Octreotide and the Novel Multireceptor Ligand Somatostatin Receptor Agonist Pasireotide (SOM230) Block the Adrenalectomy-Induced Increase in Mitotic Activity in Male Rat Anterior Pituitary

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The novel somatostatin receptor agonist pasireotide binds with high affinity to somatostatin receptors SSTR1, 2, 3, and 5. Acting principally through the latter, it inhibits basal and CRH-stimulated ACTH secretion from the AtT20 corticotroph cell line and ACTH release from a proportion of human corticotroph adenomas both in vitro and in vivo. Data supporting an additional antiproliferative effect has led to pasireotide being explored as a potential therapy for patients with Cushing’s disease. We have compared the effects of pasireotide and octreotide on adrenalectomy-induced mitotic and apoptotic activity in the male rat anterior pituitary. Adrenalectomized rats were treated with daily sc injections of vehicle, pasireotide, or octreotide. Changes in proliferation and apoptosis were determined 2–6 d postoperatively. Pasireotide and octreotide had no effect on baseline pituitary cell turnover and no measurable effects on apoptosis. However, the wave of increased mitotic activity normally seen in the pituitary after adrenalectomy was completely abolished. Nevertheless, pasireotide and octreotide did not diminish the increase in ACTH-immunopositive cell index after adrenalectomy, indicating that cell division and differentiation of hormonally null cells in the pituitary are under independent control. In conclusion, basal cell turnover in the pituitary is not inhibited by pasireotide or octreotide. Bilateral adrenalectomy stimulates differentiation of preexisting null cells into ACTH-positive cells. Cell division after bilateral adrenalectomy occurs in a specific subpopulation of hormonally null cells that are equally sensitive to the antiproliferative effects of pasireotide and octreotide, implicating SSTR2 receptors in this antiproliferative response.

SOMATOSTATIN ANALOGS are well-established anti-secretory treatments for neuroendocrine malignancies such as carcinoid tumors and human somatotroph adenomas. Somatostatin analogs have also been shown to have antiproliferative effects in acromegaly, controlling growth in the majority for the duration of treatment, and in a proportion of cases inducing tumor regression (1, 2). Antiproliferative effects may be limited by the relative proportions of somatostatin receptor subtypes expressed, and seem to be independent of somatostatin analog-induced changes in secretory activity (2–6). Mechanistically, the trophic effects of somatostatin analogs on pituitary tumors, if they occur, appear to be mediated by cell cycle arrest and a reduction in mitotic activity rather than the induction of apoptosis (3, 7).

Somatotroph adenomas predominantly express SSTR2 and SSTR5 somatostatin receptors. Prolactinomas tend to express SSTR1 and SSTR5, and corticotroph adenomas predominantly express SSTR5 in approximately 50% with low levels of SSTR2 in about one third (8). Most recent reports suggest an even higher percentage of SSTR5 expression in patients with primary Cushing’s disease (9). The presence of SSTR5 receptors on corticotroph adenomas and the numerous pathways of somatostatin action, which include phototyrosine phosphatases, potassium and calcium channel modulation, phospholipase C, and adenylyl cyclase second messenger pathways, suggests that somatostatin analogs with modified receptor subtype affinities have the potential to be developed as new treatment modalities for corticotroph adenomas (10).

With few exceptions (4), SSTR1, 2, 3, and 5 have been found to be antiproliferative in endocrinologically inactive pituitary adenoma cells in vitro and in nondocrine tumors (11, 12). Octreotide, which acts predominantly on SSTR2 receptors, has not proven effective in inhibiting ACTH secretion in patients with Cushing’s disease (9, 10, 13, 14). Nevertheless, the multiligand somatostatin analog pasireotide is potentially of interest in the therapeutics of Cushing’s disease as it has 30–40 times the binding affinity of octreotide at the type 1 and type 5 receptors and occupies the receptors for 11 h compared with 90 min with octreotide (15–17). In a small study of six human corticotroph adenoma cell samples studied in vitro, pasireotide reduced total cell proliferation measured by quantification of fluorescent vital stain uptake, by between 10–70% after 48–96 h (5), suggesting that a useful therapeutic effect might be anticipated in a subgroup of patients with this condition. However, studies of this nature are difficult to perform and interpret because the amount of tissue available is usually very small and may be contaminated with fragments of normal pituitary. In addition, tumor phenotypes even within the same principal secretory subgroup are highly variable, and almost without exception,
human pituitary adenomas do not grow in vitro so, therefore, cannot be used easily as an experimental model of trophic activity (18). Pituitary cell lines such as mouse corticotroph AtT20 cells are equally unhelpful in this context as they are already abnormal from a trophic point of view and isolated from the effects of surrounding cells and normal physiological influences. Use of well-validated in vivo animal models in which mitotic activity in normal anterior pituitary cells can be manipulated reproducibly over a reasonably short time frame is a useful alternative strategy to begin to examine the general antiproliferative effects of somatostatin analogs that might be relevant to their clinical use.

In the present study, we have examined the effects of pasireotide and octreotide on mitotic and apoptotic activity and changes in ACTH labeling index in the male rat pituitary under basal conditions and after surgical bilateral adrenalectomy as a model of nonneoplastic, enhanced trophic activity. This pathophysiological stimulus induces a highly reproducible wave of increased mitotic activity in normal rat pituitary cells that reaches significance within 48 h of surgery before returning to baseline after between 7–14 d (19).

Materials and Methods

Animals and treatments

Male Wistar rats weighing 125–175 g were allowed to acclimatize for 1 wk in the animal holding facility before surgical adrenalectomy or sham surgery under fluorothane anesthesia. For postoperative pain relief, antinociceptive analgesics were administered through the intraperitoneal injection of 30 μg/kg hydrocodone (Suppository Fusion, Kent, UK) 4 mg/kg body weight in a total volume of 0.2 ml diluted in saline. Adrenalectomized rats were given 0.9% saline to drink after surgery. Groups of adrenalectomy and sham-operated rats were given sc injections of 30 μg pasireotide (Novartis Pharma AG, Basel, Switzerland) in 0.25 ml saline—a dose toward the higher end of that known to be tolerated without risk of gastrointestinal side effects—at the time of surgery followed by a single daily sc injection of 60 μg pasireotide. Groups of rats received equal volumes of saline vehicle and were killed with treatment groups 2, 4, and 6 d after surgery. To further define the somatostatin receptor subtype implicated in the mitotic and apoptotic effects of pasireotide, an additional experiment was carried out, in which groups of adrenalectomized and sham-operated rats were treated with either a single injection of pasireotide as above or twice daily injections of 30 μg octreotide (Novartis Pharma AG), an analog with high affinity for SSTR2 but low affinity for SSTR1, 4, and 5. Groups of rats were weighed 5 d postoperatively, and pituitary glands were harvested 6 d after adrenalectomy. Trunk blood was collected in heparinized tubes and centrifuged at 3000 rpm for 10 min. Plasma was stored at −20°C and used to assay plasma ACTH levels with an ACTH immunoradiometric assay kit according to the manufacturer’s instructions (DiaSorin ACTH IRMA; DiaSorin Ltd., Buckingham, UK). As an additional indicator of mitotic rate, all animals were given a single 200 mg/kg of body weight ip injection of bromodeoxyuridine (BrdU; 10 mg/ml in 0.007 M NaOH/0.9% NaCl, Roche, Welwyn Garden City, UK) 24 h before being killed. Rats were not injected on the day they were killed, which occurred between 0800 and 0900 h. All procedures were carried out in accordance with UK Home Office animal welfare regulations.

BrdU and ACTH immunohistochemistry

Standard pituitary tissue sections for trophic analysis and immunohistochemistry were prepared exactly as described (20). Pituitary sections were processed for BrdU immunohistochemistry according to a previously published protocol (21) with minor modifications (22). Briefly, dewaxed and rehydrated sections were transferred to a hot antigen unmasking solution (0.01 M citric acid in water; pH 6.0) and incubated for 10 min in a microwave oven on a power setting that maintained the solution just below its boiling point. Sections were then cooled in water to room temperature before permeabilization for 10 min in 0.001% trypsin (Roche) diluted in 0.1% CaCl2/20 mM Tris buffer (pH 7.5). After three washes in PBS, the sections were denatured in 2 N HCl in PBS for 30 min, washed again in PBS, gently agitated for 30 min in blocking serum (3% normal horse serum, 0.5% Triton X-100 in PBS) and incubated overnight at 4°C with monoclonal anti-BrdU antibody (Becton Dickinson no. 347980; 1/100 diluted in blocking serum; Becton Dickinson, Oxford, UK). Sections were incubated for 1 h at room temperature with biotinylated antimouse IgG (Vector Labs, Peterborough, UK; 1/200 diluted in blocking serum), washed again in fresh PBS before blocking endogenous peroxidases for 30 min with 0.6% (vol/vol) hydrogen peroxide in PBS. After a further three washes in PBS, sections were incubated with R.T.U. Vectastain Elite ABC reagent (PK-7100; Vector Labs) for 1 h at room temperature, rinsed in PBS, and developed for approximately 8 min in diaminobenzidine substrate according to the manufacturer’s instructions (SK-4100; Vector Labs). The resulting brown color reaction was stopped in water, and sections were rinsed three times in PBS, gently agitated for 30 min in blocking serum (3% normal goat serum, 0.5% Triton X-100 in PBS), and incubated overnight at 4°C with either rabbit polyclonal anti-ACTH (National Institute of Diabetes and Digestive and Kidney Diseases; 1/2000 diluted in blocking serum). Sections were washed in three changes of PBS, incubated for 1 h at room temperature with biotinylated antirabbit IgG (Vector Labs; 1/200 diluted in blocking serum), washed in a further three changes of PBS, incubated with R.T.U. Vectastain Elite ABC reagent for 1 h at room temperature, rinsed in PBS, and developed for approximately 2 min in VIP substrate according to the manufacturer’s instructions (SK-4600; Vector Labs). The resulting purple color reaction was stopped in water, and the sections were lightly counterstained with hematoxylin.

Image analysis for trophic activity

Apogetic and mitotic event prevalence was analyzed on 2-μm-thick hematoxylin-and-eosin-stained pituitary sections at ×1000 magnification (19) using a real-time system [AxioHOME Zeiss (23)] that projects an image of a computer screen fractionally above the histological section. Identifier tags placed over manually identified cells and trophic events remain in registration with the targets irrespective of subsequent stage movements and magnification changes. For each animal, three random areas of approximately 47,000 μm² were scored for the presence of mitotic and apoptotic figures. By defining counting boundaries at low power and counting events at high power, selection bias and double scoring were eliminated, allowing the error in quantifying the number of normal cells surrounding these events to be limited to 2% or less and the overall error in estimating the prevalence of trophic events to be reduced to approximately 0.01%.

Histological characteristics used to define apoptotic cells were clusters of two or more extremely dense round or oval structures varying in diameter from approximately 0.7–4 μm and surrounded by normal cells (24). Earlier stages of apoptosis cannot be visualized using hematoxylin and eosin staining and light microscopy. Results were expressed as a percentage of the total cell numbers counted for each animal.

Immunopositive cell counts were performed at ×1000 magnification. For each animal, three areas totaling 0.15 mm², containing an average of 1000 cells were scored as positive or negative for the presence of immunoreactive BrdU and/or ACTH. In this study, no specific mathematical corrections were made for anticipated increases in corticotroph cell size after adrenalectomy (25). Therefore, changes in corticotroph number are likely to be overestimated by 20–35% (19). All slides were coded and counted by one blinded observer (L.A.N.), and the results expressed as the mean ± se with differences between groups evaluated using one-way ANOVA followed by Tukey-Kramer multiple comparison post tests. P < 0.05 was considered statistically significant.

Results

Effect of somatostatin analogs on body weight gain

As expected, adrenalectomy alone resulted in a slight reduction in body weight gained over the first 5 d after surgery compared with sham-operated, vehicle-treated animals (Fig. 1). Concurrent administration of either pasireotide or oct-
reotide to adrenalectomized animals caused a further small reduction in body weight over the same time period, as did octreotide alone in sham-operated animals (Fig. 1).

**The effects of pasireotide on baseline and adrenalectomy-induced mitosis in the anterior pituitary**

Two days after surgery, the baseline apoptotic and mitotic indices measured in sham-operated, vehicle-treated male rat anterior pituitary were $0.038 \pm 0.008\%$ and $0.108 \pm 0.016\%$, respectively. These values did not vary significantly between the individual time points throughout the 6-d experimental period (Fig. 2; $n = 8$ in all groups). As expected, a numerically small but nevertheless highly significant increase in mitotic activity was observed in adrenalectomized animals treated with vehicle at 2, 4, and 6 d after surgery ($0.316 \pm 0.027\%$ at d 6; Fig. 2, bottom panel). Pasireotide completely blocked this wave of adrenalectomy-induced increase in mitotic activity, although baseline mitotic activity was not suppressed at any time point. Pasireotide administered to adrenal-intact rats had no effect on mitotic index when compared with intact rats treated with vehicle alone (Fig. 2, bottom panel). There was no measurable effect of pasireotide and/or surgery on the apoptotic index at any time point (Fig. 2, top panel). The effects of octreotide on anterior pituitary trophic activity were indistinguishable from those of pasireotide at the dose, route, and time point studied (Fig. 3).

**The effects of pasireotide on adrenalectomy-induced changes in ACTH labeling index**

Four days after surgery, the proportion of ACTH-positive cells in the anterior pituitary significantly increased in adrenalectomized rats compared with sham-operated controls irrespective of whether they were concurrently treated with pasireotide or vehicle alone (Fig. 4, top panel). As pasireotide completely blocked the wave of increased anterior pituitary mitotic activity that normally follows adrenalectomy, these data indicate that differentiation of preexisting ACTH-immunonegative cells rather than cell division is largely responsible for the observed increase in ACTH-immunopositive cells after adrenalectomy. Six days after surgery, the...
proportion of ACTH-positive cells, although increased compared with values after 4 d, was not significantly different in adrenalectomized rats treated with vehicle, pasireotide, or octreotide (13.55 ± 0.45% vs. 12.96 ± 0.55% vs. 12.79 ± 0.39%, respectively; \( n = 6 \)). The percentages of ACTH-positive cells in sham-operated animals treated with vehicle or octreotide were not significantly different either from each other (7.33 ± 0.14% vs. 8 ± 0.37%, respectively) or from sham-operated animals treated with vehicle or pasireotide for 4 d.

The BrdU labeling index represents cell proliferation during the period of BrdU availability for incorporation into DNA minus apoptosis of BrdU-labeled cells (Fig. 4, bottom panel). In sham-operated rats treated with vehicle or octreotide (13.55 ± 0.45% vs. 12.96 ± 0.55% vs. 12.79 ± 0.39%, respectively; \( n = 6 \)). The percentages of ACTH-positive cells in sham-operated animals treated with vehicle or octreotide were not significantly different either from each other (7.33 ± 0.14% vs. 8 ± 0.37%, respectively) or from sham-operated animals treated with vehicle or pasireotide for 4 d.

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The proportion of BrdU-labeled cells that was also immunopositive for ACTH was extremely low in all groups (Fig. 4, bottom panel), again supporting the observation that the observed increase in ACTH labeling index after adrenalectomy does not result from division of differentiated, hormone-producing cells.

The effects of pasireotide and octreotide on plasma ACTH

Six days after surgery, adrenalectomy resulted in a more than 9-fold increase in plasma ACTH compared with controls (\( P < 0.001 \)) (Fig. 5). Concurrent treatment of adrenalectomized animals with either pasireotide or octreotide suppressed plasma ACTH levels by 41% (\( P < 0.01 \)) and 34% (\( P < 0.05 \)) of vehicle-treated controls, respectively (Fig. 5). No suppression of baseline ACTH secretion was seen in sham-operated animals treated with octreotide.

Discussion

Removal of glucocorticoids after adrenalectomy and excess endogenous or exogenous glucocorticoids may both affect the expression of cellular somatostatin receptors (26).
In the male rat anterior pituitary, it has been shown that, 8 d after adrenalectomy, SSTR2 is specifically up-regulated, suggesting that pituitary SSTR2 synthesis is inhibited tonically by physiological levels of endogenous glucocorticoids (26). High-dose dexamethasone in vivo resulted in an overall decrease in SSTR1–4 transcripts and an increase in SSTR5 transcripts in rat anterior pituitary cells (26). High circulating cortisol levels in patients with Cushing’s disease may also result in somatostatin receptor down-regulation on relevant cell populations resulting in decreased efficacy of selective somatostatin receptor agonist treatments (9). Indeed, octreotide was unable to suppress CRH-induced ACTH secretion in human corticotroph adenoma cells in vitro (14) and in normal corticotrophs, ACTH levels are not affected by infusion of somatostatin or octreotide in the presence of glucocorticoids (9). However, the SSTR5 receptor subtype may be more resistant to receptor down-regulation by glucocorticoids (9, 27). In our model system using adrenalectomized rats, glucocorticoid levels are very low or absent and CRH/arginine vasopressin levels are increased. Six days after the start of treatment with octreotide, the degree of suppression of plasma ACTH in octreotide-treated rats (34%) is comparable with that seen in adrenal-intact rats treated in the short-term with CRH (28). Pasireotide treatment resulted in a higher degree of suppression been identified in either normal or adenomatous human corticotrophs. Pasireotide binds to multiple somatostatin receptors with high affinity (SSTR5 > 2 > 3 > 1) and has a more pronounced inhibitory effect on ACTH secretion from AtT20 mouse corticotroph tumor cells than octreotide (9, 27). It has not been shown to have any effects on AtT20 cell proliferation or rates of apoptosis (9), but a reduction in cell proliferation of from 10–70% in six of six human corticotroph adenoma cell samples maintained in vitro has been observed within the 1–100 nm concentration range (5). As might be expected, the effects of pasireotide on ACTH secretion and cell proliferation appear to be relatively independent (5) in the same way that in human somatotroph adenomas, reduction in GH secretion and tumor shrinkage in response to somatostatin analogs are poorly correlated (3, 6).

After adrenalectomy in the male rat there is a modest increase in the anterior pituitary mitotic index that peaks within 2–6 d of surgery and returns to baseline levels over the next 14 d or so, despite the continued absence of circulating glucocorticoids (19). The transient nature of the trophic response which results principally from glucocorticoid withdrawal at the level of the pituitary (31) contrasts with the sustained ACTH secretory response that is induced by increased CRH stimulation (31). In the current study, we have shown that concurrent treatment with pasireotide or octreotide blocks the adrenalectomy-induced increase in male rat anterior pituitary mitosis but had no measurable effects on basal cell turnover throughout the 6-d time period after surgery.

Previous studies have shown that, after adrenalectomy, most newly formed ACTH cells are derived from differentiation of preexisting hormonally undifferentiated cells and that the same progenitor-like population responds mitotically to both adrenalectomy and gonadectomy (32). In the present study, double immunochemistry with antibodies against BrdU and ACTH was used to quantify the proportion of dividing cells with hormonal identity. Four days after adrenalectomy, the proportion of double-labeled cells remained extremely low irrespective of whether the animals were adrenalectomized or whether they received pasireotide treatment. The very small increase in the proportion of dividing cells that also labeled for ACTH presumably represents rare division in recently differentiated corticotrophs reaching the end of their proliferative potential. The data presented in the current study indicate that, whereas pasireotide suppresses division in a subpopulation of anterior pituitary cells, these cells are not preexisting corticotrophs, neither are they destined to become differentiated corticotrophs in response to adrenalectomy in this rat model.

In light of previously published effects of pasireotide and a primarily antiproliferative effect of octreotide on these cells.
octreotide on the inhibition of secretion of GH and cell proliferation in human somatotroph adenomas, it has been suggested that their inhibitory effect on cell proliferation in the rat anterior pituitary could be directly mediated through differentiated somatotrophs (2, 6). However, our own unpublished data quantifying the proportion of GH-positive cells in the anterior pituitary at either 2, 4, or 7 d after adrenalectomy show no significant change compared with that found in adenalin-intact controls (26.4 ± 1.34% vs. 31.8 ± 1.66%, respectively, at 4 d postoperatively). These data indicate that the mitotic wave induced by adrenalectomy alone does not occur within the differentiated somatotroph population.

The findings in the present study suggest that basal cell turnover in the rat anterior pituitary is mediated by a small subpopulation of progenitor cells, the activity of which is not influenced by exposure to either pasireotide or octreotide. Daughter cells generated by progenitor cell division that do not undergo early apoptosis, remain hormonally null in the short-term. These nascent cells respond to multiple stimuli including acute reduction in circulating corticosterone levels by further limited division and concurrent somatostatin receptor expression. Cells derived from this pool may undergo apoptosis or differentiate further into new hormone-positive cells, which presumably include corticotrophs. From the present study and others, it seems likely that the differentiation pathway is unaffected by octreotide or pasireotide. The very small increase in BrdU/ACTH double-labeled cells after adrenalectomy occurs in recently differentiated corticotrophs that are approaching the end of their capacity to divide. They represent an extremely small fraction of the BrdU-only-labeled cells and the ACTH-only-labeled cells. In this model, somatostatin analogs only have a small window of opportunity to influence cell proliferation and can only do so during limited and specific periods of cell cycling. In summary, surgical adrenalectomy in the male rat stimulates cell division in a specific subpopulation of hormonally null cells that are equally sensitive to the antiproliferative effects of pasireotide and octreotide, suggesting that this effect is primarily mediated by the SSTR2 receptor subtype. In line with these data is the clinical observation that patients with Nelson’s disease but not primary pituitary-dependent Cushing’s show responsiveness to octreotide (33). Basal cell turnover, apoptotic index, and division and differentiation of ACTH-positive cells in the anterior pituitary are unaffected by treatment with either of these somatostatin receptor agonists used in the present study.

In conclusion, the presence and amplitude of ACTH antisecretory and antiproliferative responses to the somatostatin analogs pasireotide and octreotide depends on current and recent changes in anterior pituitary progenitor cell hormonal microenvironment. Base line anterior pituitary cell turnover is unaffected by pasireotide and octreotide, but progenitor cell mitotic activity induced by corticosterone withdrawal is blocked by these somatostatin analogs. Basal ACTH secretion is unaffected by pasireotide and octreotide, but the secretory response to bilateral adrenalectomy is greatly reduced.

Acknowledgments

We thank Dr. Helen Atkinson for technical support.

Received December 20, 2006. Accepted February 27, 2007.

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We would like to acknowledge financial support from Novartis and The Wellcome Trust.

Disclosure Statement: L.A.N and A.L. received grant support (January 2006 to February 2007) from Novartis Pharma. H.A.S. is employed by Novartis Pharma.

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