Human γ -melanotropin precursor potentiates corticotropin-induced adrenal steroidogenesis by stimulating mRNA synthesis

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(Received 23 October 1981/Accepted 8 December 1981)

Circulating human γ -melanotropin precursor (76 amino acids) [*N*-POC(1–76)] enhances the corticosterone and aldosterone response to corticotropin by isolated perfused rat adrenal cells [Al-Dujaili, Hope, Estivariz, Lowry & Edwards (1981) *Nature (London)* **291**, 156–159]. Actinomycin D (4×10^{-6} mol/litre) did not significantly affect corticotropin-induced corticosterone or aldosterone outputs, but completely inhibited the potentiating action of γ -melanotropin precursor on rat adrenal cells. This suggests that the precursor enhances corticotropin-induced steroidogenesis via an increase in available mRNA produced from DNA transcription.

We have recently reported that a naturally circulating intact human pituitary γ -melanotropin (melanocyte-stimulating hormone, 'MSH') precursor, *N*-POC(1–76) (Estivariz *et al.*, 1980), enhances the adrenal response to corticotropin (adrenocorticotrophic hormone, 'ACTH') in a perfused cell column system *in vitro* (Al-Dujaili *et al.*, 1981a). It has also been shown that a 16 kDa (kilodalton) glycopeptide containing the γ -melanotropin sequence (Drouin & Goodman, 1980) isolated from mouse pituitary tumour cells when treated with trypsin potentiates corticotropin-induced corticosterone biosynthesis *in vitro* and stimulates cholesterol ester hydrolase activity (Pedersen & Brownie, 1980). However, the potentiation produced by *N*-POC(1–76) was only manifest after exposure of the adrenal cells to at least 15 min continuous infusion. No potentiating effect was seen when bolus doses of *N*-POC(1–76) were given simultaneously with corticotropin. This delayed potentiating action of the glycopeptide suggested the involvement of a slow activation mechanism distinct from the fast corticotropin-mediated response. To investigate this, experiments were carried out *in vitro* on isolated perfused rat adrenal cells with actinomycin D, Mithramycin or Cordycepin (inhibitors of DNA transcription).

Materials and methods

The studies were performed with isolated perfused rat adrenal cells by using the system pre-

Abbreviation used: *N*-POC(1–76), melanotropin-(1–76)-peptide ('*N*-terminal pro-opiocortin'; 'pro- γ -melanotropin').

viously described (Lowry & McMartin, 1974). Whole adrenals obtained from female Wistar rats (190–210 g body wt.) were used to prepare isolated cells by digestion with trypsin (Al-Dujaili *et al.*, 1981a). The ratio of glomerulosa cells to total was 1:5. The cells were perfused with Earle's solution (Gibco-Europe, Paisley, Renfrewshire, Scotland, U.K.) containing 2.5 g of bovine serum albumin/litre (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) and 60 mg of lima-bean (*Phaseolus limensis*) trypsin inhibitor/litre (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) at a flow rate of 0.5 ml/min in a water bath at 37°C and gassed with O₂/CO₂ (19:1). Two or four columns were prepared for each experiment; one column was always used as a control.

The cells were perfused with incremental bolus doses of corticotropin-(1–24)-peptide (Synacthen; 1–5 ng/litre; Ciba, Horsham, Sussex, U.K.) alone or during *N*-POC(1–76) infusion (0.8–2 nmol/litre). To study the effect of actinomycin D on corticotropin-induced steroidogenesis by isolated perfused rat adrenal cells, four columns were prepared; the first was infused with *N*-POC(1–76) (2 nmol/litre), the second with actinomycin D (4 μ mol/litre; Sigma), the third with *N*-POC(1–76) (2 nmol/litre) and actinomycin D (4 μ mol/litre) and the fourth was used as a control. Actinomycin D and/or *N*-POC(1–76) were infused for 30 min before the bolus doses of corticotropin were introduced. The effect of Mithramycin (2 μ mole/litre) and Cordycepin (8 μ mole/litre) (Sigma) on corticotropin-induced corticosterone and aldosterone production was studied in an identical manner. Aldosterone and

corticosterone outputs were measured by highly specific direct radioimmunoassays (Al-Dujaili & Edwards, 1981; Al-Dujaili *et al.*, 1981b). Responses were calculated as peak above basal output produced by a 10 min pulse of corticotropin. Experiments involving the static incubation of adrenal cells with [3 H]uridine (sp. radioactivity 40–60 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) to determine the rate of synthesis of mRNA were carried out as described by Brattin & Portanova (1978). Incremental doses of *N*-POC(1–76) with or without the presence of actinomycin D ($4 \mu\text{mol/litre}$) were incubated with $(0.8\text{--}1.4) \times 10^5$ cells at 37°C for 30 min in 1 ml of Earle's solution containing $2 \mu\text{Ci}$ of [3 H]uridine, 10 mg of bovine serum albumin and 1 mg of lima-bean trypsin inhibitor.

Results

Corticotropin-(1–24)-peptide produced a dose-dependent increase in aldosterone and corticosterone production by perfused rat adrenal cells as shown in Figs. 1 and 2. The experiments show potentiation of aldosterone and corticosterone responses to corticotropin by *N*-POC(1–76) continuous infusion. This potentiation was completely inhibited by simultaneous infusion of actinomycin D. These findings were reproducible, demonstrating a 1–2-fold increase in aldosterone ($P < 0.001$) and 3–5-fold increase in corticosterone secretion ($P < 0.001$) by corticotropin plus *N*-POC(1–76) infusion compared with corticotropin alone (Fig. 2). There was no statistically significant difference between responses obtained by

Fig. 2. Dose-response curves of (a) aldosterone and (b) corticosterone produced by bolus doses of corticotropin-(1–24)-peptide alone (●), corticotropin-(1–24)-peptide with an infusion of *N*-POC(1–76) (2 nmol/litre , △), corticotropin-(1–24)-peptide with an infusion of actinomycin-D ($4 \mu\text{mol/litre}$, ○) and corticotropin-(1–24)-peptide with simultaneous infusions of *N*-POC(1–76) (2 nmol/litre) and actinomycin D ($4 \mu\text{mol/litre}$) (■).

Values are means $\pm$ S.E.M. ($n = 8$). The results show a 1–2-fold increase in aldosterone ($P < 0.001$) and a 3–5-fold increase in corticosterone secretion ($P < 0.001$) by corticotropin [(1–24)-peptide] plus *N*-POC(1–76) infusion compared with corticotropin alone. Responses obtained by corticotropin plus actinomycin D, corticotropin plus *N*-POC(1–76) and actinomycin D or corticotropin alone were not significantly different.

Fig. 1. Aldosterone (a) and corticosterone (b) secretion by isolated perfused rat adrenal cells in response to pulses of corticotropin-(1–24)-peptide.

Infusions of *N*-POC(1–76) and/or actinomycin D were given between fractions 14 and 78. ●, Control column (corticotropin alone); △, with an infusion of *N*-POC(1–76) (2 nmol/litre); ○, with an infusion of actinomycin D ($4 \mu\text{mol/litre}$); ×, with simultaneous infusions of *N*-POC(1–76) (2 nmol/litre) and actinomycin D ($4 \mu\text{mol/litre}$). Corticotropin-(1–24)-peptide concentrations: □, 1.0 ng/litre ; ▤, 2.5 ng/litre ; ▨, 5.0 ng/litre .

corticotropin plus actinomycin D, corticotropin plus *N*-POC(1–76) and actinomycin D or corticotropin alone. Mithramycin and Cordycepin produced similar inhibition of the potentiating action of *N*-POC(1–76) on corticotropin-induced corticosterone and aldosterone production ($P < 0.001$) (Table 1). The results of the static incubation experiments of adrenal cells with [^3H]uridine to determine the rate of increase in mRNA synthesis due to *N*-POC(1–76) are shown in Table 2. Incremental doses of the glycopeptide produced a progressive significant increase in total cellular [^3H]uridine incorporation ($P < 0.001$) that was inhibited by actinomycin D ($4\text{ }\mu\text{mol/litre}$) ($P < 0.001$).

Discussion

Human *N*-POC(1–76) markedly potentiates corticotropin-induced aldosterone and corticosterone production by perfused rat adrenal cells (Al-Dujaili

et al., 1981a). The potentiation was completely inhibited by actinomycin D. It is therefore concluded that the *N*-POC(1–76) enhances corticotropin-induced steroidogenesis by increasing available mRNA produced from DNA transcription and that the glycopeptide may play an important role in modulating the action of corticotropin on the adrenal gland. The time delay for transcription to take place would explain the slow onset of action observed in our previous report (Al-Dujaili *et al.*, 1981a). Pedersen & Brownie (1980) demonstrated that part of the *N*-terminal portion of pro-opiocortin (corticotropin/ β -lipotropin precursor) (16 kDa fragment) isolated from mouse pituitary tumour cells had, after treatment with trypsin, a significant potentiating effect on corticotropin action as tested in a static incubation system. They observed an increase in cholesterol ester hydrolase activity caused by trypsin-treated 16 kDa fragment and also by a synthetic γ -melanotropin

Table 1. Effect of Mithramycin and Cordycepin on the potentiating action of *N*-POC(1–76) on aldosterone and corticosterone production by isolated perfused rat adrenal cells in response to pulses of corticotropin-(1–24)-peptide

Secretagogue	Concn. (ng/litre)	[Aldosterone] (pg/ml) (mean $\pm$ S.E.M.)	[Corticosterone] (ng/ml) (mean $\pm$ S.E.M.)
Basal		76.0 $\pm$ 6.4	1.2 $\pm$ 0.5
Corticotropin-(1–24)-peptide	1.0	179.2 $\pm$ 5.7	9.7 $\pm$ 0.6
	2.5	288.8 $\pm$ 10.6	21.2 $\pm$ 1.8
	5.0	467.5 $\pm$ 9.8	40.4 $\pm$ 2.8
Corticotropin-(1–24)-peptide + <i>N</i> -POC(1–76) (2 nmol/litre)	1.0	330.5 $\pm$ 16.5*	25.5 $\pm$ 2.5*
	2.5	495.6 $\pm$ 56.0*	46.5 $\pm$ 6.6*
	5.0	796.0 $\pm$ 68.5*	88.6 $\pm$ 10.5*
Corticotropin-(1–24)-peptide + <i>N</i> -POC(1–76) (2 nmol/litre) + Mithramycin (2 $\mu\text{mol/litre}$)	1.0	180.5 $\pm$ 8.5†	11.4 $\pm$ 0.8†
	2.5	305.0 $\pm$ 18.5†	24.1 $\pm$ 1.4†
	5.0	482.6 $\pm$ 26.0†	44.5 $\pm$ 1.9†
Corticotropin-(1–24)-peptide + <i>N</i> -POC(1–76) (2 nmol/litre) + Cordycepin (8 $\mu\text{mol/litre}$)	1.0	183.3 $\pm$ 9.5†	10.5 $\pm$ 0.5†
	2.5	290.0 $\pm$ 14.6†	23.2 $\pm$ 1.4†
	5.0	501.0 $\pm$ 28.2†	45.0 $\pm$ 2.2†

* $P < 0.001$; potentiation by corticotropin-(1–24)-peptide and *N*-POC(1–76) versus corticotropin-(1–24)-peptide alone.

† No statistically significant differences were found between these results and those produced by corticotropin-(1–24)-peptide alone.

Table 2. Results of static incubation of rat adrenal cells with [^3H]uridine

	Total [^3H]uridine incorporation/(0.8–1.4) $\times 10^5$ adrenal cells [mean $\pm$ S.E.M. (c.p.m.) (n)]	
	With no actinomycin D	With actinomycin D (4 $\mu\text{mol/litre}$)
Basal	5994.2 $\pm$ 67.3 (10)	3958.6 $\pm$ 78.5† (10)
<i>N</i> -POC(1–76) (0.4 nmol/litre)	7720.3 $\pm$ 88.8* (8)	3816.6 $\pm$ 114.7† (8)
<i>N</i> -POC(1–76) (0.8 nmol/litre)	8842.5 $\pm$ 130.2* (8)	3891.5 $\pm$ 98.2† (8)
<i>N</i> -POC(1–76) (4 nmol/litre)	10 718.5 $\pm$ 350.8* (10)	3873.0 $\pm$ 100.7† (10)

* $P < 0.001$; basal versus *N*-POC(1–76).

† $P < 0.001$; results of [^3H]uridine incorporation without actinomycin D versus those with the inhibitor.

(27 amino acids, γ_3 -melanotropin) (Pedersen *et al.*, 1980), but not by corticotropin. In our system, the potentiation of corticotropin-induced corticosterone and aldosterone secretion by *N*-POC(1-76) was only apparent when the cells were exposed to a priming infusion for 30 min, followed by continuous infusion of the precursor. One possible explanation for this was that the potentiating action of *N*-POC(1-76) required DNA transcription. This hypothesis was supported by the finding that actinomycin D (at a dose that did not significantly affect corticotropin-induced corticosterone and aldosterone outputs) completely inhibited the potentiating action of the glycopeptide. This inhibition was also produced by other inhibitors of DNA transcription, such as Mithramycin and Cordycepin. In addition, we have shown in a static incubation system that incremental doses of *N*-POC(1-76) produced a significant increase in total cellular [3 H]-uridine incorporation which was completely inhibited by actinomycin D.

These results suggest that *N*-POC(1-76), which is known to be released into the circulation at the same time as corticotropin (Hope *et al.*, 1981), being under the same hypothalamic control (Gillies & Lowry, 1981), can potentiate its action. This latter effect requires transcription. Our results are compatible with the hypothesis that corticotropin stimulates cyclic AMP production, induces phosphorylation and uses mRNA to synthesize a labile protein postulated to be required for the rate-limiting step of steroidogenesis (Garren *et al.*, 1971; Simpson *et al.*, 1978; Kimura, 1981). Human *N*-POC(1-76) increases the cellular level of mRNA and thereby the rate of synthesis of the labile protein. This conclusion does not appear to contradict the findings

reported by Pedersen & Brownie (1980), but rather it explains the early stage of the mechanism of action of *N*-POC(1-76).

This work was supported by the British Heart Foundation and the Medical Research Council. The technical assistance of Mr. Iain Gow is gratefully acknowledged.

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