A Nitric Oxide Synthesis Inhibitor Prevents the ACTH-Stimulated Production of Aldosterone in Rat Adrenal Gland

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Abstract. The synthesis of nitric oxide (NO) has been demonstrated in the vascular endothelial cells and other tissues including in rat and bovine adrenal gland. To determine whether NO mediates aldosterone production in the adrenals, we evaluated the basal production of aldosterone, and the ACTH or angiotensin II (A II)-stimulated aldosterone in the presence of L-N^G monomethyl arginine (L-NMMA) or L-arginine or L-NMMA + L-arginine in isolated rat adrenal capsular tissue by an *ex vivo* perfusion technique. ACTH increased the release of aldosterone by 40%. Such release of aldosterone was inhibited by L-NMMA, but not by a mixture of L-NMMA and L-arginine. A II stimulated aldosterone release by 40%. The increase in aldosterone release in response to A II was inhibited by L-NMMA or L-arginine. The increase in aldosterone release stimulated by A II was also inhibited by L-NMMA with L-arginine. L-glutamine did not inhibit the A II stimulated aldosterone release. In conclusion, the inhibition of NO synthesis prevented the release of aldosterone stimulated by ACTH, suggesting NO is required for the action of ACTH on aldosterone production.

Key words: Nitric oxide, Aldosterone, Adrenal gland, Perfusion, ACTH

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NITRIC oxide (NO) is a ubiquitous intercellular messenger synthesized from the amino acid L-arginine by various isoforms of NO synthase (NOS) in different tissues. NO stimulates soluble guanylate cyclase to produce cGMP, and this in turn may stimulate the intracellular pathway. There are two isoforms of NOS that are classified by the expression of the enzymes. Constitutive NOS (cNOS) are present in the cerebellum [1], the anterior cerebrum [2], the peripheral nerves [3] and the vascular endothelial cells [4–6], and share common biochemical and pharmacological features. And, inducible NOS (iNOS) is produced *de novo* by a stimulation as cytokine in the macrophages [7–9], the neutro-

phil [10], the endothelial cells [11] and other tissues.

It has recently been shown that L-arginine and both the NOS inhibitors L-N^G-monomethyl arginine (L-NMMA) and L-N^G-nitro arginine (L-NNA) stimulate pancreatic islet insulin release *in vitro*, but L-NNA did not stimulate insulin release in the perfused pancreas [12]. Furthermore, Kato showed that GH-releasing hormone (GHRH)-induced GH secretion was augmented by L-NMMA in rat pituitary cells, and suggested that GHRH stimulated the synthesis of NO at least partly through cAMP, thereby partially inhibiting GHRH-induced GH secretion [13].

Palacios *et al.* [14] identified L-arginine: NO synthase in rat and bovine adrenal glands and its stimulation of the production of soluble guanylate cyclase and guanosine 3', 5'-monophosphate (cyclic GMP) production. Although neuronal cNOS mRNA [15] and endothelial cNOS mRNA [16] were expressed in adrenal glands, immunohisto-chemi-

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cal reactivity of neuronal cNOS was not localized to rat adrenal capsular layers [17].

L-NMMA was reported to increase the serum corticosterone level in rats [18], and L-arginine administration decreases the serum aldosterone level in man [19]. The role of the NO-NOS system in aldosterone production has not been fully elucidated. To demonstrate that NO or NO-induced cyclic GMP mediates aldosterone production in the adrenal gland, we determined the aldosterone production stimulated by ACTH or angiotensin II (A II) in the perfused adrenal capsular tissue after the administration of L-arginine or L-NMMA.

Materials and Methods

Materials

Synthetic ACTH (1-24) and L-arginine were obtained from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). A II and L-glutamine were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). L-NMMA was obtained from Ajinomoto Co., Inc. (Tokyo, Japan).

Animals

Nine-week-old male Wistar rats (300–350g) were housed at a constant temperature of 24 °C and under artificial light (12 h of light; i.e; lights on from 0600 to 1800 h). They received a diet containing a normal level of sodium 0.26% and potassium 0.85% and water *ad libitum*.

Perfusion technique

The animals were killed by decapitation. The adrenal glands were quickly removed and separated into the capsular and non-capsular portions. The capsular portions (mainly the zona glomerulosa) were bathed in Krebs-Ringer bicarbonate glucose albumin buffer (KRBGA). This buffer contained 119 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.7 mM CaCl₂, 11 mM glucose and 0.2% bovine serum albumin. The perfusion system was previously described [20]. The KRBGA was gassed continuously with a 95% O₂–5% CO₂ mixture, adjusted to pH 7.4, 37 °C, and pumped at a flow rate of 0.2 ml/min. One capsular portion was cut into four

pieces and applied onto a column. After 30 min of perfusion to achieve wash out and stabilization, effluents were collected every 10 min for the determination of aldosterone. The sample to which no agents were added to the KRBGA throughout the experiment was used as the control. After a 20-min baseline sampling, the tissue was superfused with L-NMMA (100 µM), L-arginine (300 µM), or L-NMMA (100 µM) with L-arginine (300 µM) dissolved in KRBGA for 60 min. After the tissues were superfused with L-NMMA (100 µM), L-arginine (300 µM), or L-NMMA (100 µM) with L-arginine (300 µM) containing KRBGA for 30 min, ACTH (10⁻⁸ M) or A II (5 × 10⁻⁸ M) was added to each medium for 30 min to investigate the effect of L-NMMA, L-arginine and L-NMMA with L-arginine on the stimulation of aldosterone production by ACTH and A II. To investigate the specific effects of amino acids on the stimulation of aldosterone production by A II, L-glutamine (300 µM) was used instead of L-arginine.

Analytical methods

Levels of aldosterone in the medium were determined with a SPAC-S RIA Kit (Daiichi Pure Chemical Co., Ltd. Tokyo, Japan) [21]. The protein content in the adrenal gland was determined according to Lowry *et al.* [22].

Statistical analysis

Values are presented as the means ± SEM. One-way analysis of variance was used for statistical analysis. Student's *t*-test was used as appropriate. A level of *P* < 0.05 was considered statistically significant.

Results

Aldosterone concentrations in the effluent KRBGA medium after baseline stabilization collected from the perfused rat adrenal gland were 177 ± 56 pg/mg protein/min. The release of aldosterone after baseline stabilization did not differ significantly between all groups.

The release of aldosterone in the control group decreased gradually during the period of observation, as shown in Fig. 1. Neither L-NMMA (100 µM) nor L-arginine (300 µM) affected the release

of aldosterone over the six fractions *vs.* control. L-NMMA (100 μ M) with L-arginine (300 μ M) tended to reduce the release of aldosterone with significant differences apparent at 20–30 min *vs.* control.

ACTH (10^{-8} M) stimulated the release of aldosterone from the 50 min, and significantly stimulated it for a period of 60–70 min (Fig. 2). This ACTH-stimulated release of aldosterone was inhibited by L-NMMA (100 μ M), but not by L-arginine (300 μ M) alone, or by L-NMMA (100 μ M) with L-arginine (300 μ M).

As shown in Fig. 3, A II (5×10^{-8} M) stimulated the release of aldosterone over the four fractions during its addition. This response was earlier than that of ACTH caused by an 1 fraction (10 min). The increase in the release of aldosterone in response to A II was inhibited by L-NMMA (100 μ M) and L-arginine (300 μ M). There was no significant difference in these two groups as compared with the control throughout the experiment. The increase in aldosterone release in response to A II was also inhibited by L-NMMA (100 μ M) with L-arginine (300 μ M) after the significant inhibition of aldosterone release by L-NMMA with L-arginine. A II stimulated the release of aldosterone in the presence of L-glutamine (300 μ M), showing significant differences in a 50–70 min period.

Discussion

The increase in aldosterone release caused by ACTH in our experiment was inhibited by L-NMMA, but not by L-NMMA with L-arginine, which indicates that the NO system in the adrenal glomerular layer is involved in the production of aldosterone via ACTH. There are at least two pathways of aldosterone release via ACTH. One is via cyclic AMP, and the other is via Ca^{2+} [23]. The former is affected by a pharmacologic dose of ACTH (more than 10^{-9} M) while the latter is affected by a physiological concentration of ACTH (less than 10^{-10} M). The dose of 10^{-8} M of ACTH in our experiment stimulated adenylyl cyclase and cyclic AMP. McFarland *et al.* [24] reported that cyclic GMP-stimulated phosphodiesterase mediated the ANP-induced decreases in the production of cyclic AMP and aldosterone. Therefore in the adrenal NO system, some factor, perhaps NO rather than cyclic GMP, may stimulate the cyclic AMP pathway. Alternatively, basal levels of NO may

Fig. 1. Effects of L-arginine, L-NMMA and L-NMMA with L-arginine on the release aldosterone. Adrenal slices were superfused with medium containing KRBGA alone (control, $\circ$), L-arginine ($\blacktriangle$), L-NMMA ($\square$) and L-NMMA with L-arginine ($\blacksquare$) for 60 min. Results are presented as the mean $\pm$ SEM, expressed as the percentage of each basal aldosterone release at the 0 min. * $P < 0.05$ *vs.* control (baseline).

be essential for aldosterone production stimulated by ACTH. It is not likely that ACTH stimulate the production of aldosterone directly, because ACTH is able to stimulate the particulate guanylate cyclase, but not the soluble guanylate cyclase in rat adrenal gland [25]. Furthermore, Rosenberg *et al.* [26] reported that sodium nitroprusside inhibited ACTH-stimulated aldosterone secretion. These reports and our data may indicate that NO does not stimulate the production of aldosterone, but the process of aldosterone production stimulated by ACTH requires NO.

The increase in aldosterone release in response

Fig. 2. Effects of L-arginine, L-NMMA and L-NMMA with L-arginine on the release of aldosterone in response to ACTH. ACTH was added for 30 min after the superfusion with a medium containing KRBGA alone (Δ), L-arginine ($\blacktriangle$), L-NMMA ($\square$) and L-NMMA with L-arginine ($\blacksquare$) for 30 min. Each point is the mean $\pm$ SEM. * P <0.05 vs. control (baseline, $\circ$).

to A II was inhibited by L-NMMA, L-arginine and L-NMMA with L-arginine. To confirm whether L-arginine and L-NMMA suppressed aldosterone production directly or through the NO system, we studied the effect of L-glutamine which was not a NO donor. The increase in aldosterone release in response to A II was not inhibited by L-glutamine. The NO-cyclic GMP pathway was known to inhibit the Ca^{2+} phosphoinositide messenger system [27, 28], and Wink *et al.* [29] reported that microsomal cytochrome P450 steroidogenic enzymes can be directly inhibited by NO. These results suggest that the NO system may inhibit aldosterone release in response to A II by the pathway of NO-cyclic GMP or by the inhibitory effect of NO on P450. Since L-NMMA inhibited aldosterone re-

Fig. 3. Effects of L-NMMA, L-arginine, L-NMMA with L-arginine and L-glutamine on the release of aldosterone in response to angiotensin II (A II). A II was added for 30 min after superfusion with a medium containing KRBGA alone (Δ), L-arginine ($\blacktriangle$), L-glutamine ($\bullet$), L-NMMA ($\square$) and L-NMMA with L-arginine ($\blacksquare$) for 30 min. Each point is the mean $\pm$ SEM. * P <0.05 vs. control (baseline, $\circ$).

lease in response to A II, L-NMMA may have a specific effect. This effect may make it difficult to assess the role of the NO system on the production of aldosterone stimulated by A II. Our findings may point out another possibility: that the NO system has not a major effect on the release of aldosterone in response to A II, and that L-NMMA and L-arginine directly suppress the release of aldosterone in response to A II.

Since NO inhibits the Ca^{2+} phosphoinositide messenger system [27, 28], the production of aldosterone via A II would be inhibited if cyclic GMP were produced by the NO system in the adrenal gland to affect aldosterone production, but our results were not consistent with this hypothesis. Our findings suggested that the NO system is involved in the release of aldosterone in response to A II, and that L-NMMA and L-arginine directly sup-

press the release of aldosterone in response to A II. Cyclic GMP is a second messenger that modifies the effects of cyclic AMP. It was recently recognized to be a messenger of atrial natriuretic peptide (ANP) and NO. An increase [30], no effect [31], or a decrease [24] in aldosterone production has variously been reported, concerning the effects of cyclic GMP on steroid production following exposure to a cyclic GMP analogue. Some investigators report no effect or a decreased effect of A II stimulation on aldosterone production by a cyclic GMP analogue [31, 32]. If a NO-cyclic GMP pathway inhibits aldosterone release by A II, L-NMMA would stimulate aldosterone release, or both L-arginine and L-

NMMA may have an inhibitory effect on aldosterone production by A II. The exact reason why the basal release of aldosterone was decreased by L-arginine and L-NMMA is not certain, but this effect occurred transient in periods of the 20 and 30 min and the level of aldosterone returned to normal after that.

In conclusion, the inhibition of NO synthesis prevented the ACTH-stimulated release of aldosterone, which suggests that NO is required for the action of ACTH on aldosterone production. L-NMMA and L-arginine may directly suppress the release of aldosterone in response to A II, but further studies are needed to elucidate the role of NO in the action of A II.

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