

Paula Ocaranza*, Ximena Gaete, Rossana Román, Fernanda Morales, Germán Iñiguez and Fernando Cassorla

Phosphotyrosine phosphatases in GH-stimulated skin fibroblasts from children with idiopathic short stature

Abstract

Aim: Some cases of idiopathic short stature (ISS) may be caused by defects in the modulation of the negative feedback regulation of the growth hormone receptor (GHR)/Janus kinase (JAK)2/signal transducers and activators of transcription (STAT)5 signaling pathway. The cytosolic tyrosine phosphatases, protein tyrosine phosphatase 1B (PTP1B) and Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1), the later which translocates to the nucleus after activation, interact with JAK2 in a GH-dependent manner. The possible contribution of PTP1B and SHP-1 to GH signaling in fibroblasts from ISS patients has not been studied.

Methods: We determined the basal protein content of PTP1B and SHP-1 in the presence of recombinant human GH (rhGH) for 24 h in skin fibroblast cultures, obtained from patients with ISS, and were compared with a normal height control children group. JAK2 activation was determined in both groups.

Results: JAK2 activation was delayed in fibroblasts from ISS patients compared to controls. Under basal conditions, the protein content of SHP-1 was lower in ISS, and after incubation with rhGH, it decreased in the non-nuclear and nuclear fraction of controls, but not in ISS patients. The protein content of PTP1B, however, increased in a similar fashion in fibroblasts from both ISS and control children.

Conclusion: The delayed activation of JAK2 and the lack of response of SHP-1 after incubation with GH in fibroblasts from ISS patients, suggests that the growth retardation observed in some of these children may be mediated in part by this phosphotyrosine phosphatase.

Keywords: GH signal transduction; idiopathic short stature; PTPs.

Ximena Gaete, Rossana Román, Fernanda Morales, Germán Iñiguez and Fernando Cassorla: Institute of Maternal and Child Research, School of Medicine, University of Chile, Santiago, Chile

Introduction

The term idiopathic short stature (ISS) is applied to a broad range of short children with a variety of conditions, such as those with familial short stature, constitutional growth delay and those who are abnormally short for their parental target height. It has been suggested that some of these children may harbor an unrecognized endocrine defect (1, 2).

The syndrome of growth hormone (GH) insensitivity has been described in patients with increased concentrations of GH and decreased levels of insulin-like growth factor-I (IGF-I) (1). This condition may be caused by different factors affecting the GH signaling transduction pathway. A related syndrome of partial growth hormone insensitivity has been observed among some children with suspected ISS, who have relatively low levels of IGF-I and relatively high levels of GH (3), however, the specific cause of this partial GH insensitivity has not been defined in most of these cases.

The signal transduction cascade induced by the growth hormone receptor (GHR) triggers a complex array of biochemical events acting in a coordinated fashion, and involves a large number of distinct molecules. The GHR does not have intrinsic kinase activity, but a conformational change induced by ligand binding leads to activation of receptor-associated tyrosine kinases of the Janus kinase (JAK) family (4). JAK2 constitutively associates with the GHR, which in turn induces tyrosine phosphorylation within itself and the GHR. These tyrosines form binding sites for a number of signaling proteins, including members of the family of signal transducers and activators of transcription (STATs) (5, 6), namely STAT1, STAT3, and the two isoforms of STAT5 respond to GH stimuli. Upon GH binding, STATs are recruited to the GHR complex and

*Corresponding author: Paula Ocaranza, PhD, Institute of Maternal and Child Research, School of Medicine, University of Chile, Casilla 226-3, Santiago, Chile, Phone: +56-2-2977-0851, Fax: +56-2-2424-7240, E-mail: pauocaranza@gmail.com

undergo both tyrosine phosphorylation by JAK kinases resulting in STAT dimerisation and nuclear translocation, where they bind to specific DNA elements and initiate gene transcription (2, 3, 6, 7).

As described above, activation of GH-dependent signaling pathways is based on protein phosphorylation on tyrosine, serine or threonine residues. Mechanisms that limit and terminate GHR signaling include inactivation of JAK2 and downstream signaling molecules by dephosphorylation; GHR internalization and degradation of the GHR, and inhibition of signaling by negative regulators, such as suppressors of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS) (8–10).

Given the central role of JAK2 in initiating GH signaling, the phosphatases involved in its deactivation may be important. The candidates that have been shown to be involved in this process include the cytosolic tyrosine phosphatases Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) and SHP-2 (11–13). SHP1 interacts with activated JAK2 (11), and can dephosphorylate STAT5 in the nucleus after GH stimulation (14). SHP2 is a dual player, both promoting and inhibiting JAK/STAT-mediated signaling, acting through its catalytic and SH2 binding domains (12–16), depending on its local concentration and the cell context. The protein tyrosine phosphatase 1B (PTP1B), which is ubiquitously expressed, binds phosphorylated JAK2 in leptin- and gamma interferon (IFN- γ)-treated cells and has been shown to interact with JAK2 in a GH-dependent manner (17).

The aim of the present study was to investigate the contribution of PTP1B and SHP-1 in children with ISS compared to controls, using the *in vitro* fibroblast culture model. To test this hypothesis, we determined the protein content of these phosphatases and JAK2 activation in skin fibroblasts obtained from boys with ISS and apparent low GH sensitivity, and from control boys with normal height. The protein content in ISS and control children was assessed under basal conditions and in the presence of recombinant human GH (rhGH) stimulation.

Materials and methods

Study subjects

The study population consisted of 12 prepubertal children (7.9 ± 0.6 years of age) with ISS who met the following inclusion criteria: i) growth velocity under the 10th percentile for age and sex;

ii) a GH response to a clonidine stimulation test >15 ng/mL; iii) serum IGF-I or IGFBP-3 levels below the mean for age and sex; iv) without apparent mutations in the GHR. The control group included 12 prepubertal healthy children (6.6 ± 0.1 years of age) with normal stature and weight for age and sex who underwent elective surgery for an unrelated condition. The subjects were recruited at the San Borja-Arriarán Hospital, and the study was approved by the Ethics Committee of the University of Chile. Informed consent was obtained from the parents of the patients and the control children enrolled in the study.

Chemicals

rhGH was a gift from Dr. A. F. Parlow (National Hormone And Peptide Program; NIH, Bethesda, MD, USA). Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L of glucose, Dulbecco's phosphate buffered solution (DPBS), penicillin/streptomycin, and fungizone were purchased from Invitrogen (Grand Island, NY, USA). Heat inactivated fetal calf serum (FCS) was from Biological Industries (Kibbutz Beit Haemek, Israel). Prestained molecular mass standard proteins were from Fermentas (Burlington, Canada), and the other chemicals were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise.

GH provocative studies

After 12 h of fasting, the clonidine test was performed as described by Gil-Ad et al. (18) with minor modifications. We administered an oral dose of $100 \mu\text{g}/\text{m}^2$ and blood samples were obtained at 0, 30, 60, and 90 min post-clonidine.

Assays

Serum GH was measured by a commercial Immuno Radiometric Assay (IRMA; Izotop, Budapest, Hungary) with 0.05 ng/mL sensitivity, and intra- and interassay coefficients of variation (CVs) of 4% and 5.8%, respectively. Serum IGF-I levels were determined by RIA after an ethanol-acid sample extraction as a first step to remove the IGF-BPs, as we previously described (19). The sensitivity of this assay was 5 ng/mL, and the intra- and interassay CVs are 8.6% and 10.2%, respectively. Serum IGFBP-3 levels were measured by IRMA from Diasource (Nouvelles, Belgium). The sensitivity of this assay was 0.1 mg/L, with intra- and interassay CVs of 1.1% and 1.8%, respectively.

Cell culture

Primary fibroblast cultures were established from skin-biopsy specimens obtained from the patients (internal elbow fold) and healthy control children (at the time of surgery). This tissue ($\sim 1 \text{ mm}^3$) was cultured in DMEM supplemented with 10% FCS, 10 U/mL, penicillin/streptomycin and 0.25 $\mu\text{g}/\text{mL}$ fungizone at 37°C in a 5% CO_2 atmosphere. Fibroblast cultures were used between the third and sixth passage, in order to avoid the influence of plasma factors and senescent changes in the cellular response.

Protein extraction

Skin fibroblasts seeded on 100 mm dishes at 80%–90% confluence were washed with DPBS and serum deprived for 24 h, and treated with 200 ng/mL rhGH for 24 h. Cytosolic and nuclear protein extracts were prepared as described by Schreiber et al. (20). Treated skin fibroblasts were scraped into 125 μ L of cold lysis buffer A (20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.01 mM KCl, 1 mM DTT, 5 mM NaF, 1 mM Na_3VO_4 , 10 mM Na_2MoO_4 , 50 μ g/mL trypsin inhibitor, 1 μ g/mL leupeptin, 0.1 μ g/mL aprotinin, and 0.5 mM PMSF) with 0.05% Nonidet-P40. Samples were centrifuged at 100 g for 2 min at 4°C obtaining the cytosolic extract. The remaining pellet was resuspended in 40 μ L of cold lysis buffer C (20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.4 M NaCl, 1 mM DTT, 5 mM NaF, 1 mM Na_3VO_4 , 10 mM Na_2MoO_4 , 1 μ g/mL leupeptin, 0.1 μ g/mL aprotinin, and 0.5 mM PMSF), and the sample was sonicated. After 30 min of agitation at 4°C the cell debris was removed by centrifugation obtaining the nuclear extract. The protein content was determined by the Bio-Rad Bradford (Richmond, CA, USA) assay (21). Soluble fractions from fibroblasts were heated at 95°C with 0.33 vol of SDS-PAGE sample buffer.

Analysis of the protein content by Western immunoblotting

Samples were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). To reduce non-specific antibody binding, membranes were incubated for 1 h at room temperature in TBS (pH 7.4) containing 0.1% (v/v) Tween-20 (TBST) blocking buffer with 3% w/v BSA. The membranes were then incubated overnight at 4°C with antibodies anti-JAK2 1:2000 from Millipore (Billerica, MA, USA), anti-SHP1 1:2000, anti-PTP1B 1:2000 and anti-TFIIB 1:400 purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA); anti-phospho-JAK2 Tyr^{1007/1008} 1:1000 purchased from Cell Signaling Technology (Danvers, MA, USA), and anti- β -actin 1:100,000 obtained from Sigma-Aldrich (St. Louis, MO, USA). After washing in TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibody (1:10,000 for peroxidase conjugated anti-rabbit IgG or peroxidase-conjugated anti-mouse IgG) purchased from Rockland (Gilbertsville, PA, USA). Immunoreactive proteins were revealed by enhanced chemiluminescence (Pierce ECL Western Blotting Substrate Kit; Pierce, Rockford, IL, USA), blots were obtained by exposure using Discovery 10gD Ultralum with Ultraquant software (6.0.0344 version) and band intensities were quantified using the Image J 1.38x (NIH).

For reblotting, membranes were stripped by incubation for 2 min at room temperature with Re-blot Mild Solution (Chemicon, Tamecula, CA, USA). Blots were washed, reblotted and immunolabeled as described above. Blots were obtained by exposure using Discovery 10gD Ultralum with Ultraquant software (6.0.0344 version) and quantification of band intensities were obtained using the Image J 1.38x (NIH).

Statistical analyses

The SDS for weight and height for our patients were based on the National Center for Health Statistics (NCHS). Results are shown as

mean \pm SEM. Differences between groups were determined by the Student t-test for parametric variables, and by the Mann-Whitney test for non-parametric variables. The differences between basal and 24 h with rhGH for each subject was compared by the Kruskal-Wallis test. Statistical analysis was performed with the GraphPad Prism program version 5.00, and p-values <0.05 were considered significant.

Results

Clinical and hormonal characteristics of ISS patients and control subjects

The clinical and hormonal characteristics of the children studied are shown in Table 1. The 12 male patients with ISS had a mean age of 7.9 \pm 0.6 years, a mean height of -2.61 ± 0.24 SDS, a mean GH peak in response to clonidine of 19.9 \pm 3.5 mUI/L, and a mean serum IGF-I of -0.35 ± 0.22 SDS. The 12 male control children had a mean age 6.6 \pm 0.1 years, and a mean height of 0.06 \pm 0.04 SDS.

ISS patients have delayed JAK2 phosphorylation

To determine whether JAK2 phosphorylation was altered in patients with ISS, we studied the activation of this protein in response to GH stimulation. The cells were incubated with 200 ng/mL of rhGH for various times, and the cytoplasmic lysates were analyzed by SDS-PAGE and immunoblotting. As shown in Figure 1A, JAK2 phosphorylation peaked within 15 min of the addition of rhGH in the control subjects (0.97 \pm 0.08 vs. 1.14 \pm 0.08), and within 30 min in the ISS subjects (0.95 \pm 0.07 vs. 1.27 \pm 0.09; Figure 1B). JAK2 phosphorylation returned to basal levels in both groups after 60 min, which were similar between both groups previous to GH treatment.

PTP1B and SHP-1 protein content in ISS patients and controls

GH signaling is limited by dephosphorylation of activated mediators by specific phosphatases, such as PTP1B and SHP1 that are recruited to the activated GHR complex to extinguish the signal. Using the Western immunoblot technique, we studied the protein content of PTP1B and SHP1 in skin fibroblasts cultures from ISS patients, and in the control children.

To determine if the ISS patients had a different protein content of these enzymes compared to control children

Table 1 Clinical and hormonal characteristics of the subjects enrolled in the study.

| Subjects | Sex M | Age, years (mean±SEM) | Height z score (mean±SEM) | Weight z score (mean±SEM) | IGF-I z score (mean±SEM) | IGFBP-3 z score (mean±SEM) | GH peak, mUI/L (mean±SEM) |
|----------|----------|--------------------------|------------------------------|------------------------------|-----------------------------|-------------------------------|------------------------------|
| Controls | 12 | 6.6±0.1 | 0.06±0.041 | 0.43±0.18 | -0.55±0.38 | -1.4±0.64 | — |
| Patients | 12 | 7.9±0.6 | -2.61±0.24 ^a | -1.92±0.55 ^a | -0.35±0.22 | -0.29±0.38 | 19.9±3.5 |

Values are presented as mean±SEM. ^ap<0.05. IGF, insulin-like growth factor-I; GH, growth hormone.

with normal height, we studied these levels under basal conditions. Our results show that PTP1B protein content (Figure 2A, left panel) is similar between the ISS and control group. However, the basal protein level for SHP-1 under basal conditions was higher in the control group (1.43±0.14) compared to the ISS group (0.8±0.11; Figure 2C).

To investigate how these proteins respond to G stimulation (Figure 3), we subsequently incubated the skin fibroblast cultures with rhGH at a concentration of 200 ng/mL for 24 h, and compared the content of these enzymes in fibroblasts from ISS patients and controls.

Our results show that after incubation with rhGH, the protein content for PTP1B and SHP-1 were similar between the control and the ISS group. In addition, when the levels were compared with their respective basal content, the PTP1B protein content increased after incubation with rhGH both in the control children (0.98±0.9 vs. 1.6±0.4; Figure 3B), and in the ISS group (0.88±0.09 vs. 1.78±0.47; Figure 3C).

The SHP-1 basal protein content in both groups of children was similar. When compared with their respective basal protein content, however, we observed a reduction

(1.02±0.17) in the control group after incubation with rhGH (Figure 3E), but not in the ISS group (1.22±0.27; Figure 3F).

An important feature of SHP-1 concerns its translocation to the nucleus during activation. Our next step was to study the protein content of this phosphatase in the nuclear fraction of these fibroblasts. Under basal conditions (Figure 4A, left panel), the nuclear protein content of SHP-1 was similar in both groups of children. Following rhGH stimulation, a reduction in SHP-1 nuclear protein content was observed in the control group when compared with its respective basal content (1.32±0.12 vs. 0.85±0.16; Figure 4C). In the ISS group, however, the results showed no difference in the SHP-1 nuclear protein content when compared to its basal level (1.3±0.23 vs. 0.91±0.13; Figure 4D).

Discussion

The regulation of the intracellular growth hormone signaling pathway occurs through a complex mechanism involving the action of several positive and negative regulators.

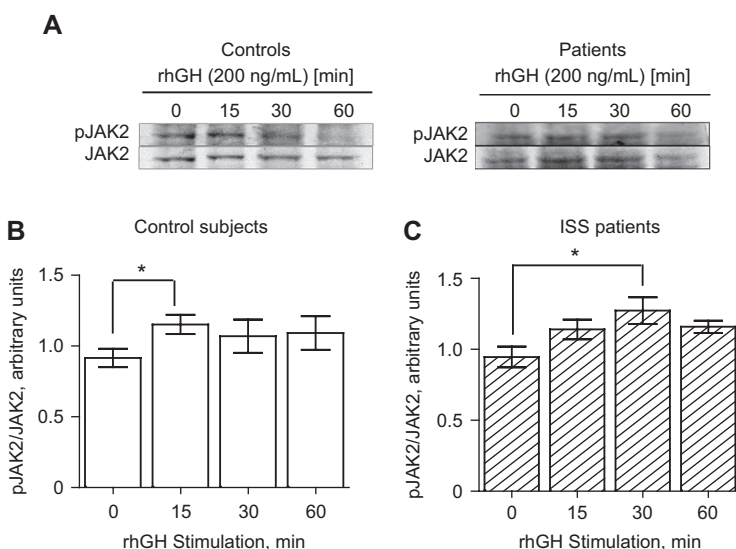


Figure 1 Time-course of Janus kinase (JAK)2 activation in skin fibroblasts after recombinant human growth hormone (rhGH) stimuli. (A) Representative Western immunoblots raised against pJAK2 and JAK2 for Controls and ISS patients. (B) JAK2 activation in control children. (C) JAK2 activation in idiopathic short stature (ISS) patients. Values are expressed as mean±SEM. *p<0.05.

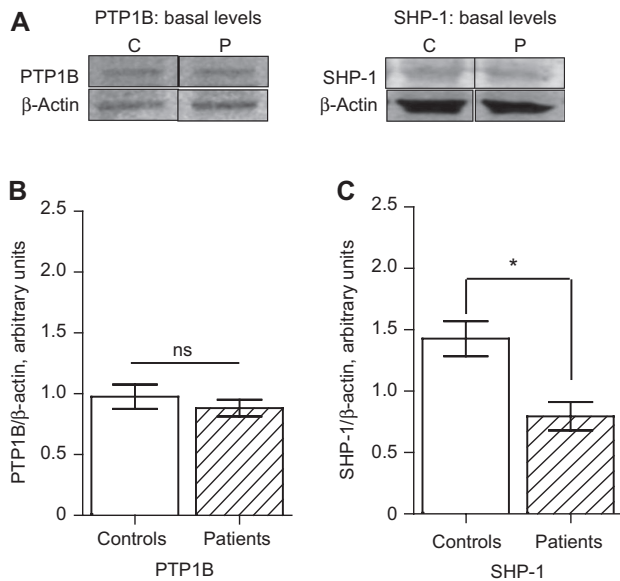


Figure 2 Protein content of the phosphatases in skin fibroblasts under basal conditions. (A) Representative Western immunoblots raised against protein tyrosine phosphatase 1B (PTP1B) and Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) for controls “C” and idiopathic short stature (ISS) patients “P”. Bands were quantified and normalized to β -actin. (B) PTP1B content in healthy controls and ISS patients under basal conditions. (C) SHP-1 content in healthy controls and ISS patients under basal conditions. Values are expressed as mean \pm SEM. * $p < 0.05$; ns, non-significant.

In a previous study, we described an increased expression of the *SOCS* genes, mainly *SOCS2*, in these patients which may cause impairment of GH signal transduction (22). It is not known, however, whether another group of negative regulators, such as phosphatases that may down-regulate the GHR signaling pathway by interacting with JAK kinases (23, 24), could be related to the growth delay observed in some patients with ISS.

The GHR/JAK2/STAT5 cascade is regarded as the main GH-activated signaling pathway. The action of phosphatases on these three proteins is critical for the cellular response to GH. Recent studies have identified several phosphatases which are involved in the specific down-regulation of GHR signaling, such as PTP1B and SHP-1. PTP1B interacts with JAK2 in a GH-dependent manner and dephosphorylates the tyrosines present in the active JAK2 molecule. It has been reported to associate to the GH-dependent phosphorylated GHR, and to induce its dephosphorylation (25). GH can activate SHP-1 and induce its translocation to the nucleus, where it binds to phosphorylated STAT5b, resulting in an attenuation of STAT5 activity (14). SHP-1 also binds GH-activated JAK2 and controls the duration of GH-dependent JAK2 phosphorylation

in the liver. Consequently, in mice lacking SHP-1 the hepatic GH signaling is prolonged (11).

JAK2 is an “activator” of GH-signaling and is essential for this signaling pathway; it is highly regulated by several negative modulators. GHR is phosphorylated by JAK2, which in turn is autophosphorylated on a related

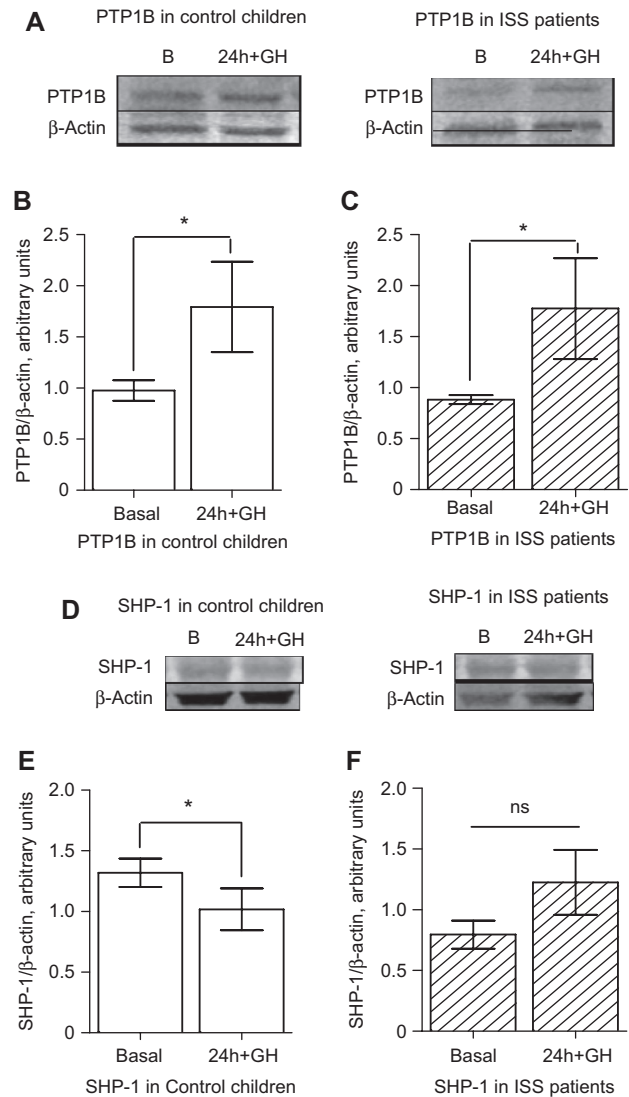


Figure 3 Protein content of the phosphatases in skin fibroblasts after recombinant human growth hormone (rhGH) stimulation. (A) Representative Western immunoblots raised against protein tyrosine phosphatase 1B (PTP1B) in controls and idiopathic short stature (ISS) patients. Bands were quantified and normalized to β -actin. (B, C) PTP1B content after the stimuli in healthy controls and ISS patients. (D) Representative Western immunoblots raised against Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) in controls and ISS patients. Bands were quantified and normalized to β -actin. (E, F) SHP-1 content after the stimuli in healthy controls and ISS patients. Values are expressed as mean \pm SEM. * $p < 0.05$; ns, non-significant.

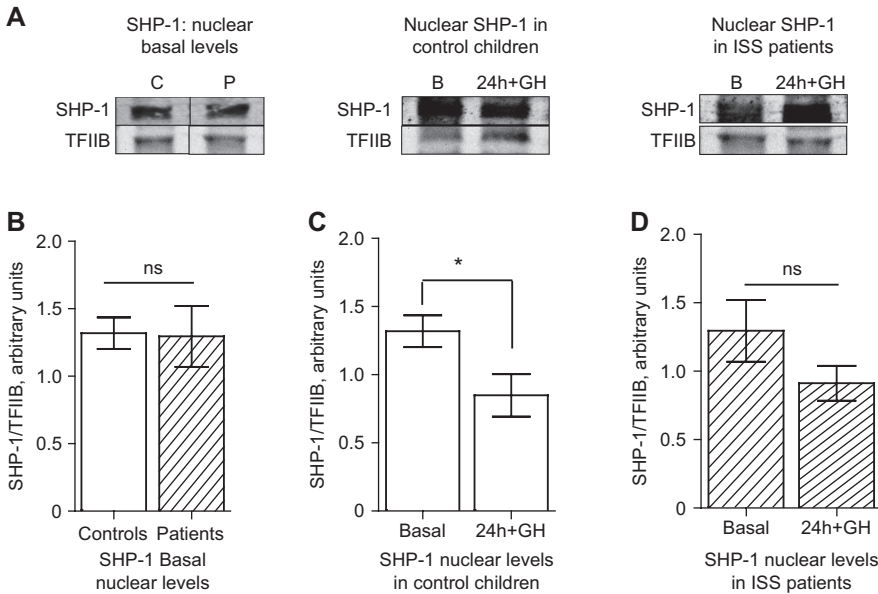


Figure 4 Nuclear protein content of SHP-1 in skin fibroblasts after recombinant human growth hormone (rhGH) stimulation. (A) Representative Western immunoblots raised against Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) in controls and idiopathic short stature (ISS) patients. Bands were quantified and normalized to transcription factor IIB (TFIIIB). SHP-1 content in healthy controls and ISS patients under basal conditions, and after the rhGH stimuli. (B) SHP-1 content in healthy controls. (C) and (D) SHP-1 nuclear content after the stimuli in healthy controls and ISS patients. Values are expressed as mean \pm SEM. * p <0.05; ns, non-significant.

target sequence (26), allowing the recruitment of a variety of signaling proteins (10). Abnormalities in the “activators” of the GH signal transduction pathway, such as STAT5 (27, 28) and STAT3 (29, 30) have been extensively studied in children with ISS, but relatively little information is available regarding possible abnormalities in the “inhibitors” of this pathway in these patients.

Recently, a disorder called growth hormone transduction defect (GHTD) has been described by Rojas-Gil et al. in children with severe growth retardation (31). The features of these patients were over-expression of the negative GH regulator CIS (cytokine inducible SH2 domain-containing protein) and its 37 kDa UbCIS in cultured fibroblasts, which caused increased GHR degradation. This in turn, retarded the activation of JAK2 and STAT5, and interfered with the activation of STAT3 (31).

In this study, we first investigated the activation of JAK2 in skin fibroblast cultures obtained from ISS patients and control children. The results of our study show that JAK2 is activated in both groups of children within 30 min, but the activation is delayed in the ISS patients when compared to controls, even though the basal content of this protein is similar in both groups. This finding suggests that the intracellular GH signaling pathway may be slightly impaired in these patients.

We elected to study ISS patients with evidence of attenuated GH sensitivity that were characterized by

relatively high serum concentrations of GH and relatively low levels of IGF-1. We postulated that the impairment in the GH intracellular signal exhibited by some of these patients may be caused by negative modulators of the signaling pathway. To investigate this hypothesis, we studied whether some of these patients have an increase in the intracellular protein content of the tyrosine phosphatases PTP1B and SHP-1. The results of our study showed that the SHP-1 basal content was lower in the ISS children compared to controls, but that the PTP1B protein content was similar in both study groups.

Our next step was to investigate whether stimulation with GH during 24 h had any differential effect on the protein content of SHP-1 and PTP1B in the patients with ISS compared to controls. PTP1B protein content increased to a similar degree in both groups of subjects in response to GH, however, SHP-1 protein content decreased in controls, but not in ISS patients. To understand the role of these various tyrosine phosphatases on GH signaling, their cellular locations need to be considered. PTP1B is a cytosolic phosphatase, therefore, it is not surprising to find an increase in the content of this negative intracellular modulator after GH administration. Most of the interactions between PTP1B and phosphorylated JAK2 occur in the membrane compartment (17). An important issue for SHP-1, however, is related to its nuclear translocation during activation, which might explain the decreased

content of its non-nuclear fraction after incubation with rhGH. We documented a different behavior of SHP-1 in ISS patients compared to controls, suggesting that a deregulation in the negative modulation of the GH signaling pathway may be observed in some of these patients.

Following GH signaling, SHP-1 is activated in the cytoplasm and is translocated to the nucleus, where it dephosphorylates STAT proteins that act as transcriptional factors by binding to specific DNA elements and initiating gene transcription (6, 7). In the control children with normal height, we observed a reduction in the SHP-1 nuclear protein content after stimulation with rhGH, reduction which was not observed in the ISS patients. These results suggest that SHP-1 may impair intracellular GH signaling in some patients with ISS.

Although PTP1B and SHP-1 are not unique in their ability to dephosphorylate JAK2, and that several PTPs may be involved in different aspects of GHR signaling (different PTPs recognize different GHR phosphotyrosines),

our results suggest that these phosphatases may modulate the action of GH in our skin fibroblast model.

In conclusion, our results suggest that the growth retardation observed in some ISS patients might be related to a delayed activation of JAK2, which affects the “activators” that need to be phosphorylated to continue the signaling process. This delay in the GH signaling pathway may contribute to the impairment in the negative phosphatase modulators, which in turn deregulate the GHR/JAK2/STAT signaling pathway. However, a reduction in the content of nuclear phosphatases after rhGH stimulation may be required to maintain GH signaling activation, and allow normal growth to proceed.

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