Octreotide, a Somatostatin Analogue, Mediates Its Antiproliferative Action in Pituitary Tumor Cells by Altering Phosphatidylinositol 3-Kinase Signaling and Inducing Zac1 Expression

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Abstract

Somatostatin limits cell growth by inhibiting the proliferative activity of growth factor receptors. In this study, it is shown that in pituitary tumor cells, the somatostatin analogue octreotide produces its antiproliferative action by inducing the expression of the tumor suppressor gene Zac1. Zac1 expression was pertussis toxin sensitive and was abolished after transfection with a dominant negative vector for SHP-1. Zac1 is a target of the phosphatidylinositol 3-kinase (PI3K) survival pathway. Octreotide treatment decreased the tyrosine phosphorylation levels of the PI3K regulatory subunit p85, induced dephosphorylation of phosphoinositide-depentent kinase 1 (PDK1) and Akt, and activated glycogen synthase kinase 3β (GSK3). Therefore, in pituitary tumor cells, somatostatin analogues produce their antiproliferative action by acting on the PI3K/Akt signaling pathway and increasing Zac1 gene expression.

Introduction

Somatostatin regulates neurotransmission, inhibition of hormone secretion, and proliferation, and is used for the treatment of neuroendocrine tumors. Somatostatin binds to a family of receptors (SSTR1-5), which belong to the seven-transmembrane-domain G-protein coupled receptors (GPCR; ref. 1), and exerts its antiproliferative action by inducing G0-G1 cell cycle arrest (2) or G2-M arrest and apoptosis (3).

Somatostatin binding to SSTR, receptor heterodimerization, G protein sequestration, and the intracellular effectors of SSTR signaling were extensively studied (reviewed in refs. 1, 4–7). Somatostatin limits cell growth by inhibiting the proliferative activity of growth factor receptors. Phosphotyrosine phosphatases (PTP) play a central role in this process by dephosphorylating the tyrosine phosphorylation levels of the PI3K regulatory subunit p85, induced dephosphorylation of phosphoinositide-dependent kinase 1 (PDK1) and Akt, and activated glycogen synthase kinase 3β (GSK3). Therefore, in pituitary tumor cells, somatostatin analogues produce their antiproliferative action by acting on the PI3K/Akt signaling pathway and increasing Zac1 gene expression.

Materials and Methods

Reagents. Cell culture materials were purchased from Life Technologies (Karlsruhe, Germany), Nunc (Wiesbaden, Germany), and Sigma (St. Louis, MO). Octreotide was provided by the American Peptide Company (Sunnyvale, CA); pertussis toxin, orthovanadate, and PD908059 were from Sigma; and LY294002, wortmannin, SB-415286, and lithium chloride were from Calbiochem (Bad Soden, Germany). Octreotide was dissolved in DMSO.
Cell culture. GH3 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% FCS, 2.2 g/L NaHCO3, 10 mmol/L HEPES, 2 mmol/L glutamine, 2.5 mg/L amphotericin B, and 105 units/L penicillin-streptomycin at 37°C and 5% CO2. Cells (4 × 105) were treated with octreotide, pertussis toxin, orthovanadate, LY294002, wortmannin SB-415286, and lithium alone or in the combination appropriate for each experiment, dissolved in serum-free DMEM. The carriers in which the substances were dissolved were used as controls. Pertussis toxin was administered 12 hours, orthovanadate 2 hours, and lithium 1 hour before octreotide treatment. Treatment time was 20 hours unless otherwise indicated.

RNA extraction and semiquantitative reverse transcription-PCR. RNA was extracted by the guanidinium-isothiocyanate phenol method. PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was done on 1 µg RNA to exclude genomic DNA contamination. One microgram of RNA was reverse transcribed using random hexanucleotides and semiquantitative radioactive PCR was done for Zac1 and GAPDH (internal control) under restrictive conditions as previously described (25). Each reverse transcription-PCR (RT-PCR) was done in RNA extracted from three independent experiments.

Western blot analysis. Cells were treated as described for 1, 3, and 6 hours. Cell lysates were separated by PAGE and blotted using standard procedures (26). Primary antibodies were against SHP-1/PTP1C, SHP-2/PTP1D (both made in mouse; Transduction Laboratories, Lexington, KY), Akt, phosphoinositide-dependent kinase 1 (PDK1), glycogen synthase kinase 3β (GSK3β), p70S6K, and AKT, phosphorylated Accord (Ser473),Akt (Thr308),PDK1 (Ser408), GSK3β (Ser21), phosphatase and tensin homologue (PTEN; Ser240), p70S6K (Ser422), and FKHR (Ser256; all made in rabbit; New England Biolabs GmbH, Frankfurt am Main, Germany). Horseradish peroxidase–conjugated secondary antibodies were used against mouse and rabbit (Amersham Pharmacia Biotech, Freiburg, Germany). Each Western blot was done in lysates obtained from three independent experiments.

Communomunoprecipitation. GH3 cells were treated with 1 µmol/L octreotide for 10, 30, and 60 minutes and collected in ice-cold lysis buffer [150 mmol/L NaCl, 50 mmol/L HEPES (pH 7.4), 1 mmol/L sodium orthovanadate, 2 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, and 1% NP40]. Protein (600 µg) was immunoprecipitated with an antibody against the p85 subunit (Upstate, Charlotteville, VA) or with a control mouse immunoglobulin G (IgG). Protein A Sepharose (Amersham Pharmacia Biotech) was used as previously described (26). The immunoprecipitates were extensively washed and protein bound to sepharose was eluted and separated by 10% SDS-PAGE (27). Western blot was done using the horseradish peroxidase–conjugated anti-phosphotyrosine 4G10 (Upstate), anti-p85, or anti-SHP-1 monoclonal antibody. Communomunoprecipitation was done in two independent experiments and was repeated using Protein G Sepharose.

Plasmids. SHP-1/C53S (SHP-1dn; ref. 17) and C/S SHP-2 (SHP-2dn; ref. 19) dominant negative mutants and the G3Jy sequester β-ARK-CT (gift of P. Voigt, Institute of Pharmacology, Charité-Medical University, Campus Benjamin Franklin, Berlin, Germany) were used. The p33-Luc construct (Mercury pathway profiling system, Clontech Laboratories, Inc., Palo Alto, CA) has the p33 responsive element upstream to the TATA box of the herpes simplex virus thymidine kinase promoter and the reporter gene luciferase.

Transfection and reporter assays. Cells were transfected using SuperFect (Qiagen GmbH, Hilden, Germany). Cells (3 × 105) were transfected for 3 hours with 1 µg of SHP-1dn, SHP-2dn, or β-ARK-CT plasmid, left in cell growth medium overnight, and treated for 20 hours with 1 µmol/L octreotide. RNA was extracted and semiquantitative RT-PCR was done for Zac1. Lysates of cells transfected with β-ARK-CT were analyzed for phosphorylated Akt by Western blot and lysates of cells transfected with SHP-1dn were immunoprecipitated with p85 as described above. Each transfection experiment was done in duplicate. To confirm the SHP-1 and 2dn incorporation, Western blot was done for expression of SHP-1 and 2dn. Incorporation of SHP-1dn was immunoprecipitated with p85 as described above. Each transfection experiment was done in duplicate. To confirm the SHP-1 and 2dn incorporation, Western blot was done for expression of SHP-1 and 2dn. Incorporation of SHP-1dn was immunoprecipitated with p85 as described above. Each transfection experiment was done in duplicate.

Statistical analysis. Differences were assessed by one-way ANOVA in combination with Schefle's test. P < 0.05 was considered as significant.

Results

Octreotide induces the Zac1 gene. The somatostatin analogue octreotide increased Zac1 gene expression at concentrations of 1 and 100 nmol/L (Fig. 1A) after 6 to 24 hours (Fig. 1B). This induction pattern is in accordance to the cell cycle kinetics previously described after octreotide treatment in which GH3 cells accumulated in G0-G1 after 24 hours but there was no effect on cell cycle and number after 48 hours (2).

Knocking down Zac1 by RNA interference abolished the antiproliferative effect of octreotide. To examine the role of Zac1 in the antiproliferative signaling of octreotide, we knocked down Zac1 using siRNA. siRNA against Zac1 increased cell proliferation, similar to what was reported in a previous study using antisense oligonucleotides (24; ref. 32). Although 24-hour octreotide treatment decreased cell viability in untransfected GH3 cells and in cells transfected with control siRNA (scramble), it had no effect in cells transfected with 50 and 100 nmol/L siRNA against Zac1 (Fig. 2B). On the other hand, octreotide decreased CAMP in untransfected and transfected cells, indicating that the inability of octreotide to decrease cell proliferation in siRNA-transfected cells is not due to a dysfunctional receptor (Fig. 2C). Furthermore, treatment with lepin,
which does not affect Zac1 expression, decreased cell proliferation in untransfected cells, as well as in scramble- and siRNA-transfected cells, as previously documented (data not shown; ref. 33), further showing that the effect of Zac1 knockdown is specific for octreotide.

The effect of octreotide on Zac1 gene expression is pertussis toxin sensitive. Preincubation with 100 ng/mL pertussis toxin for 12 hours abolished the stimulatory effect of octreotide on Zac1 transcription, indicating the involvement of Gi (Fig. 3A). When activated, Gi generates α-subunits and free βγ dimers. Transfecting GH3 cells with the Gβγ sequester β-ARK-CT did not influence the effect of octreotide on Zac1 expression, indicating the involvement of the α-subunit but not of the βγ (Fig. 3B).

A PTP mediates the stimulatory effect of octreotide on Zac1 gene expression. The PTP inhibitor orthovanadate reversed the effect of octreotide on Zac1 (Fig. 3C). Both SHP-1 and SHP-2 were expressed in GH3 cells (data not shown). Cells transfected with dominant negative SHP-1 did not respond to octreotide by increasing Zac1 expression whereas transfection with the dominant negative SHP-2 had no effect (Fig. 3D), indicating that in pituitary cells SHP-1 is required for the action of octreotide.

Octreotide inhibits the PI3K/Akt pathway. We examined which pathways link octreotide to Zac1, downstream of SHP-1. The MAPK inhibitor PD098059 had no effect on basal or octreotide-stimulated Zac1 levels (data not shown). On the other hand, cells treated with the PI3K inhibitors wortmannin and LY294002 displayed increased Zac1 gene expression (Fig. 4A), indicating that Zac1 is controlled by PI3K and that octreotide may regulate Zac1 expression by inhibiting its signaling. Octreotide had no effect on total PDK1 and Akt protein levels but it decreased PDK1 and Akt phosphorylation (Fig. 4B). These effects were pertussis toxin sensitive but were not abolished in cells transfected with β-ARK-CT, indicating involvement of the Gi α-subunit but not of the βγ dimers (data not shown).
The PI3K pathway is switched off by the lipid phosphatase and tumor suppressor PTEN. However, octreotide did not affect total or phosphorylated PTEN levels (Fig. 4B), suggesting that its inhibitory effect on PDK1/Akt is not due to PTEN activation. PI3K was also shown to be inactivated by SHP-1, which acts by dephosphorylating the PI3K regulatory subunit p85 (27). In GH3 cells, p85 coimmunoprecipitated with SHP-1, confirming the physical association between SHP-1 and p85 (Fig. 4C; ref. 34). Octreotide decreased the levels of p85 detected with the 4G10 phosphotyrosine antibody (Fig. 4C), but had no effect on p85 phosphorylation in cells transfected with SHP-1dn (Fig. 4D), indicating an important role for SHP-1 in octreotide signaling. Therefore, it is possible that octreotide treatment decreases the phosphorylation levels of the PI3K regulatory subunit p85 and subsequently those of PDK1 and Akt.

Octreotide dephosphorylates and therefore activates GSK3β. PDK1 and Akt transduce their signals by phosphorylating and activating or inactivating a number of substrates. Octreotide decreased p70/S6K, FKHR, and GSK3β phosphorylation levels without affecting their total protein levels (Fig. 5A). Blocking GSK3β with 14 μmol/L SB-415286 or 20 mmol/L lithium abolished the stimulatory effect of octreotide on Zac1, indicating that Zac1 expression after 24-hour treatment with 1 μmol/L octreotide, after 16-hour pretreatment with 100 ng/mL pertussis toxin (Fig. 4A), and with 1 μmol/L octreotide in cells transfected with the βARK-CT (B), β-ARK-CT incorporation is proved by the decrease in Ca2+ oscillation in basal and carbachol-treated GH3 cells. Each determination was done twice. RFU, relative fluorescence units. *, P < 0.05. C, effect of 2-hour pretreatment with 1 μmol/L, 100 mmol/L, and 10 nmol/L of the PTP inhibitor orthovanadate on the induced Zac1 expression of octreotide. D, effect of 1 μmol/L octreotide on Zac1 gene expression in untransfected GH3 cells (control) and in cells transfected with a dominant negative vector for SHP-1 (SHP-1dn) or SHP-2 (SHP-2dn). SHP-1dn and SHP-2dn incorporation is shown by Western blot for hemagglutinin (HA) and c-myc, respectively. Similar results were shown by two other experiments. All treatments were done in serum-free DMEM. A and B, quantitative analysis of the A Zac/A GAPDH ratio for each sample.

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up-regulation happens downstream to GSK3β (Fig. 5B). Octreotide had no effect on p53 expression, which is one of the GSK3 targets (data not shown), but it increased p53 transcriptional activity, and this effect was pertussis toxin sensitive and was reversed after cotreatment with SB-415286 or lithium (Fig. 5C). Knocking down p53 decreased Zac1 levels and abolished the effect of octreotide on Zac1 transcription (Fig. 5D). Therefore, octreotide, by blocking GSK3β phosphorylation, activates p53 and increases Zac1 gene expression (Fig. 6).

**Discussion**

In the present study, it is shown that octreotide increases the expression of the tumor suppressor gene Zac1 in a pertussis toxin–sensitive mechanism involving the PTP SHP-1. The role of this tumor suppressor gene in the antiproliferative action of octreotide is shown by the fact that cells in which Zac1 was knocked down failed to respond to the drug treatment. Therefore, Zac1 up-regulation is necessary for octreotide to exert its growth inhibiting action. The antiproliferative role of Zac1 is indicated by the fact that although highly expressed in normal adenohypophysis, it is down-regulated in most pituitary adenomas (25, 32). Furthermore, Zac1 is lost in transformed rat epithelial ovarian cells (36) and down-regulated by the EGF mitogenic signaling (37).

Herein, it is also shown that Zac1 is a downstream target of the PI3K survival pathway. PI3K and its downstream targets mediate the growth-promoting and cell survival actions of growth factors, cytokines, and GPCR ligands. On activation, class I PI3Ks phosphorylate phosphatidylinositol-4,5-biphosphates to phosphatidylinositol-3,4,5-triphosphates. Phosphatidylinositol-3,4,5-triphosphate recruits Akt to the plasma membrane and changes its conformation to facilitate its phosphorylation by PDK1 (reviewed in ref. 38). Octreotide treatment in pituitary cells inhibited both PDK1 and Akt phosphorylation.

PI3K activity can be inhibited by the lipid phosphatase and tumor suppressor PTEN. However, we show that octreotide does not affect PTEN, indicating that its inhibitory action on the PI3K pathway is not through PTEN. Still, PI3K activity is under tight regulation by mechanisms controlling its phosphorylation. Class I PI3Ks are

**Figure 6.** Proposed scheme for the signaling cascade taking place after octreotide treatment in pituitary cells. Because GH3 cells express only SSTR1 and SSTR2 (53) and octreotide can bind to SSTR2, SSTR3, and SSTR5, but not to SSTR1 (1), we assume that this pathway originates from the activated SSTR2. Gia associates with SSTR2 and SHP-1 (15). After 10-minute octreotide treatment, SHP-1 dephosphorylates the PI3K regulatory subunit p85 and probably inactivates PI3K without involving PTEN. Three hours later, PDK1, Akt, and GSK3β are dephosphorylated and GSK3β is activated. This activation results in an increase in p53 transcriptional activity and Zac1 transcription 6 hours after octreotide treatment. In contrast to a previous model (54), Gi/γ and SHP-2 were not needed for the action of octreotide in pituitary cells.
heterodimers consisting of a regulatory subunit (p85 and p101) and a catalytic subunit (p110α, β, γ, and δ). Under resting conditions, p85 stabilizes p110α and inhibits its kinase activity (39) whereas, on tyrosine phosphorylation, p85 releases its inhibitory action on p110α, leading to PI3K activation. The PTP SHP-1 was described to inhibit PI3K by dephosphorylating p85 (27). In accordance to what was reported before, in pituitary cells, SHP-1 was found to physically associate with p85, implying that because octreotide can activate SHP-1, it may also control PI3K. Indeed, in this study, it is shown that octreotide decreases p85 tyrosine phosphorylation and that SHP-1 plays an important role in this process. Therefore, we speculate that octreotide initiates its antiproliferative signaling by dephosphorylating p85, through SHP-1, and subsequently decreasing the phosphorylation levels of members of the PI3K pathway (Fig. 6).

An interesting observation is the time lapse between the dependent p85 and Akt dephosphorylation of octreotide. Although there is an increasing amount of information about Akt activation, very little is known about the mechanisms governing Akt inactivation (40). There is evidence that Akt resides in lipid rafts where it can remain constitutively active (41, 42). Akt trapped in a constitutively active form could explain the time lapse noticed in the present study, but this is a speculation which remains to be examined.

GPCR activates PI3Kβ and PI3Kγ, but not PI3Kα, through Gβγγ, and this association is always stimulatory (43, 44). Furthermore, Gi coupled receptors activate PI3Kβ also through Gβγγ (45). In the case of somatostatin, SSTR1 and SSTR2 were shown to activate PI3K signaling in a mechanism involving Gβγγ and SHP-2 (20–22). However, in the present study, the effect of octreotide is most probably Gβγγ independent because sequestering the Gβγ subunits with β-ARK-CT did not abolish its effect on Akt phosphorylation. These data suggest that Gi-linked GPCR can interact with and inhibit PI3K through the Gi α-subunit, revealing a novel way of how GPCR, in general, and SSTRs, in particular, can restrict cell growth. Akt mediates the ant apoptotic and cell survival effects of growth factors by phosphorylating and subsequently inhibiting FKHR and GSK3β (46–48). The inhibitory action of octreotide on PDK1 and Akt resulted, as expected, in decreased FKHR and GSK3β phosphorylation levels. GSK3β inhibition abolished the stimulatory effect of octreotide on Zac1 gene expression, indicating that this tumor suppressor gene is downstream to GSK3β. GSK3β regulates cell cycle progression by affecting cyclin E and cyclin D1 proteolysis and subcellular localization, members of the Forkhead family of transcription factors, the tumor suppressor tuberin (TSC2), and p27/Kip1 (reviewed in refs. 48, 49). Furthermore, GSK3β phosphorylates p53 and activates its transcriptional activity (50). Because p53 activates Zac1 transcription (51), it is possible that the effect of octreotide on Zac1 is due to up-regulation of p53 transcriptional activity. Indeed, knocking down p53 abolished the stimulatory effect of octreotide on Zac1 transcription.

The present study suggests a novel mechanism of octreotide action through direct inhibition of components of the PI3K pathway. This observation contrasts with previous studies in pancreatic tumor cells, in which octreotide did not affect basal, but it inhibited growth factor–induced Akt phosphorylation (23, 52), indicating that octreotide signaling can vary among different cell types. Octreotide signaling as described herein initiates by inhibiting the phosphorylation of the PI3K regulatory subunit p85 through the Gi α-subunit and SHP-1. Inhibition of the PI3K pathway leads to GSK3β activation, increased p53 transcriptional activity, and subsequently Zac1 up-regulation. Zac1 regulates cell growth and its presence is required for octreotide to stop cell cycle progression, because cells in which Zac1 is knocked down cannot respond to the antiproliferative action of this somatostatin analogue.

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