Corticotrophin-releasing activity of desmopressin in Cushing's disease: lack of correlation between *in vivo* and *in vitro* responsiveness

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Abstract

Desmopressin (DDAVP), an arginine vasopressin analogue, markedly stimulates ACTH secretion in patients with Cushing's disease, in contrast to its minimal effect in normal subjects. However, little is known about the mechanisms underlying this action and it appeared to be of interest to evaluate the effect of DDAVP on ACTH-secreting pituitary adenomas *in vitro*, in comparison with its effect in the same patients *in vivo*. Pituitary adenomas from 14 patients with Cushing's disease were incubated with DDAVP, corticotrophin-releasing hormone (CRH) and DDAVP together with vasopressin receptor antagonists or CRH. Incubation with DDAVP induced a modest dose-dependent increase in ACTH concentrations which appeared maximal at 10 nM. CRH stimulated ACTH to a greater extent compared with DDAVP and potentiated the effect of DDAVP alone. The DDAVP-induced

ACTH increase appeared blunted by vasopressin V₂ and V₃ receptor antagonists. V₃ receptor gene expression was detected by RT-PCR in all adenoma samples except for two which were not responsive to DDAVP *in vitro* but responsive to the peptide *in vivo*. Surprisingly, no difference in the *in vitro* ACTH secretory response was observed between *in vivo* DDAVP-responsive (ACTH peak >150% baseline) and -unresponsive (ACTH peak <120% baseline) patients, suggesting that the pituitary adenoma is not the sole mediator of the ACTH-releasing effect of DDAVP. In conclusion, the marked stimulatory effect of DDAVP observed in patients with Cushing's disease appears to be mainly dependent on an extrapituitary action, possibly the inhibition of a corticotrophin release-inhibitory factor.

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Introduction

Desmopressin (DDAVP), the long-acting analogue of arginine vasopressin (AVP), has recently been shown to markedly stimulate corticotrophin (ACTH) and cortisol secretion in patients with Cushing's disease (Malerbi *et al.* 1993, Colombo *et al.* 1997, Sakai *et al.* 1997, Moro *et al.* 2000). This behaviour is typical for patients with an ACTH-secreting pituitary tumour as DDAVP exerts only a modest stimulatory effect in normal subjects (Andersson *et al.* 1972, Gaillard *et al.* 1988) and in patients with pseudoCushing syndrome (i.e. patients with alcoholism, obesity and depression, presenting clinical and/or biochemical features resembling Cushing's disease) (Malerbi *et al.* 1996, Tsagarakis *et al.* 1999, Coiro *et al.* 2000, Moro *et al.* 2000). On the basis of this evidence, we recently recommended the use of DDAVP in the differential diagnosis between mild forms of Cushing's disease and pseudoCushing syndrome (Moro *et al.* 2000).

The mechanisms whereby DDAVP selectively stimulates ACTH secretion in patients with Cushing's disease are not yet fully understood. DDAVP is a known agonist for the V₂ receptor (i.e. kidney receptor) but has also been shown to bind the V₃ receptor isoform (Thibonnier *et al.* 1998). This latter is a characteristic of ACTH-secreting cells, both within and without the pituitary (Lolait *et al.* 1995, De Keyzer *et al.* 1996), and is apparently over-expressed in tumoural corticotrophs (Dahia *et al.* 1996, De Keyzer *et al.* 1998), thus possibly subserving the marked response to DDAVP observed in patients with Cushing's disease.

The aim of the present study was to evaluate the response to DDAVP in pituitary ACTH-secreting adenomas *in vitro*, in comparison with the response observed *in vivo* in the same patients prior to surgery. In addition, we investigated the AVP receptor subtype involved in this response by performing co-incubation experiments with DDAVP and AVP receptor antagonists and by evaluating

V₃ receptor mRNA in individual tumour cultures. Lastly, the effect of DDAVP on ACTH was compared with the selfsame response to corticotrophin-releasing hormone (CRH), alone and together with DDAVP itself.

Materials and Methods

Patients

Fourteen patients with an ACTH-secreting pituitary adenoma (eleven females and three males; age 40.0 ± 3.39 years, range 20–68) were studied. Diagnosis of Cushing's disease was established by standard criteria (Invitti *et al.* 1999a), including stimulation with CRH in six patients, and confirmed by results of pituitary pathology. Tumour size was greater than 1 cm in patients 1, 2, 4, 9–11 and 13, the remainder presenting a microadenoma (size range 6–9 mm).

In vivo studies

Preoperative testing with DDAVP was performed as previously described (Moro *et al.* 2000). In short, DDAVP (10 µg; Minirin, Ferring Pharmaceuticals Ltd, Malmö, Sweden) was injected in the morning after an overnight fast and serum samples were collected prior to and 15, 30, 45, 60, 90 and 120 min after the injection. Plasma ACTH levels were measured by immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA, USA). In our laboratory, intra- and interassay coefficients of variation are 3.2 and 8.2% respectively. In order to clearly differentiate DDAVP-responsive and -unresponsive patients we adopted stringent response criteria: a peak increase in ACTH levels greater than 150% of baseline identified responders whereas an ACTH peak less than 120% of baseline identified non-responders. These separate cut-offs were purposely chosen to allow a clear characterization of the *in vivo* DDAVP response pattern.

In vitro studies

ACTH-secreting pituitary adenomas were collected during trans-sphenoidal surgery and established in culture according to our usual protocol (Invitti *et al.* 1999b). Briefly, adenomas were enzymatically dispersed, checked for viability, plated at a density of 2×10^5 cells/well, and incubated in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum and antibiotics for 3–5 days. The following treatments were performed according to the abundance of the tumour specimen: (1) 1 nM–10 µM DDAVP, (2) 10 nM CRH, (3) 10 nM CRH and 10 nM–1 µM DDAVP together, (4) 10 µM V₁

receptor antagonist ((3-(4-azidophenyl)propionyl¹, D-Tyr(Me)², Arg⁶, Arg⁸, Tyr-NH₂⁹)-vasopressin) and 100 nM DDAVP, (5) 10 µM V₂ receptor antagonist ((d(CH₂)₅¹, D-Ile², Ile⁴, Arg⁸, Ala-NH₂⁹)-vasopressin) and 100 nM DDAVP, (6) 10 µM V₃ receptor antagonist (des-Gly⁹-(β-mercapto-β,β-cyclopentamethylenepropionyl¹, O-Et-Tyr², Val⁴, Arg⁸)-vasopressin) and 100 nM DDAVP and (7) each antagonist alone. The concentration of DDAVP for co-incubation experiments was purposely high in order to obtain the greatest possible ACTH increase. Control wells were incubated with DMEM and 0.1% bovine serum albumin. Each treatment was performed in triplicate or quadruplicate. Test agents were purchased from Bachem Feinchemikalien AG, Bubendorf, Switzerland except for the V₃ receptor antagonist (Sigma Chemical Co., St Louis, MO, USA).

ACTH was measured by radioimmunoassay (Pecori Giraldi & Cavagnini 1998) after 1- and 4-h incubations with test agents. Sensitivity, intra- and interassay coefficients of variation of this assay are 0.5 pg/ml, 5.9% and 6.8% respectively.

Total RNA was extracted from pituitary adenoma primary cultures using RNazol B (Tel Test Inc., Friendswood, TX, USA). RNA was reverse transcribed in the presence of 0.5 mM deoxynucleoside triphosphate, 10 µM random primers, 25 U RNase inhibitor and 200 U murine leukaemia virus reverse transcriptase for 45 min at 37 °C. The resulting cDNA was amplified with 500 nM intron-spanning primers specific to the human V₃ receptor gene (upstream: bp 2420–2440, downstream bp 8185–8205; Genbank Accession #AF152238) and Taq DNA polymerase according to the following protocol: initial denaturing at 95 °C for 2 min, then 95 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s for 35 cycles, followed by a 5-min extension at 72 °C. The identity of the V₃ receptor PCR product was confirmed by Southern blotting with a digoxigenin-labelled oligonucleotide (bp 7871–7892 of the human V₃ receptor gene) according to the manufacturer's instructions (DIG oligonucleotide tailing kit; Boehringer Mannheim GmbH, Mannheim, Germany). RNA from a growth hormone-secreting adenoma was used as a negative control. The integrity of RNA was assessed by amplification of the hypoxanthine phosphoribosyltransferase (HPRT) gene with 250 nM intron-spanning primers (upstream: bp 570–589, downstream: bp 647–667; Genbank Accession #NM000194) according to the same PCR protocol except for annealing at 53 °C. Reagents for reverse transcription, PCR and restriction enzyme digestion were provided by Promega Corp., Madison, WI, USA.

For statistical analysis, experiments were normalized as a percentage of control and treatments were compared using Wilcoxon's statistic for non-parametric data. Qualitative data were analysed by the chi square statistic. Data are expressed as means $\pm$ S.E.M. Statistical significance was accepted at $P < 0.05$.

Figure 1 Effect of DDAVP on ACTH secretion by individual ACTH-secreting pituitary tumours *in vivo* and *in vitro*. The left-hand axis of each figure represents ACTH medium concentrations in tumour cultures: control wells (=control), maximal increase induced by DDAVP (=DD) and 10 nM CRH (=CRH). The shaded bars represent ACTH concentrations after 1 h of incubation and the open bars after 4 h of incubation. The right-hand most bars (=in vivo) and the right-hand axes indicate the effect of 10 µg DDAVP in the same patient prior to surgery: the solid area (baseline secretion set at 100%; % bas) superimposed on the open area indicating ACTH % peak. Note that DDAVP administration was followed by a decrease in ACTH in patient 1 *in vivo* (right-hand most bar).

Results

Testing with DDAVP *in vivo* identified nine patients (2, 3, 5–7, 9–11 and 14) in whom DDAVP induced a clear ACTH increase (peak >150% baseline) and five non-responsive patients (1, 4, 8, 12 and 13), as may occur in

some patients with Cushing's disease (Moro *et al.* 2000). There was no difference between these two groups as regards age, size of the adenoma at pituitary imaging, and basal ACTH levels.

Figure 1 shows maximal DDAVP-stimulated ACTH concentrations in individual pituitary adenoma cultures

Figure 2 Mean percent variation of ACTH concentrations in tumour cultures from *in vivo* DDAVP-responsive (shaded bars) and -unresponsive (open bars) patients. Control secretion is set at 100%; CRH was incubated at 10 nM.

ordered according to the strength of the *in vitro* ACTH response with patient 1 presenting the lowest and patient 14 the greatest response. As can be seen, the stimulatory effect of DDAVP, if present, was already apparent after 1 h of incubation; by 4 h of incubation this increase in ACTH concentration was further magnified. The stimulatory effect of DDAVP appeared to be dose-dependent (maximal stimulation at 10 nM; Fig. 2) and slightly, although not significantly, greater in micro- than in macroadenomas (maximal ACTH increase at 1 h: 190.9 ± 34.37 vs $164.8 \pm 25.58\%$ of control, not significant (NS) and at 4 h: 174.2 ± 21.10 vs $150.8 \pm 20.18\%$ of control, NS respectively). Surprisingly, no response could be observed in cultures from patients 2, 3, 5 and 6 in whom DDAVP had elicited a marked increase in ACTH levels *in vivo* (Fig. 1). Further, there appeared to be no correlation between the *in vivo* and *in vitro* DDAVP responsiveness as a clear-cut increase in ACTH concentrations (i.e. peak at least 50% higher than control secre-

Figure 3 Mean percent variation of ACTH concentrations in tumour cultures incubated with 10 nM CRH and 10 nM–1 µM DDAVP. Open bars indicate treatment with CRH with or without DDAVP and the shaded bars treatment with DDAVP alone. Control secretion is set at 100%.

tion) was observed in five out of nine DDAVP responders (patients 7, 9–11 and 14) and three out of five DDAVP non-responders (patients 8, 12 and 13). Overall, no difference could be detected in the mean DDAVP-induced ACTH increase between *in vivo* DDAVP responders and non-responders (Fig. 2).

All tumours incubated with CRH released substantial amounts of ACTH and a comparison of DDAVP and CRH responses in individual tumour cultures showed that ACTH concentrations after DDAVP were mostly smaller than those after CRH, except for patient 14 (Fig. 1); this difference was already apparent 1 h after incubation with the test agent. Overall, the mean DDAVP-induced increase was clearly smaller than the mean CRH-evoked response (Fig. 2) whereas, in the same patients, the peak ACTH response to the two stimuli was comparable *in vivo* ($483.9 \pm 179.15\%$ vs $344.8 \pm 135.82\%$ of baseline, NS for DDAVP and CRH respectively). The effect of CRH incubation *in vitro* could be compared with the ACTH response to CRH prior to surgery in patients 1, 3, 7, 10, 12 and 14. All these patients were responsive to CRH *in vivo* (peak ACTH ranging from 161% to 1267% of baseline) and their tumours gave rise to a secretory response to CRH *in vitro* (Fig. 1).

Incubation of DDAVP and CRH together was performed in five pituitary adenomas (patients 4 and 9–12). In all experiments, co-incubation with CRH increased the DDAVP-induced ACTH response, even in the one patient who was unresponsive to DDAVP alone (patient 4). ACTH concentrations during incubation with DDAVP+CRH were significantly higher than with DDAVP alone but not different from those with CRH alone (Fig. 3), thus showing that CRH strongly increases the ACTH response to DDAVP but the latter only slightly enhances the ACTH response to CRH.

Figure 4 Mean percent variation of ACTH concentrations in tumour cultures incubated with 100 nM DDAVP and AVP receptor antagonists. The shaded bars indicate co-incubation of DDAVP with and without receptor antagonists and the open bars incubation with antagonists alone. Control secretion is set at 100%.

Incubation with AVP receptor antagonists was performed in seven tumour cultures (patients 1–3, 6–8 and 14) and did not affect ACTH concentrations (all NS). Co-incubation of DDAVP and V_2 or V_3 receptor antagonists in three tumours responsive to DDAVP *in vitro* (patients 7, 8 and 14) blunted the ACTH increase, although this effect did not reach statistical significance ($P=0.062$ for V_2 and $P=0.057$ for V_3 ; Fig. 4). No clear-cut change in ACTH medium concentrations was observed during co-incubation with the V_1 receptor antagonist (NS; Fig. 4).

V_3 receptor gene expression was detected in most patients, regardless of their *in vivo* response status (data not shown); tumour mRNA tested positive for V_3 receptor in all cultures in which DDAVP stimulated ACTH *in vitro* (patients 7–14) and also in some specimens unresponsive to DDAVP *in vitro* (patients 1–4). The integrity of mRNA in tumour cultures which failed to express the V_3 gene was established by positive amplification of the HPRT gene. The small amount of RNA obtained from each sample did not allow us to seek evidence for V_2 receptor gene expression.

Discussion

Recent years have seen a renewed interest in the action of DDAVP, a V_2 receptor agonist, on ACTH secretion. In fact, evidence collected by us and others has demonstrated that most patients with Cushing's disease present a marked increase in plasma ACTH levels following i.v. administration of DDAVP (Malerbi *et al.* 1993, Colombo *et al.* 1997, Sakai *et al.* 1997, Moro *et al.* 2000), in contrast with the barely detectable response observed in normal subjects

(Andersson *et al.* 1972, Gaillard *et al.* 1988) and patients with pseudoCushing syndrome (Malerbi *et al.* 1996, Tsagarakis *et al.* 1999, Coiro *et al.* 2000, Moro *et al.* 2000). On the basis of mRNA quantification studies, it had been hypothesized that the hyper-response observed in patients with Cushing's disease was due to an overexpression of the pituitary AVP receptor subtype (i.e. V_3) by the ACTH-secreting pituitary adenoma (Dahia *et al.* 1996, De Keyzer *et al.* 1998). This hypothesis relied on the fact that DDAVP, while presenting high affinity for the V_2 receptor subtype (K_i 2.7 nM), also interacts with the V_3 receptor (K_i 22 nM) (Thibonnier *et al.* 1998) and thus could directly stimulate adenomatous corticotrophs.

To our knowledge, only one study has been published reporting the effect of DDAVP on primary cultures from ACTH-secreting pituitary adenomas (Sakai *et al.* 1997). In this series of three adenomas, DDAVP induced a variable increase in ACTH concentrations, overall far less than the release evoked by CRH. The authors concluded that DDAVP directly stimulates ACTH secretion by tumoural corticotrophs, although no comparison with the *in vivo* response to DDAVP was performed (Sakai *et al.* 1997). An additional recent case report described a stimulatory effect of 100 nM DDAVP on long-term cultures from an ACTH-secreting macroadenoma (Abe *et al.* 2000).

The evidence collected in our larger series of experiments indicates that DDAVP exerts only a modest stimulatory effect (barely 1.5-fold) on ACTH-secreting pituitary adenomas *in vitro*. Surprisingly, the response to DDAVP *in vitro* was of modest magnitude even in patients exhibiting an ACTH increase over 200% of baseline in response to DDAVP *in vivo*. Indeed, incubation with DDAVP did not elicit a greater ACTH increase in adenomas from patients responsive to DDAVP compared with adenomas excised from DDAVP non-responsive patients. These data suggest a dissociation of the *in vitro* and *in vivo* ACTH responsiveness to DDAVP.

By comparison, CRH induced a far greater increase in ACTH concentrations in all but one specimen and this disparity is noteworthy given the fact that, *in vivo*, CRH and DDAVP elicit largely comparable ACTH increases in patients with Cushing's disease, both in terms of peak and timing of the ACTH response (Sakai *et al.* 1997, Putignano *et al.* 1999). Results of co-incubation experiments indicate that CRH and DDAVP exert additive effects on ACTH release *in vitro*.

It is worth recalling that the ACTH increase elicited by DDAVP in patients with Cushing's disease was unique to the analogue and distinct from the ACTH-stimulatory effect of AVP, the parent peptide. In fact, the stimulatory effect of AVP in patients with an ACTH-secreting pituitary adenoma is markedly smaller than the response of the same subjects to CRH (Liu *et al.* 1983, Catania *et al.* 1984, Dickstein *et al.* 1996, Sakai *et al.* 1997). Further, *in vitro* data from rat pituitary primary cultures demonstrated a different ACTH responsiveness to AVP and DDAVP

(Antoni 1984, Knepel *et al.* 1984, Buckingham 1987). Evidently, DDAVP and AVP do not appear to act in the same manner on both tumoural and non-tumoural corticotrophs.

Our incubation data point towards both V_2 and V_3 receptor subtypes subserving the effect of DDAVP on tumoural corticotrophs. Either receptor antagonist in fact blunted the DDAVP-induced ACTH release *in vitro*, an observation in keeping with the expression of both receptors in tumoural corticotrophs (Dahia *et al.* 1996). The faint response to DDAVP *per se* and the involvement of both receptors may have hampered the attainment of statistical significance for individual receptor antagonists alone. In our hands, V_3 receptor gene expression was detected in all tumours which exhibited a response to DDAVP *in vitro* as well as in primary cultures which did not respond to DDAVP. V_3 receptor mRNA was not detected in two specimens, both from patients in whom DDAVP failed to stimulate ACTH secretion *in vitro* but were clearly responsive to DDAVP *in vivo*.

The direct comparison of corticotroph secretory activity *in vivo* and *in vitro* may be biased by the inherent diversity of the two experimental conditions and the possible problems related to tumour explantation and culture, such as cell damage, loss of receptor and heterogeneity of the excised specimen. In our hands, the maintained responsiveness of cultured corticotrophs to CRH, for which there was a complete concordance between *in vivo* and *in vitro* stimulation, argues against a possible receptor loss or damage to the secretory apparatus during tumour isolation. Further, CRH responsiveness indicates the presence of adenomatous corticotrophs as neighbouring, normal corticotrophs are suppressed and unresponsive to CRH (Avgerinos *et al.* 1987, Invitti *et al.* 1999a). In addition, corticotroph secretory capacity was by no means exhausted by DDAVP, as indicated by the fact that CRH alone or with DDAVP was capable of inducing a significantly greater ACTH release. Therefore, even using appropriate circumspection while translating *in vivo* findings to *in vitro* conditions, our findings showed a superimposable *in vitro* ACTH response to DDAVP in patients who presented a clearly different response pattern *in vivo*. Evidently, the removal of the tumoural corticotroph from its milieu interfered with DDAVP responsiveness and some factor other than the direct stimulatory action of DDAVP on adenomatous corticotrophs must contribute to *in vivo* responsiveness. In this context, several scenarios can be drawn.

Theoretically, DDAVP could act at the hypothalamic level in order to stimulate CRH, as occurs for AVP (Hedge *et al.* 1966, Rivier & Vale 1983, Kjær *et al.* 1992). This hypothesis, however, is not unchallenged as other investigators have reported findings suggesting an inhibitory circuit from AVP to CRH (Plotsky *et al.* 1984). Further, normal subjects fail to respond to DDAVP *in vivo* and thus it is unlikely that CRH mediates the marked

DDAVP-induced increase in patients with Cushing's disease.

In an alternative hypothesis, DDAVP could act through the blunting of an endogenous inhibitor of ACTH secretion. The existence of a non-corticosteroid inhibitor of ACTH secretion has long been postulated and was recently reviewed (Engler *et al.* 1999, Jessop 1999). Several candidate corticotrophin-release inhibitory factors (CRIF), have been studied, such as preprothyrotrophin-releasing hormone (178–199), atrial natriuretic peptide, dopamine, lipocortin and endogenous opioids, to list but a few, although conclusive evidence for one of these factors is as yet lacking. Even oxytocin, the 'sister' neurohormone to AVP, has been postulated to inhibit ACTH secretion (Legros 2001). In view of the existence of a corticotrophin-release inhibitory tone, it stands to reason that this system might be hyperactivated in Cushing's disease. DDAVP might interfere with this inhibitor and thus cause ACTH release, much as it occurs for dopamine antagonists in hyperprolactinaemia.

In conclusion, the present study has demonstrated a discrepancy between the ACTH response to DDAVP observed *in vivo* in patients with Cushing's disease and the effect of DDAVP on pituitary adenoma cultures from the same patients. Our findings support the view that the bulk of the ACTH response triggered by DDAVP in patients with Cushing's disease is due to an extrapituitary mechanism, possibly inhibition of the fabled CRIF.

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