Interferon- γ inhibits cellular proliferation and ACTH production in corticotroph tumor cells through a novel janus kinases–signal transducer and activator of transcription 1/nuclear factor-kappa B inhibitory signaling pathway

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Abstract

Interferon- γ (IFNG) is a cytokine that exerts potent anti-proliferative and tumoricidal effects in a variety of cancers. Moreover, IFNG modulates normal pituitary hormone secretion, and was shown to inhibit the expression of the ACTH precursor *POMC* in murine ACTH-secreting AtT-20 tumor cells. We have studied the functional role of IFNG on pituitary tumor cells, focusing on the involvement of IFNG in the molecular events leading to the control of *POMC* transcriptional repression. Herein, it is shown that IFNG inhibits AtT-20 tumor cell proliferation without inducing apoptosis. Unexpectedly, an activated janus kinases–signal transducer and activator of transcription (JAK–STAT1) cascade is required for

IFNG inhibitory action on *POMC* promoter activity. Factor-kappa B (NF- κ B) is necessary for the inhibitory action of IFNG on *Pomc* transcription, since loss of NF- κ B activity with I κ B super-repressor abolishes this effect. In addition, 1 and 2 IFNG receptor immunoreactivity was detected in human corticotropinoma cells. Interestingly, IFNG inhibits ACTH production from these cells in primary cell culture, without affecting basal ACTH biosynthesis in normal non-tumoral pituitary cells. In conclusion, our data show for the first time that *POMC* transcription can be negatively regulated by a JAK–STAT1 and NF- κ B-dependent pathway.

Journal of Endocrinology (2008) **199**, 177–189

Introduction

Cushing's disease is a severe clinical condition caused by hypersecretion of corticosteroids due to excessive adrenocorticotrophin (ACTH) secretion from a pituitary adenoma. In the last few years, there has been little progress elucidating the molecular mechanisms responsible for constitutive and autonomous ACTH secretion of pituitary corticotropinomas. As a consequence, no effective drug therapy is currently available, particularly if surgical excision is not successful.

ACTH biosynthesis is coordinately controlled by different corticotrophin-releasing hormone (CRH)-triggered transcription factors at the level of the proopiomelanocortin (*Pomc*) promoter. Two Nur DNA-binding sites have been identified on the *Pomc* promoter. The proximal binding sequence named NUR77-binding response element (NBRE) binds NUR77 or Nurr1 monomers. The distal Nur response element (NurRE), constituted two everted NBRE-related sites, binds NUR77

homodimers or NUR77/NURR1 heterodimers, and plays a dominant role in mediating stimulation by CRH (Murphy & Conneely 1997, Philips *et al.* 1997a, Maira *et al.* 1999). CRH also induces transcriptional activity of activating protein 1 (AP1) and cAMP-responsive element-binding protein 1 (CREB1), which have been proposed to be involved in *POMC* transcription at the level of the AP1 site located in the first exon (Boutillier *et al.* 1995, 1998). Recent studies have provided evidence that, in corticotrophs, the inhibition of nuclear factor-kappa B (NF- κ B) binding at its consensus binding site on the *POMC* promoter is required for the transcriptional activation of the *POMC* gene by CRH (Karalis *et al.* 2004). Additionally, a functional signal transducer and activator of transcription 1/3 (STAT1/3) low-affinity binding site overlapping in part with the NurRE was identified in the distal region of the *Pomc* promoter (Bousquet *et al.* 2000). A synergistic signaling by CRH and the pleiotropic cytokine, leukemia inhibitory factor (LIF), bridged by phosphorylated CREB1 at the NurRE–STAT element of the

POMC promoter was already described (Mynard *et al.* 2004). Transcription factors PITX1 and TBX19 are critical for terminal differentiation and identity of corticotroph cells. TBX19 activates *POMC* transcription in cooperation with PITX homeoproteins (Lamolet *et al.* 2001).

Cytokines play an important role in modulating normal pituitary hormone secretion (Besedovsky & del Rey 1996, Ray & Melmed 1997, Auernhammer & Melmed 2000, Arzt 2001, Nudi *et al.* 2005). Previous reports showed that interferon- γ (IFNG), a potent immunostimulatory cytokine, also plays a regulatory role in the adenohipophysis. Vankelecom *et al.* (1990, 1992) described that IFNG inhibits agonist-stimulated ACTH, prolactin (PRL), and growth hormone secretion in normal rat pituitary cell cultures via folliculostellate cells. On the other side, it was shown that IFNG increased PRL and IL6 production (Yamaguchi *et al.* 1991), but had no effect on unstimulated ACTH secretion (Holsboer *et al.* 1988) in rat pituitary cell cultures. *In vivo*, IFNG enhances human cortisol secretion without a comparable rise in ACTH secretion (Holsboer *et al.* 1988).

Beyond the modulatory role exerted by IFNG on normal pituitary hormone secretion, this cytokine displays anti-proliferative (Buszello 1995, Wu *et al.* 1996, Suk *et al.* 2001, Ikeda *et al.* 2002, Wall *et al.* 2003) and tumoricidal (Saiki *et al.* 1992, Belardelli 1995, Billiau 1996, Boehm *et al.* 1997) effects in a variety of cancers. However, the potential role of IFNG on tumor pituitary cells is poorly understood. It has been described that in corticotroph AtT-20 tumor cell line, IFNG decreases *POMC* promoter-driven expression of luciferase reporter gene (Katahira *et al.* 1998). Pointing to a role for IFNG as a para/autocrine pituitary factor, *Ifng* mRNA was detected by RT-PCR in unstimulated and lipopolysaccharide (LPS)-treated normal murine pituitary cells (Pitossi *et al.* 1997). Moreover, mRNA for IFNG receptor was expressed after LPS stimulation in the pituitary gland from young and old mice (Utsuyama & Hirokawa 2002).

IFNG acts through a transmembrane receptor composed by two subunits: the ligand-binding subunit 1 (IFN- γ R1 and the signal-transducing subunit 2 (IFN- γ R2; Aguet *et al.* 1988, Kumar *et al.* 1989, Hemmi *et al.* 1994). Binding of IFNG to its receptor activates the receptor-associated Janus kinases (JAK1 and JAK2), allowing the recruitment and phosphorylation of STAT1. Phosphorylated STAT1 undergoes dimerization, translocates to the nucleus, and regulates gene expression (Ramana *et al.* 2002, Platanias 2005). Negative regulation of the JAK-STAT pathway involves several inhibitory mechanisms, such as binding to receptor sites and inactivation of JAKs by suppressor of cytokine (SOCS; Aaronson & Horvath 2002). STAT3 can also be weakly activated by IFNG (Qing & Stark 2004). STAT1 and STAT3 have similar structures, both are phosphorylated upon cytokine stimulation, and both form dimers, move to the nucleus, bind to γ -activated sequence elements, and activate transcription of many genes (Schindler & Darnell 1995, Darnell 1997, Levy & Darnell 2002). STAT1 and 3 dimers bind selectively to very similar but not identical elements (Horvath *et al.* 1995, Seidel *et al.* 1995).

In this work, we studied the role of IFNG on the molecular events leading to the control of *POMC* transcriptional repression. The rationale for choosing IFNG was both its inhibitory action on *POMC* transcription and its potent tumoricidal/antiproliferative effects. Herein, we demonstrated that in the pituitary corticotroph tumor cell line AtT-20, IFNG-induced activation of the JAK-STAT1 cascade results in *Pomc* promoter repression through a NF- κ B-mediated mechanism. Moreover, IFNG inhibits ACTH production in AtT-20 cells and in human corticotropinomas, but not in the primary culture of normal murine pituitary cells. Besides that, IFNG inhibits proliferation in AtT-20 cells.

Materials and Methods

Cell culture

Cells were kept at 37 °C in 5% CO₂. AtT-20 pituitary corticotroph tumor cells (Utsuyama & Hirokawa 2002) were cultured in 45 cm³ culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 10⁵ U/l penicillin/streptomycin. AtT-20 cells were distributed for cell growth and hormone secretion studies in 96-well plates (1×10⁵ cells/ml). For western blot analysis and luciferase assay, the cells were seeded onto 6-well plates (3.5×10⁵ cells/ml).

ACTH-secreting pituitary tumors were obtained from patients with Cushing's disease. Pituitary cell culture from human pituitary tumors or mice pituitary glands obtained from adult mice (20 g) was performed as described previously (Paez-Pereda *et al.* 2001). The tissue was washed with preparation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 10 mM glucose, and 15 mM HEPES (pH 7.3)). Sliced fragments were dispersed in preparation buffer containing 4 g/l collagenase (Cooper Biochemicals, Malvern, PA, USA), 10 mg/l DNase II, 0.1 g/l soybean trypsin inhibitor, and 1 g/l hyaluronidase. Dispersed cells were centrifuged and resuspended in DMEM supplemented with 2 mM essential vitamins, 5 mg/l insulin, 20 mg/l selenium, 5 mg/l transferrin, 30 nM triiodothyronine (Henning, Berlin, Germany), 10% FCS, and 1×10⁵ U/l penicillin-streptomycin. Mice and human pituitary cells were seeded onto 48-well (1×10⁵ cells/ml) and 96-well plates (1×10⁵ cells/ml) respectively.

The cells were treated as indicated in each experiment with recombinant human or mouse IFNG (Roche) and human/rat CRH (Bachem, Heidelberg, Germany). Cycloheximide was obtained from Sigma-Aldrich.

Immunohistochemistry

Human pituitary tissue was obtained from autopsies performed 12–16 h post-mortem in healthy subjects after accidental death. Experiments were performed according to the guidelines of the Ethical Committee of the Max Planck Institute for using autopsy and biopsy materials. Eight micrometer sections were

cut in a cryostat and fixed in cold 4% phosphate-buffered paraformaldehyde (Sigma). IFNG receptor subunits were detected using anti-IFN- γ R α (1:1500; Acris, Hiddenhausen, Germany) and anti-IFN- γ R β (1:800; Santa Cruz Biotechnology, Santa Cruz, CA, USA), in combination with anti-rabbit biotinylated IgG (1:800; Santa Cruz Biotechnology) and avidin–biotin–peroxidase complex with diaminobenzidine (Vector Laboratories, Burlingame, CA, USA).

ACTH was detected with a mouse mAb (Dako, Hamburg, Germany) in combination with anti-mouse IgG and mouse alkaline phosphatase (AP)–anti-AP complex (Sigma) with Vector Red, according to the manufacturer's instructions.

Cell proliferation and viability assays

After plating for 24 h, the cells were washed and incubated in DMEM with 2% FCS. The next day the cells were stimulated with 1–100 U/ml murine recombinant IFNG, between 1 and 5 days, as indicated. A cell proliferation reagent WST-1 assay (Roche Molecular Biochemicals) was used to measure cell proliferation and viability, following the manufacturer's instructions (Paez-Pereda *et al.* 2000). Acridine orange–ethidium bromide staining was used to rule out toxic effects.

Hormone measurements

After plating for 48 (AtT-20 cells) or 72 h (murine and human pituitary cells), the cells were washed and incubated in DMEM with 2% FCS. The next day the medium was changed and the cells were stimulated with 1–100 U/ml murine recombinant IFNG for 24 h as indicated. Samples were stored at -80°C until measurement of ACTH was performed. ACTH was measured by RIA as described previously (Paez-Pereda *et al.* 2000).

Transfection assays

The *Pomc*-Luc plasmid, kindly provided by Dr Low, Oregon Health and Science University, Portland, OR, USA (Liu *et al.* 1995), containing the luciferase gene under the control of 770 bp of the rat *Pomc* promoter includes all the necessary sequences for the expression and regulation of *Pomc*. The AP1–Luc construct contains seven repeats of the AP1-responsive sequence (Stratagene, La Jolla, CA, USA). The NurRE–Luc and NBRE–Luc constructs contain three copies of the NurRE or NBRE coupled to the minimal *Pomc* promoter ($-34/+63$) and the complete *Pomc*-mut–NurRE promoter contains a mutant NurRE. The NurRE, NBRE, and the *Pomc*-mut–NurRE–Luc plasmid were provided by Dr Drouin, Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de Montréal, Québec, Canada. The activity of NF- κ B was specifically suppressed employing transfections with the I κ B α super-repressor construct containing a mutated site at Ser 32 and Ser 36, which impedes phosphorylation and proteolysis and therefore prevents nuclear translocation of NF- κ B (Dr

altschmidt, Institut für Neurobiochemie, University of Witten/Herdecke, Witten, Germany). The *Pomc*-mut–NF- κ B, kindly provided by Dr Karalis, Division of Endocrinology, Children's Hospital, Boston, MA, USA (Karalis *et al.* 2004), contains a mutated NF- κ B-binding site. SOCS1 expression vector was kindly provided by Willson, The Walter and Eliza Hall Institute of Medical Research and the Cooperative Research Centre for Cellular Growth Factors, PO Royal Melbourne Hospital, Parkville, Australia (Starr *et al.* 1997). κ B–Luc was provided by Dr Bell, Mayo Clinic, Rochester, MN, USA. STAT–Luc reporter plasmid containing three repeated STAT sites from the *Ly6e* promoter cloned into pZLuc–TK plasmid (Addgene plasmid 8689) and STAT3 pcDNA3 (Addgene plasmid 8706), provided by Darnell (Zhong *et al.* 1994, Wen *et al.* 1995), were obtained from Addgene, Cambridge, MA, USA. The pEFBOS–HA–STAT1 plasmid was kindly provided by Dr Hirano, Osaka University Medical School, Osaka, Japan (Nakajima *et al.* 1996). Site-directed mutagenesis was performed in order to obtain the *Pomc*-mut–STAT plasmid. The sequence of the STAT-binding site on the *Pomc* promoter was, for the STAT-binding site, TGCCAG–GAA, and for the mutated binding site, TGCCAGCGG (Mynard *et al.* 2004). The mutated plasmid was sequenced for verification.

Cell transfection was performed using Lipofectamine (Invitrogen). Twenty-four hours after plating, the cells were transfected for 6 h in OptiMEM medium using 10 μ l Lipofectamine per well and a 1.5 μ g total plasmid DNA. Transfection efficiency was determined using a plasmid containing the CMV promoter driving the Renilla reporter gene. After 18 h in the culture medium, the cells were incubated for 6, 18, or 24 h with IFNG, CRH, or both. At the end of the treatment, the protein lysate was collected and luciferase and Renilla activities were measured by the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. The results are ratios of luciferase to Renilla activity. Results are expressed as fold induction with respect to basal.

Real-time quantitative reverse transcription PCR

Total RNA was isolated from AtT-20 cells after 30-min cycloheximide (10 μ g/ml) and 18-h IFNG stimulations, as well as from control cells (18-h IFNG). Each group consisted of three experimental independent subjects. RNA was extracted using TRIzol reagent (Invitrogen), following the manufacturer's instructions. cDNA was synthesized from 1 μ g total RNA using the Superscript II Reverse Transcriptase (Invitrogen). PCR amplifications were performed using the LightCycler 2.0 Real-Time PCR Systems and the QuantiFast SYBR Green PCR (Qiagen), according to the manufacturer's protocol. The primers used for the *Pomc* quantification are as follows: forward, ATAGATGTGTGGAGCTGGTGC; reverse, GGCTCTGG–ACTGCCATCTC. Quantification was carried out using a dilution standard from pooled sample probes. Relative expression was determined by normalization to hypoxanthine guanine phosphoribosyl transferase (*Hprt*; forward,

ACCTCTCGAAGTGTTGGATACAGG; reverse, CTTGC-GCTCATCTTAGGCTTTG).

Western blot

Western blot was performed as described previously (Paez-Pereda *et al.* 2001, Giacomini *et al.* 2006). Briefly, AtT-20 cells were washed with PBS and cell lysates were collected and analyzed by western blot. Equal levels of protein were electrophoresed by 10% SDS-PAGE. Proteins were blotted onto nitrocellulose blotting membranes (Sigma) using standard procedures and the following antibodies were added: IFN- γ R1 (CD119; Acris) and IFN- γ R2 (Santa Cruz Biotechnology). For the expression of P-STAT1 and P-STAT3 (Santa Cruz Biotechnology), AtT-20 cells were first incubated with IFNG (10 U/ml) for 30 min or 3 h. β -ACTIN levels were analyzed using a monoclonal anti- β -ACTIN antibody (Chemicon, Temecula, CA, USA).

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Differences were assessed by one-way ANOVA in combination with Scheffé's test, considering $P < 0.05$ as significant.

Results

Expression of IFNG receptors and activation of JAK-STAT pathway by IFNG in AtT-20 corticotroph cells

The expression of IFN- γ R1 and 2 in the corticotroph cell line AtT-20 was examined by western blot analysis. Both components of the receptor complex were expressed on AtT-20 pituitary corticotroph tumor cells, as shown in Fig. 1A.

To determine the functional significance of pituitary IFNG receptors, we first analyzed the effects of IFNG on the activation of the JAK-STAT cascade. IFNG treatment for up to 3 h stimulated tyrosine phosphorylation of corticotroph STAT1 (Fig. 1B). STAT3 was also transiently phosphorylated in response to IFNG in AtT-20 cells but its phosphorylation was less prolonged than the STAT1 phosphorylation (Fig. 1B).

Accordingly, IFNG increased STAT-dependent transcriptional activity (Fig. 1C).

IFNG inhibits *Pomc* transcription in AtT-20 cells

Since a functional STAT1/3 low-affinity binding site was identified in the *Pomc* promoter and we found that STAT1 was activated by IFNG in these cells, we decided to investigate the effect of IFNG on *Pomc* transcriptional activity. AtT-20 cells were transiently transfected with the *Pomc* promoter reporter plasmid (*Pomc*-Luc) and treated with IFNG for different stimulation times, as indicated in Fig. 2A.

IFNG treatment from 18 h onward, and in a time-dependent manner, inhibits *Pomc* promoter-dependent

Figure 1 Expression of IFNG receptors and STAT activation by IFNG in tumor mouse corticotroph AtT-20 cells. (A) Expression of IFNG receptor 1 and 2 was determined by western blot using cell lysates from AtT-20 cells and splenocytes as positive control. One representative experiment is shown ($n = 3$). (B) Expression of P-STAT1 and P-STAT3 was determined by western blot analysis after treatment with IFNG (10 U/ml) for 30 min and 3 h. One representative experiment is shown ($n = 3$). (C) Transcriptional activity of STAT-Luc in transiently transfected AtT-20 cells after IFNG treatment for 6 h. *** $P < 0.001$. Values indicate the mean $\pm$ S.D. of triplicates from one representative experiment ($n = 3$). Results are expressed as the fold induction over the respective basal value, arbitrarily taken as 1.

transcription (Fig. 2A). A dose-response curve was obtained after treatment for 24 h. Even using a low IFNG dose of 1 U/ml, there is a significant inhibition of the transcriptional activity driven by the *Pomc* promoter (Fig. 2B).

In parallel, we performed a reverse transcriptase real-time PCR of endogenous *Pomc* mRNA from AtT-20 cells treated with IFNG (10 U/ml). Eighteen hours of treatment with IFNG reduced endogenous *Pomc* gene transcription (*Pomc*/*Hprt1* (mRNA): basal 0.83 ± 0.02 versus IFNG 0.62 ± 0.09 , $P < 0.05$).

Figure 2 IFNG inhibits *Pomc* transcription in AtT-20 cells. Cells were transiently transfected with *Pomc*-Luc and treated with (A) 10 U/ml IFNG in a time-course-dependent manner. * $P < 0.05$, ** $P < 0.01$ versus basal time point. (B) IFNG (1, 10, and 100 U/ml) for 24 h. ** $P < 0.01$, *** $P < 0.001$ versus basal. Luciferase activity was measured as described in the Materials and Methods section. Values indicate the mean $\pm$ s.d. of triplicates coming from one representative experiment ($n = 3$). Results are expressed as the fold induction over the basal value, arbitrarily taken as 1.

Inhibition of *Pomc* gene expression by IFNG is JAK–STAT dependent

To analyze whether the inhibition on *Pomc* transcriptional activity by IFNG is mediated by the JAK–STAT pathway, AtT-20 cells were transfected with an SOCS1 expression vector, which inhibits cytokine-induced JAK–STAT. SOCS1 overexpression in AtT-20 cells significantly blocked the IFNG induced inhibition of the *Pomc* promoter activity (Fig. 3A), indicating that this effect is JAK–STAT dependent.

To test the role of STAT1 and STAT3 in *Pomc* promoter transcriptional activity in response to IFNG, AtT-20 cells were cotransfected with the *Pomc*-Luc reporter plasmid and the STAT1 and/or STAT3 expression plasmids and the cells were treated with IFNG for 24 h. Luciferase activity showed that STAT1 but not STAT3 inhibits the *Pomc* transcriptional activity and this effect was significantly enhanced in the

Figure 3 IFNG inhibition on *Pomc* transcription is reverted by SOCS1 and is STAT1 dependent. AtT-20 cells were cotransfected with (A) *Pomc*-Luc and SOCS1 expression vector. The empty vector was used as control. Cells were treated with IFNG (10 U/ml) for 24 h. *** $P < 0.001$; IFNG versus basal and SOCS1 + IFNG versus IFNG alone. (B) *Pomc*-Luc and STAT 1 and/or STAT3 expression plasmids. Cells were treated with IFNG (10 U/ml) for 24 h. *** $P < 0.001$ IFNG or LIF versus respective basal and STAT1 versus control plasmid in basal conditions. ** $P < 0.01$ and LIF versus respective basal. (C) *Pomc*-Luc or *Pomc*-mut-STAT and treated with IFNG (10 U/ml) for 24 h. *** $P < 0.001$ versus basal. Luciferase activity was measured as described in the Materials and Methods section. Values indicate the mean $\pm$ s.d. of triplicates from one representative experiment ($n = 3$). Results are expressed as the fold induction over basal value, arbitrarily taken as 1.

presence of IFNG (Fig. 3B). Even more STAT3 overexpression blocked the IFNG-mediated inhibition of *Pomc*. Concomitant STAT1 and STAT3 overexpression abolished the STAT1 inhibitory effects on basal *Pomc* transcription. Moreover, STAT3 overexpression also abolished STAT1-mediated inhibitory action of IFNG on the *Pomc* promoter (Fig. 3B). These results suggest a dual action of STAT1 and STAT3 in corticotroph. Interestingly, despite the STAT3 dependency for LIF action on corticotroph (Bousquet & Melmed 1999), in the presence of STAT1, LIF increased *Pomc* transcriptional activity (Fig. 3B). Incubation for 24 h with LIF did not increase *Pomc* promoter activity, since LIF activation has a maximal effect between 2 and 6 h after treatment

(Mynard *et al.* 2002). Nevertheless, STAT3 but not STAT1 overexpression sensitized the cells to LIF, demonstrating a STAT3 dependency for long-lasting effects of LIF.

Next, we analyzed whether STAT1-binding activity to the *Pomc* promoter is important for the inhibition of the *Pomc* gene expression by IFNG. For this purpose, the *Pomc* promoter from the *Pomc*-Luc construct was mutated at the STAT-binding site according to Mynard *et al.* (2004). AtT-20 cells were transfected with either the intact or mutated reporter gene. The mutation at the STAT-binding site of the *Pomc* promoter does not abolish the inhibition of the transcriptional activation of the *Pomc* gene by IFNG treatment (Fig. 3C). Thus, *Pomc* gene repression by IFNG does not involve STAT1 binding to target DNA consensus sequences on the *Pomc* promoter.

IFNG does not affect basal NurRE, NBRE, AP1, and CRE transcriptional activity in AtT-20 cells

To elucidate whether IFNG also modulates elements on the *Pomc* promoter, which are involved in *Pomc* induction by CRH, we used luciferase reporters for NurRE, NBRE, AP1, and CRE. In all these experiments, CRH was used as positive control for reporter induction. We found that 6-h incubation with IFNG inhibits CRH-induced NurRE and NBRE transcriptional activities without affecting the basal

transcription (Fig. 4A and B). Similar results were obtained when the cells were treated during 18 h (data not shown). Furthermore, using a reporter containing the *Pomc* promoter mutated on the NurRE site, it was observed that IFNG still inhibits basal *Pomc* transcription (Fig. 4C). These data indicate that the inhibition of the *Pomc* promoter by IFNG is mediated, at least in part, by NUR77/Nurr1 when CRH is present but not under basal conditions.

The AP1- and CRE-dependent transcription was not affected by IFNG in AtT-20 cells in any condition tested (Fig. 4D and E respectively).

*NF- κ B is associated with the transcriptional inhibition of *Pomc* by IFNG*

Considering that NF- κ B has been described to exert transcriptional inhibitory action on the CRH-induced *Pomc* promoter (Karalis *et al.* 2004) and to elucidate the possible role of NF- κ B on IFNG inhibitory effects, we evaluate the *Pomc* promoter transcriptional activity in tumor corticotroph AtT-20 cells in the presence of a super-repressor form of I κ B α , resistant to both phosphorylation and proteolytic degradation. The overexpression of the repressor abolished completely the IFNG-mediated inhibition of *Pomc* transcriptional activity in basal conditions (Fig. 5A). Furthermore, the inhibition of NF- κ B by the pharmacological inhibitor BAY 11 7082 ((E)-3-(4-

Figure 4 In AtT-20 cells, IFNG inhibits NurRE- and NBRE-dependent transcription in the presence of CRH without affecting basal transcription. Cells were transiently transfected with (A) NBRE-Luc, (B) NurRE-Luc, (C) *Pomc*-mut-NurRE-Luc, (D) AP1-Luc, and (E) CRE-Luc, and treated with IFNG (10 U/ml) and/or CRH (100 nM) for 6 h except *Pomc*-mut-NurRE-Luc-transfected cells that were treated for 24 h. *** $P < 0.001$ CRH versus basal and IFNG versus basal, ** $P < 0.01$; CRH + IFNG versus CRH. Luciferase activity was measured as described in the Materials and Methods section. Values indicate the mean $\pm$ s.d. of triplicates from one representative experiment ($n = 3$). Results are expressed as the fold induction over basal value, arbitrarily taken as 1.

Figure 5 NF-κB mediates *Pomc* transcriptional inhibition. AtT-20 cells were cotransfected with (A) *Pomc*-Luc + IkB-SR expression vector or the empty control vector and treated with IFNG (10 U/ml) for 24 h. *** $P < 0.001$; IFNG versus basal, IkB-SR + IFNG versus IFNG. (B) *Pomc*-Luc or *Pomc*-mut-NF-κB and treated with IFNG (10 U/ml) for 24 h. ** $P < 0.01$ versus basal. (C) *Pomc*-Luc + IkB-SR and/or STAT1 expression vector or empty control vector and treated with IFNG (10 U/ml) for 24 h. ** $P < 0.01$ with respect to control plasmid. (D) NF-κB-Luc and treated with IFNG (10 U/ml) for 18 h. * $P < 0.05$ versus basal. Luciferase activity was measured as described in the Materials and Methods section. Values indicate the mean $\pm$ s.d. of triplicates from one representative experiment ($n=3$). Results are expressed as the fold induction over the basal value (control plasmid), arbitrarily taken as 1.

Methylphenyl-sulfonyl)-2-propenenitrile), which inhibits NF-κB activation by the inhibition of IkBα phosphorylation (Gutierrez *et al.* 2005), reversed the inhibitory effect of IFNG on the *Pomc* promoter transcriptional activity (data not shown). These data indicate that NF-κB mediates the transcriptional inhibition of *Pomc* by IFNG. To test whether NF-κB-binding activity to the *Pomc* promoter is an important step for the inhibition of the pituitary *Pomc* gene expression, we transfected AtT-20 cells with a plasmid containing the *Pomc* promoter, either intact or mutated at the NF-κB-binding site, coupled to the luciferase reporter gene (Fig. 5B). IFNG treatment (24 h) of the AtT-20 cells transfected with the mutated construct resulted in an inhibition of the transcriptional activation of the *Pomc* gene to the same extent as the one observed using the wild-type construct. The same results were obtained when AtT-20 cells were treated for 18 h with IFNG (data not shown). These data indicate that NF-κB binding to its cognate site is not necessary for the transcriptional inhibition of *Pomc* by IFNG. To further understand the inhibitory effect of STAT1 and NF-κB on IFNG response, we cotransfected AtT-20 cells with STAT1 and IkBα super-repressor expression plasmids. Figure 5C shows the abrogation of the STAT1-mediated inhibition of the *Pomc* promoter transcriptional activity in the presence of IkBα super-repressor. Furthermore, treatment of AtT-20 cells with IFNG induces NF-κB transcriptional activity (Fig. 5D).

As shown above, mutations at the STAT1- and NF-κB-binding sites do not abolish IFNG inhibitory action on the *Pomc* promoter, indicating that the binding of these factors to their specific DNA sequences is not required. To test whether IFNG inhibition of endogenous *Pomc* transcription requires *de novo* synthesis of transcription factors, AtT-20 cells were treated with or without IFNG in the presence or absence of the protein synthesis inhibitor cycloheximide. By reverse transcriptase real-time PCR, we observed that the inhibition of endogenous *Pomc* transcription by IFNG was abolished in the presence of cycloheximide (percentage of inhibition after IFNG: $25.3 \pm 3.7\%$ versus IFNG + cycloheximide: $5.2 \pm 0.6\%$, $P < 0.05$). Thus, IFNG may inhibit transcription indirectly by stimulating *de novo* synthesis of a repressor-like molecule induced by STAT1/NF-κB or by a negative interaction of STAT1/NF-κB with newly synthesized transcription factors recruited to the *Pomc* promoter.

IFNG inhibits ACTH production in AtT-20 cells and in human corticotroph tumors but not in normal murine pituitary cells

To determine whether the observed inhibition in *Pomc* transcription results in ACTH inhibition, we examined the functional role of IFNG on ACTH secretion. IFNG treatment for 24 h decreased ACTH production in tumoral mouse corticotroph AtT-20 cells (Fig. 6A) but not in normal murine pituitary cells (Fig. 6B). To evaluate the role of STAT1 on ACTH production, AtT-20 cells were transfected with STAT1 expression vector. The cells were treated with IFNG versus basal and the supernatant was taken for ACTH determination. In agreement with our data obtained using the *Pomc*-Luc reporter plasmid, STAT1 inhibits ACTH production and this inhibition was even more pronounced in the presence of IFNG (ACTH in ng/ml, control plasmid: basal 26.0 ± 1.2 versus IFNG 16.0 ± 1.2 , $P < 0.001$; STAT1 expression plasmid: basal 20.0 ± 1.6 versus IFNG 12.0 ± 2.0 , $P < 0.05$).

To evaluate whether IFNG exerts similar inhibitory effects on the ACTH production in Cushing's tumors in the primary culture, the cells were treated with different concentrations of IFNG. Twenty-four hours of IFNG treatment of tumor cells led to a 20–60% reduction in ACTH production with respect to untreated cells in six out of seven tumors treated (Fig. 7).

Normal and tumoral human corticotroph cells express IFN-γR1 and 2

To analyze whether different expression levels of the receptor complex on the corticotropinoma cells may account for the different response to IFNG on ACTH production, the expression of IFN-γR1 and 2 in corticotroph adenoma tissue was evaluated by immunohistochemistry. IFN-γR1 and 2 expression in human adult normal pituitary gland as well as in corticotroph adenoma tissue was observed (Fig. 8). No differences were observed between all corticotropinoma

Figure 6 IFNG inhibits ACTH production in tumoral but not in normal murine pituitary cells. (A) AtT-20 or (B) normal murine pituitary cells were treated with IFNG (1, 10, and 100 U/ml). After 24 h, ACTH was measured by RIA in the supernatants. Values indicate the mean $\pm$ s.d. of triplicates coming from one representative experiment ($n=3$). *** $P<0.001$ versus corresponding basal.

tissues tested (data not shown). The IFN- γ R1 and 2 signals were localized in ACTH-immunoreactive cells (Fig. 8).

IFNG inhibits proliferation but does not influence apoptosis in AtT-20 cells

The ability of IFNG to modulate cell proliferation in AtT-20 cells was tested. A dose-response inhibition of AtT-20 cell proliferation was observed in the WST-1 assay on day 4 (Fig. 9A).

The maximal inhibitory effect reached by 10 U/ml IFNG on AtT-20 cells proliferation was observed on day 5 (Fig. 9B).

To analyze whether the antiproliferative effect of IFNG was secondary to an increase in apoptosis, we carried out in parallel a flow cytometry analysis of DNA fragmentation, which is the molecular hallmark of apoptosis. The cells were treated with IFNG at different time points from 1 to 5 days every 24 h. These studies clearly indicate that IFNG does not influence cell death in corticotrophs at any time point analyzed (data not shown).

Discussion

The aim of this work was to study the role of IFNG in ACTH-secreting AtT-20 tumor cells. We focused on the involvement of IFNG in the molecular events leading to the control of *Pomc* transcriptional repression. In the present study, we report for the first time that IFNG exerts an inhibitory effect on important tumoral corticotroph functions, such as ACTH production and cellular growth. Moreover, our data demonstrate that *Pomc* transcription can be negatively regulated by a JAK-STAT1- and NF- κ B-dependent pathway.

IFNG receptor subunits 1 and 2 are expressed on AtT-20 cells. The receptor expression indicates a possible direct IFNG action on these cells and not only via folliculostellate cells, as suggested previously (Vankelecom *et al.* 1990, 1992). Binding of IFNG to its receptor induces phosphorylation of STAT1 and, to a lesser extent, STAT3, in agreement with previous reports in other cellular models (Aaronson & Horvath 2002, Ramana *et al.* 2002, Qing & Stark 2004). Furthermore, IFNG increased STAT-dependent transcriptional activity. These data indicate that the IFNG signaling pathway is functional in AtT-20 cells.

Our results showed that IFNG inhibits *Pomc* transcriptional activity in a time- and dose-dependent manner. Interestingly, this inhibitory effect of IFNG is unique among cytokines, which were reported to stimulate *Pomc* transcription, and the intracellular signaling mechanisms leading to this inhibition are unknown. Herein, we show that activation of corticotroph JAK-STAT is essential for IFNG-mediated inhibition of basal *Pomc* gene expression, since this effect can be abolished by the overexpression of the JAK-STAT inhibitor SOCS1. To our knowledge, there are no earlier reports demonstrating the inhibition of *Pomc* promoter by JAK-STAT signaling. Conversely, LIF activates the *Pomc* promoter by inducing JAK-STAT (Auernhammer *et al.* 1998, Auernhammer & Melmed 2000). Whereas LIF preferentially promotes STAT3 homodimerization and binding to their consensus sites (Mynard *et al.* 2002), IFNG induces the formation of STAT1 complexes (Ramana *et al.* 2002, Platanias 2005). Thus, the binding of distinct homodimers to the STAT1/3 site present in the *Pomc* promoter may trigger opposite responses in terms of *Pomc* transcription. Accordingly, data from the literature showed that STAT1 and STAT3 have opposing biological effects, with STAT3 acting as an oncogene (Bromberg *et al.* 1999, Bromberg 2002) and STAT1 as a tumor suppressor (Bromberg *et al.* 1996, Chin *et al.* 1996). Herein, we also showed that mutation at the STAT1-binding sites did not abolish IFNG inhibitory action, indicating that STAT1 binding to its cognate site at the *Pomc* promoter is not necessary for the inhibition in *Pomc* transcriptional activity by IFNG.

In addition to the STAT pathway, we show that NF- κ B is also involved in the IFNG-mediated inhibition of *Pomc* gene expression. Under CRH-activating conditions, Karalis *et al.* (2004) found that the transcriptional activation of the *Pomc* gene is associated with the inhibition of NF- κ B DNA binding.

Figure 7 IFNG inhibits ACTH production in human pituitary adenoma cells from patients with Cushing's disease. Primary cell cultures of human corticotropinomas were treated with IFNG (1, 10, or 100 U/ml). After 24 h, ACTH was measured by RIA in the supernatants. Bars indicate percentages of basal ACTH values (100%) after IFNG treatment (mean $\pm$ s.d. of at least triplicates). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with the corresponding basal values.

In the present work, loss of NF- κ B activity by using the I κ B super-repressor prevents the IFNG-mediated inhibition of the *Pomc* promoter under basal conditions. Furthermore, the presence of the I κ B super-repressor abolishes the inhibition mediated by the STAT1 overexpression on *Pomc* transcriptional activity. Moreover, IFNG induces NF- κ B transcriptional activity. It has been proposed that IFNs can activate NF- κ B directly as a component of IFN-stimulated gene expression (Sizemore *et al.* 2004). In this direction, Deb *et al.* (2001) showed that IFNG alone can activate NF- κ B by a JAK1-mediated mechanism. Thus, NF- κ B acting downstream to STAT1 is necessary for *Pomc* transcriptional repression. Interestingly,

IFNG inhibited the promoter activity of *Pomc* bearing a mutation in the NF- κ B-binding site. These data indicate that an intact NF- κ B DNA-binding activity is not necessary for IFNG action. Therefore, *Pomc* gene repression is NF- κ B dependent but does not involve NF- κ B binding to target DNA consensus sequences on the *Pomc* promoter. Hence, a direct interaction between STAT1 or NF- κ B and the *Pomc* promoter is not necessary for the IFNG-mediated inhibition of *Pomc*. These results together with the fact that the inhibition of the promoter activity took place after 18 h indicate an indirect effect of the IFNG pathway on the *Pomc* promoter. Indeed, the effect of IFNG on endogenous *Pomc* was abolished by cycloheximide,

Figure 8 Expression of IFN- γ R1 and IFN- γ R2 in the human adult pituitary gland. Colocalization with ACTH. Immunohistochemical analysis of autopsy-derived normal human and corticotrophinoma tissues, using immunoperoxidase (IFN- γ R1 and IFN- γ R2 are indicated by filled arrows) Double immunohistochemistry of the receptors with ACTH (red color) is indicated by thin arrows. Nuclei were counterstained with toluidine blue. Data are representative of at least three different human adult pituitary samples and at least three independent experiments.

Figure 9 IFNG inhibits proliferation of tumor mouse corticotroph AtT-20 cells. (A) Cells were treated with IFNG (0.1, 1, 10, and 100 U/ml) for 96 h. Proliferation was measured using the WST-1 assay. Averages of three wells per treatment and SDS from one representative experiment out of three are shown. ** $P < 0.01$, compared with the basal value. (B) AtT-20 cells were treated with 10 U/ml IFNG. Proliferation was measured after 24, 72, and 120 h using the WST-1 assay. Averages of three wells per treatment and SDS from one representative experiment out of three are shown. *** $P < 0.001$ compared with the corresponding basal values.

indicating the involvement of new protein synthesis. Thus, IFNG inhibits transcription indirectly by stimulating *de novo* synthesis of a repressor-like molecule through STAT1/NF- κ B or by a negative interaction of STAT1/NF- κ B with new synthesized transcription factors recruited to the *Pomc* promoter. In other cellular models, like lung adenocarcinoma cells, direct STAT/NF- κ B interactions have been described (Marks-Konczalik *et al.* 1998, Ganster *et al.* 2005). Whether STAT1 interacts with NF- κ B and/or whether there is a recruitment of the complex together with other transcription factors to the *Pomc* promoter, which may elicit transcriptional repression of *Pomc*, remains to be elucidated. At present, the genes targeted by IFNG in AtT-20 cells remain unknown. Microarray or Chip-on-Chip experiments could specify which are these genes, and would allow gaining more insight into the STAT1 and NF- κ B interactions on consensus binding sites.

CRH induces AP1 and CREB1 transcriptional activity, which have been proposed to be involved in *Pomc* transcription (Boutillier *et al.* 1995, 1998). However, the main mediators of CRH action on *Pomc* transcription are the nuclear orphan receptors NUR77 and Nurr1, which are acting on the NurRE and NBRE sites (Philips *et al.* 1997a,b, Maira *et al.* 1999,

Kovalovsky *et al.* 2002). We found that IFNG inhibited the NUR77/Nurr1 transcriptional activities in AtT-20 cells stimulated with CRH without affecting AP1- and CRE-driven luciferase activities. By contrast, IFNG did not affect the basal transcriptional activity of these transcription factors. Thus, in tumoral corticotroph cells, IFNG inhibits basal *Pomc* transcriptional activity without affecting the factors involved in CRH-induced *Pomc* transcription. Since pituitary corticotropinomas are poorly sensitive to CRH and *Pomc* is not under CRH control in these cells, the inability of IFNG to affect these factors is of no consequence for its inhibitory action on ACTH synthesis. Taken together, these data reinforce the fact that IFNG inhibition on *Pomc* promoter is mediated by the factors different from the main mediators of CRH. Repression of *Pomc* transcription by interference with PITX/TPIT was described for the transcription factor Smad (Nudi *et al.* 2005). Further experiments are needed to evaluate whether TPIT is also involved in IFNG STAT1-mediated inhibition of *Pomc* transcriptional activity.

We found that IFNG not only affected *Pomc* transcriptional activity but also endogenous ACTH production in AtT-20 cells. In normal murine pituitary cells, the basal secretion of ACTH remained unaffected by IFNG treatment pointing to a tumor-specific *Pomc* inhibitory action of IFNG. Accordingly, a previous clinical study showed that IFNG administration to healthy males does not affect ACTH levels (Holsboer *et al.* 1988). However, in corticotropinomas derived from patients with Cushing's disease, we found that IFNG inhibits hormone production in six out of seven tumors in primary cell cultures. Essentially, the same results were found in AtT-20 cells. These results clearly indicate the specific inhibitory action of IFNG on ACTH production in tumors from patients with Cushing's disease and not in normal tissues. It is well known that the expression of STAT1 and STAT3 is often deregulated in different tumors and tumor cell lines (Schindler 2002, Calo *et al.* 2003). In such cases, the responses to IFNG may be different to the normal response (Qing & Stark 2004).

In agreement with our data obtained using the *Pomc*-Luc reporter plasmid, STAT1 inhibits ACTH production and this inhibition is even more pronounced in the presence of IFNG. Thus, inhibition of hormone release and gene transcription by IFNG occurs through a STAT1-dependent pathway.

In addition, we showed that IFNG reduces endogenous *Pomc* mRNA, though to a lesser extent, than the transcriptional activity directed from the *Pomc* reporter plasmid. Nevertheless, it has been described that changes in endogenous *Pomc* mRNA levels do not reflect changes in *Pomc* transcription, most probably due to *Pomc* mRNA stability and accumulation (Levin *et al.* 1989, Boutillier *et al.* 1998). Even in the presence of CRH or cAMP, Gagner & Drouin (1987) showed a slight induction of *Pomc* mRNA levels after treatment of AtT-20 or primary rat anterior pituitary cells with respect to basal. In the same direction, Lundblad & Roberts (1988) observed that the rapidity of the effects of stimulators or inhibitors of *Pomc* peptide release on *Pomc* gene transcription is sharply contrasted against the changes observed in mRNA

levels in the cytoplasm. Thus, changes in *Pomc* mRNA levels may be physiologically relevant despite its low magnitude. In this direction, IFNG inhibits ACTH production in about $39.5 \pm 4.2\%$, showing the physiological importance of IFNG in setting ACTH levels despite the endogenous *Pomc* mRNA inhibition of $25.3 \pm 3.7\%$.

We report here that IFNG receptor subunits α and β are expressed in normal pituitary corticotroph cells, being colocalized with ACTH-immunoreactive cells. Furthermore, the expression of both subunits is also found in corticotroph adenoma tissue from patients with Cushing's disease. IFN- γ R1 and 2 expression levels in all seven corticotroph pituitary tumors examined was similar to that detected in normal pituitary tissues. These data indicate that the receptor levels may not account for the different response to IFNG between tumoral and non-tumoral corticotroph cells.

In addition to ACTH production and gene expression, we demonstrated here for the first time that IFNG inhibited corticotroph tumor cell proliferation. A dose-dependent inhibition of AtT-20 cell growth was observed. Similar results of an IFNG-dependent inhibition of cellular proliferation have been observed in ovarian cancer, fibrosarcomas, and murine fibroblasts, but not in mutagenized human cells lacking STAT1 or in murine cells derived from STAT1-deficient mice (Ikeda *et al.* 2002). On the basis of the presented data, it can be proposed that the antiproliferative effect of IFNG on AtT-20 cells may be mediated by the JAK-STAT1 pathway. The IFNG/STAT1 cascade has been implicated in promoting tumor cell apoptosis (Suk *et al.* 2001). Furthermore, the robust inhibitory effect of IFNG suggests that the reduction in cell number might be due to apoptotic cell death. Nevertheless, recent reports demonstrate that IFNG arrests the cell cycle in different cellular models by the inhibition of the expression of *Cyclin D1* and induction of the cyclin-dependent kinase inhibitor *p27-Kip1* (Harvat *et al.* 1997, Mandal *et al.* 1998, Chew *et al.* 2005). Flow cytometry studies analyzing DNA fragmentation did not reveal apoptotic cell death, strongly indicating that IFNG acts by causing cell cycle arrest.

In summary, our data show for the first time that IFNG-induced STAT1 activation inhibits *Pomc* promoter transcriptional activity in AtT-20 corticotroph cells. IFNG induces the phosphorylation of STAT1 and, to a lesser extent, STAT3. STAT3 overexpression abolished the STAT1 effect on *Pomc*, indicating that alternative activation of STAT1 or STAT3 has opposing biological effects. Furthermore, we found that NF- κ B, acting downstream to STAT1, is necessary for the IFNG STAT1-mediated inhibitory effect on *Pomc* transcription.

Thus, we demonstrate for the first time a novel JAK-STAT1/NF- κ B-dependent inhibitory pathway acting on basal *Pomc* promoter activity. Pharmacological treatments that ameliorate the hypercortisolemic features of Cushing's disease do not decrease pituitary ACTH levels or tumor growth. Signaling pathways triggered by IFNG inhibit pituitary tumor cell proliferation and ACTH synthesis and secretion. Thus, the development of therapeutic agents that target this novel IFNG-JAK-STAT1/NF- κ B pathway might provide a valuable approach for treating Cushing's disease.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of the research reported.

Funding

Dr D Refojo is supported by the European Molecular Biology Organization (EMBO).

Acknowledgements

We are thankful to Dr M Low, Dr J Drouin, Dr B Kaltschmidt, Dr M Bell, Dr K Karalis, T Willson, and Dr T Hirano for various plasmids.

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Received in final form 5 August 2008

Accepted 19 August 2008

Made available online as an Accepted Preprint
19 August 2008