Dual effects of suppressor of cytokine signaling (SOCS-2) on growth hormone signal transduction

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Abstract A family of suppressors of cytokine signaling (SOCS) has recently been identified of which two members have been shown to block growth hormone (GH) signaling. Dose-response experiments were conducted in 293 cells and SOCS-1 and SOCS-3 were shown to inhibit the transcriptional activation of a GH-responsive element and suppressed Jak2 tyrosine kinase activity. SOCS-2 had two opposite effects: at low concentrations it inhibited GH-induced STAT5-dependent gene transcription, but restoration of GH signaling was observed at higher concentrations. In cotransfection studies, SOCS-2 was able to block the inhibitory effect of SOCS-1 but not that of SOCS-3 on GH signaling. These findings suggest that a major function for SOCS-2 is to restore the sensitivity to GH by overcoming the initial inhibitory effects of other endogenous SOCS molecules.

Key words: Growth hormone; Suppressor of cytokine signaling; Jak2 kinase

1. Introduction

Growth hormone (GH) is the major regulator of postnatal body growth [1]. It exerts diverse and pleiotropic effects on the growth, differentiation and metabolism of cells. GH initiates its biological actions, including the regulation of expression of a broad range of genes in mammalian tissues, by interacting with a specific membrane-bound receptor. GH receptor (GHR) signaling involves ligand-induced receptor homodimerization and activation of the tyrosine kinase Jak2 [2,3]. These events initiate a cascade of phosphorylation of cellular proteins including the kinase itself and the receptor, thereby providing docking sites for src homology domain 2 (SH2)-containing molecules such as the latent cytoplasmic signal transducer and activator of transcription 5 (STAT5) [4]. Jak2 activation results in the phosphorylation, dimerization and nuclear translocation of activated STAT factors which bind to their DNA-responsive elements to activate gene transcription.

The mechanism by which GH signaling is switched off is less known. Recent studies [5–13] have identified a novel family of cytokine-inducible inhibitors of signaling (SOCS) which includes eight members (SOCS-1, -2, -3, -4, -5, -6, -7 and CIS) that act in a classical negative feedback loop to regulate cytokine signal transduction. Transcription of the SOCS-1 gene is induced in response to a variety of cytokines and the protein has been shown to interact with and to inhibit the tyrosine phosphorylation of the members of the Jak kinase family as well as other tyrosine kinases. CIS, on the other hand, appears to negatively regulate cytokine signaling by competing with STAT5 for tyrosine docking sites on the erythropoietin and interleukin-3 (IL-3) receptors [5]. SOCS-1 and SOCS-3 have been shown to block IL-6, GH, prolactin (PRL) signaling as well as interferon-mediated antiviral and antiproliferative activities [14–18], though with different regulatory roles and different potencies. Although SOCS-3 also binds to the Jak2 tyrosine kinase domain (JH1), it is not clear whether it can inhibit its tyrosine kinase activity [14,17]. The regulatory role of SOCS-2 is less defined; it surprisingly appeared to facilitate the superinduction of a reporter gene in response to GH [15] and was shown to interact with the insulin-like growth factor-I (IGF-1) receptor [19]. The present study was conducted to clarify the effect of SOCS-2 on GH signaling and its interaction with other SOCS molecules.

2. Materials and methods

2.1. Hormone

Recombinant human GH was kindly provided by Serono-Ares Laboratories (Geneva, Switzerland). Anti-Flag monoclonal antibody M2 is a product of IBI-Kodak. The anti-phosphotyrosine antibody and the anti-Jak2 antibody were purchased from Upstate Biotechnology, Inc.

2.2. Cell culture and transfection

Human kidney fibroblast 293 cells were grown in Dulbecco's modified Eagle's medium nut F12 medium (Gibco) containing 10% fetal bovine serum. Several hours before transfection, cells were plated in two-thirds Dulbecco's modified Eagle's medium nut F12, one-third Dulbecco's modified Eagle's medium 4.5 g/l glucose medium containing 10% fetal bovine serum.

For GH-dependent induction of reporter construct, 293 cells were plated in 6 well plates before being transiently cotransfected by the calcium phosphate technique as previously described [20,21] with 100 ng of the β-galactosidase-encoding vector, 100 ng of the lactogenic hormone responsive element (LHRE)-TK-luciferase reporter gene (fusion gene carrying six copies of the LHRE and the thymidine kinase (TK) minimal promoter linked to the coding region of the luciferase gene (LHRE is a STAT5 binding element of the β-casein promoter), 50 ng of plasmid pcDNA3/monkey (mk) GHR [20] and increasing concentrations (0–250 ng DNA) of Flag epitope-tagged SOCS-1, SOCS-2, SOCS-3 or CIS, also referred to as CIS1, in pEF-BOS expression vector (kindly provided by Drs. D.J. Hilton and R. Starr). One day after transfection, cells were incubated with serum-free medium containing 50 nM hGH for 24 h. Cells were then lysed in lysis buffer (Pharmacia) and whole cell extracts were used for determination of luciferase and β-galactosidase activities. Aliquots of lysates were analyzed by Western blot using anti-Flag monoclonal antibody M2 and confirmed that increasing the concentration of transfected SOCS plasmids resulted in increased expression of SOCS proteins (data not shown).

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2.3. Immunoprecipitation and Western blot analysis

Each 100 mm culture dish of 293 cells was cotransfected with 4 μg of GHR cDNA, 0.2 μg of the cDNA encoding Jak2 (kindly provided by Dr. J. Ihle) and 8 μg of each form of SOCS-encoding plasmid. In these conditions Jak2 is tyrosine phosphorylated and active in the absence of GH stimulation in 293 cells because of its high level of expression [7,14]. Cells were subsequently lysed as previously described [22] and the lysates were incubated with anti-Jak2 antibody (1 μg/ml) and collected using protein A-agarose. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (7%), transferred onto polyvinylidene difluoride transfer membrane (Polyscreen, DuPont NEN) and immunodetected with appropriate antibodies to Jak2 (UBI, 1/1000) and anti-phosphotyrosine (UBI, 1/10 000) and visualized by chemiluminescence (ECL kit from Amersham).

3. Results

3.1. Effects of SOCS molecules on GH-induced transactivation of transcription

GH can activate transcription of the chimeric LHRE reporter gene in the absence of constitutive expression of SOCS genes in 293 cells. A maximal activation of 13.3 ± 0.7 ($n = 7$) times was obtained upon GH stimulation when SOCS plasmids were not transfected. Luciferase activity obtained in dose-response experiments was normalized to these maximal fold induction values and is expressed as a percentage of these values. Dose-response experiments with SOCS molecules were conducted to analyze in detail the effect of each SOCS. The constitutive expression of the SOCS-1 (Fig. 1) or SOCS-3 gene (Fig. 2) at increasing concentrations resulted in the total inhibition of the activation of transcriptional activity and this inhibition was effective starting with very low doses of transfected cDNA (5 ng/well which represents a 1:0.1 ratio of GHR to SOCS). Both SOCS molecules displayed similar potency in inhibiting signaling. Surprisingly, expression of the SOCS-2 gene at increasing concentrations resulted in two opposite effects (Fig. 3); the first was a 50% inhibitory effect that was obtained with low doses of transfected cDNA in the range of 5–25 ng/well (corresponding to a 1:0.1 to a 1:0.5 ratio of GHR to SOCS-2); however, a second effect was obtained with higher concentrations of transfected SOCS-2 plasmid and was characterized by a dose-related restoration of transactivation of transcription reaching even a superinduction (200%) of the maximal activation obtained in the absence of constitutive expression of SOCS genes (Fig. 3). As expected, constitutive expression of the CIS gene did not affect activation of gene transcription (data not shown).

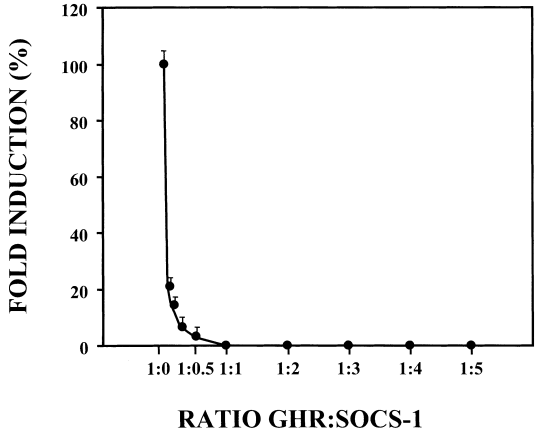

Fig. 1. Effect of constitutive expression of SOCS-1 on induction of the LHRE-luciferase reporter gene by hGH. 293 cells were transfected and assayed as described in Section 2. Results of luciferase activity were assayed in cells transfected with different ratios (1:0, 1:0.05, 1:0.1, 1:0.25, 1:0.5 to 1:5) of mkGHR to SOCS-1 cDNA. The dose-response curve is expressed in percentage of control activity (in the absence of cotransfected SOCS-1). 100% corresponds to a control luciferase induction of 13.3-fold. Values are the means ± S.E.M. of four to seven independent experiments.

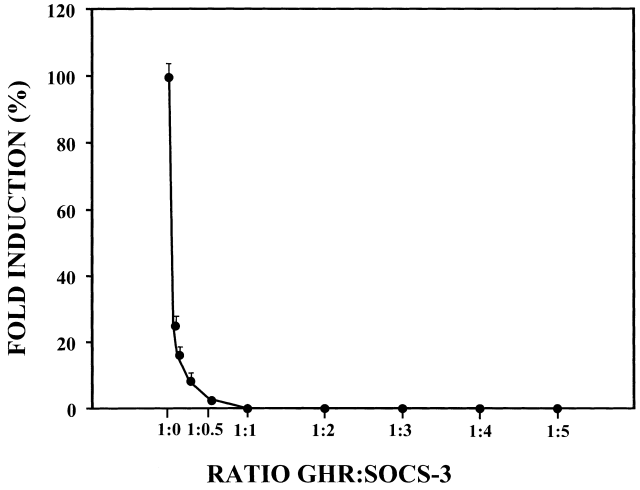

Fig. 2. Effect of constitutive expression of SOCS-3 on GH-induced transactivation of LHRE-TK promoter. 293 cells were transfected and assayed as described in Fig. 1. The same ratios of mkGHR to SOCS-3 cDNA (1:0 to 1:5) as described in Fig. 1 were used. The dose-response curve is expressed in percentage of control activity (in the absence of cotransfected SOCS-3). Values are the means ± S.E.M. of three to seven independent experiments.

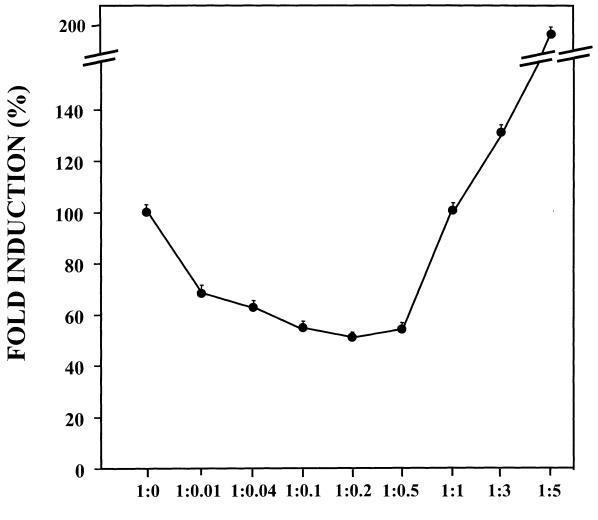

Fig. 3. Dual effect of constitutive expression of SOCS-2 on GH-induced activation of LHRE-TK promoter. 293 cells were transfected and assayed as described in Fig. 1. Different ratios (1:0, 1:0.02, 1:0.04, 1:0.1, 1:0.2, 1:0.5 to 1:5) of mkGHR to SOCS-2 cDNA were used in transfection studies. The dose-response curve is expressed in percentage of control activity (in the absence of cotransfected SOCS-2). Values are the mean ± S.E.M. of four to seven experiments.

3.2. Effects of co-expression of different SOCS molecules on GH-dependent activation of gene transcription

To analyze the mechanism by which SOCS-2 was able to restore activation at higher concentrations, experiments were designed to analyze the interactions of SOCS molecules in GH signaling, based on the hypothesis that SOCS-2 may inhibit endogenous SOCS that are induced by GH and which are required to inhibit GH signaling itself. As SOCS-1 and SOCS-3 were shown to be potent inhibitors of GH-mediated activation of gene transfection in the previous experiments, their respective cDNAs encoding plasmids were cotransfected together with that encoding SOCS-2 protein. As shown in Fig. 4, the constitutive expression of SOCS-1 and SOCS-2 at increasing concentrations resulted, in a dose-dependent manner, in the restoration of the transactivation of transcription that had been inhibited by SOCS-1. This effect appeared to be specific to SOCS-1 since the same experiments conducted with SOCS-3 and increasing concentrations of transfected SOCS-2 plasmid did not show any reactivation of the transcriptional activity that had been inhibited by SOCS-3.

3.3. Activation of Jak2 kinase upon constitutive expression of SOCS genes

Immunoblot analysis of 293 cell lysates that were immunoprecipitated with Jak2 antibody revealed that, upon constitutive expression of SOCS-1 or SOCS-3, tyrosine phosphorylation of Jak2 was abolished (Fig. 5). On the other hand, Jak2 tyrosine activity was not affected by the constitutive expression of SOCS-2- or CIS-encoding plasmids.

4. Discussion

Key events in GH signal transduction are well defined and involve mainly the activation of the Jak2-STAT5 pathway. The mechanisms by which signaling is switched off are just beginning to be understood with the recent identification of a family of cytokine-inducible inhibitors of signaling [5–13].

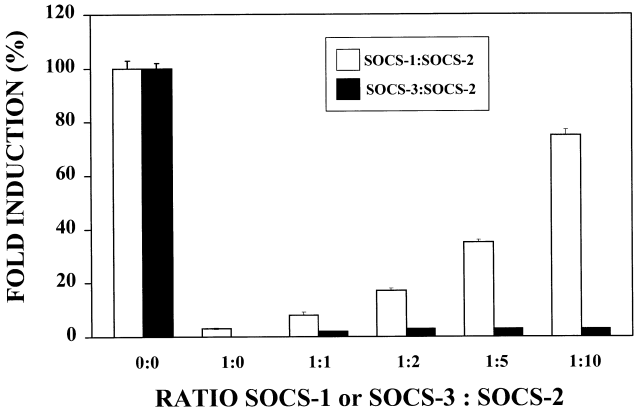

Fig. 4. Effect of constitutive co-expression of SOCS-1 and SOCS-2 or SOCS-3 and SOCS-2 on GH-induced transactivation of LHRE-TK promoter. 293 cells were transfected and assayed as described in Fig. 1. Different ratios (0:0 to 1:10) of SOCS-1 or SOCS-3 to SOCS-2 cDNA were used for transfection studies. A ratio of 1:1 of mkGHR to SOCS-1 or to SOCS-3 was used (50 ng of each plasmid) since this ratio was shown in Figs. 1 and 2 to result in a complete inhibition of GH-induced transactivation of the LHRE promoter. The dose-response curves are expressed in percentage of control activity (in the absence of SOCS). Values are the means ± S.E.M. of four independent experiments.

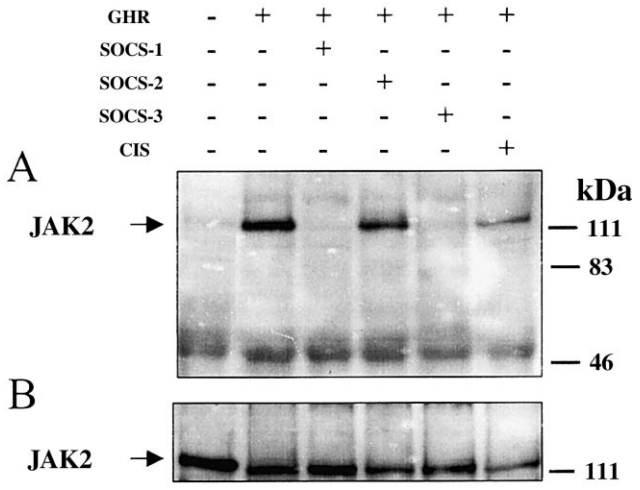

Fig. 5. Inhibition of tyrosine phosphorylation of Jak2 by SOCS-1 and SOCS-3. 293 cells expressing mkGHR (+), Jak2 and various SOCS constructs (+) were lysed and immunoprecipitated with anti-Jak2 antibody. Immunoprecipitated proteins were Western blotted with anti-phosphotyrosine antibody (A) or anti-Jak2 antibody (B). The position of Jak2 (130 kDa) is indicated on the left. Molecular masses of protein standards are indicated on the right in kDa.

Among them, SOCS-1 and SOCS-3 have been shown to block GH-induced transactivation of a GH-responsive gene promoter [15]; we now assess the implication of SOCS-2 in GH signaling.

The inhibitory effect of SOCS-2 on GH signaling has not been reported previously and was observed in the present study only at low concentrations of transfected cDNA, stressing the importance of using a large spectrum of concentrations to study the effect of SOCS molecules. In fact, only one high concentration of transfected SOCS-2 has been used previously [15], impeding the characterization of this effect. In view of the general mechanism of action of this family of proteins, this inhibitory effect is not surprising but it represents to our knowledge the first demonstration of a role of SOCS-2 in attenuating signaling of a member of the cytokine receptor superfamily. Indeed, similar results have been obtained recently with the closely related PRL receptor (Pezet et al., submitted); whether this applies to other cytokines needs to be evaluated by using similar dose-response experiments. The inhibitory effects of SOCS-1 and SOCS-3 are similar to those observed previously on GH as well as on signaling of several cytokines [14–18]. Moreover, SOCS-1 and -3 are shown to be very potent inhibitors even when their expression levels are comparable to endogenous ones, a situation which also occurs in IL-6 signaling [17]. Although SOCS-3 has been shown to be a weaker inhibitor than SOCS-1 against the signaling of several cytokines [5,12,17], this does not appear to be the case for GH-mediated activation of gene transcription. Constitutive expression of these two inhibitors resulted also in a dramatic reduction of Jak2 tyrosine kinase activity, confirming the mechanism of action of these inhibitors as described for other cytokines [11].

Another feature associated with SOCS-2 resided in its capacity to restore, in a dose-dependent manner, the GH receptor functional activity that had been inhibited by SOCS-1. Indeed, this effect appeared to be specific for the interaction between SOCS-1 and -2 since it was not observed between

SOCS-3 and -2 suggesting that the mechanisms of inhibition of SOCS-1 and -3 are different, in agreement with a recent report on leukemia inhibitory factor (LIF) and IL-6 signal transduction [14]. The capacity of SOCS-2, when expressed alone and at high concentrations, to restore and even to result in a superinduction of reporter gene activity supports the hypothesis that it can inhibit endogenous SOCS as illustrated for SOCS-1. The present results thus demonstrate that SOCS-2 has a dual effect by acting as an inhibitor of GH signaling but also by restoring signaling through inhibition of other SOCS molecules. Given the pattern of sequential expression of the genes encoding the SOCS molecules following GH administration [15], SOCS-1 and -3 being expressed early and SOCS-2 expressed later, it is tempting to speculate that, in addition to its inhibitory effect, a major function of SOCS-2 would be to restore late the sensitivity of cells to GH action by inhibition of endogenous SOCS following their initial inhibitory effects. Generation of mice lacking one or more genes for SOCS [23] will be necessary to study in detail the role and the functions of SOCS molecules in vivo and their implication in GH signaling.

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