Differential regulation of human dopamine D₂ and somatostatin receptor subtype expression by glucocorticoids *in vitro*

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Abstract

Dopamine agonists (DA) and somatostatin (SS) analogues have been proposed in the treatment of ACTH-producing neuro-endocrine tumours that cause Cushing's syndrome. Inversely, glucocorticoids (GCs) can differentially influence DA receptor D₂ or SS receptor subtype (sst) expression in rodent models. If this also occurs in human neuro-endocrine cells, then cortisol-lowering therapy could directly affect the expression of these target receptors. In this study, we investigated the effects of the GC dexamethasone (DEX) on D₂ and sst expression in three human neuro-endocrine cell lines: BON (carcinoid) and TT (medullary thyroid carcinoma) versus DMS (small cell lung cancer), which is severely GC resistant. In BON and TT, sst₂ mRNA was strongly down-regulated in a dose-dependent manner (IC₅₀ 0.84 nM and 0.16 nM), whereas sst₅ and especially D₂ were much more resistant to DEX treatment. Sst₂ down-regulation was abrogated by a GC receptor antagonist and reversible in time upon GC withdrawal. At the protein level, DEX also induced a decrease in the total number of SS (–52%) and sst₂-specific (–42%) binding sites. Pretreatment with DEX abrogated calcitonin inhibition by sst₂-preferring analogue octreotide in TT. In DMS, DEX did not cause significant changes in the expression of these receptor subtypes. In conclusion, we show that GCs selectively down-regulate sst₂, but not D₂ and only to a minor degree sst₅ in human neuro-endocrine BON and TT cells. This mechanism may also be responsible for the low expression of sst₂ in corticotroph adenomas and underwrite the current interest in sst₅ and D₂ as possible therapeutic targets for a medical treatment of Cushing's disease.

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Introduction

Somatostatin (SS) and dopamine (DA) are small molecules with a variety of functions throughout the human body, including neurotransmission and inhibition of hormone release (Missale *et al.* 1998, Barnett 2003). They bind to high-affinity receptors that belong to the family of G-protein coupled receptors. Both for SS and DA multiple receptor subtypes have been identified: sst_{1–5} and D_{1–5} (Missale *et al.* 1998, Patel 1999). The presence of these receptors has been demonstrated on a number of neuro-endocrine tumours and therefore SS analogues and DA agonists could play an important role in the medical treatment of these tumours (Heaney & Melmed 2004). Recent reports have shown significant efficacy of selective D₂-receptor agonists, such as cabergoline, and of a multiligand SS analogue with high sst₅ affinity (pasireotide/SOM230) in the medical management of pituitary-dependent Cushing's syndrome (Pivonello *et al.* 2004, Boscaro *et al.* 2008, Pivonello *et al.* 2008). The use of sst₂- or D₂-targeting agonists has also been advocated in cases of Cushing's syndrome due to ectopic ACTH production by neuro-endocrine tumours (von Werder *et al.* 1996, Pivonello *et al.* 2007).

The hallmark of Cushing's syndrome, regardless of its cause, is a profound and sustained overproduction

of glucocorticoids (GCs) by the adrenal glands. It is also known that GCs can have (in)direct effects on receptor expression patterns of different neurotransmitters and hormones in the human body. Therefore, when considering the clinical use of DA agonists and/or SS analogues to lower cortisol levels in a patient, it is important to investigate how alterations in GC levels (i.e. response to treatment) can secondarily affect the expression of the target SS and DA receptors in ACTH-producing neuro-endocrine tumours. If such a (in)direct relationship exists, then this could first of all explain our current knowledge on SS and DA receptor expression patterns in human corticotroph adenomas as it has been described before by different groups (Pivonello *et al.* 2004, Hofland *et al.* 2005, Batista *et al.* 2006). Secondly, perhaps more importantly, it could help us to predict what happens to the expression of the target SS and DA receptors, when medical therapy lowers circulating GC levels. This may have implications for the type and timing of additional SS- or DA-based medical treatment in these patients.

A considerable number of studies have investigated the effects of GCs on DA receptor expression, mainly within the central nervous system and associated neuropsychiatric disorders, but not in human neuro-endocrine tumour cells (Lee *et al.* 1995, Lammers *et al.* 1999, Sigala *et al.* 2000, Czyrak *et al.* 2003, Van Craenenbroeck *et al.* 2005).

Most of these studies report no or only minor effects of GC exposure on D₂-receptor expression. Several other studies have shown that GCs can influence SS receptor subtype (sst) expression in a differential manner in rat or murine pituitary cells and cell lines (Xu *et al.* 1995, Park *et al.* 2003, van der Hoek *et al.* 2005). No study thus far, however, has concomitantly evaluated D₂ and SS receptor subtype expression and their regulation by GCs within the same, human-derived neuro-endocrine cell system.

For that reason we conducted the present study. We investigated the time- and dose-dependent effects of the synthetic GC dexamethasone (DEX) on sst₂, sst₅ and D₂-receptor expression *in vitro* in three different human neuro-endocrine cell lines (BON, TT and DMS-79), for which evidence of direct transcriptional regulation by GCs has been reported (Ikeda *et al.* 1989, Evers *et al.* 1995, Gaitan *et al.* 1995). BON (pancreatic carcinoid) and TT (medullary thyroid carcinoma) cells are not known to have any defects in their GC regulation and signalling pathway. By using these two different neuro-endocrine cell lines, we aimed to find general patterns in GC-mediated effects that are representative for the larger group of neuro-endocrine tumours as a whole. Since BON and TT cells do not necessarily reflect ectopic ACTH-producing tumours that display severe GC resistance, we compared these results with a third and completely different neuro-endocrine cell line: the human ectopic ACTH-producing small cell lung carcinoma cell line DMS-79, which harbours distinct mutations in the GC receptor gene and is known for its severe GC-resistant properties (Ray *et al.* 1994, Gaitan *et al.* 1995).

Materials and methods

Cell culture

BON cells were routinely grown in 75 cm² flasks containing DMEM-F12 Glutamax medium supplemented with 10% FCS, penicillin (1 × 10⁵ U/l) and fungizone (0.25 mg/l). Cells were cultured in a 5% CO₂ incubator at 37 °C and routinely passaged by trypsinization (trypsin 0.05–EDTA 0.02%). Medium was refreshed twice a week and cell viability always exceeded >95% as measured by trypan blue staining. TT cells were cultured following the same protocol with F-12K Nutrient Mixture (Kaighn's Modification). DMS cells were grown in suspension under the same conditions with 10% FCS, glutamine and penicillin in RPMI 1640 medium (ATCC 30-2001). All cell lines were confirmed to be mycoplasma-free. Media and supplements were obtained from Invitrogen unless otherwise stated.

Cell treatment for mRNA expression studies

For the experiments, cells were trypsinized, counted in a standard haemocytometer and seeded at a density of 15 000 (BON) or 100 000 (TT, DMS) cells/well in 24-well plates (Corning, Cambridge, MA, USA) in 1 ml medium. After 72 h, media were refreshed and incubations started without or with different doses of DEX and/or the GC receptor antagonist RU-486. At different time points, media were removed and cells were lysed on ice with a buffer containing 100 mM Tris-HCl (pH 8), 500 mM LiCl, 10 mM EDTA (pH 8), 5 mM DTT and 1% LiDS (HT Biotechnology Ltd, Cambridge, UK) and stored at –80 °C until further analysis. All experimental conditions were performed in quadruplicates.

Quantitative PCR

Quantitative PCR was performed according to a previously published method (Hofland *et al.* 2004). In short, poly(A⁺) mRNA was isolated from the lysed cells with the use of Dynabeads Oligo (dT)₂₅ (DynaL AS, Oslo, Norway). The poly (A⁺) mRNA was eluted in H₂O (65 °C) for 2 × 2 min and used for cDNA synthesis in a Tris buffer (50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM DTT and 10 mM MgCl₂) with 10 units RNase inhibitor, 2 units avian myeloblastosis virus Super Reverse Transcriptase and 1 mM of each deoxynucleotide triphosphate in a final volume of 40 µl. This was incubated for 1 h at 42 °C and the resulting cDNA was diluted fivefold in 160 µl sterile H₂O.

One twentieth of the total cDNA library was used for quantification of hypoxanthine phosphoribosyltransferase (HPRT), sst₂, sst₅ and D₂ mRNA levels. The total reaction volume (25 µl) consisted of 10 µl cDNA and 15 µl TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ, USA) with primers–probes in the following concentrations: HPRT, sst₂ and sst₅ 500–500–100 nM and D₂ 300–300–200 nM for forward primer, reverse primer and probe respectively. The primer and probe sequences that were used for HPRT, sst₂ and sst₅ have been published previously (Hofland *et al.* 2004). D₂ primer and probe sequences were: forward 5'-GCCACT-CAGATGCTCGCC-3', reverse 5'-ATGTGTGTGATGAA-GAAGGGCA-3' and probe 5'-FAM-TTGTCTCTCGG-CGTGTTTCATCATCTGC-TAMRA-3'. This primer–probe set measures total D₂ expression (D₂ long + short isoform). All primers and probes were purchased from Sigma-Aldrich. Real-time quantitative PCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems) and the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA, USA). After two initial heating steps at 50 °C (2 min) and 95 °C (10 min), samples were subjected to 40 cycles of denaturation at 95 °C (15 s)

and annealing at 60 °C (60 s). All samples were assayed in duplicate. Values were normalized against the expression of the housekeeping gene *HPRT*. Dilution curves were constructed to calculate PCR efficiencies (E) for every primer–probe set (Rasmussen 2001). Efficiencies were: *sst*₂ 1·92, *sst*₅ 1·92, D₂ 1·94 and *HPRT* 1·93. Estimated copy numbers were calculated using the comparative threshold method with efficiency correction, as described previously (Pfaffl 2001). To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template, in parallel with cDNA samples.

Cell proliferation and DNA fragmentation assays

After trypsinization, the cells were counted, seeded and treated with DEX in similar fashion as described above in the experiments for qPCR analysis. At different time points, media were aspirated and cells were collected for DNA measurement. Measurement of total DNA contents, representative for the number of cells, was done using the bisbenzimidazole fluorescent dye (Hoechst 33258; Boehringer Diagnostics, La Jolla, CA, USA) as described previously (Hofland *et al.* 1990). Parallel to this, the induction of apoptosis was evaluated in these cells by DNA fragmentation analysis, using a commercially available ELISA kit (Cell Death Detection ELISA^{plus}, Roche Diagnostics GmbH) according to the manufacturer's protocol. Experiments were done in quadruplicates.

Membrane-binding studies with [¹²⁵I-Tyr¹¹]-SS-14 and [¹²⁵I-Tyr³]-OCT

Membrane-binding experiments were performed according to a previously published protocol (Hofland *et al.* 1992). In brief, BON cells were grown to 40–50% confluency and subsequently treated with DEX 10 nM for 72 h. At 72 h cells were collected and homogenized with a Polytron Homogenizer (Kinematica). Membrane fractions were obtained by centrifugation at 14 000 r.p.m for 30' and protein content was determined by Bradford analysis. Fifty microlitres of membrane homogenates were incubated for 45 min at room temperature with 25 µl of increasing amounts of [¹²⁵I-Tyr¹¹]-SS-14 (GE Healthcare, Brussels, Belgium) or [¹²⁵I-Tyr³]-OCT (Novartis) tracer, with or without excess (1 µM) of unlabelled SS-14 or OCT respectively, in 25 µl HEPES buffer (10 mM HEPES, 5 mM MgCl₂ and 0·02 g/l bacitracin, pH 7·6) containing 0·2% BSA. Incubation was terminated by the addition of 1 ml ice-cold HEPES–BSA buffer, and membrane-bound radioactivity was separated from unbound by centrifugation for 2 min at

14 000/min. The remaining pellets were washed twice in HEPES buffer, air-dried and counted in a liquid scintillation γ-counter for 1 min. Specific binding was regarded as total binding minus the binding in the presence of excess (1 µM) unlabelled SS-14 or OCT. Experimental conditions were in duplicate and the experiments were performed at least twice.

Hormone release

After trypsinization, TT and DMS cells were pretreated with DEX 10 nM or vehicle control for 72 h. Cells were then washed and subsequently treated with OCT 10 nM for 72 h. After that time, media were collected and stored at –20 °C. In DMS cells, aprotinin (4 × 10⁵ IU/ml medium; Bayer) was added to the media prior to storage to prevent ACTH degradation. ACTH and calcitonin levels were measured by commercially available, non-isotopic, automatic, chemiluminescence immunoassay systems (DPC Immulite, Los Angeles, CA, USA). Intra- and interassay coefficients of variation were 5·6 and 7·8% for ACTH, and 2·0 and 3·5% for calcitonin respectively. Sensitivity thresholds were 2·0 pg/ml (calcitonin) and 5·0 pg/ml (ACTH).

Test substances

DEX and octreotide were obtained from the hospital pharmacy, Erasmus Medical Center, aliquotted and stored at 4 °C. SS-14 and the GC receptor antagonist RU-38486 (mifepristone) were obtained from Sigma–Aldrich.

Statistical analysis

Each experimental condition was run in quadruplicate and experiments were performed at least twice, independently of each other. Statistical analysis was done using GraphPad Prism software v.3.02 (San Diego, CA, USA). Average values per group were compared by ANOVA. When significant differences were found, the Newman–Keuls test was used to make comparisons between groups. IC₅₀ values of dose–response curves were calculated by non-linear curve fitting. Data of membrane-binding studies were analysed by the method of Scatchard. *P* < 0·05 was considered statistically significant. Data are reported as mean ± s.e.m.

Results

Baseline mRNA expression levels

The baseline expression levels of *sst*₂, *sst*₅ and D₂ mRNA in the different cell lines are depicted in Fig. 1. All three cell lines expressed all receptors of interest, albeit in

Figure 1 Sst₂, sst₅ and D₂ mRNA expression in the three different neuro-endocrine cell lines used in this study. Expression levels of receptor subtypes were normalized against the housekeeping gene HPRT. Values represent the mean $\pm$ S.E.M. of ≥ 3 independent experiments per cell line. sst₂ ■; sst₅ ▨; D₂ □.

different ratios. BON cells expressed relatively high amounts of sst₅ (0.43 ± 0.09 , mean $\pm$ S.E.M.), followed by D₂ (0.22 ± 0.05) and sst₂ (0.08 ± 0.01); TT cells predominantly expressed D₂ (1.26 ± 0.13), followed by sst₂ (0.40 ± 0.06) and sst₅ (0.34 ± 0.03); DMS cells had overall lower expression levels: sst₂ (0.15 ± 0.03), D₂ (0.12 ± 0.01) and sst₅ (0.04 ± 0.01).

Cell proliferation and DNA fragmentation studies

We investigated whether DEX treatment (0.1–100 nM) caused significant changes at 24, 72 and 168 h in total DNA contents, HPRT expression per nanogram RNA and the induction of apoptosis compared with untreated cells (data not shown). In the BON and DMS cells, we did not observe significant differences with different DEX doses at any time point. In TT cells, however, the highest DEX dose (100 nM) caused a significant decrease in DNA contents and an increase in apoptosis already after 72 h, which was not present at 24 h. For that reason, we performed full dose–response experiments (0.1–100 nM) in TT cells at the 24 h time

point instead of the 72 h time point, as for BON and DMS cells.

Quantitative PCR

In BON cells, sst₂ was dose-dependently down-regulated by DEX (0.1–100 nM, 72 h) with an IC_{max} of -85% at 100 nM ($P < 0.001$ vs control) and an IC₅₀ just below 1 nM (0.84 nM; see Fig. 2). Sst₅ was less sensitive to down-regulation with a tenfold higher IC₅₀ value (10.0 nM) than sst₂ and only showed significant down-regulation in the highest dose of DEX 100 nM (-50% , $P < 0.01$). D₂ did not show any down-regulation at any of the tested concentrations; in fact, at the highest concentration (100 nM) D₂ was slightly up-regulated ($+33\%$, $P < 0.05$). In TT cells (24 h), a similar dose-dependent down-regulation for sst₂ was observed with an IC_{max} of -84% ($P < 0.001$) and a subnanomolar IC₅₀ (0.16 nM). In TT cells sst₅, nor D₂ showed any signs of down-regulation in any of the concentrations tested. In DMS cells (72 h), no significant effects were observed by DEX treatment.

Based on the presence of DEX-induced effects in BON cells and the absence of growth inhibition or apoptosis induction at any treatment duration or dose, we investigated in these cells the effects of different treatment periods (24 and 168 h) on DEX-induced receptor down-regulation. We found that at 24 and 168 h, DEX treatment produced similar dose-dependent decreases in sst₂ mRNA expression as those observed at 72 h. IC_{max} and IC₅₀ values were respectively -74% ($P < 0.001$) and 0.60 nM at 24 h, and -80% ($P < 0.001$) and 0.84 nM at 168 h (see Fig. 3). Also for sst₅, comparable dose-dependent effects were observed at these time points: IC_{max} -45% ($P < 0.01$) and IC₅₀ 4.0 nM at 24 h, and -52% ($P < 0.01$) and 5.4 nM at 168 h. For D₂, no down-regulation was demonstrable at any DEX dose at these time points. The observed D₂ up-regulation after 72 h with DEX 100 nM was not demonstrable at 24 h or 168 h.

Figure 2 Dose-dependent effect of DEX treatment on sst₂, sst₅ and D₂ mRNA expression in BON, TT and DMS cells. Cells were treated with DEX (0.1–100 nM) for 72 h (BON and DMS) or 24 h (TT). Subsequently, cells were lysed and sst and D₂ mRNA expression levels were determined. Expression levels were normalized against the housekeeping gene HPRT. Values represent per cent change relative to control. IC₅₀ values were calculated for the dose–response effect of DEX on sst and D₂ mRNA expression and are indicated in the results section.

Figure 3 Effect of treatment duration (DEX) on sst_2 , sst_5 and D_2 mRNA expression in BON cells. Cells were treated with DEX (0.1–100 nM) for either 24 or 168 h. Subsequently, cells were lysed and sst and D_2 mRNA expression levels were determined. Expression levels were normalized against the housekeeping gene HPRT. Values represent per cent change relative to control (untreated cells). IC_{50} values were calculated for the dose–response effect of DEX on sst and D_2 mRNA expression and are indicated in the results section.

To investigate whether DEX-induced down-regulation of sst_2 and to a lesser extent sst_5 was a GC receptor-specific effect, we also performed these experiments with the addition of the GC receptor antagonist RU-38486 in all cell lines. The down-regulation after 72 h by DEX 10 nM of sst_2 (–71%, $P < 0.001$) and sst_5 (–22%, $P < 0.05$) in BON cells, of sst_2 (–69%, $P < 0.001$) in TT cells, and sst_5 (–24%, $P < 0.05$) in DMS cells, was completely abrogated by co-incubation with RU-38486 (see Fig. 4).

Reversibility of GC-mediated sst_2 down-regulation was also investigated. BON and TT cells were pretreated with DEX 10 nM for 24 h. After changing the medium into DEX-free medium ($t = \text{day } 0$), the cells were subsequently cultured for another 2–4 days. After an initial sst_2 down-regulation due to DEX treatment

(BON –68%, $P < 0.001$; TT –66%, $P < 0.001$ at day 0), a complete reappearance of sst_2 expression was observed in both BON and TT cells after 2–4 days of culture in DEX-free medium (BON –2% after 4 days; TT +8% after 2 days; see Fig. 5).

Membrane-binding studies

To assess whether the observed sst_2 mRNA down-regulation also occurred at the protein level, membrane-binding studies were performed on BON membrane homogenates with the radiolabelled SS analogues [$^{125}\text{I-Tyr}^{11}$]-SS-14 and [$^{125}\text{I-Tyr}^3$]-octreotide. Treatment of BON cells with DEX 10 nM for 72 h led to a 42% decrease in total sst receptor-binding sites, as measured by [$^{125}\text{I-Tyr}^{11}$]-SS-14 binding: B_{max} (DEX) 60 fmol/mg vs B_{max} (control) 104 fmol/mg with unchanged binding affinity (K_d 1.2 nM). The decrease in total sst -binding sites included a marked decrease (–52%) in specific sst_2 -binding sites as shown by [$^{125}\text{I-Tyr}^3$]-octreotide binding: B_{max} (DEX): 40 fmol/mg vs B_{max} (control): 84 fmol/mg, with unchanged binding affinity (K_d 0.2 nM; see Fig. 6).

Hormone release data

In TT cells, 72 h treatment with the sst_2 -preferring analogue octreotide 10 nM induced a significant decrease in calcitonin release compared with control (–32%, $P < 0.001$; see Fig. 7). However, when TT cells were pretreated with DEX 10 nM for 72 h, the octreotide-mediated inhibition of calcitonin release was completely abolished (–3%, $P > 0.05$). In DMS cells, treatment with OCT 10 nM for 72 h did not significantly inhibit ACTH release in either control or DEX pretreated cells.

Discussion

In this study, we have shown that somatostatin receptor subtypes and the dopamine D_2 receptor, natively co-expressed by human neuro-endocrine cell lines, show a differential pattern of response to GC exposure *in vitro*.

In both BON and TT cells, sst_2 is highly sensitive to GC-induced down-regulation in a dose-dependent manner, whereas the sst_5 is significantly less sensitive. Moreover, D_2 is fully insensitive to this type of down-regulation. The phenomenon appears to be GC receptor (GR)-specific, as addition of the GR antagonist RU-38486 can completely abrogate these effects and because sst_2 down-regulation is fully reversible in time upon GC withdrawal. The functional relevance of this sst_2 down-regulation could be demonstrated by the fact that the sst_2 -preferring

Figure 4 Antagonism of DEX-induced changes in D₂, sst₂ and sst₅ mRNA expression by co-incubation with the glucocorticoid receptor antagonist RU-38486. Cells were treated for 72 h with DEX 10 nM, RU-38486 100 nM or their combination. Subsequently, cells were lysed and sst and D₂ mRNA expression levels were determined. Expression levels were normalized against the housekeeping gene HPRT. CT, control; DEX, dexamethasone 10 nM; RU, glucocorticoid antagonist RU-38486 at 100 nM (BON, TT) or 1 μ M (DMS), D + R = DEX 10 nM + RU-38486; Values represent per cent change $\pm$ s.e.m. relative to control and are the mean of ≥ 2 independent experiments. * $P < 0.05$, ** $P < 0.001$ vs control.

analogue octreotide lost its efficacy in DEX pretreated TT cells. In DMS cells, derived from a severely GC-resistant tumour type *in vivo*, these GC-mediated effects on sst₂ expression were not demonstrable.

The results in BON and TT cells could indirectly explain the low sst₂ expression found specifically in human corticotroph adenomas (Hofland *et al.* 2005, Batista *et al.* 2006), whereas this receptor subtype is abundantly expressed in other pituitary adenomas, such as somatotroph and non-functioning adenomas (Jaquet *et al.* 2000, Taboada *et al.* 2007). Based on our results, one can speculate that high endogenous GC levels in patients with Cushing's disease (CD) are at least partially responsible for this observed difference in sst₂ expression between adenoma types. It would also explain the low clinical efficacy of octreotide in the treatment of CD: most studies on the use of this sst₂-preferring compound in CD report no to limited effect (Lamberts *et al.* 1989, Ambrosi *et al.* 1990, Stalla *et al.* 1994).

At the same time, these data do support the interest in the potential use of sst₅ and D₂ selective agents in the medical treatment of CD. Apparently, these receptor subtypes are fully (D₂) or partially (sst₅) resistant to high GC pressure and thus may still be of functional value in the regulation of ACTH release in corticotroph

adenomatous cells. Several clinical studies have been performed already with these agents and have shown promising results in subsets of patients. Pivonello *et al.* (2004) showed that 3-month treatment with the D₂-specific agonist cabergoline could significantly reduce urinary-free cortisol (UFC) levels in 60% of patients and even induce complete normalization of UFC in 40% of patients with CD. A report on the use of the multiligand SS analogue pasireotide (SOM230), which has sub-nanomolar affinity for sst₂, sst₃ and sst₅, showed equally interesting results: 15-day treatment with pasireotide 600 μ g twice daily, led to a decrease in UFC in 76% (22/29) of CD patients, including complete normalization of UFC in 17% (5/29) of patients, probably mediated through sst₅ receptor activation (Boscaro *et al.* 2008). Considering the reported efficacy of both cabergoline and pasireotide in subsets of CD patients, combination treatment with these agents is an interesting future option. For that reason, studying the effects of GCs on a single cell system from a neuro-endocrine origin that expresses sst₂, sst₅ and D₂, could help us to understand some more of the biological backgrounds of these clinical observations.

This is the first study to describe a differential pattern of GC responsiveness regarding both D₂ and SS receptor

Figure 5 Reappearance of *sst*₂ expression after GC withdrawal in BON and TT cells. Cells were pretreated for 24 h with DEX 10 nM. Part of the cells were lysed at that time (*t* = day 0), whereas the remainder of the cells underwent prolonged culturing in normal, DEX-free medium. These cells were subsequently lysed either at day 2 or at day 4 and analysed for *sst*₂ expansion. Cell lysates were analysed for *sst*₂ mRNA expression at different time points. Values are represented as per cent change $\pm$ S.E.M. relative to that in the control wells on the specific time points. **P* < 0.001 vs control.

subtypes co-expressed in human neuro-endocrine cell lines. The observed pattern closely resembles the one seen earlier in the murine corticotroph AtT-20 cells (van der Hoek *et al.* 2005). In these cells, *sst*₂ is sensitive whereas *sst*₅ is largely insensitive to GC-induced down-regulation. Unfortunately, D₂ was not expressed at sufficiently high levels in these cells to allow for investigation of this receptor subtype (unpublished observations). Nevertheless, one could hypothesize that a similar pattern of GC responsiveness exists in different human and murine neuro-endocrine cell lines.

Our findings are in line with earlier studies that describe the presence of distinct GC-responsive elements (GREs) in the murine *sst*₂ gene, whereas the murine *sst*₅ gene only contains multiple GRE half-sites (Kraus *et al.* 1998, 1999, Gordon *et al.* 1999). Moreover, in transfection experiments it was shown that the

human *sst*₂ gene promoter is under direct control of GCs, whereas the *sst*₅ promoter is not (Petersenn *et al.* 1999, 2002). To our knowledge, comparable GC-responsive regulatory sequences have thus far not been identified within the D₂ gene.

Previously, Petersenn *et al.* (2002) found in GH4 cells (rat pituitary adenoma cell line) that hydrocortisone 100 nM did not influence *sst*₅ promoter activity, whereas we do find moderate but significant down-regulation of *sst*₅ at higher DEX doses (100 nM) in BON cells. We believe that this difference can be ascribed to the difference in relative *in vitro* potency of hydrocortisone compared with DEX. In previous studies on differential regulatory mechanisms by GCs it was found that DEX has a 14-fold higher potency *in vitro* compared with hydrocortisone (GILZ EC₅₀ of 4.1 and 56.7 nM respectively; Smit *et al.* 2005). Therefore, the dose used by Petersenn *et al.* would be the equivalent of approximately 7 nM DEX in our study. At this dose, we did not observe any significant effects on *sst*₅ mRNA expression either.

Even though corticotroph adenoma cells are GC resistant to a certain degree, GC regulation of SS receptors apparently remains intact in AtT-20 cells. Interestingly, BON and TT cells, derived from tumours that do not show GC resistance *in vivo*, have indeed a higher degree of *sst*₂ down-regulation when exposed to DEX 10 nM, compared with the partially GC-resistant corticotroph adenoma cell line AtT-20 (van der Hoek *et al.* 2005). In the severely GC-resistant DMS cells, derived from an ectopic ACTH-producing small cell lung cancer, most of the GC-induced *sst*₂ down-regulation is lost, as would be expected. The latter *in vitro* observation correlates with two interesting clinical observations. First of all, many ectopic ACTH-producing tumours are positive on ¹¹¹In-pentetreotide receptor scintigraphy (Tsagarakis *et al.* 2003, Uwaifo *et al.* 2003, Ilias *et al.* 2005, Isidori *et al.* 2006), despite the hypercortisolic environment they are exposed to. And secondly, octreotide has been shown to be effective in controlling tumour size and cortisol production in some of these patients as opposed to patients with pituitary-derived CD (Lamberts *et al.* 1988, Phlipponneau *et al.* 1994).

Another aspect we have observed in this study is the reappearance of *sst*₂ expression within 2–4 days after withdrawing GC exposure. This observation could be of clinical interest. When it is possible to lower GC levels in CD patients with the use of, for instance, *sst*₅ or D₂ selective agents, then this state of normocortisolaemia could lead to a re-expression of *sst*₂ in the corticotroph adenomas of these patients. Return of *sst*₂ expression *in vivo* would result in a strong increase in the efficacy of traditional SS analogues such as octreotide (*sst*₂) as well as pasireotide (*sst*₂ + *sst*₅) and thus expand the pharmacological options to maximally inhibit ACTH production. Obviously, the question remains to which extent these

Figure 6 Membrane-binding studies. (A) Scatchard analysis of [¹²⁵I-Tyr¹¹]-SS-14 binding to cell membranes of BON cells, cultured in the absence (K_d 1.2 nM, B_{max} 104 fmol/mg) or the presence of 10 nM DEX (72 h; K_d 1.2 nM, B_{max} 60 fmol/mg). (B) Scatchard analysis of [¹²⁵I-Tyr³]-OCT binding to cell membranes of BON cells cultured in the absence (K_d 0.2 nM, B_{max} 84 fmol/mg) or the presence of 10 nM DEX (72 h; K_d 0.2 nM, B_{max} 40 fmol/mg).

in vitro observations can model the clinical situation of a CD patient in whom long-term GC overexposure is relieved through cortisol-lowering therapy. Clinical evidence in favour of a return of ss_{t2} expression *in vivo* does exist upon GC withdrawal, however. In patients with Nelson's syndrome, inoperable or recurrent corticotroph adenomas (low ss_{t2} expression) necessitate bilateral adrenalectomy, leading to chronic hypo-/or normocortisolaemia. By removing most of the excessive negative feedback loop on the pituitary, the ACTH-producing pituitary adenomas left *in situ* may expand with time and can even show invasive growth in surrounding tissues. Some of these Nelson adenomas, however, are visible on ¹¹¹In-pentetreotide receptor scintigraphy (Octreoscan), whereas most primary corticotroph adenomas are not (de Herder *et al.* 1994, de Herder & Lamberts 1996).

Moreover, octreotide has been effective in some of these Nelson patients by lowering ACTH levels and stabilizing tumour growth (Lamberts *et al.* 1989, Petrini *et al.* 1994, Kelestimur *et al.* 1996). Most likely, this reappearance of functional ss_{t2} receptor expression is a direct effect of removing chronic hypercortisolism in these patients.

In conclusion, we show that GCs selectively down-regulate ss_{t2} , but not D_2 and only to a minor degree ss_{t5} in human neuro-endocrine BON and TT cells. If this is a common regulatory mechanism in human neuro-endocrine cells, then these data would support the hypothesis that chronically elevated GC levels in Cushing's disease may be directly responsible for the low expression of ss_{t2} in corticotroph adenomas. It also suggests that ss_{t5} and D_2 are interesting candidate receptors in the search for a medical treatment of CD,

Figure 7 Effect of DEX pretreatment on inhibition of hormone release by octreotide in TT cells. Cells were pretreated for 72 h with DEX 10 nM (preDEX+) or not (preDEX-). At that time ($t=0$), cells were washed, media refreshed and incubations started for 72 h with octreotide 10 nM. After 72 h, media were aspirated and analysed for calcitonin levels. All experiments were performed in quadruplicates. Values represent per cent change relative to control (=no OCT treatment). * $P<0.001$ vs control. CT, control; OCT, octreotide.

due to their (relative) resistance to GC-induced down-regulation. It needs to be emphasized, however, that these data require confirmation, preferably in primary cultures of human corticotroph adenomas.

Declaration of interest

No conflict of interest.

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