Octreotide exerts different effects in vivo and in vitro in Cushing’s disease

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The effect of the long-acting somatostatin analog octreotide (SMS 201-995) on adrenocorticotropic (ACTH) secretion was studied in five patients with untreated Cushing’s disease in vivo and in six human corticotropin adenoma cell cultures in vitro. For the in vivo study, 100 µg of octreotide sc was given 30 and 180 min after cannulation of the cubital vein and 100 µg of corticotropin-releasing hormone (CRH) was injected iv at 210 min. Serum ACTH and cortisol levels were measured for 390 min. In vivo, octreotide had no significant effect either on basal or CRH-stimulated ACTH levels and did not influence cortisol levels. The in vitro studies were conducted with corticotropic adenoma cell cultures derived from adenoma tissue obtained from six patients with Cushing’s disease. In four of six cell cultures, octreotide (1 nmol/l–1 µmol/l) inhibited basal ACTH secretion in a dose-dependent manner. The inhibition ranged from 70 to 92% for 1 nmol/l octreotide to 14–46% for 1 µmol/l octreotide as compared to controls (100%). In three of three octreotide-responsive adenoma cell cultures investigated, CRH-stimulated ACTH secretion was suppressed by octreotide. Hydrocortisone pretreatment in vitro abolished the inhibitory effect of octreotide on ACTH secretion in one octreotide-responsive corticotropin adenoma cell culture. In conclusion, we showed that octreotide in most cases could inhibit the ACTH release from human corticotropic adenoma cells in vitro but had no suppressive effect on ACTH levels of patients with Cushing’s disease in vivo. This discrepancy could be due to a somatostatin receptor down-regulation by cortisol at the hypercortisolemic state in vivo.

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Somatostatin (SRIF), the physiological growth-inhibiting hormone, and its long-acting analog octreotide (SMS 201-995) are substances that are well known for inhibiting growth hormone hypersecretion in most patients with acromegaly (1, 2). Furthermore, octreotide often reduces the size of somatotrophic adenomas and in some cases also that of non-functioning pituitary adenomas, indicating that these substances inhibit pituitary adenoma growth (1, 2). With respect to normal pituitary function, SRIF and octreotide inhibit TRH-stimulated TSH secretion but have no effect on basal or stimulated LH, FSH or prolactin secretion (3, 4). Octreotide did not affect corticotropin-releasing hormone (CRH) or hypoglycemia-induced ACTH secretion or CRH-stimulated β-endorphin or lipotropin secretion (5, 6).

In Cushing’s disease octreotide has no effect on basal or CRH-stimulated ACTH release (7–9), and transsphe- nodal microsurgery of the corticotropin adenomas is still the first therapeutic approach. However, octreotide suppressed ACTH serum levels in patients with adrenal insufficiency (Addison’s disease) (10) and in patients with Nelson’s syndrome (9, 11). The latter indicates that human corticotropin adenoma cells contain somatostatin receptors, although corresponding somatostatin receptor scintigraphic studies have not been done yet (1). Because Addison’s disease and Nelson’s syndrome are accompanied by hypocortisolism, the missing effect of octreotide in patients with Cushing’s disease could be due to a down-regulation of the somatostatin receptor at the hypercortisolemic state. The glucocorticoid-induced down-regulation of SRIF receptors was demonstrated with normal rat pituitary cell cultures (12) and GH4C1 rat pituitary tumor cells (13, 14), where it was shown that glucocorticoid pretreatment abolished the inhibitory effect of octreotide on ACTH secretion or GH release, respectively.

In order to support the hypothesis that the missing inhibitory effect of octreotide on ACTH secretion in patients with Cushing’s disease is due to a down-regulation of SRIF receptors as a consequence of increased plasma cortisol levels, we have investigated the effect of octreotide on ACTH secretion of corticotropic adenomas both in vivo and under cortisol-reduced cell culture conditions in vitro.
Patients, material and methods

Patients

The in vivo effect of octreotide on ACTH and cortisol serum levels was studied in five patients (four females and one male); the corticotropic tumor tissue was obtained from six patients (five females and one male). The patients presented with typical features of chronic hypercortisolism, including facial and truncal obesity as well as hypertension. The diagnosis of Cushing’s disease was made by the following endocrine evaluation: in all patients the serum cortisol levels were not suppressed sufficiently after the low-dose (2 mg/day) dexamethasone test, and preoperative determination of plasma cortisol concentrations at 4–5-h intervals over 24 h showed a blunted diurnal rhythm of secretion. Plasma levels for cortisol ranged from 266 to 946 nmol/l (normal, 140–550 nmol/l) and for ACTH from 12.2 to 56.3 pmol/l (normal, 2.2–11 pmol/l). In all subjects only the high (8 mg/day)-dose dexamethasone test significantly suppressed cortisol secretion. None of the patients showed an appropriate response of cortisol secretion under insulin-induced hypoglycemia (0.15 IU/kg body wt). Radiological investigation of the patients consisted of a cranial NMR scan and additionally every patient was subjected to a sinus-petrous-inferior catheterization in combination with a CRH stimulation test to determine the location of the corticotropic adenoma within the pituitary.

In vivo studies with octreotide

The in vivo studies consisted of a CRH test without octreotide (placebo) on day 1 and with octreotide on day 2. Informed consent was gained from all patients. After overnight rest and fasting, the cubital vein was cannuled at 08.30 h. The iv catheter was kept open by infusion of 0.9% saline. Placebo or 100 μg of octreotide (Sandoz, Basel, Switzerland) was injected subcutaneously 30 and 180 min later. Corticotropin-releasing hormone (100 μg; Corticobiss, Bissendorf, Germany) were injected iv at 210 min. Blood samples for ACTH and cortisol measurements were drawn every 15 or 30 min for 390 min, as indicated. This protocol was designed to investigate the influence of octreotide on both basal and CRH-stimulated serum ACTH levels.

Corticotropic adenoma cell culture

Except where stated, materials and reagents for the cell culture were obtained from Flow Laboratories (Meckenheim), Gibco Europe (Karlsruhe), Seromed (Berlin), Falcon (Heidelberg) or Nunc (Wiesbaden) in Germany.

From six patients with Cushing’s disease, corticotropic pituitary adenoma tissue (numbered TU1–TU6) was obtained at transsphenoidal microsurgery and transferred to the laboratory in sterile culture medium (see below). Routinely, the transport media as well as 24-h conditioned media from the cell cultures were assayed for all pituitary hormones. In none of the cases could LH, FSH, TSH, GH or prolactin be detected, while excessively high amounts of ACTH were always present. In three of the six cases enough material was available to perform immunohistochemical characterization to confirm that more than 95% of the cells were corticotropes. Adenoma cell cultures were performed with minor modifications, as described earlier (15). The adenoma tissue was washed several times with preparation buffer (137 mmol/l NaCl, 5 mmol/l KCl, 0.7 mmol/l Na2HPO4, 10 mmol/l glucose, 15 mmol/l HEPES (pH 7.3; 2.5 mg/l amphotericin B and 10 μg/m l penicillin/streptomycin). Dissected tissue fragments were dispersed mechanically and enzymatically in preparation buffer containing 1000 mU/l collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA), 4 g/l BSA, 10 mg/l DNAase II, 1 g/l soybean trypsin inhibitor and 1 g/l hyaluronidase. Dispersed cells were centrifuged and resuspended in Dulbecco’s modified Eagle’s medium (DMEM), pH 7.3, containing 10% fetal calf serum (FCS), 2.2 g/l NaHCO3, 10 mmol/l HEPES, 2 mmol/l glutamine, 10 μl/ml non-essential amino acids, 10 ml/l MEM vitamins, 2.5 mg/l amphotericin B and 10 μg/m l penicillin/streptomycin, 5 mg/l insulin, 5 μg/l transferrin, 20 mg/l sodium selenite and 30 μmol/l T3 (Henning, Berlin, Germany). Between 1.5 × 106 and 4 × 106 adenoma cells with a viability of 93–98% (acridine orange/ethidium bromide staining) were isolated and cultured in 48-well plates (104 cells in 0.5 ml of culture medium) at 37°C in 5% CO2. Cells of the adenomas attached to the multiwell plates within 48 h.

In vitro studies with octreotide

For the in vitro experiments cells were used 3–4 days after plating. The initial culture medium was removed by aspiration and the attached cells were washed twice with stimulation medium (DMEM, pH 7.3, supplemented with 2.2 g/l NaHCO3, 10 mmol/l HEPES, 2 mmol/l glutamine, 1 g/l BSA and 30 mg/l ascorbic acid). Octreotide and CRH were freshly diluted for each experiment in stimulation medium and used at the concentrations indicated. The final volume of stimulation medium was 0.5 ml per well in each case. After an incubation period of 4 h the supernatants were removed and the ACTH content was determined. For the investigation of the effect of hydrocortisone pretreatment on the action of octreotide on ACTH secretion in vitro, cells were grown over 3 days before the stimulation procedure in culture medium supplemented with 1 μmol/l hydrocortisone and were then treated as described above for 4 h in the absence of hydrocortisone. Owing to the limited amount of adenoma cells, not all in vitro experiments could be done in parallel with each corticotropic adenoma cell culture.
**Hormone measurement**

Plasma ACTH was measured with a commercial IRMA kit from Nichols Institute (San Juan Capistrano, CA, USA). Intra- and interassay variabilities were 6.9% and 13.3%. The detection limit was 1.0 pmol/l. The ACTH levels in the cell culture supernatants were evaluated by a specific radioimmunoassay (16) with intra- and interassay variabilities of 8.3% or 9.1%, respectively, and a detection limit of 120 pmol/l. Plasma cortisol was measured by a specific radioimmunoassay (17) with intra- and interassay variabilities of 3.6% or 5%, respectively, and a detection limit of 30 nmol/l.

**Statistical analysis**

Statistics were performed by analysis of variance in combination with the Scheffé test. p values of <0.05 were considered to be significant.

**Results**

**Effect of octreotide on ACTH secretion in vivo**

In vivo, octreotide did not suppress significantly the ACTH or cortisol secretion of the five patients with Cushing’s disease investigated. Figure 1 summarizes the results for all five patients and shows that neither the basal ACTH nor the cortisol secretion were changed by octreotide. The CRH-induced ACTH and cortisol secretion also were not changed by octreotide pretreatment. The area under the curve (AUC; mean±SD) for ACTH after stimulation with CRH without or with octreotide pretreatment was 333.7±94.8 or 456.6±123.4 (pmol/l within 6 h), respectively. For cortisol, the respective AUCs were 12 287±4702 and 14 347±4829. This was observed in all the patients investigated, in spite of the interindividual variations of basal (12.2–38.5 pmol/l) and CRH-stimulated (33.6–110.9 pmol/l) ACTH levels.
In vitro characteristics of corticotropic adenoma cell cultures.*

<table>
<thead>
<tr>
<th>Tumor number</th>
<th>ACTH secretion (ng·10^5 cells⁻¹·4 h⁻¹)</th>
<th>CRH stimulation (% of basal)</th>
<th>Responsive to octreotide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>After 1 nmol/l CRH</td>
<td></td>
</tr>
<tr>
<td>TU1</td>
<td>14.5 ± 0.4</td>
<td>64.8 ± 11</td>
<td>447</td>
</tr>
<tr>
<td>TU2</td>
<td>9.8 ± 1.3</td>
<td>42.9 ± 3.3</td>
<td>438</td>
</tr>
<tr>
<td>TU3</td>
<td>55.2 ± 7.7</td>
<td>96.5 ± 9.1</td>
<td>175</td>
</tr>
<tr>
<td>TU4</td>
<td>4.9 ± 0.1</td>
<td>10.0 ± 1.0</td>
<td>204</td>
</tr>
<tr>
<td>TU5</td>
<td>8.8 ± 1.2</td>
<td>29.5 ± 3.9</td>
<td>335</td>
</tr>
<tr>
<td>TU6</td>
<td>22.6 ± 1.6</td>
<td>45.4 ± 2.1</td>
<td>201</td>
</tr>
</tbody>
</table>

* In vitro characteristics of the six corticotropic adenoma cell cultures (TU1–TU6) investigated. The absolute values for ACTH secretion under basal and corticotropin-releasing hormone (CRH, 1 nmol/l)-stimulated conditions are listed, as well as the percentage increase after CRH stimulation.

b Responsiveness to octreotide determined only for basal conditions.

c Responsiveness to octreotide determined for both basal and CRH-stimulated conditions.

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**Effect of octreotide on ACTH secretion in vitro**

The influence of octreotide on ACTH secretion was investigated in six corticotropic adenomas at concentrations ranging from 1 nmol/l to 1 µmol/l. The six corticotropic adenoma cell cultures showed marked variations of basal ACTH secretion in vitro, ranging from 4.9 to 55.2 ng ACTH per 10^5 cells over 4 h (Table 1). In all cases 1 nmol/l CRH significantly stimulated ACTH secretion (range: 175–447% of basal ACTH release; see Table 1). Octreotide significantly suppressed ACTH secretion in four out of six corticotropic cell cultures (Fig. 2). A 1 nmol/l dose of octreotide significantly suppressed ACTH secretion only in TU1 (70 ± 10% vs control (100%)). In three adenoma cultures 10 nmol/l octreotide inhibited ACTH release (range: 55 ± 12–81 ± 6%) and in all octreotide-responsive cell cultures significant inhibition of ACTH secretion was observed with 100
nmol/l octreotide (range: 27 ± 8–71 ± 6%) as well as with 1000 nmol/l (14 ± 6–60 ± 9%). In the two octreotide non-responsive cell cultures, which had the lowest basal ACTH secretion, even the highest concentration of octreotide had no suppressive effect on ACTH release. In three of the four octreotide-responsive adenoma cultures enough cells were available to study also the effect of 10 and 100 nmol/l octreotide on CRH (1 nmol/l)-induced ACTH secretion (Fig. 2). The corresponding ACTH values after stimulation in the absence of octreotide were set to 100% (control) and in all cases CRH-stimulated ACTH secretion was inhibited significantly with 10 nmol/l (range: 67 ± 9–78 ± 8 vs control) and with 100 nmol/l octreotide (range: 48 ± 6–63 ± 8%).

In one octreotide-responsive adenoma cell culture enough material was available to compare the effect of octreotide on ACTH secretion in hydrocortisone-pretreated (1 mg/l hydrocortisone for 3 days) corticotrophic adenoma cells and untreated control cells. Hydrocortisone treatment reduced both basal and CRH-stimulated ACTH secretion and abolished the inhibitory effect of octreotide (Fig. 3).

To be sure that the octreotide and hydrocortisone concentrations used are not cytotoxic for the cells, at the end of the stimulation experiments the cells were stained for viability (acridine orange/ethidium bromide) and in no case was a decrease of cell viability observed.

Discussion
We have shown in vitro that the long-acting somatostatin analog octreotide suppresses basal ACTH secretion in four of six cases and suppresses CRH-stimulated ACTH release in three cases of Cushing’s disease using corticotrophic cell cultures derived from adenoma tissue. In vivo we could observe no inhibitory effect of octreotide on basal or CRH-induced ACTH secretion in five patients with Cushing’s disease. The latter confirms previous reports of others that in Cushing’s disease octreotide treatment is of no value (7–9). In contrast, it has been
reported that in diseases accompanied by hypocortisolism, such as Nelson’s syndrome and Addison’s disease, somatostatin or analogs like octreotide could reduce ACTH serum levels (9–11).

In vitro hydrocortisone pretreatment abolishes the inhibitory effect of octreotide on ACTH secretion of normal rat pituitary corticotropes (12) or on GH secretion of the rat pituitary tumor GH4C1 cell line (13, 14) and it was speculated that glucocorticoids down-regulate the somatostatin receptor. Therefore, it was assumed that the missing effect of octreotide on ACTH secretion in patients with Cushing’s disease in vivo is due to the down-regulation of somatostatin receptors in corticotropic adenoma cells at the hypercortisolemic state. This hypothesis was supported by the results of Spada et al. (18), who showed that, in vitro, under glucocorticoid-reduced conditions octreotide could suppress cAMP levels in corticotropic adenoma cell cultures. Because cAMP is the most important second messenger with respect to ACTH secretion, the data of Spada et al. indicate that octreotide in vitro also might suppress the ACTH release from corticotropic adenoma cells. To confirm this we have studied the effect of octreotide on the ACTH secretion of corticotropic adenoma cells in vitro, cultured under glucocorticoid-reduced conditions. We found that in four out of six adenomas octreotide dose-dependently suppressed the basal ACTH secretion and in three of these cases suppressed the CRH-stimulated ACTH secretion. The suppressive effects of octreotide became significant at low nanomolar concentrations, which correspond to the Kₐ of octreotide with respect to its inhibitory effect on GH secretion of somatotropes (2). At these low concentrations octreotide also is known to exert no specific cytotoxic effects on the cells, which is confirmed by cell viability staining.

In two adenoma cell cultures even the highest concentration of octreotide tested did not affect the ACTH secretion. It is unlikely that in these cases small amounts of contaminating glucocorticoids, added to the medium with the application of FCS, still suppress the up-regulation of the somatostatin receptor. It could be possible that an octreotide non-responsive subtype of corticotropic adenomas exists that does not express sufficient amounts of the somatostatin receptor independently from the cortisolemic state. Such a correlation of the somatostatin receptor status with the response to octreotide is known also for octreotide non-responsive somatotropic adenoma cells (19). Another explanation for the missing effect of octreotide on corticotropic adenoma cells could be that these cells express an altered somatostatin receptor with low or no affinity for octreotide. In the last few years at least five subtypes of the somatostatin receptor have been described, which differ in their affinity to SRIF or SRIF analogs. For example, the mouse AT2 20 corticotropic cell line mainly carries a 57-kD somatostatin receptor, whilst in somatotropic cell lines the 27-kD receptor is expressed, which has the highest affinity for octreotide (20, 21).

If the missing effect of octreotide in vivo is due to somatostatin receptor down-regulation at the hypercortisolemic state, pretreatment with glucocorticoids should abolish the inhibitory effect of octreotide on ACTH secretion in vitro. Unfortunately, because all the corticotropic tumors investigated were microadenomas, only in one octreotide-responsive case was there enough cell culture material available for this study. Hydrocortisone pretreatment itself, as expected, reduced both basal and CRH-stimulated ACTH secretion, but in parallel abolished the inhibitory effect of octreotide on ACTH secretion. Although this single observation supports the hypothesis of glucocorticoid-induced somatostatin receptor down-regulation, much more work with a number of corticotropic adenomas is necessary to confirm this preliminary result. Furthermore, somatostatin binding studies on corticotropic cells, pretreated without or with glucocorticoids, are necessary to demonstrate the down-regulation of the somatostatin receptor directly.

Octreotide has been used successfully in the treatment of patients with Cushing’s disease caused by ectopic ACTH-secreting tumors. With one exception (22), it was shown that octreotide reduced ACTH levels in these patients in spite of hypercortisolism (23–26). Furthermore, preoperative somatostatin scintigraphical studies conducted on ectopic ACTH-secreting tumors have shown that ectopic ACTH-secreting tumors contain somatostatin receptors (1). Whether this is due to the expression of a different somatostatin receptor subtype whose expression is not changed by glucocorticoids or whether these ectopic tumors in general respond differently to hypercortisolism as compared to pituitary corticotropic adenomas is still unclear. Nevertheless, the observation that despite hypercortisolism somatostatin or its analogs could influence ACTH secretion in vivo, together with our finding that corticotropic adenoma cells in vitro are sensitive to octreotide, might indicate that octreotide or other, more potent somatostatin analogs, in combination with drugs reducing the serum cortisol levels, may be of benefit for patients with Cushing’s disease.

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