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ORIGINAL ARTICLE

PI3K/Akt/mTOR pathway involvement in regulating growth hormone secretion in a rat pituitary adenoma cell line

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Abstract
Purpose Insulin-like growth factor 1 (IGF1) controls growth hormone (GH) secretion via a negative feed-back loop that may disclose novel mechanisms possibly useful to control GH hyper-secretion. Our aim was to understand whether PI3K/Akt/mTOR pathway is involved in IGF1 negative feedback on GH secretion.
Methods Cell viability, GH secretion, Akt, and Erk 1/2 phosphorylation levels in the rat GH3 cell line were assessed under treatment with IGF1 and/or everolimus, an mTOR inhitior.
Results We found that IGF1 improves rat GH3 somatotroph cell viability via the PI3K/Akt/mTOR pathway and confirmed that IGF1 exerts a negative feed-back on GH secretion by a transcriptional mechanism. We demonstrated that the negative IGF1 loop on GH secretion requires Akt activation that seems to play a pivotal role in the control of GH secretion. Furthermore, Akt activation is independent of PI3K and probably mediated by mTORC2. In addition, we found that Erk 1/2 is not involved in GH3 cell viability regulation, but may have a role in controlling GH secretion, independently of IGF1.
Conclusion Our data confirm that mTOR inhibitors may be useful to reduce pituitary adenoma cell viability, while Erk 1/2 pathway may be considered as a useful therapeutic target to control GH secretion. Our results open the field for further studies searching for effective drugs to control GH hyper-secretion.

Keywords Growth hormone · Insulin-like growth factor 1 · PI3K/Akt/mTOR pathway · MAPK pathway

Introduction

The growth hormone (GH)/insulin-like growth factor 1 (IGF1) axis is regulated by hypothalamic, pituitary and endocrine factors [1]. IGF1 is the primary factor that regulates homeostatic control of GH synthesis and release [2], exerting an inhibitory control by a feed-back loop [3]. Several studies on rat pituitary cell lines and primary cultures have demonstrated that IGF1 is able to reduce GH gene expression and hormone secretion [4–6]. Similarly, insulin has been shown to suppress somatotroph function in a primate model [7]. The capability of IGF1 to physiologically exert a negative feedback on GH secretion, which is thought to be partially lost in the settings of GH hypersecretion by neoplastic somatotroph, is an interesting objective of study. In this context, the effect of IGF1 on GH secretion could be closely related to the mechanisms underlying resistance to medical therapy in acromegaly [8]. A better understanding of the mechanisms by which IGF1 can control GH secretion may help in identifying new potential medical approaches to GH hypersecretion.

It has been already demonstrated that somatotroph pituitary cells express both insulin and IGF1 receptors (IGF1R), that activate at least two different intracellular pathways, PI3 Kinase (PI3K)-Akt-mTOR and MAP Kinase pathways [9], leading to the phosphorylation of numerous downstream effectors, including transcription factors [10]. In rat pituitary cells IGF1 has been shown to promote

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survival and protect from apoptosis, possibly through Akt activation [11, 12]. Similarly, IGF1 was found to induce cell viability in MtT/E cells and in non functioning pituitary adenoma primary cultures [13]. In addition, IGF1 regulates prolactin gene expression by Erk 1/2 activation in rat GH4C1 cells [14] and inhibits GH release through PI3K pathway in teleost pituitary primary cells [15].

The involvement of PI3K/Akt/mTOR pathway in the pathophysiology of pituitary adenomas has already been proposed [16–18] and recent studies reported that mTOR inhibitors, such as rapamycin and Everolimus, have anti-proliferative effects on rat GH3 and MtT/S cells [19]. Sajjad et al. demonstrated that mTOR activity is increased in all subtypes of human pituitary adenomas, suggesting that mTOR inhibition may represent a therapeutic approach for patients resistant to current medical treatment [20]. This hypothesis is further supported by our previous results, showing the capability of Everolimus to reduce cell viability and promote apoptosis in primary cultures of human non functioning pituitary adenomas with a mechanism that involves IGF1 signaling [21]. A similar mechanism has been reported also in other endocrine tumors [22].

The purpose of this study is to understand whether PI3K/Akt/mTOR pathway is involved in the IGF1 negative feedback on GH secretion. Specific PI3K/Akt/mTOR and MAPK inhibitors have been used in order to identify the key proteins necessary for IGF1 actions.

Materials and methods

Compounds

Everolimus and NVP-BEZ235 were provided by Novartis (Basel, Switzerland). Everolimus is reported to specifically inhibit mTORC1, while NVP-BEZ235 inhibits mTORC1, mTORC2 and PI3K [23]. IGF1 was purchased by PeproTek Inc (Rocky Hill, NJ, USA), SCH772984 from Selleckchem (Boston, MA, USA) and LY294002 from Cell Signaling (Danvers, MA, USA). All other reagents, if not otherwise specified, were purchased from Sigma Aldrich.

Cell culture

The GH3 and GH4C1 rat pituitary adenoma cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in the Ham's F12K and in the Ham's F10 medium, respectively, enriched with 15% horse serum (HS) and 2.5% fetal bovine serum, as previously described [24]. The cell lines were used after the fourth passage.

Viable cell number assessment

Cell viability was assessed by the ATPlite kit (Promega, Milano, Italy), as described previously [25]. Briefly cells were seeded at 2×10^4 cells/well in 96-well black plates and treated with the indicated substances, renewing the treatments every 24 h of incubation. After 72 h, cell viability was evaluated by Envision Multilabel Reader (Perkin Elmer, Monza, Italy). Results were obtained by determining the mean value ± standard error of the mean (SEM) vs. untreated control cells from three independent experiments in six replicates.

GH secretion

GH secretion was evaluated by measuring rat GH immunoreactivity in the culture medium by a specific ELISA Kit (Growth Hormone Rat ELISA Kit #KRC5311; Invitrogen, Carlsbad, CA, USA). The assay was performed in duplicate after appropriate sample dilutions. The detection limit was <0.5 ng/ml, with intra- and inter-assay coefficients of variation of 5.0 and 5.8, respectively. The assay results were normalized by viable cell number, as determined from ATPlite assay. Results are expressed as the mean value ± SEM percent GH concentration vs. untreated control cells in three experiments in duplicate.

RNA extraction and quantitative PCR

Total RNA was isolated with TRIzol reagent (Invitrogen). To prevent DNA contamination, RNA was treated with RNase-free DNase (Promega). The Experion Automated Electrophoresis System (Bio-Rad Laboratories, Milano, Italy) was used to determine the concentration and integrity of RNA samples, as previously reported [26]. Only samples with RNA quality index >9 were processed. Using a first strand cDNA synthesis kit (SuperScript Pre-amplification System for First Strand cDNA Synthesis, Invitrogen), 1 µg total RNA was reverse-transcribed with random hexamers according to the manufacturer's protocol, as previously reported [27]. In order to test retro transcription quality, all cDNAs were amplified with 18S Taqman Assays. All CTs were comparable with that of control cDNA, showing good retro transcription efficiency. Furthermore, all minus retrotranscriptase (RT-) showed no amplification, indicating the absence of genomic DNA contamination. GH expression levels (GH1-001) were evaluated by performing relative quantitative real-time PCR (qPCR) by using the specific TaqMan gene expression assay (ID assay rn01495894_g1; amplicon length 60 bp) (Thermo Fisher Scientific, Monza, Italy) according to TaqMan® Gene Expression Assays Protocol Thermo Fisher Scientific). Samples were run in triplicate on Applied

Biosystem 7900 HT and analyzed with the SDS 1.9 Software (Applied Biosystems, Monza, Italy) by applying the method described by Pfaffl [28]. The house keeping gene 18S rRNA (ID assay 4319413E) (Thermo Fisher Scientific) was used to normalize gene signals [24]. Results are expressed as the mean value ± SEM percent GH expression vs. untreated control cells in three independent experiments in three replicates.

Western blot

For Western blot analysis, GH3 cells were dissolved in RIPA Buffer (Thermolab Inc., Waltham, MA, USA), kept in ice for 30 min and then centrifuged at 13,000 rpm for 20 min at 4 °C. Protein concentration was measured by BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA), as previously described [29, 30]. Proteins were fractionated on 12% SDS-PAGE, as previously described, and transferred by electrophoresis to Nitrocellulose Transfer Membrane (PROTRAN, Dassel, Germany), as described previously [31]. Membranes were incubated with the following antibodies: 1:1000 polyclonal rabbit anti phospho (Ser473) Akt (Cell Signaling Technology), 1:1000 polyclonal rabbit anti Erk 1/2 and phospho (Thr202/Thy204) Erk 1/2 (Cell Signaling Technology). Immunoreactive bands were detected using 1:5000 anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Dako Italia, Milano, Italy). Immunoreactivity was revealed using the ECL Western Blotting Detection Reagents (GE Healthcare Europe GmbH, Milano, Italy). The blots were then stripped and used for further blotting with 1:1000 anti-GAPDH antibody (Cell Signaling Technology), for normalization purposes. Experiments were performed at least twice.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using student *T*-test or one-way ANOVA or Kruskal-Wallis after proof of normality test. Data were analyzed using GraphPad (Prism v-6.0). *P* values <0.05 were considered as statistically significant.

Results

Effects of IGF1, Everolimus and NVP-BEZ235 on GH3 cells

In order to explore the effects of IGF1, Everolimus, and NVP-BEZ235 on GH3 and GH4C1 cell viability cells were treated for 72 h without or with 100 nM Everolimus or NVP-BEZ235, in the presence or in the absence of 100 nM IGF1. Compounds concentrations were chosen on the basis of previous studies [32, 33]. As shown in Fig. 1a, GH3 cell viability was significantly induced by IGF1 (+30%; $P < 0.01$ vs. untreated control cells) and reduced by Everolimus and NVP-BEZ235 up to 30% ($P < 0.01$ vs. untreated control cells). Both compounds blocked the stimulatory effects of IGF1 on cell viability. On the contrary, as shown in Fig. 1b, GH4C1 cell viability was not influenced by IGF1, but was significantly reduced by Everolimus (−60%; $P < 0.01$ vs. control treated cells) and by NVP-BEZ235 (−46%; $P < 0.01$ vs. untreated control cells); both effects were not counteracted by co-treatment with IGF1.

To determine the effects of Everolimus, NVP-BEZ235 and IGF1 on GH secretion, GH levels were assessed in conditioned medium from GH3 and GH4C1 cells treated for 72 h in the same conditions. As shown in Fig. 1c, Everolimus and NVP-BEZ235 did not significantly influence GH secretion in GH3 cells, after normalizing for the viable cell number. On the contrary, IGF1 significantly inhibited GH secretion, (−40%; $P < 0.01$ vs. untreated control cells), an effect not influenced by Everolimus, but reduced by NVP-BEZ235 ($P < 0.05$ vs. cells treated with IGF-1). GH4C1 did not secrete a measurable amount of GH in the conditioned medium, therefore, the following experiments were performed only with GH3 cells.

These results indicate that IGF1 improves somatotroph cell viability via the PI3K/Akt/mTOR pathway and support the hypothesis that PI3 Kinase (PI3K) pathway is probably involved in the inhibitory effect of IGF1 on GH secretion.

Effects of IGF1, Everolimus, and NVP-BEZ235 on GH expression

To determine whether IGF1 may affect GH secretion by modulating GH expression, GH mRNA levels were assessed in GH3 cells treated with 100 nM IGF-1 for up to 72 h. Figure 2a shows that IGF1 significantly reduces GH mRNA levels after 4 h (−54%; $P < 0.01$ vs. time 0), without significant modifications at other time points. Therefore, we measured GH mRNA levels after a 4 h treatment without or with 100 nM Everolimus or NVP-BEZ235 in the presence or in the absence of 100 nM IGF1. The data in Fig. 2b confirmed that IGF1 reduces GH mRNA expression (−37%; $P < 0.01$ vs. untreated control cells). On the contrary, Everolimus and NVP-BEZ235 did not influence GH mRNA levels, but blocked the inhibitory effects of IGF1.

These data support the hypothesis that IGF1 exerts its inhibitory action on GH at transcriptional level through the PI3K/Akt/mTOR pathway.

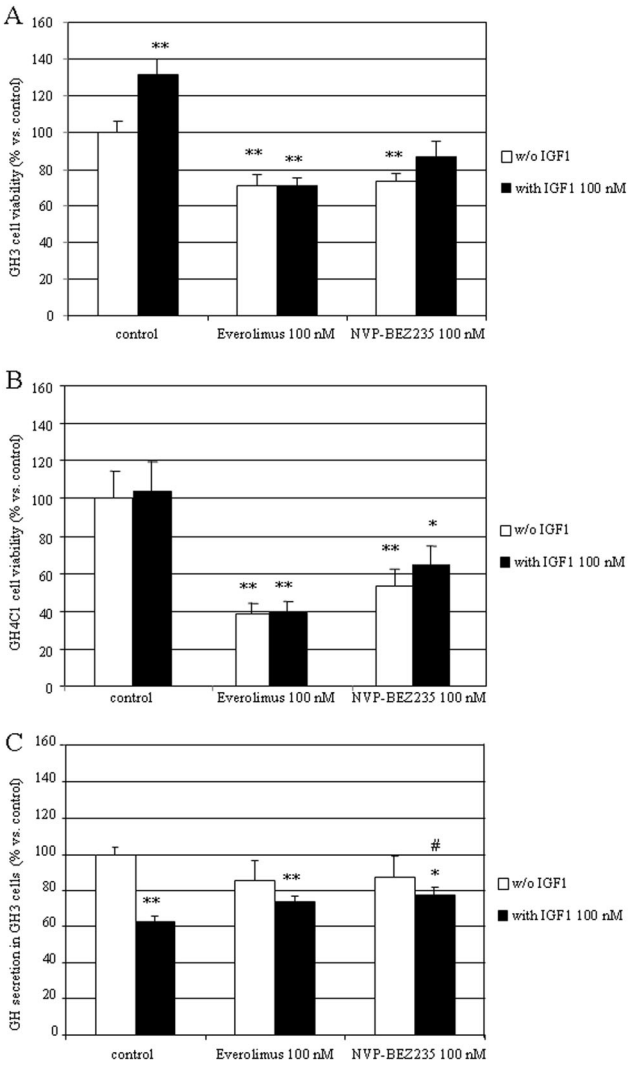

Fig. 1 Effects of IGF1, Everolimus and NVP-BEZ235 on GH3 cell line viability and GH secretion. GH3 (**a**) and GH4C1 cells (**b**) were incubated in 96-well plates for 72 h in a culture medium supplemented with 100 nM Everolimus or 100 nM NVP-BEZ235 without (white bars) or with 100 nM IGF1 (black bars); control cells were treated with vehicle solution. Cell viability was measured as a luminescent signal in three independent experiments with six replicates each, and expressed as the mean ± SEM percent cell viability vs. untreated control cells. $*P < 0.05$ and $**P < 0.01$ vs. untreated control cells. **c** GH3 cells were incubated for 72 h with 100 nM Everolimus or 100 nM NVP-BEZ235 without (white bars) or with 100 nM IGF1 (black bars). Medium was collected and GH secretion was evaluated by ELISA in three independent experiments in duplicate, expressed as the mean ± SEM percent GH secretion vs. untreated control cells. $*P < 0.05$ and $**P < 0.01$ vs. untreated control cells; $\#P < 0.05$ vs. IGF-1 treated cells

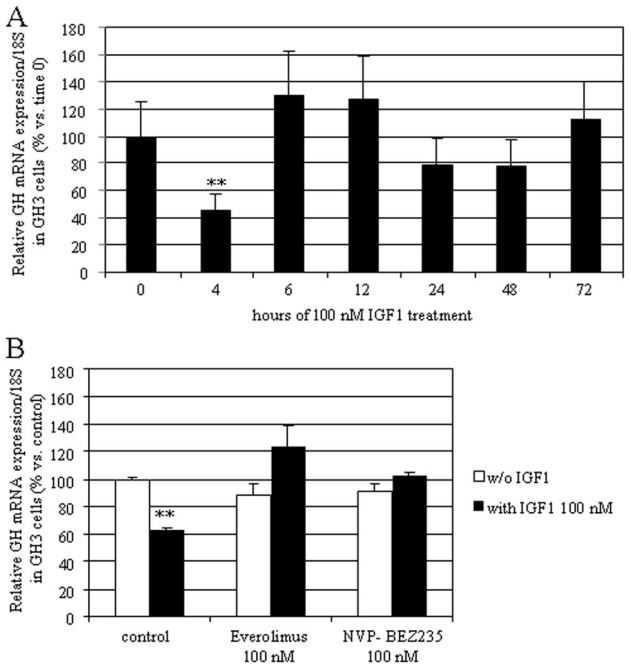

Fig. 2 Effects of IGF1, Everolimus and NVP-BEZ235 on GH expression in GH3 cells. **a** GH3 cells were incubated for 0, 4, 6, 12, 24, 48, and 72 h with 100 nM IGF1. Total RNA was extracted and relative qPCR for GH expression was performed. Time 0 was considered as control. Results are expressed as the mean ± SEM percent GH expression vs. time 0. $**P < 0.01$ vs. time 0. **b** GH3 cells were treated for 4 h with 100 nM Everolimus or 100 nM NVP-BEZ235 without (white bars) or whit 100 nM IGF1 (black bars). Total RNA was extracted and relative qPCR for GH expression was performed. Results are expressed as the mean ± SEM percent GH expression vs. untreated control cells. $**P < 0.01$ vs. untreated control cells

Effects of IGF1 and PI3K/Akt/mTOR inhibitors on Akt phosphorylation

In order to understand whether mTOR pathway could have a role in modulating the effects of IGF1 on GH3 cells, we evaluated Akt activation. Akt is one of the main proteins, upstream in the mTOR pathway, which activation regulates many downstream effectors, such as mTOR, controlling translation, metabolism and proliferation [34]. As show in Fig. 3a phosphorylated Akt (pAkt) protein levels were induced by treatment with IGF1. Similarly, Everolimus induced an increase in pAkt levels and did not influence IGF1 effects. NVP-BEZ235 did not affect basal pAkt, but completely abolished the stimulatory effects of IGF1 on this parameter. Therefore, Akt may be involved in the actions of IGF1 in GH-producing cells. Akt activation also depends on PI3K activity; therefore we employed a specific PI3K inhibitor, LY294002, in order to investigate the involvement of PI3K. As shown in Fig. 3b, LY294002 did not modify basal levels of both pAkt and total Akt, nor influenced the stimulatory effects of IGF1 on pAkt. Moreover, NVP-BEZ235 did not influence pAkt levels in the presence of LY294002, without or with IGF1. On the other hand, in keeping with previous results, Everolimus induced pAkt, an effect completely abolished by LY294002. At the same time, pAKT was rescued by co-treatment with IGF1, supporting the hypothesis that PI3K is not involved in IGF1 signaling through Akt in GH3 cells.

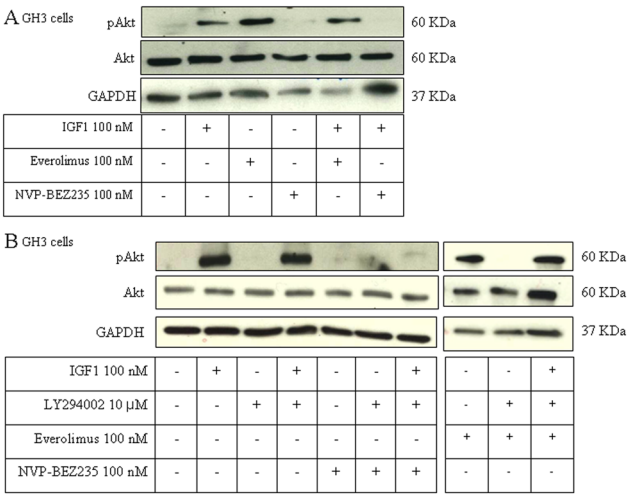

Fig. 3 Effects of IGF1 and PI3K/Akt/mTOR inhibitors on Akt phosphorylation in GH3 cells. **a** GH3 cells were incubated for 4 h with 100 nM IGF1, 100 nM Everolimus or 100 nM NVP-BEZ235, alone or in combination. Control cells were treated with a vehicle solution. Total proteins were isolated and total Akt as well as pAkt protein levels were assessed by Western blot analysis. GAPDH is shown as a loading control. The displayed images refer to two different Western blot membranes. **b** GH3 cells were incubated for 4 h in a culture medium supplemented with 100 nM IGF1, 100 nM Everolimus, 100 nM NVP-BEZ235 and 10 µM LY294002, alone or in combination; control cells were treated with vehicle solution. Total proteins were isolated and total and pAkt protein levels were assessed by Western blot analysis. GAPDH is shown as a loading control

Effects of IGF1 and an Erk inhibitor on GH3 cells

IGF1, upon binding the IGF1 receptor, exerts its actions through the activation of Ras/Raf/MAPK pathway. In order to understand whether MAPK signaling cascade could be involved in IGF1 effects on GH3 cells, we treated GH3 cells for 72 h with 10 nM SCH772984, a selective Erk inhibitor, without or with 100 nM IGF1 and evaluated cell viability as well as GH secretion. We observed that SCH772984 did not affect cell viability and did not influence the stimulatory effects of IGF1 on this parameter (Fig. 4a). Furthermore, we tested SCH772984 in combination with Everolimus and NVP-BEZ235. We found that SCH772984 did not modify the inhibitory effects of Everolimus and NVP-BEZ235 on cell viability (Fig. 4b). Subsequently, an ELISA assay was performed to measure GH in the conditioned media. As shown in Fig. 4c, SCH772984 significantly reduced GH secretion (−20%; $P < 0.05$ vs. untreated control cells), but did not influence IGF1 effects. Finally, as shown in Fig. 4d, Everolimus and NVP-BEZ235 blocked the inhibitory effect of SCH772984 on GH secretion.

These data suggest that Erk 1/2 is not involved in GH3 cell viability regulation, but may have a role in regulating GH secretion, independently of IGF1.

Effects of an Erk inhibitor and PI3K/Akt/mTOR inhibitors on Erk phosphorylation

To better understand the involvement of Erk proteins, we evaluated their phosphorylation levels in the same conditions. As shown in Fig. 5, phosphorylated Erk (pErk) levels were induced by IGF1, while SCH772984 drastically reduced pErk and abrogated the stimulatory effects of IGF1 on this parameter. Everolimus and NVP-BEZ235 enhanced pErk levels and their effect was not influenced by IGF1 or by SCH772984. These data indicate the presence of a cross-talk between PI3K and MAPK pathways, where increased pErk may be a consequence of mTORC1 inhibition, leading to an increase in MAPK activation [35].

Discussion

Our data confirm that IGF1 directly inhibits GH secretion, involving PI3K/Akt/mTOR but not MAPK pathway. Our findings are consistent with previous literature showing that IGF1 increases pituitary cell survival [36], which is on the contrary reduced by mTOR inhibitors [16]. In addition, the evidence that IGF1 is not capable of rescuing rat pituitary cells from the inhibitory effects of mTOR inhibitors on cell viability indicates that the proliferative effects of this growth factor are mediated by the PI3K/Akt/mTOR pathway. This hypothesis is further strengthened by the evidence that the employed Erk inhibitor does not influence IGF1 proliferative effects, indicating that the activation of the MAPK/Erk pathway may not be crucial for pituitary cell survival in response to IGF1. These data are in keeping with previous evidence showing the lack of correlation between MAPK and Cyclin D1 levels in human pituitary adenomas, indicating that an increased MAPK activity does not correspond to a proliferative drive in pituitary adenomas [18]. Our results confirm previous evidence showing the efficacy of mTOR inhibitors in reducing viability of pituitary cell lines and primary cultures [19, 21, 37], supporting the putative role of similar drugs in the medical therapy of aggressive pituitary tumors. Tretment with mTOR inhibitors may result in a reduced GH secretion due to the negative impact of mTOR inhibitors on somatotroph cell viability. However, we found that mTOR inhibitors do not directly affect GH secretion, when the results are normalized according to the number of viable somatotrope cells. The lack of efficacy of the employed mTOR inhibitors in reducing GH secretion prompts further research to identify novel strategies to limit GH hyper-secretion by neoplastic somatotrophs.

The previously demonstrated reduced expression of IGF1 receptor in somatotroph tumors [38], and the lack of evidence for IGF1 receptor mutations as putative drivers of

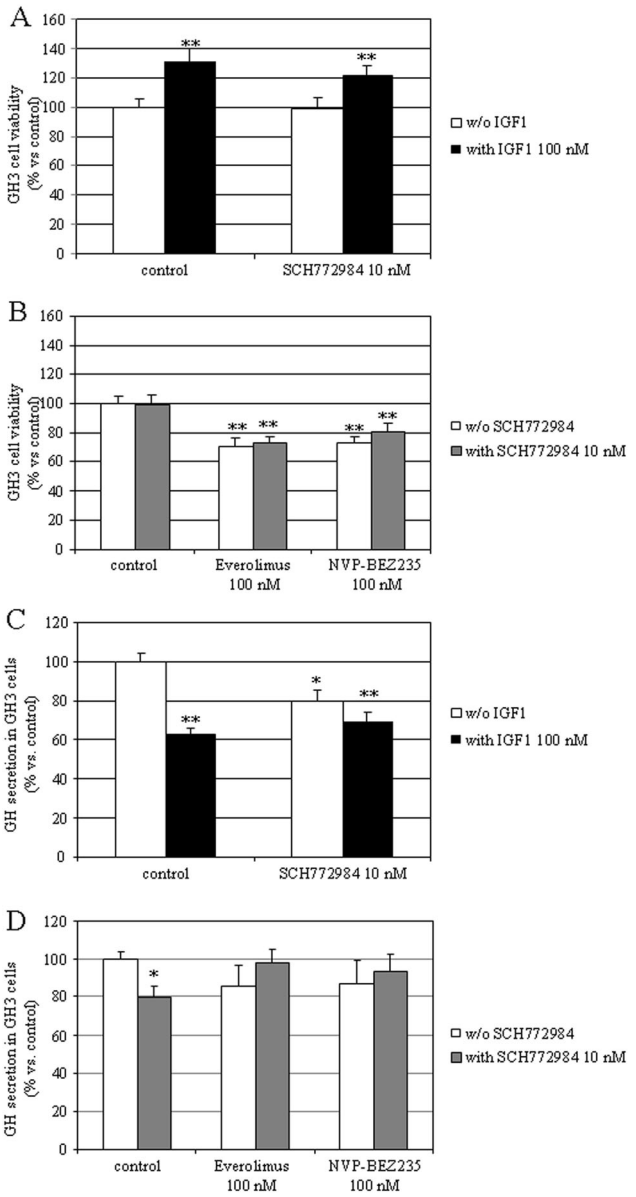

Fig. 4 Effects of IGF1 and of SCH772984 on cell viability and GH secretion in GH3 cells. GH3 cells were incubated in 96-well plates for 72 h in a culture medium supplemented without or with 10 nM SCH772984 in the absence (white bar) or in the presence of 100 nM IGF1 (black bars). **a** Cell viability was assessed as described in the Materials and methods section, and expressed as the mean ± SEM percent cell viability vs. untreated control cells. $^{**}P < 0.01$ vs. untreated control cells. **c** Medium was collected and GH secretion was evaluated by ELISA in three independent experiments in duplicate and expressed as the mean ± SEM percent GH secretion vs. untreated control cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. untreated control cells. GH3 cells were incubated in 96-well plates for 72 h in a culture medium supplemented without or with 100 nM Everolimus or 100 nM NVP-BEZ235 in the absence (white bar) or in the presence of 10 nM SCH772984 (gray bars). **b** Cell viability was assessed as described in the Materials and Methods section, and expressed as the mean ± SEM percent cell viability vs. untreated control cells. $^{**}P < 0.01$ vs. untreated control cells. **d** Medium was collected and GH secretion was evaluated by ELISA in three independent experiments in duplicate and expressed as the mean ± SEM percent GH secretion vs. untreated control cells. $^{*}P < 0.05$ vs. untreated control cells

acromegaly development [39] suggest that IGF1 receptor signaling is conserved. In these settings, the negative feed-back exerted by IGF1 on GH secretion may become a tool to disclose innovative approaches by exploring receptor down-stream signaling.

We therefore further tested IGF1 effects on GH secretion, confirming previous findings showing that IGF1 reduces GH secretion by rat pituitary cells [40], and that this reduction in secretory activity is mirrored by a reduction in GH mRNA levels. Romero et al. indeed previously showed that IGF1 inhibits GH expression by modulating GH promoter activity, providing evidence that cyclic AMP (cAMP) response element binding protein (CREB) binding protein (CBP) is a target of IGF1R signaling [32]. Since PI3K/Akt/mTOR pathway is a down-stream effector of IGF1, we employed two different mTOR inhibitors that were capable of blocking the inhibitory effects of IGF1 on GH mRNA. However, these drugs did not directly affect GH mRNA levels and secretion, indicating that they might not be useful to control GH hypersecretion. On the other hand, NVP-BEZ235, a dual PI3K-mTOR inhibitor, partially blocks the inhibitory effects of IGF1 on GH secretion and this effect is not observed after Everolimus treatment, suggesting a differential involvement of the PI3K/Akt/mTOR pathway in the negative feedback of IGF1 on GH expression and secretion. We therefore took advantage of this differential interaction to dissect the pathway and identify the key proteins necessary for IGF1 negative feed-back.

Previous studies showed that IGF1 rapidly induces Akt phosphorylation, which can be blocked by LY294002, a specific PI3K inhibitor [41]. We found similar results also in rat pituitary GH3 cells, where, in keeping with previous literature [42], we observed a rebound increase in pAkt levels after treatment with Everolimus, but not with NVP-BEZ235. The different findings of Gorshtein et al. [19] might be explained by the longer incubation time they employed when assessing pAkt levels in GH3 cells. In our settings, the effects of IGF1 and of mTOR inhibitors on pAkt levels were not affected by the PI3K inhibitor, supporting the hypothesis that PI3K is not involved in IGF1 signaling through Akt in GH3 cells. These data support the hypothesis that Akt may be directly activated by mTORC2, independently of PI3K, and that Akt activity is crucial in regulating IGF1 negative feed-back on GH secretion.

On the basis of these findings, we searched for the possible involvement of alternative pathways down-stream of IGF1R. We therefore investigated the involvement of the MAPK pathway by using a specific Erk inhibitor, SCH772984, and found that it does not influence IGF1 inhibitory effects on GH secretion. On the other hand, the Erk inhibitor directly reduced GH secretion by GH3 cells, confirming that the MAPK pathway has an important role in

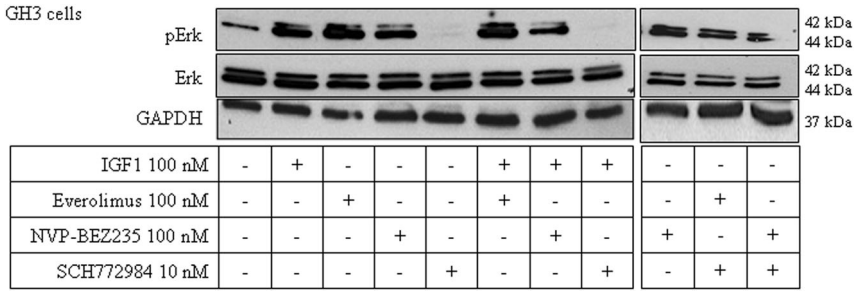

Fig. 5 Effects of IGF1 and of SCH772984 on Erk phosphorylation in GH3 cells. GH3 cells were incubated for 24 h in a culture medium supplemented with 100 nM IGF1, 100 nM Everolimus, 100 nM di NVP-BEZ235 or 10 nM SCH772984, alone or in combination; control cells were treated with vehicle solution. Total proteins were isolated and total and phosphorylated Erk protein levels were assessed by Western blot analysis. GAPDH is shown as a loading control. The displayed images refer to two different Western blot membranes

the regulation of pituitary hormone secretion [43]. Therefore, these data indicate that the MAPK pathway is not involved in the IGF1 negative feed-back loop, but support an independent role of this pathway in the regulation of GH secretion, as previously reported [44]. As a consequence, Erk may represent a druggable target in the medical treatment of GH excess. In addition, our data show that mTOR inhibitors interfere with the inhibitory effects of the Erk inhibitor on GH secretion, indicating the presence of a cross-talk between PI3K/Akt/mTOR and MAPK pathways [35]. This hypothesis is further strengthened by the evidence that the employed mTOR inhibitors induce an increase in pErk levels, which is not influenced by co-treatment with the Erk inhibitor. At the same time, our data suggest that a combination therapy with Erk inhibitors and mTOR inhibitors may not achieve a better control of hormone secretion in GH-secreting pituitary adenomas. In conclusion, we found that the negative IGF1 effect on GH secretion requires Akt activation, independently of PI3K. Despite the MAPK pathway is not involved in IGF1 negative feed-back on GH, it may represent a useful target for the therapeutic approach of GH excess. Moreover, our results indicate that mTOR inhibitors may be useful to control pituitary cell proliferation.

Further clarifying the mechanism of action of IGF1 on cell proliferation and hormonal secretion may be helpful to find a key molecule that mediates the effect of IGF1 on GH secretion, which modulation by a specific compound may represent an innovative pharmacological treatment.

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Compliance with ethical standards

Conflict of interest MCZ has received consultant fees from Novartis and Genzyme. EdU has received consultant fees from Novartis and grant support from Sanofi. The remaining authors declare that they have no competing interests.

References

1. N. Goldenberg, A. Barkan, Factors regulating growth hormone secretion in humans. Endocrinol. Metab. Clin. N. Am. **36**, 37–55 (2007)
2. M.D. Gahete, J. Córdoba-Chacón, Q. Lin, J.C. Brüning, C.R. Kahn, J.P. Castaño, H. Christian, R.M. Luque, R.D. Kineman, Insulin and IGF-I inhibit GH synthesis and release in vitro and in vivo by separate mechanisms. Endocrinology **154**, 2410–2420 (2013). https://doi.org/10.1210/en.2013-1261
3. A. Niiori-Onishi, Y. Iwasaki, N. Mutsuga, Y. Oiso, K. Inoue, H. Saito, Molecular mechanisms of the negative effect of insulin-like growth factor-I on growth hormone gene expression in MtT/S somatotroph cells. Endocrinology **140**, 344–349 (1999)
4. H. Sugihara, N. Emoto, H. Tamura, J. Kamegai, T. Shibasaki, S. Minami, I. Wakabayashi, Effect of insulin-like growth factor-I on growth hormone-releasing factor receptor expression in primary rat anterior pituitary cell culture. Neurosci. Lett. **276**, 87–90 (1999)
5. S. Yamashita, S. Melmed, Insulin like growth factor I regulation of growth hormone gene transcription in primary rat pituitary cells. J. Clin. Invest. **79**, 449–452 (1987)
6. S. Melmed, S. Yamashita, H. Yamasaki, J. Fagin, H. Namba, H. Yamamoto, M. Weber, S. Morita, J. Webster, D. Prager, IGF-I receptor signaling: lessons from the somatotroph. Recent Prog. Horm. Res. **51**, 189–215 (1996)
7. R.M. Luque, M.D. Gahete, R.J. Valentine, R.D. Kineman, Examination of the direct effects of metabolic factors on somatotrope function in a non-human primate model, Papio Anubis. J. Mol. Endocrinol. **37**(1), 25–38 (2006)
8. A. Colao, R.S. Auriemma, G. Lombardi, R. Pivonello, Resistance to somatostatin analogs in acromegaly. Endocr. Rev. **32**, 247–271 (2011). https://doi.org/10.1210/er.2010-0002
9. J.W. Unger, M. Betz, Insulin receptors and signal transduction proteins in the hypothalamo-hypophyseal system: a review on morphological findings and functional implications. Histol. Histopathol. **13**, 1215–1224 (1998)

10. A.I. Castillo, A. Aranda, Differential regulation of pituitary-specific gene expression by insulin-like growth factor 1 in rat pituitary GH4C1 and GH3 cells. Endocrinology **138**, 5442–5451 (1997)
11. M. Fernández, F. Sánchez-Franco, N. Palacios, I. Sánchez, G. Villuendas, L. Cacicedo, Involvement of vasoactive intestinal peptide on insulin-like growth factor I-induced proliferation of rat pituitary lactotropes in primary culture: evidence for an autocrine and/or paracrine regulatory system. Neuroendocrinology **77**, 341–352 (2003)
12. M. Fernández, F. Sánchez-Franco, N. Palacios, I. Sánchez, C. Fernández, L. Cacicedo, IGF-I inhibits apoptosis through the activation of the phosphatidylinositol 3-kinase/Akt pathway in pituitary cells. J. Mol. Endocrinol. **33**, 155–163 (2004)
13. H. Rubinfeld, A. Kammer, O. Cohen, A. Gorshtein, Z.R. Cohen, M. Hadani, H. Werner, I. Shimon, IGF1 induces cell proliferation in human pituitary tumors—functional blockade of IGF1 receptor as a novel therapeutic approach in non-functioning tumors. Mol. Cell. Endocrinol. **390**, 93–101 (2014). https://doi.org/10.1016/j.mce.2014.04.007
14. D. Romano, M. Pertuit, R. Rasolonjanahary, J.V. Barnier, K. Magalon, A. Enjalbert, C. Gerard, Regulation of the RAP1/RAF-1/extracellularly regulated kinase-1/2 cascade and prolactin release by the phosphoinositide 3-kinase/AKT pathway in pituitary cells. Endocrinology **147**, 6036–6045 (2006)
15. A.I. Castillo, R.M. Tolon, A. Aranda, Insulin-like growth factor-1 stimulates rat prolactin gene expression by a Ras, ETS and phosphatidylinositol 3-kinase dependent mechanism. Oncogene **16**, 1981–1991 (1998)
16. E. Monsalves, K. Juraschka, T. Tateno, S. Agnihotri, S.L. Asa, S. Ezzat, G. Zadeh, The PI3K/AKT/mTOR pathway in the pathophysiology and treatment of pituitary adenomas. Endocr. Relat. Cancer **21**, R331–R344 (2014). https://doi.org/10.1530/ERC-14-0188
17. M. Musat, M. Korbonits, B. Kola, N. Borboli, M.R. Hanson, A.M. Nanzer, J. Grigson, S. Jordan, D.G. Morris, M. Gueorguiev, M. Coculescu, S. Basuand, A.B. Grossman, Enhanced protein kinase B/Akt signaling in pituitary tumors. Endocr. Relat. Cancer **12**, 423–433 (2005). https://doi.org/10.1677/erc.1.00949
18. D. Dworakowska, E. Wlodek, C.A. Leontiou, S. Igreja, M. Cakir, M. Teng, M.I. Prodromou, N. Góth, S. Grozinsky-Glasberg, M. Gueorguiev, B. Kola, M. Korbonits, A.B. Grossman, Activation of RAF/MEK/ERK and PI3K/AKT/mTOR pathways in pituitary adenomas and their effects on downstream effectors. Endocr. Relat. Cancer. **16**, 1329–1338 (2009)
19. A. Gorshtein, H. Rubinfeld, E. Kendler, M. Theodoropoulou, V. Cerovac, G.K. Stalla, Z.R. Cohen, M. Hadani, I. Shimon, Mammalian target of rapamycin inhibitors rapamycin and RAD001 (everolimus) induce anti-proliferative effects in GH-secreting pituitary tumor cells in vitro. Endocr. Relat. Cancer **16**, 1017–1027 (2009). https://doi.org/10.1677/ERC-08-0269
20. E.A. Sajjad, G. Zieliński, M. Maksymowicz, Ł. Hutnik, T. Bednarczuk, P. Włodarski, mTOR is frequently active in GH-secreting pituitary adenomas without influencing their morphopathological features. Endocr. Pathol. **24**, 11–19 (2013). https://doi.org/10.1007/s12022-012-9230-y
21. M.C. Zatelli, M. Minoia, C. Filieri, F. Tagliati, M. Buratto, M.R. Ambrosio, M. Lapparelli, M. Scanarini, E.C. degli Uberti, Effect of everolimus on cell viability in nonfunctioning pituitary adenomas. J. Clin. Endocrinol. Metab. **95**, 968–976 (2010). https://doi.org/10.1210/jc.2009-1641
22. E. Gentilin, C. Di Pasquale, M. Rossi, F. Tagliati, T. Gagliano, R. Rossi, M. Pelizzo, I. Merante Boschin, E.C. degli Uberti, M.C. Zatelli, Igf-I influences everolimus activity in medullary thyroid carcinoma. Front. Endocrinol **6**, 63 (2015). https://doi.org/10.3389/fendo.2015.00063
23. T. Gagliano, M. Bellio, E. Gentilin, D. Molè, F. Tagliati, M. Schiavon, N.G. Cavallesco, L.G. Andriolo, M.R. Ambrosio, F. Rea, E. degli Uberti, M.C. Zatelli, mTOR, p70S6K, AKT, and ERK1/2 levels predict sensitivity to mTOR and PI3K/mTOR inhibitors in human bronchial carcinoids. Endocr. Relat. Cancer **20**, 463–475 (2013). https://doi.org/10.1530/ERC-13-0042
24. F. Tagliati, T. Gagliano, E. Gentilin, M. Minoia, D. Molè, E.C. degli Uberti, M.C. Zatelli, Magmas overexpression inhibits staurosporine induced apoptosis in rat pituitary adenoma cell lines. PLoS One **8**, e75194 (2013). https://doi.org/10.1371/annotation/3af6faef-d942-4221-8d1a-47ce279e462b
25. E. Gentilin, D. Molè, T. Gagliano, M. Minoia, M.R. Ambrosio, E. C. degli Uberti, M.C. Zatelli, Inhibitory effects of mitotane on viability and secretory activity in mouse gonadotroph cell lines. Reprod. Toxicol. **45**, 71–76 (2014). https://doi.org/10.1016/j.reprotox.2014.01.008
26. E. Gentilin, F. Tagliati, M. Terzolo, M. Zoli, M. Lapparelli, M. Minoia, M.R. Ambrosio, E.C. degli Uberti, M.C. Zatelli, Mitotane reduces human and mouse ACTH-secreting pituitary cell viability and function. J. Endocrinol. **218**, 275–285 (2013). https://doi.org/10.1530/JOE-13-0210
27. M.C. Zatelli, D. Piccin, C. Vignali, F. Tagliati, M.R. Ambrosio, M. Bondanelli, V. Cimino, A. Bianchi, H.A. Schmid, M. Scanarini, A. Pontecorvi, L. De Marinis, G. Maira, E.C. degli Uberti, Pasireotide, a multiple somatostatin receptor subtypes ligand, reduces cell viability in non-functioning pituitary adenomas by inhibiting vascular endothelial growth factor secretion. Endocr. Relat. Cancer **14**, 91–102 (2007)
28. M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **623**, 29 e45 (2001)
29. E. Gentilin, C. Di Pasquale, T. Gagliano, F. Tagliati, K. Benfini, M.R. Ambrosio, M. Bondanelli, E.C. degli Uberti, M.C. Zatelli, Protein Kinase C Delta restrains growth in ACTH-secreting pituitary adenoma cells. Mol. Cell. Endocrinol. **419**, 252–258 (2016). https://doi.org/10.1016/j.mce.2015.10.025
30. T. Gagliano, E. Gentilin, K. Benfini, C. Di Pasquale, M. Tassinari, S. Falletta, C. Feo, F. Tagliati, E. degli Uberti, M.C. Zatelli, Mitotane enhances doxorubicin cytotoxic activity by inhibiting P-gp in human adrenocortical carcinoma cells. Endocrine **47**, 943–951 (2014). https://doi.org/10.1007/s12020-014-0374-z
31. T. Gagliano, E. Gentilin, F. Tagliati, K. Benfini, C. Di Pasquale, C. Feo, S. Falletta, E. Riva, E. degli Uberti, M.C. Zatelli, Inhibition of epithelial growth factor receptor can play an important role in reducing cell growth and survival in adrenocortical tumors. Biochem. Pharmacol. **98**, 639–648 (2015). https://doi.org/10.1016/j.bcp.2015.10.012
32. C.J. Romero, E. Pine-Twaddell, D.I. Sima, R.S. Miller, L. He, F. Wondisford, S. Radovick, Insulin-like growth factor 1 mediates negative feedback to somatotroph GH expression via POU1F1/CREB binding protein interactions. Mol. Cell. Biol. **32**, 4258–4269 (2012). https://doi.org/10.1128/MCB.00171-12
33. M.C. Zatelli, M. Minoia, C. Martini, F. Tagliati, M.R. Ambrosio, M. Schiavon, M. Buratto, F. Calabrese, E. Gentilin, G. Cavallesco, L. Berdondini, F. Rea, E.C. degli Uberti, Everolimus as a new potential antiproliferative agent in aggressive human bronchial carcinoids. Endocr. Relat. Cancer **17**, 719–729 (2010). https://doi.org/10.1677/ERC-10-0097
34. I. Hers, E.E. Vincent, J.M. Tavaré, Akt signaling in health and disease. Cell. Signal. **23**, 1515–1527 (2011). https://doi.org/10.1016/j.cellsig.2011.05.004
35. A. Carracedo, L. Ma, J. Teruya-Feldstein, F. Rojo, L. Salmena, A. Alimonti, A. Egia, A.T. Sasaki, G. Thomas, S.C. Kozma, A. Papa, C. Nardella, L.C. Cantley, J. Baselga, P.P. Pandolfi, Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J. Clin. Invest. **118**, 3065–3074 (2008). https://doi.org/10.1172/JCI34739

36. S.L. Atkin, A.M. Landolt, R.V. Jeffreys, L. Hipkin, J. Radcliffe, C.R. Squire, M.C. White, Differential effects of insulin-like growth factor 1 on the hormonal product and proliferation of glycoprotein-secreting human pituitary adenomas. J. Clin. Endocrinol. Metab. **77**, 1059–1066 (1993)
37. V. Cerovac, J. Monteserin-Garcia, H. Rubinfeld, M. Buchfelder, M. Losa, T. Florio, M. Paez-Pereda, G.K. Stalla, M. Theodoropoulou, The somatostatin analog octreotide confers sensitivity to rapamycin treatment on pituitary tumor cell. Cancer Res. **70**, 666–674 (2010). https://doi.org/10.1158/0008-5472.CAN-09-2951
38. B. Kola, M. Korbonits, S. Diaz-Cano, G. Kaltsas, D.G. Morris, S. Jordan, L. Metherell, M. Powell, S. Czirják, G. Arnaldi, S. Bustin, M. Boscaro, F. Mantero, A.B. Grossman, Reduced expression of the growth hormone and type 1 insulin-like growth factor receptors in human somatotroph tumors and an analysis of possible mutations of the growth hormone receptor. Clin. Endocrinol. **59**, 328–338 (2003)
39. Y. Greenman, D. Prager, S. Melmed, The IGF-I receptor submembrane domain is intact in GH-secreting pituitary tumors. Clin. Endocrinol. **42**, 169–172 (1995)
40. S. Morita, S. Yamashita, S. Melmed, Insulin-like growth factor I action on rat anterior pituitary cells: effects of intracellular messengers on growth hormone secretion and messenger ribonucleic acid levels. Endocrinology **121**, 2000–2006 (1987)
41. R. Yao, G.M. Cooper, Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science **267**, 2003–2006 (1995)
42. K.E. O'Reilly, F. Rojo, Q.B. She, D. Solit, G.B. Mills, D. Smith, H. Lane, F. Hofmann, D.J. Hicklin, D.L. Ludwig, J. Baselga, N. Rosen, mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. **66**, 1500–1508 (2006)
43. A.I. Arroba, L.M. Frago, J. Argente, J.A. Chowen, Estrogen requires the insulin-like growth factor-I receptor for stimulation of prolactin synthesis via mitogen-activated protein kinase. J. Neuroendocrinol. **17**, 97–104 (2005)
44. F.Y. Gong, J.Y. Deng, Y.F. Shi, Mek and p38 MAPK-dependant pathways are involoved in the positive effect of interleukin-6 on human growth hormone gene expression in rat MtT/S somatotroph cells. Chin. Med. Sci. J. **23**, 73–80 (2008)