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β -arrestin expression in corticotroph tumor cells is modulated by glucocorticoids

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Abstract

Pituitary-directed medical treatment for Cushing's disease (CD) is currently represented by membrane receptor targeting drugs (somatostatin analogs and dopamine agonists). Somatostatin and dopamine receptors are regulated by β -arrestins, which have been shown to be differentially regulated by glucocorticoids in non-neuroendocrine cells. In this study we investigated the effects of glucocorticoids on β -arrestin expression in corticotroph tumor cells. First, AtT20 cells, a mouse model of CD, were exposed to dexamethasone (Dex) at different time points and β -arrestin expression was evaluated at mRNA and protein levels. Furthermore, β -arrestin mRNA expression was evaluated in 17 human corticotroph adenoma samples and correlated to patients' pre-operative cortisol levels. We observed that Dex treatment induced a time-dependent increase in β -arrestin 1 mRNA expression and a decrease in β -arrestin 2. The same modulation pattern was observed at protein level. Dex-mediated modulation of β -arrestins was abolished by co-treatment with mifepristone, and Dex withdrawal restored β -arrestin expression to basal levels after 72 h. The evaluation of β -arrestin mRNA in corticotroph adenomas from CD patients with variable disease activity showed a significant positive correlation between β -arrestin 1 mRNA and urinary cortisol levels. The effect of glucocorticoids on β -arrestin levels was confirmed by the analysis of two samples from a single patient, which underwent adenomectomy twice, with different pre-operative cortisol levels. In conclusion, glucocorticoids induce an inverse modulation of the two β -arrestin isoforms in corticotroph tumor cells. Since β -arrestins regulate membrane receptor functions, this finding may help to better understand the variable response to pituitary-targeting drugs in patients with Cushing's disease.

Key Words

- ▶ β -arrestins
- ▶ Cushing's disease
- ▶ corticotroph adenoma
- ▶ glucocorticoids
- ▶ AtT20 cells

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Introduction

The presence of an ACTH-secreting pituitary adenoma (corticotroph adenoma) in the anterior or intermediate lobe of the pituitary gland is the cause of Cushing's disease (CD), a severe systemic condition characterized by

a chronic state of hypercortisolism (Lacroix *et al.* 2015). Patients with CD display a number of peculiar clinical signs and symptoms, as well as an increased morbidity and mortality (Dekkers *et al.* 2007). Treatment of choice

for ACTH-secreting adenomas is nowadays represented by transsphenoidal adenomectomy, performed by an experienced neurosurgeon (Biller *et al.* 2008). Overall remission rate after first surgical approach ranges between 60 and 90%; however, recurrence rate is about 10–35% (Hassan-Smith *et al.* 2012, Dimopoulou *et al.* 2014). As such, it is clear that additional treatment options, other than surgery (e.g. radiotherapy, medical treatment, or even bilateral adrenalectomy), are often needed to achieve remission or, at least, disease control (Biller *et al.* 2008, Pivonello *et al.* 2015). Current available medical therapies for CD can be classified into three different groups, according to the targets: adrenal-blocking drugs, glucocorticoid receptor-blocking agents and pituitary-directed drugs (Feelders & Hofland 2013). In this context, it is well recognized that the ideal medical therapy should be represented by an effective pituitary-directed drug, able to control hormonal hyper-secretion and tumor growth and/or reduce the adenoma mass (the primary cause of the disease).

Treatment with somatostatin analogs (SSAs) and/or dopamine agonists (DAs) may result in the inhibition of ACTH secretion by the pituitary adenoma and, therefore, represent attractive drugs for CD treatment (Fleseriu 2014).

The presence of somatostatin receptors (SSTs, particularly SST₅) and dopamine receptors (DRs, with D2R as the predominant subtype) on the corticotroph adenoma cell membrane represents the pathophysiological rationale for SSA and DA treatment (Pivonello *et al.* 2004, de Bruin *et al.* 2009b, Chinezu *et al.* 2014). Noteworthy, both SSTs and DRs belong to the superfamily of G-protein-coupled receptors (GPCRs), which is known to be tightly regulated by β -arrestins in its desensitization and trafficking processes (Gurevich & Gurevich 2006, Gatto & Hofland 2011). Briefly, after ligand-mediated receptor phosphorylation by GPCR-related kinases, β -arrestins are recruited to the cell membrane, disrupt the coupling between the receptor and its related G-proteins, and subsequently drive the receptor to the endocytic machinery. As such, these molecules affect the rate of receptor internalization/recycling (Kim *et al.* 2004, Tulipano *et al.* 2004, Shenoy & Lefkowitz 2011). Furthermore, β -arrestins are also able to exert G-protein-independent signaling properties, such as the activation of the MAP kinase pathways (Shenoy & Lefkowitz 2011).

Interestingly, Oakley and co-workers demonstrated that glucocorticoids induce β -arrestin 1 and decrease β -arrestin 2 expression in multiple cell types, through direct effects on gene transcription (Oakley *et al.* 2012).

These authors also showed that the modulation of β -arrestins mediated by glucocorticoids may result in a shift of GPCR signaling from a G-protein-dependent setting to a more β -arrestin-dependent signaling (and *vice versa*), based on the peculiar affinity of the activated membrane receptor for the two β -arrestin isoforms (Oakley *et al.* 2012).

In this context, corticotroph adenoma cells of patients harboring an ACTH-secreting pituitary adenoma are exposed (by definition) to high levels of endogenous glucocorticoids. Moreover, patients with persistent active disease despite surgery, often undergo medical treatment in order to reduce/normalize the high circulating levels of glucocorticoids (Feelders & Hofland 2013). Therefore, during the management of patients with CD, corticotroph tumor cells can be exposed to different levels of endogenous glucocorticoids, which may affect the molecular characteristics of the adenoma, including the receptor pattern and the cognate intracellular pathways. Indeed, previous preclinical studies already showed that glucocorticoids induce a downregulation of SST₂ mRNA levels in ACTH-secreting adenoma cells (in both mouse cell models and human samples) (van der Hoek *et al.* 2005, van der Pas *et al.* 2013).

Since β -arrestins are key players in the regulation of GPCR functions, in the present study we aimed to investigate whether β -arrestin expression is modulated by glucocorticoid exposure in AtT20 corticotroph adenoma cells. Furthermore, we evaluated whether this process was reversible and directly dependent on glucocorticoid receptor activation. Finally, we characterized β -arrestin 1 and 2 expression in human corticotroph adenoma samples, and correlated expression with the disease activity (cortisol levels) at the time of surgical procedure.

Materials, methods and patients

Reagents

Dexamethasone (Dex) was obtained from the hospital pharmacy of the Erasmus Medical Center. The glucocorticoid-receptor (GR) antagonist mifepristone (RU-486) and the steroidogenesis inhibitor ketoconazole were purchased from Sigma-Aldrich. Of note, ketoconazole concentrations that were used (1 or 5 μ M) were 10- and 2-fold lower, respectively, than clinically achievable serum concentrations (approximately 10 μ M, although a significant proportion is protein bound) (Herrera-Martinez *et al.* 2019).

The anti- β -arrestin 1 polyclonal antibody was purchased from Invitrogen (Invitrogen Corporation), while the anti- β -arrestin 2 monoclonal antibody, the anti-SST₂ monoclonal antibody (clone UMB-1) and the anti-SST₅ monoclonal antibody (clone UMB-4) were purchased from Abcam (Abcam). The HRP-conjugated anti- β -actin monoclonal antibody was obtained from Santa-Cruz (Santa Cruz Biotechnology).

AtT20 cell culture

Murine corticotroph AtT20-D16v cells were purchased from ATCC and routinely grown in 75 cm² flasks (Corning Costar) containing Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (1 $\times$ 10⁵ U/L) and L-glutamine (2 mmol/L). Cells were cultured in a 5% CO₂ incubator at 37°C, medium was refreshed twice a week and cells were routinely passaged by trypsinization (trypsin 0.05%-EDTA 0.53 mM) weekly. Medium and supplements were obtained from Invitrogen.

AtT20 cell treatment for mRNA and protein expression studies

Cells were trypsinized, counted in a standard hemocytometer and seeded at different densities in 6- or 12-well plates (Corning), depending on the experimental design.

For 6–24 h incubation experiments, cells were seeded at a density of 4 $\times$ 10⁵ cells/well in 12-well plates, in 2 mL of culture medium. When longer incubation time was needed, 1.5 $\times$ 10⁵ cells/well (48–72 h incubation) or 7.5 $\times$ 10⁴ cells/well (72+72 h, medium and compounds refreshed at 72 h) were plated. After 24-h cell growth, medium was refreshed and incubations were started without (control, CTR) or with test substances (Dex 10 nM and/or RU-486 100 nM). The choice for Dex and RU-486 concentrations was based on our previously published studies, in which the effect of these compounds was investigated in neuroendocrine and corticotroph tumor cell lines (van der Hoek *et al.* 2005, de Bruin *et al.* 2009a). At different time points, medium was removed, cells were lysed on ice with a commercially available buffer (Roche Applied Science) and stored at –80°C until RNA isolation and mRNA analysis. For the evaluation of protein expression, cells were seeded at a density of 2 $\times$ 10⁵ cells/well in six-well plates and incubated without or with Dex 10 nM for 72 h. Medium was then removed and cells were lysed on ice using a specific lysis buffer

(see the 'Intracellular protein extraction' section). All experimental conditions were performed in triplicate and experiments repeated three times.

Patients, adenoma tissues and corticotroph primary cultures

Seventeen corticotroph pituitary adenoma tissues from 16 patients with CD were available after transsphenoidal surgery (one patient underwent surgery twice). The diagnosis of CD was based on clinical features, biochemical evaluation (elevated 24-h urinary free cortisol (UFC) and/or absence of a physiological circadian cortisol rhythm) and dynamic tests (e.g. inadequate suppression of plasma cortisol levels after administration of 1 mg Dex). The concomitant presence of a pituitary adenoma was confirmed by magnetic resonance imaging (MRI) or, when MRI was inconclusive (one case), by bilateral inferior petrosal sinus sampling.

Fourteen out of 16 patients (88%) received pre-surgical medical treatment with ketoconazole (alone or in combination). In our center, most patients are medically pre-treated with ketoconazole for 3 months before surgery to lower UFC levels. More in detail, 11 patients underwent ketoconazole monotherapy, 1 patient was treated with the combination of ketoconazole and cabergoline, while 2 patients included in this study participated in a clinical trial in which stepwise medical therapy with pasireotide, cabergoline, and ketoconazole was applied (Feelders *et al.* 2010).

Due to the different pre-surgical medical management of the patients and to their variable response to medical treatment, we had 11 samples from patients with pre-operatively normalized UFC ($\leq$ 1 upper limit of normality, ULN) and 5 samples from patients with elevated UFC ($>$ 1 xULN) before surgery. A further sample was obtained from a patient (n.15) untreated before surgery and with active disease (elevated salivary cortisol), although showing normal UFC due to impaired renal function. Therefore, in our analysis we included this patient in the hypercortisolemic group, together with those subjects showing a UFC $>$ 1 xULN (Table 1).

Approval from the Medical Ethical Committee of the Erasmus Medical Center, as well as informed consent to use the tumor tissues for research purposes were obtained. To check for purity of corticotroph adenoma tissues, growth hormone (GH)/pro-opiomelanocortin (POMC) mRNA expression ratios were calculated. All the adenomas included in the study had a GH/POMC ratio $<$ 10% compared with normal pituitary tissue and were

Table 1 General and biochemical characteristics of patients with Cushing's disease included in the study.

Patient	Gender	Age (years)	MRI	Pretreatment	Drug(s)	Pre-op UFC (xULN)
1	F	37	Micro	Yes	Keto	0.92
2	F	51	Micro	Yes	Keto	0.77
3	F	66	Micro	Yes	Keto + Cab	0.76
4	M	47	Micro	Yes	Pas + Cab + Keto	0.27
5	F	51	Micro	Yes	Keto	0.78
6	F	45	Micro	Yes	Keto	0.59
7	M	36	Macro	Yes	Keto	0.47
8	M	39	Macro	Yes	Keto	0.79
9	F	42	Micro	Yes	Pas + Cab + Keto	0.78
10	F	37	Micro	Yes	Keto	0.47
11	F	41	Macro	Yes	Keto	1.06
12 ^a	F	48	Macro	Yes	Keto	1.8 (I)
				Yes	Keto	4.9 (II)
13	M	70	Undetectable	Yes	Keto	1.3
14	F	41	Macro	Yes	Keto	0.26
15	F	63	Macro	No	–	0.51 ^b
16	F	56	Micro	No	–	2.1

Patient 15 had active disease at the time of surgery with evidence of elevated salivary cortisol levels and lack of plasma cortisol suppression after 1 mg dexamethasone test overnight. Bold values indicate patients with UFC levels >1 xULN at time of surgery.

^aPatient 12 underwent adenectomy twice: I, first surgery; II, second surgery. ^bLow UFC because of impaired renal function.

Cab, cabergoline; F, female; Keto, ketoconazole; M, male; Macro, macroadenoma; Micro, microadenoma; MRI, MRI at the time of diagnosis; Pas, pasireotide; Pre-op, preoperative; UFC, urinary free cortisol; ULN, upper limit of normality.

therefore considered as pure corticotroph adenoma tissues (de Bruin *et al.* 2009b).

Quantitative PCR

Quantitative PCR was performed according to a previously described method (Hofland *et al.* 2004). Briefly, to perform β -arrestin evaluation (intron spanning prime-probe sequences) in mouse AtT20 cells, total mRNA isolation was performed using the commercially available High Pure Isolation RNA Kit (Roche Applied Science). cDNA was synthesized from 500 ng of total mRNA, eluted in adequate amount of H₂O to reach a 20 μ L volume, using Oligo (dT)_{12–18} Primer. From human adenoma tissues we isolated poly A⁺ mRNA, instead of total mRNA (in order to have the possibility to evaluate SST expression later on, if necessary), using Dynabeads Oligo (dT)₂₅ (DynaL AS). cDNA was synthesized using the poly A⁺ mRNA, which was eluted from the beads in 40 μ L H₂O twice for 2 min

at 65°C, using Oligo (dT)_{12–18} Primer (Invitrogen), as previously described in detail (Hofland *et al.* 2004). Samples were analyzed on an ABI Prism 7900 Sequence Detection System (PerkinElmer) for real-time amplifications, according to manufacturer's protocol. The primer-probe sequences and the efficiencies of mouse β -arrestin 1 and 2 are reported in Table 2, while sequences, efficiencies and reaction conditions used for the detection of human β -arrestins, GH and POMC, as well as both human and mouse hypoxanthine guanine phosphoribosyl transferase (hppt) have been previously described (Hofland *et al.* 2004, de Bruin *et al.* 2009b, Gatto *et al.* 2013).

Intracellular protein extraction

After 24-h growth and a 72-h incubation without or with Dex (10 nM), AtT20 cells were lysed in lysis buffer (20 mM Tris-HCl, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.50% NP-40, 200 μ M Na₂VO₄, 200 μ M

Table 2 Primer-probe sequences for mouse β -arrestin 1 and β -arrestin 2.

Primer		Sequence (5'–3')	Efficiency
β -arrestin 1 (mouse)	fwd	CCATGGAGGAGGCTGATGA	2.00
	rev	CAGGAAGGGAGTCAGCGTATAGA	
	probe	FAM-TGGCACCCAGCTCAACATTCTGCA-TAMRA	
β -arrestin 2 (mouse)	fwd	CTGTGAGACAGTACGCCGACAT	1.89
	rev	TGAGCCACGGGACACTTGT	
	probe	FAM-TGCCTCTTCAGCACCGCGCAG-TAMRA	

fwd, forward; rev, reverse.

PMSF, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, pH 7.6) and subsequently centrifuged for 15 min (16,000g) at 4°C. The supernatant was collected and protein content was measured by use of Bio-Rad Protein assay (Bio-Rad).

Immunoblot analysis

Forty micrograms of intracellular proteins were fractioned on 10% SDS-PAGE and electrophoretically transferred to Hybond-C extra nitrocellulose membrane (GE Healthcare). Non-specific binding sites were blocked by treating membranes with Tris-buffered saline-Tween (TBS-T: 0.02 M Tris, 0.137 M NaCl, and 0.5% Tween 20; pH 7.6 with 1 M HCl), containing 10% BSA, for 1 h at 22°C on a rotating shaker. After blocking, membranes were incubated for 16 h at 4°C with primary antibodies to β -arrestin 1 (dilution 1:500), β -arrestin 2 (1:250), SST₂ (1:1000) and SST₅ (1:1000). Membranes were washed with TBS-T and then incubated for 1 h at 22°C with 1:5000 dilution of horseradish peroxidase (HRP)-linked anti-rabbit/mouse IgG.

For β -actin evaluation (used to normalize β -arrestin and SST expression), after blocking non-specific sites, membranes were incubated for 30 min, at room temperature, with a primary antibody (dilution 1:10.000) already conjugated with HRP.

Finally, blots were washed in TBS-T, immersed for 5 min in a detection solution and analyzed using a dedicated chemiluminescence imaging system (UVITEC Alliance, UVITEC, Cambridge, UK). Quantification of the digitized bands was performed by UN-SCAN-IT software (Silk Scientific, Orem, UT, USA) according to manufacturer's instructions.

Statistical analysis

Data were statistically analyzed using SPSS software, version 21.0 for Windows (SPSS), while graphs and figures were drawn by use of GraphPad Prism software, version 5.02 (GraphPad Software). Quantitative data are presented as mean $\pm$ s.e.m. for repeated experiments performed on AtT20 cell line, while median with range (minimum-maximum) was used for data from patients and adenoma samples. In this context, Kolmogorov–Smirnov test was used in order to check the normality of distribution of continuous variables. Between-group comparisons were analyzed by the two-tailed *t* test (normal distribution of the data), the Mann–Whitney *U* test (nonparametric data), or using one-way ANOVA followed by Newman–Keuls *post hoc* test (normal distribution, comparison between

more than two groups). Correlation coefficients were calculated by the Spearman rank order *R* (nonparametric data). Differences were taken to be statistically significant at $P < 0.05$.

Results

Effect of dexamethasone treatment on β -arrestin mRNA and protein expression

Basal β -arrestin mRNA expression in AtT20 cells is depicted in Fig. 1 (panel A). β -arrestin 1 mRNA levels are about 3-fold lower compared to β -arrestin 2 (ratio over *HPRT* 0.068 ± 0.006 vs 0.208 ± 0.01).

Dex treatment resulted in a time-dependent increase of β -arrestin 1 mRNA expression and a concomitant decrease in β -arrestin 2 mRNA levels (Fig. 1, panel B and C). In both cases, the maximum effect was observed after 72-h Dex incubation, with a β -arrestin 1 increase of $+76\% \pm 5.0$ ($P < 0.0001$) and a slight, but statistically significant, repression of β -arrestin 2 mRNA transcript ($-26\% \pm 2.0$, $P < 0.001$).

Figure 1

Basal β -arrestin mRNA expression in AtT20 cells (panel A) and β -arrestin mRNA modulation after dexamethasone (Dex) treatment (panels B–C). AtT20 cells were treated with Dex 10 nM for the indicated times, and β -arrestin expression was evaluated by RT-qPCR technique. Values represent mean $\pm$ s.e.m. β -arrestin mRNA expression in AtT20 cells (panel A) and percent mean change $\pm$ s.e.m. relative to control (panels B and C). Statistically relevant differences are reported in each graph as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. β -arrestin mRNA expression levels are normalized against the housekeeping gene *HPRT*. Experiments were repeated three times, in triplicate. CTR, control; DEX 10 nM, dexamethasone 10 nM; β -arr 1, β -arrestin 1; β -arr 2, β -arrestin 2.

The same trend of modulation was observed at protein level, evaluated by Western blot technique. AtT20 cells were treated for 72 h without or with Dex 10 nM. We observed an increase of β -arrestin 1 protein expression (normalized to β -actin) of $+37\% \pm 5.8$ ($P < 0.01$), and a lowering in β -arrestin 2 expression of $-35\% \pm 8.5$ ($P < 0.05$) (Fig. 2, panel A, B, C and D).

Interestingly, using the same experimental design described above, we found that somatostatin receptor subtype 2 (SST₂) protein expression was significantly downregulated after 72-h Dex exposure ($-35\% \pm 10$, $P < 0.01$), while SST₅ was not significantly affected ($-2\% \pm 7.1$) (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article). These data confirmed at the protein level previous results by our group, evaluating somatostatin receptor mRNA expression in AtT20 cells after glucocorticoid exposure ([van der Hoek *et al.* 2005](#)).

Of note, Dex treatment did not result in a significant modulation of both *hprt* mRNA and β -actin protein expression (housekeeping genes) (data not shown).

Figure 2

β -arrestin protein modulation in AtT20 cells after treatment with 72 h dexamethasone (10 nM) (panels A, B and C). β -arrestin protein expression was evaluated by Western blot technique. Values represent mean percent change $\pm$ S.E.M. relative to control. In panels B and D, a representative immunoblot for both β -arrestin 1 and 2 expression and modulation in AtT20 cells is shown. Each immunoblot represents a representative single experiment performed in triplicate. Experiments were repeated three times, in triplicate. Statistically relevant differences are reported in each graph as: * $P < 0.05$; ** $P < 0.01$. β -arrestin protein expression levels are normalized to β -actin. CTR, control; DEX 10 nM, dexamethasone 10 nM; β -arr 1, β -arrestin 1; β -arr 2, β -arrestin 2; W.B., Western Blot; KDa, kilodalton.

Effect of glucocorticoid receptor antagonist treatment on Dex-mediated β -arrestin modulation

AtT20 cells were treated for 72 h with Dex 10 nM, RU-486 100 nM or the combination of the two compounds. As shown in Fig. 3, co-treatment with the glucocorticoid receptor antagonist RU-486 was able to abolish the Dex-induced changes in β -arrestin mRNA expression. The significant upregulation of β -arrestin 1 mRNA and the decrease of β -arrestin 2 observed after Dex treatment was fully restored to control level in cells treated with the combination of Dex and RU-486.

In detail, β -arrestin 1 mRNA relative expression vs control was $+88\% \pm 13$ after 72 h Dex treatment and $+27\% \pm 2$ after Dex+RU-486 co-incubation ($P < 0.001$). On the other hand, Dex treatment resulted in a decrease of β -arrestin 2 levels vs control ($-16\% \pm 2$), which was completely abolished by RU-486 co-treatment ($+8\% \pm 2$, $P < 0.01$). Treatment with RU-486 alone did not result in significant changes of β -arrestin mRNA levels.

Reversibility of dexamethasone effect

To test whether the modulatory effect of Dex was reversible, we treated AtT20 cells for 72 h with or without Dex, after which the medium was refreshed and cells (both Dex treated and controls) were cultured for an additional 72 h in standard culture medium (washout period). As internal control for the effect of Dex, after the first 72 h part of Dex-treated and -untreated cells were lysed and β -arrestin mRNA levels were measured.

Figure 3

Antagonism of Dex-induced changes in β -arrestin mRNA expression in AtT20 cells by co-incubation with the glucocorticoid receptor antagonist RU-486. Cells were treated for 72 h without or with Dex 10 nM, RU-486 100 nM or their combination. Subsequently, β -arrestin mRNA levels were determined. Values represent mean percent change $\pm$ S.E.M. relative to control. Statistically relevant differences are reported in each graph as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. β -arrestin mRNA expression levels are normalized against the housekeeping gene *HPRT*. Experiments were repeated three times, in triplicate. CTR, control; DEX 10 nM, dexamethasone 10 nM; RU-486 100 nM, glucocorticoid receptor antagonist RU-486 100 nM; DEX + RU-486, DEX 10 nM co-incubated with RU-486 100 nM.

As shown in Fig. 4, we observed that β -arrestin mRNA levels approximated control values (untreated cells) after the 72 h washout from Dex. In detail, β -arrestin 1 mRNA expression increased up to $+76\% \pm 5$ vs control ($P < 0.001$) after 72 h Dex treatment, while after additional 72-h washout, the difference with related control was not statistically significant anymore ($+27\% \pm 6$). Likewise, Dex induced a significant reduction of β -arrestin 2 mRNA ($-31\% \pm 3$ vs control, $P < 0.01$), while at the end of washout period the basal expression was completely restored ($+5\% \pm 3$ vs control). Of note, β -arrestin 1 and β -arrestin 2 expression after 72-h Dex exposure (Dex 72 h) was statistically significant different (higher β -arrestin 1 and lower β -arrestin 2) compared to the evaluation performed after 72-h Dex treatment followed by 72-h washout (Dex 72 h + W.O. 72 h; Fig. 4).

Effect of ketoconazole and pasireotide on β -arrestin mRNA expression in AtT20 cells

Since in our center most patients with CD undergo pre-surgical treatment with ketoconazole (Table 1), we investigated whether medical treatment with this compound could directly affect β -arrestin expression in corticotroph cells. Therefore, AtT20 cells were treated with or without different concentrations of ketoconazole (1 μ M and 5 μ M, based on previous studies from our group

Figure 4

Reversibility of effect of Dex on β -arrestin modulation after 72 h glucocorticoid withdrawal in AtT20 cells. Cells were incubated with or without Dex 10 nM for 72 h. Part of the cells were lysed at that time, whereas other cells underwent prolonged culturing in normal, Dex-free medium, for additional 72 h. These cells were subsequently lysed (72 + 72 h time point). Cell lysates were analyzed for β -arrestin 1 and 2 mRNA expression at different time points. Each time point had its internal control condition. Values represent mean percent change $\pm$ S.E.M. relative to control. Statistically relevant differences are reported in each graph as: ** $P < 0.01$; *** $P < 0.001$. β -arrestin mRNA expression levels are normalized against the housekeeping gene *HPRT*. Experiments were repeated three times, in triplicate. CTR I, control 72 h; DEX 72 h, dexamethasone 10 nM for 72 h; CTR II, control 72 + 72 h; DEX + W.O., 72-h treatment with dexamethasone 10 nM followed by 72-h washout (incubation in standard culture medium); n.s., not statistically significant ($P \geq 0.05$).

Figure 5

Effect of ketoconazole on β -arrestin mRNA expression in AtT20 cells. Cells were incubated with or without ketoconazole at the concentration of 5 μ M or 1 μ M for 72 h. Ketoconazole was added in cells cultured in standard medium (panel A and B) or co-incubated with Dex 10 nM (panel C and D). Values represent percent mean change $\pm$ S.E.M. relative to control. β -arrestin mRNA expression levels are normalized against the housekeeping gene *HPRT*. Experiments were repeated three times, in triplicate. Differences were taken to be statistically significant at $P < 0.05$. n.s., not statistically significant. CTR, control; DEX 72 h, dexamethasone 10 nM; KETO, ketoconazole.

(van der Pas *et al.* 2012, Creemers *et al.* 2019, Herrera-Martinez *et al.* 2019)). As depicted in Fig. 5 (panels A and B), we observed that 72-h ketoconazole treatment did not significantly modulate β -arrestin mRNA expression in the AtT20 cell model. Furthermore, since ketoconazole has been demonstrated to act as a glucocorticoid antagonist (Loose *et al.* 1983) besides its properties as steroidogenesis inhibitor, we investigated its potential role in the inhibition of Dex-induced modulation of β -arrestin transcription. As depicted in panels C and D of Fig. 5, treatment with 1 μ M and 5 μ M ketoconazole did not significantly counteract the observed inverse modulation of β -arrestin 1 and 2 mRNA expression induced by Dex (72-h treatment).

Interestingly, two patients included in our series where also treated with pasireotide (in combination with ketoconazole and cabergoline). Since AtT20 express SSTs on cell membrane, we investigated whether different concentrations of pasireotide (1 nM and 10 nM) could affect β -arrestin mRNA expression. We found that 72 h pasireotide treatment did not significantly modulate β -arrestin 1 or β -arrestin 2 mRNA expression (Supplementary Fig. 2).

Of note, we did not test the potential effect of cabergoline, since wild-type AtT20 cells are known to have a lack of functional D2 receptor (Farrell *et al.* 1992, Ben-Shlomo *et al.* 2009, Occhi *et al.* 2014).

β -arrestin mRNA expression in human corticotroph adenoma cells

We measured β -arrestin mRNA levels (relative expression, normalized to *HPRT*) in 17 samples of human corticotroph adenomas obtained from 16 patients with CD. Of note, β -arrestin 2 mRNA was not measured in the adenoma sample from patient n.9 because of lack of sufficient material. As observed in mouse AtT20 cells, median (minimum to maximum) β -arrestin 1 levels were significantly lower compared to β -arrestin 2 (0.16 (0.007–0.87) vs 7.87 (0.42–28.5), respectively), although the expression of both molecules (particularly β -arrestin 2) was highly heterogeneous between the different samples (Fig. 6, panel A). Interestingly, we observed that in our corticotroph adenoma cohort the expression of β -arrestins was significantly higher compared to the evaluation of the same molecules previously performed by our group in a sample of somatotroph adenomas ($n=35$; published in Gatto *et al.* 2016, see Fig. 6, panels B and C).

However, based on the observations in AtT20 cells and to better understand whether the variable β -arrestin expression observed in our cohort was affected by UFC levels, we divided our samples into two groups: samples from patients with biochemical control before adenectomy (normalized UFC, ≤ 1 xULN; $n=11$) and samples from patients with elevated cortisol levels (pre-operative UFC >1 xULN, $n=5$, and elevated salivary cortisol level in 1 patient with impaired renal function; total $n=6$). Noteworthy, we observed that median β -arrestin 1 mRNA level was lower, although not reaching

statistical significance, in tumor tissues from patients with pre-operative biochemical control compared to those of patients with elevated pre-operative cortisol levels (relative expression, normalized to *HPRT*: 0.11 vs 0.27, $P=0.079$; Fig. 6, panel D). Of interest, despite the small sample size, setting the cutoff for active disease up to UFC >1.5 xULN (often used as reference values for patients' inclusion in clinical trials (Colao *et al.* 2012, Bertagna *et al.* 2014, Fleseriu *et al.* 2016)), β -arrestin 1 levels came out as significantly different in patients with active vs not active disease ($n=3$ vs 14; $P=0.031$). Furthermore, β -arrestin 1 mRNA expression was directly and significantly correlated with UFC level before surgery (Spearman's $\rho=0.50$, $P=0.047$; Fig. 6, panel F). On the other hand, median β -arrestin 2 mRNA expression was almost comparable in samples from patients with normal or elevated cortisol levels (relative expression, normalized to *HPRT*: 6.7 vs 8.0, $P=0.79$; Fig. 6, panel E). Moreover, no correlation was found between β -arrestin 2 mRNA and UFC at time of surgery (Spearman's $\rho=0.18$, $P=0.53$; data not shown). In this context, we have to highlight that β -arrestin 2 mRNA levels ranged from 0.42/*HPRT* up to 28.5/*HPRT*, thus showing a nearly 70-fold variability between the minimum and the maximum value observed in the different tumor samples analyzed.

As indicated above (Table 1), most of our patients ($n=14$, 88%) received pre-surgical medical treatment with ketoconazole, alone ($n=11$) or in combination with cabergoline ($n=1$) and cabergoline plus pasireotide ($n=2$). In order to investigate whether cabergoline and/or pasireotide treatment could affect β -arrestin mRNA expression in adenoma samples, we compared

Figure 6

β -arrestin mRNA expression in the seventeen corticotroph adenoma samples included in our study, together with its correlation with disease activity. Overall median (range) β -arrestin 1 and 2 mRNA expression is depicted in panel A. In panels B–C, a comparison between β -arrestin expression in corticotroph adenomas and in a series of somatotroph adenomas ($n=35$; see Gatto *et al.* 2016) is reported. panels D and E: β -arrestin 1 and 2 levels in adenoma samples from patients with normalized cortisol levels before surgery vs patients with active disease. panel F: significant positive correlation between β -arrestin 1 mRNA levels and patients' UFC before adenectomy. β -arrestin mRNA expression levels are normalized against the housekeeping gene *HPRT*. Differences were taken to be statistically significant at $P < 0.05$. n.s., not statistically significant; β -arr 1, β -arrestin 1; β -arr 2, β -arrestin 2; UFC, urinary free cortisol; ULN, upper limit of normality.

data from patients treated with ketoconazole alone with patients which underwent combined medical therapy (ketoconazole+cabergoline+pasireotide in 2 cases and ketoconazole+cabergoline in 1 case). We did not find a statistically significant difference between ketoconazole and combination therapy treated patients, although the number of patients treated with the combination medical therapy ($n=3$) was low. This analysis was performed considering all patients included in the study ($P=0.94$ for β -arrestin 1 and $P=0.78$ for β -arrestin 2) as well as including only patients with normal UFC levels before surgery, in order to minimize the impact of high cortisol levels on β -arrestin modulation ($P=0.92$ for β -arrestin 1 and $P=0.89$ for β -arrestin 2).

Finally, we were able to investigate β -arrestin expression in two different tumor samples obtained from a patient who underwent adenomectomy twice, during different phases of glucocorticoid exposure (mild first (UFC 1.8 ULN), and then severe hypercortisolism (UFC 4.9 ULN)). In line with the findings observed in AtT20 cells, we found a clear increase in β -arrestin 1 mRNA levels (about four-fold) and a 50% decrease in β -arrestin 2 levels in the second sample (severe hypercortisolism before surgery) compared to the first one (mild disease activity before adenomectomy) (Fig. 7, panels A and B).

Discussion

In this study, we characterized the expression of β -arrestin 1 and 2 in a murine corticotroph cell model, AtT20 cell line, as well as in seventeen samples of cultured human corticotroph adenoma cells. Moreover, we demonstrated in AtT20 cells that the glucocorticoid dexamethasone

(Dex) caused an inverse modulation of the two β -arrestin isoforms (namely, an increase of β -arrestin 1 and a decrease of β -arrestin 2 expression). This finding is in line with the previous report from Oakley and co-workers, which elegantly showed that glucocorticoids differently regulate β -arrestin gene expression in non-endocrine cell lines A549 and U2OS, as well as in mouse embryonic fibroblasts (Oakley *et al.* 2012). The observation that co-treatment with the glucocorticoid receptor antagonist RU-486 is able to abolish the effect of Dex in AtT20 cells, is in line with previous observations showing that, in non-neuroendocrine cell models, β -arrestin genes are direct targets of GR regulation (Oakley *et al.* 2012). Furthermore, we observed that Dex-mediated β -arrestin modulation in AtT20 cells is reversible upon withdrawal of glucocorticoid treatment. This latest finding is of particular interest from a translational perspective, since we can reasonably hypothesize that β -arrestin levels change in human corticotroph adenoma cells as well, after treatment and following lowering/normalization of cortisol levels in CD patients. Although we did not reproduce the Dex effects on AtT20 by use of other glucocorticoids, such as corticosterone or cortisol, it could be of interest aiming to further investigate the potential role of endogenous cortisol in human corticotroph adenoma cells.

In this context, we characterized β -arrestin mRNA expression in adenoma samples from a cohort of CD patients with a variable disease activity at time of surgery (measured by UFC levels in all but one case). We found that β -arrestin 1 levels directly correlated with patients' UFC values (x ULN), with higher β -arrestin 1 expression observed in corticotroph adenomas from patients with more active disease. Interestingly, this observation was in line with the above-described findings in AtT20 cells.

However, this was not the case for β -arrestin 2. Indeed, no significant differences were found between samples from patients with active or controlled disease. Of note, this observation is in contrast with the results found in AtT20 cells (higher cortisol levels, lower β -arrestin 2 mRNA) and with the expected modulation of β -arrestin 1 levels observed in corticotroph cells. A possible explanation for this discrepancy may reside in the considerable variability of β -arrestin 2 mRNA levels we found in the different corticotroph adenoma samples, irrespective of pre-surgical UFC levels. For example, even within the group of adenoma samples from biochemically-controlled patients, β -arrestin 2 levels showed a nearly 70-fold difference between the minimum and the maximum value observed. To date, we are not able to provide a clear-cut explanation for this finding, since no correlation was

Figure 7

Relative changes of β -arrestin expression in two adenoma samples obtained from patient $n = 12$, who underwent adenomectomy twice, at different pre-operative cortisol levels (first adenomectomy (I), pre-operative UFC 1.8 xULN; second surgery (II), UFC 4.9 xULN). β -arrestin mRNA expression levels are normalized against the housekeeping gene *HPRT*. UFC, urinary free cortisol; ULN, upper limit of normality.

found between β -arrestin 2 mRNA levels and patients' age, sex and/or tumor size. However, it is well known that CD is a condition caused by a partial glucocorticoid resistance at the pituitary level, resulting in the loss of POMC transcription suppression by circulating cortisol levels. In this light, we could hypothesize (although without a direct evidence) that some patients may harbor a more pronounced glucocorticoid resistance (Riebold *et al.* 2015) and that the expected downregulation of β -arrestin 2 in corticotroph cells could be lost in these subjects, since the resistance to glucocorticoid action is more reflected by a loss of GR-induced gene repression than activation (Barnes 2011).

On the other hand, we report that, in a single patient who underwent surgery twice during different phases of glucocorticoid exposure (thus excluding the 'inter-sample' variability) the modulation of β -arrestin 2 mRNA expression (as well as of β -arrestin 1) followed the same pattern as observed in murine AtT20 cells.

Furthermore, since 15 out of 17 adenoma samples included in our study underwent ketoconazole treatment (alone or in combination) before surgery, we tested AtT20 cells in order to investigate whether medical pre-treatment could have an impact on β -arrestin mRNA expression. We observed that ketoconazole did not significantly modulate β -arrestin expression in mouse corticotroph cells, both in basal conditions and co-incubated with Dex. Therefore, despite ketoconazole has been demonstrated to act also as a glucocorticoid-antagonist, it is less potent compared to mifepristone (Svec 1988). On the basis of our results we conclude that ketoconazole does not counteract the Dex-induced modulation of β -arrestin transcription in our experimental setting.

In wider terms, aiming to investigate the effects of glucocorticoid exposure on endocrine cells, previous studies from our group have already demonstrated, in both AtT20 cells and in corticotroph adenomas from CD patients, that exposure to high levels of glucocorticoids results in a down-regulation of SST₂ mRNA, while SST₅ and D2R mRNA expression seem not, or to a lesser extent, affected (van der Hoek *et al.* 2005, van der Pas *et al.* 2013). Of note, data on SST₂ modulation in AtT20 cells after Dex exposure was confirmed in the present study also at protein level, by use of Western blot technique.

In this light, low SST₂ expression in corticotroph adenomas has been the proposed biological basis to explain the lack of efficacy of first-generation SSAs in the management of CD.

However, since it is well established that β -arrestin expression may affect the responsiveness to

GPCR-targeting drugs (Bjork *et al.* 2013, Gatto *et al.* 2013), regulating membrane receptor desensitization and intracellular trafficking (Tulipano & Schulz 2007, Shenoy & Lefkowitz 2011), we could hypothesize that β -arrestin modulation, occurring in the presence of different glucocorticoid levels during the natural history (and the clinical management) of CD, might play a role in mediating tumor responsiveness to pituitary GPCR targeting drugs. In this context, these novel findings could help us to better explain some previous *in vitro* and *in vivo* experimental observations. Indeed, after Dex pre-treatment of AtT20 cell model, van der Hoek and co-workers observed a loss of efficacy of octreotide in reducing ACTH secretion, while the inhibitory effect of a selective SST₅ agonist was significantly increased (van der Hoek *et al.* 2005). The differential efficacy of octreotide was explained by a down-regulation of SST₂, but no clear explanation was provided for the enhanced activity of the SST₅-targeting agent. In this context, we want to highlight that SST₅ is considered a class A GPCR, known to mainly interact with β -arrestin 2, while SST₂ strongly interacts with both β -arrestin 1 and 2 (class B GPCR) (Tulipano *et al.* 2004). Accordingly, SST₅ binding to β -arrestin 2 but not to β -arrestin 1, and subsequent internalization have been demonstrated in a pituitary tumor cell model (Peverelli *et al.* 2008). Therefore, we could speculate that the enhanced effect observed for the SST₅ selective ligand might be due to the glucocorticoid related down-regulation of β -arrestin 2 in AtT20 cells, which may result in a reduced receptor desensitization and internalization. Moreover, in the same study, the authors show that the anti-secretory effect of the novel SST panligand pasireotide is not affected by Dex-pretreatment of AtT20 cells (van der Hoek *et al.* 2005). Since elegant *in vitro* studies have already demonstrated that SST activation by pasireotide results in a very low β -arrestin recruitment on cell membrane, compared to both octreotide and naïve somatostatin (Lesche *et al.* 2009, Kao *et al.* 2011), this could be a further explanation for the greater efficacy of pasireotide, compared to first-generation SSAs, observed in the medical treatment of CD (Colao *et al.* 2012). Obviously, the above hypotheses need to be confirmed in functional studies using β -arrestin- knockdown models and/or corticotroph tumor cells.

Furthermore, we have to highlight that β -arrestin 1 and 2 mRNA expression in corticotroph adenomas, even considering only those samples from patients with normalized pre-operative UFC, is significantly higher (about 10-fold) compared to β -arrestin levels measured in somatotroph adenomas ($n=35$) in a previous study

by our group (Gatto *et al.* 2016). Although corticotroph and somatotroph adenoma samples were not run in the same experiment, thus possibly increasing the variability between the two measurements, the values observed (by use the same technique, in the same laboratory and by the same operator) are supporting a reliable significant difference (e.g. median β -arrestin 1 mRNA expression in corticotroph adenomas 0.16/HPRT vs 0.013/HPRT in somatotropinomas).

Since we have demonstrated that high β -arrestin mRNA levels correlate with a reduced responsiveness to octreotide treatment in somatotropinomas (Gatto *et al.* 2013, 2016), the relatively high β -arrestin level found in corticotroph adenomas might contribute to explain, beyond the variable expression of SST₂ on cell membrane, the lack of efficacy observed for treatment with first-generation SSAs in CD patients.

Finally, a recent elegant study from Petrillo and colleagues demonstrated that β -arrestin 1 also acts as a regulator of GR, stabilizing GR protein expression throughout a fine-tuning modulation of receptor degradation (Petrillo *et al.* 2019). Therefore, in the setting of active CD, β -arrestin 1 upregulation may contribute to prevent glucocorticoid-induced GR downregulation, thus sustaining the detrimental effects of prolonged exposure to high cortisol levels in different cell types. On the contrary, the reduction/normalization of cortisol levels potentially achieved after CD treatment should result in a down-regulation of β -arrestin 1, leading to an increased GR turnover with a less effective ligand-bound receptor activity (Petrillo *et al.* 2019).

We are aware that our study has some limitations. First, although CD represents a rare disease, the relatively low number of human samples included in the study may limit the statistical power of the analyses performed in subgroups of patients (e.g. β -arrestin 1 mRNA expression in biochemically controlled vs uncontrolled patients). Furthermore, future studies will be needed to confirm also at protein level the direct positive correlation between β -arrestin 1 and UFC levels we observed as mRNA expression in our CD samples. In this context, Western blot technique could represent a first approach to investigate the modulation of β -arrestin protein expression in human corticotroph adenomas, although the paucity of fresh tissue usually available from CD patients represents a limitation. On the other hand, the use of immunohistochemistry would need a careful validation of different β -arrestin antibodies in a larger number of tissue samples, possibly comparing antibodies specific for a single β -arrestin isoform with antibodies

recognizing both β -arrestin isoforms, as performed by other authors in different tissues (El-Khoury *et al.* 2018).

Although AtT20 cells are a well-accepted cell model for corticotroph adenomas, translation of molecular findings from an immortalized cell line to its human tumor counterpart always needs to be carefully weighted. Particularly, in the present study we observed that ketoconazole treatment does not affect β -arrestin mRNA expression, both in basal conditions or in cells treated with Dex. However, ketoconazole has been shown to inhibit cell function as well as to exert pleiotropic effects in rat corticotroph cells (Stalla *et al.* 1988). Herein, also due to the high percentage of patients pretreated before adenomectomy, we were not able to provide direct evidences for a potential role of ketoconazole in the modulation of β -arrestin expression in human corticotroph primary cultures. Therefore, this point remains an open issue to be further investigated.

In conclusion, in this study we have demonstrated a differential glucocorticoid-mediated modulation of β -arrestin expression in murine corticotroph adenoma cells and, to the best of our knowledge, we have characterized for the first time β -arrestin mRNA levels in human corticotroph adenoma samples. It is well recognized (and confirmed in the present study) that the exposure to high levels of glucocorticoids can modulate SST₂ expression in corticotroph cells and herein we show that glucocorticoids can also significantly affect the expression of receptor-interacting molecules, such as β -arrestins. Moreover, due to the wide spectrum of action of activated GRs in the modulation of gene transcription, we cannot exclude that other molecular changes, possibly affecting GPCR targeting, may occur during the natural history of CD. Although all these findings need additional studies to be confirmed and deeper investigated, nowadays, when facing CD, we should think of a 'multi-faced' tumor, with peculiar molecular characteristics in the different clinical phases of the disease. This knowledge may be crucial in the planning of therapeutic strategies in this severe illness.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0311>.

Declaration of interest

F G has been a speaker for Novartis and has participated on advisory boards of Novartis, AMCo Ltd, and IONIS Pharmaceuticals. R A F received honoraria from Novartis and Ipsen. D F has been a speaker for and participated on advisory boards and received research grants from

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