In Vivo and *in Vitro* Effects of Angiotensin II on the Release of β -Endorphin-Like Immunoreactivity

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Abstract

The effect of angiotensin II (A II) on the release of β -endorphin-like immunoreactivity (β -END-LI) in rats was studied *in vivo* and *in vitro*. Intravenous injection of 1 μ g/100 g body weight of A II resulted in significant increase in the plasma β -END-LI level after 10 and 20 min. Intraventricular injection of 1 ng/100 g body weight of A II also resulted in significant increase in the plasma β -END-LI level after 10 min. A II at concentrations of 10^{-12} M- 10^{-10} M caused dose-dependent stimulation of β -END-LI release from dispersed cells of rat anterior pituitary. On gel chromatography, the β -END-LI released by incubation of the cells with 10^{-10} M A II separated into two components which eluted in the same positions as human β -lipotropin and human β -endorphin, respectively. The ratio of β -LPH to β -END in these fractions was 5:1 on a molar basis. A II did not stimulate β -END-LI release in Ca^{++} -free-medium. [Sar¹, Ala⁸]-A II at concentrations of 10^{-9} M- 10^{-7} M did not stimulate β -END-LI release from the cells. Addition of [Sar¹, Ala⁸]-A II to the incubation medium inhibited A II-induced β -END-LI release from the cells.

These results indicate that A II acts, at least in part, directly on anterior pituitary cells to stimulate β -END-LI release and that calcium ion is involved in the mechanism of this effect.

β -Endorphin (β -END), an endogenous opioid peptide is known to be localized in the pituitary gland, brain, gut and pancreas (Rossier *et al.*, 1977; Orwoll and Kendall, 1980; Bruni *et al.*, 1979). Release of β -END from the pituitary gland is thought to be controlled by the same mechanism as that of adrenocorticotrophic hormone (ACTH)

and β -lipotropin and, like these components, β -END is thought to act as a stress hormone. Angiotensin II (A II) has a variety of actions in the central nervous system: it controls fluid intake (Esteyn *et al.*, 1970), electrolyte balance (Freeman and Davis, 1979; Ploth and Naval, 1979), blood pressure (Sirett *et al.*, 1979), and the releases of arginine vasopressin (ADH) (Share, 1979) and ACTH (Sobel and Vagnucci, 1982). In

the brain, it is present at high concentration in the anterior hypothalamus and the median eminence, suggesting that it acts as a neurotransmitter or neuromodulator. Recently, it has reported that binding sites for A II exist in the anterior pituitary cells (Mukherjee *et al.*, 1982; Capponi *et al.*, 1982). In fact, Sobel and Vagnucci (1982) and Schramme and Denef (1983) reported that A II stimulates the release of ACTH and prolactin from rat pituitary. But its action on β -END secretion from the anterior pituitary is still unknown.

In the present study, we examined the effect of A II on the release of β -endorphin-like immunoreactivity (β -END-LI) *in vivo* and its release from dispersed cells of rat anterior pituitary. The mechanism of A II-induced β -END-LI release was studied in relation to calcium.

Materials and Methods

Male Wistar strain rats weighing 200–250 g were housed under controlled conditions of light (0700–1900 h) and temperature with free access to laboratory rat chow and water. They were divided into 5 groups of 5 rats each and injected intraperitoneally with pentobarbital (5 mg/100 g BW, Abbott Laboratories, USA). Three groups were injected with physiological saline (0.1 ml/100 g BW intravenously or 2 μ l/100 g BW intraventricularly), two groups were given A II (Protein Foundation Minoo, Japan; 5 mg/0.1 ml physiological saline/100 g BW) via the tail vein and two groups were given A II (1 ng/2 μ l physiological saline/100 g BW) intraventricularly using a 10- μ l Hamilton microsyringe. The rats were killed by decapitation 0, 10, and 20 min after injection of physiological saline and 10 and 20 min after injection of A II and blood samples for determination of β -END-LI were put into test tubes containing EDTA (1 mg per 1 ml blood) and Trasylol (500 KIU per 1 ml blood). The time of sacrifice was adjusted to 45 min after injection of pentobarbital. Blood plasma was separated by centrifugation at 2,000 $\times$ g for 15 min at 4°C and kept –20°C until examined.

Preparation of dispersed cells from the anterior pituitary gland

Cells of the anterior pituitary were dispersed by a modification of the method of Yeo *et al.* (1979) as reported previously (Matsumura *et al.*, 1982 b). In brief, the anterior pituitaries of 15 adult male Wistar rats weighing 200–250 g were minced with fine scissors and transferred to a spinner bottle (Bellco Glass Inc., Vineland, NJ, USA) with crystalline trypsin (2.5 mg/ml, Worthington Biochemical Corp.) and deoxyribonuclease (DNase, 40 μ g/ml, Sigma Chemical Co.). The tissues were stirred gently at 37°C for 20 min in an atmosphere of 95% O₂ and 5% CO₂. The mixture was then pipetted back and forth about 20 times and transferred to a test tube, and the cloudy supernatant containing the dispersed cells was carefully separated. This dispersion procedure was repeated a total of four times. The four supernatants obtained were centrifuged at 4,000 $\times$ g for 45 sec at room temperature and the supernatant was discarded. The precipitated cells were resuspended in 2 ml of Earle's balanced salts solution (EBSS) containing lima bean trypsin inhibitor (0.5 mg/ml, Worthington Biochemical Corp.), centrifuged and resuspended in EBSS containing bovine serum albumin (2.5 mg/ml) (EBSS-BSA). The latter procedure was repeated three times more. Before the final centrifugation, the number of dispersed cells was determined. The resulting suspension of cells was preincubated at 37°C for 2 h and used for incubation as described below.

Incubation of dispersed cells of rat anterior pituitary

A suspension of 1×10^5 dispersed cells in 1 ml of incubation medium was first incubated in a shaking water bath at 37°C for 60 min in an atmosphere of 95% O₂ and 5% CO₂. The medium was then changed to medium containing a test material in EBSS-BSA. After incubation for 60 min, the cells were precipitated by centrifugation, and the medium was stored at –20°C for β -END assay.

Radioimmunoassay of β -endorphin

Anti-human β -END (β_h -END) serum was prepared by the method of Guillemín *et al.* (1977) and proved to be highly specific for β_h -END as reported previously (Matsumura *et al.*, 1982 a). It showed only 4.5% cross-reactivity with human β -lipotropin (β_h -LPH) on a molar

basis and did not cross-react with other pro-opiocortin-related peptides tested, including human enkephalins, human endorphins (α , γ and δ), human γ -LPH and human¹⁻³⁹ACTH. Synthetic β_h -END was radioiodinated with ¹²⁵I by the chloramine T method (Hunter and Greenwood, 1962), and [¹²⁵I] β_h -END was separated on a column (1×18 cm) of Sephadex G-25 (medium). The procedure for β_h -END radioimmunoassay was reported in detail previously (Matsumura *et al.*, 1982 a). [¹²⁵I] β_h -END bound to the antibody was released by unlabeled hormone in a dose-related manner in the range of 1.1–73.1 fmole/tube. Plasma β -END-LI was measured by radioimmunoassay after its extraction with acid-acetone and petroleum ether (Matsumura *et al.*, 1982 c).

Gel-filtration chromatography of β_h -END-LI released into the incubation medium

After incubation for 60 min, 1 ml of medium was lyophilized and dissolved in 0.2 N HCl. The solution was heated in a boiling water bath for 10 min and applied to a column (0.9×45 cm) of Sephadex G-50 (fine) and materials were eluted with 0.1 N acetic acid containing 0.1% BSA at a flow rate of 15 ml/h at room temperature. Fractions of 1 ml of eluate were collected, lyophilized, dissolved in the assay buffer and subjected to radioimmunoassay. The column was calibrated with blue dextran, synthetic β_h -LPH, synthetic β_h -END and Na ¹²⁵I.

Statistics

The statistical significance of differences was analyzed by Student's *t*-test.

Results

Effect of A II on β -END-LI release in vivo

As shown in Figure 1, there was no significant change in the plasma β -END-LI level 10 and 20 min after intravenous administration of physiological saline (0.1 ml/100 g body weight). However, after intravenous injection of 1 μ g/100 g body weight of A II the plasma β -END-LI level rose significantly from a basal level of 13.1 ± 1.7 to 39.3 ± 2.4 pmol/l (mean $\pm$ SEM, $p < 0.001$) at 10 min and 31.6 ± 1.6 pmol/l ($p < 0.001$) at 20 min.

Effect of intraventricular injection of A II on β -END-LI release in vivo

As shown in Figure 2, there was no significant change in the plasma β -END-LI level 10 or 20 min after intraventricular injection of physiological saline (2 μ l/100 g

Fig. 1. Plasma β -END-LI levels after iv injection of physiological saline (0.1 ml BW) or A II (1 μ g/100 g BW).

Values are means $\pm$ SEM. Figures in parentheses indicate numbers of rats.

* $p < 0.001$ vs control.

Fig. 2. Plasma β -END-LI level after intraventricular injection of physiological saline (1 μ l/100 g BW) or A II (1 ng/100 g BW).

Values are means $\pm$ SEM. Figures in parentheses indicate numbers of rats.

* $p < 0.005$ vs control.

BW). However, the plasma β -END-LI level rose significantly after intraventricular injection of 1 ng/100 g BW of A II from a basal level of 15.3 ± 0.9 to 18.6 ± 0.9 pmol/l (mean $\pm$ SFM, $p < 0.005$) at 10 min, although it was not significantly increased at 20 min.

Time course of β -END-LI release from dispersed cells on incubation with A II

β -END-LI release from dispersed cells of rat anterior pituitary into the medium was significantly increased from 60 min after addition of A II at a concentration of 10^{-10} M to at least the end of the observation period at 180 min (Fig. 3).

Effect of A II on the release of β -END-LI from dispersed cells

The addition of A II at concentrations of 10^{-12} – 10^{-10} M to the incubation medium caused a dose-dependent increase in the release of β -END-LI from dispersed cells of rat anterior pituitary. However, the addition of A II at concentrations of 10^{-10} – 10^{-7} M to the incubation medium caused a dose-dependent decrease in the release of β -END-LI from dispersed cells of rat anterior pituitary (Table 1).

Fig. 3. Time course of release of β -END-LI from dispersed cells of rat anterior pituitary in the absence (○) and presence (●) of 10^{-10} M A II. Points and bars are means $\pm$ SEM for 4 experiments.

* $p < 0.01$ vs the corresponding control value.

Size heterogeneity of β -END-LI released from dispersed cells

After the incubation of dispersed cells with 10^{-10} M A II, the medium was chromatographed on a column (0.9 $\times$ 45 cm) of Sephadex G-50. As shown in Figure 4, two components of β -END-LI were detected in the medium: one was eluted in the same position as β_h -LPH, and the other in the same position as β_h -END. The ratio

Table 1. Dose-response to A II of β -END-LI release from dispersed cells of rat anterior pituitary

A II Concentration (M)	β -END-LI released (fmole/60 min/ 10^5 cells)
0	194.0 ± 8.0
10^{-12}	$296.0 \pm 10.6^{**}$
10^{-11}	$375.1 \pm 12.9^{**}$
10^{-10}	$432.9 \pm 18.6^{**}$
10^{-9}	$342.9 \pm 20.9^{**}$
10^{-8}	$277.1 \pm 5.1^{**}$
10^{-7}	$256.6 \pm 25.1^*$

Values are means $\pm$ SEM for five experiments.

* $p < 0.01$ vs control.

** $p < 0.001$ vs control.

Fig. 4. Gel-filtration chromatography on Sephadex G-50 of β -END-LI released from dispersed cells of rat anterior pituitary in the presence of 10^{-10} M A II.

The column (0.9 $\times$ 45 cm) was calibrated with synthetic β_h -LPH and synthetic β_h -END.

of β -LPH to β -END in these fractions was 5:1 on a molar basis.

Effect of calcium ion on A II induced β -END-LI release from dispersed cells

Enhancement of β -END-LI release from dispersed cells by incubation for 60 min with 10^{-10} M A II was completely prevented by omission of Ca^{++} from the incubation medium. Addition of Ca^{++} to the medium at concentrations of 1.25–2.5 mM caused a linear increase in the release of β -END-LI induced by A II. At concentrations of above 2.5 mM Ca^{++} , the release reached a plateau (Fig. 5).

Effect of Co^{++} on A II-induced β -END-LI release from dispersed cells

The addition of 2 mM CoCl_2 alone to

the incubation medium had no effect on β -END-LI release from dispersed cells during 60 min incubation period, but completely inhibited A II-induced β -END-LI release (Table 2).

Table 2. Effect of CoCl_2 on A II-induced β -END-LI release from dispersed cells of rat anterior pituitary

Addition	β -END-LI released (fmole/60 min/ 10^5 cells)
None	225.7 $\pm$ 5.7
CoCl_2 (2 mM)	203.1 $\pm$ 6.9
A II (10^{-10} M)	451.4 $\pm$ 5.7*
A II (10^{-10} M) plus CoCl_2 (2 mM)	202.6 $\pm$ 5.1†

Values are means $\pm$ SEM for five experiments.

* $p < 0.001$ vs control.

† $p < 0.001$ vs value with A II.

Fig. 5. Effect of calcium ion on β -END-LI release from dispersed cells of rat anterior pituitary induced by 10^{-10} M A II

The cells were suspended in EBSS-BSA (Ca^{++} free, no EGTA) and then used for experiments with or without addition of calcium ion and A II. The cells were incubated at 37°C for 60 min in medium containing 0 mM, 1.25 mM, 2.5 mM, 5 mM Ca^{++} without A II ($\square$): 0 mM, 1.25 mM, 2.5 mM, 5 mM Ca^{++} with 10^{-10} M A II (hatched). Values are means $\pm$ SEM for 4 experiments.

* < 0.05 vs control.

** $p < 0.001$ vs control.

Table 3. Dose response to $[\text{Sar}^1, \text{Ala}^8]\text{-A II}$ of β -END-LI release from dispersed cells of rat anterior pituitary

Addition	β -END-LI released (fmole/180 min/ 10^5 cells)
None	244.6 $\pm$ 5.7
$[\text{Sar}^1, \text{Ala}^8]\text{-A II}$	
10^{-9}	272.0 $\pm$ 5.4
10^{-8}	267.7 $\pm$ 5.4
10^{-7}	259.4 $\pm$ 4.9

Values are means $\pm$ SEM for five experiments.

Table 4. Effect of $[\text{Sar}^1, \text{Ala}^8]\text{-A II}$ on A II-induced β -END-LI release from dispersed cells of rat anterior pituitary

Addition	β -END-LI released (fmole/180 min/ 10^5 cells)
None	246.6 $\pm$ 9.4
$[\text{Sar}^1, \text{Ala}^8]\text{-A II}$ (10^{-7} M)	259.4 $\pm$ 8.6
A II (10^{-10} M)	410.9 $\pm$ 6.3*
A II (10^{-10} M) plus $[\text{Sar}^1, \text{Ala}^8]\text{-A II}$ (10^{-7} M)	266.0 $\pm$ 6.3†

Values are means $\pm$ SEM for four experiments.

* $p < 0.001$ vs control.

† $p < 0.001$ vs value with A II.

Effect of [Sar¹, Ala⁸]-A II on A II-induced release of β -END-LI from dispersed cells

The addition of [Sar¹, Ala⁸]-A II at concentrations 10^{-9} M to 10^{-7} M to the incubation medium did not stimulate the release of β -END-LI from dispersed cells of rat anterior pituitary, as shown in Table 3. However, addition of 10^{-7} M [Sar¹, Ala⁸]-A II to the incubation medium inhibited A II-induced β -END-LI release, as shown in Table 4.

Discussion

A II has a wide variety of biological effects on the central nervous system (CNS), adrenal gland, liver, kidney, vascular smooth muscle, etc. (Peach, 1977). In the pituitary gland, it stimulates the releases of PRL, ACTH and ADH and inhibits those of GH and LH, probably due to its central action and/or direct action on the pituitary (Sobel and Vagnucci, 1982; Fitzsimons, 1972; Schrame and Denef, 1983; Steel *et al.*, 1981).

The present work showed that intravenous and intraventricular administration of A II stimulated β -END and β -LPH release in rats. The source of the β -END-LI released into the circulation was not clear, because β -END-LI is present in various tissues besides the pituitary (Orwoll and Kendall, 1980; Bruni *et al.*, 1979; Nakai *et al.*, 1978). In addition, it was not clear whether the *in vivo* effect of A II on β -END-LI release is physiological. Therefore, we performed *in vitro* studies and found that A II at concentrations of 10^{-12} M to 10^{-10} M induced dose-dependent β -END-LI release from dispersed cells of rat anterior pituitary, although doses higher than 10^{-10} M A II showed less stimulatory effect on β -END-LI release, which suggests that large doses of A II may have a toxic effect on the hormone release, but this remains to be studied. These findings indicate that A II

can act directly on the pituitary.

The concentration of A II in the hypophyseal portal vein is unknown. A II may have a physiological role in pituitary hormone release when its concentration in the hypophyseal portal blood exceeds 10^{-12} M.

Although these *in vivo* and *in vitro* experiments show that A II may act directly on the pituitary gland and induce β -END-LI release, we cannot eliminate the possibility that CNS may play a role in promoting β -END-LI release.

β -END, an endogenous opioid, has the same amino acid sequence as residues 61–91 of β -LPH, derived from the same precursor as ACTH. These three peptides are concomitantly released from the pituitary by stress, insulin hypoglycemia, and administrations of vasopressin and corticotropin releasing factor (CRF) (Wardlaw and Frantz, 1979; Przewlocki *et al.*, 1979; Rivier *et al.*, 1982). *In vitro* experiments have also shown that CRF, vasopressin and high extracellular K⁺ concentration augment the release of these peptides (Vale *et al.*, 1978; Przewlocki *et al.*, 1979; Matsumura *et al.*, 1982 b).

We found that β -END-LI release from dispersed cells *in vitro* was inhibited in Ca⁺⁺-free medium, but that ouabain, which promotes calcium uptake by tissues through its interaction with membrane-bound Na⁺-K⁺-ATPase (Vizi, 1978), stimulates β -END-LI release (Matsumura *et al.*, 1982 b). On the other hand, it has been demonstrated that elevation of the extracellular K⁺ concentration stimulates the releases of ACTH (Kraicer *et al.*, 1969), GH (MacLeod and Fontham, 1970), LH (Wakabayashi *et al.*, 1969), TSH (Schwartz *et al.*, 1972) and β -END-LI (Matsumura *et al.*, 1982 b) from pituitary cells. High K⁺ is thought to act by depolarizing the plasma membrane, resulting in an increase in Ca⁺⁺ influx, which would then induce hormone release.

The addition of cobalt ion, a competitive antagonist of calcium in transmembrane

transport (Weakly, 1973), to the incubation medium inhibited A II-induced β -END-LI release. Since cobalt ion is known to prevent calcium influx into the cell, this finding suggests that A II stimulates β -END-LI release from the pituitary cells at least in part by a Ca^{++} -dependent mechanism.

The addition of $[\text{Sar}^1, \text{Ala}^8]$ -A II, a competitive antagonist of A II for the receptor, to the incubation medium inhibited A II-induced β -END-LI release. Capponi *et al.* (1982) and Saltman *et al.* (1975) reported that $[\text{Sar}^1, \text{Ala}^8]$ -A II, which is able to block the A II-induced ACTH release completely, was as effective as A II in displacing the labeled tracer, a result which is in agreement with the affinity of other target tissues for this analogue. This finding suggests that the stimulatory action of A II on β -END-LI release by dispersed rat pituitary cells may be mediated by specific receptors for the octapeptide.

The present work showed that A II stimulated β -LPH and β -END release in rats. *In vitro* studies using dispersed cells from the anterior pituitary also indicated that A II could act directly on the pituitary to stimulate β -LPH and β -END release, and that Ca^{++} played an important role in their actions. The effect of A II on β -END-LI release from extrapituitary tissues and the significance of A II-induced β -END-LI release further studies.

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