). Thus NO may nitrosylate and either enhance or suppress the activity of proteins regulating aldosterone synthesis and secretion, such as protein kinase C (11), or endogenous porphyrins, such as heme (14).

In view of the minor positive effect obtained with L-NAME in these studies, it is difficult to mount a case for a major influence of NO in the regulation of aldosterone secretion. The results do confirm, however, that NO has a direct effect on the adrenal gland, and they provide evidence for the abilities of NO as another potential regulator of adrenal gland function. The effects of NO blockage may, at least in part, explain the secretion of aldosterone that occurs in Na+ depletion, where a dissociation between circulating ANG II and aldosterone secretion has been observed, and may constitute a part, or all, of the actions of the putative sodium status factor.

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