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Growth hormone signaling in fibroblasts from newborn boys and prepubertal boys

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ABSTRACT

Background/aim: Responsiveness to GH in target cells is mediated by its receptor, which activates the Janus kinase-2 (JAK2) and STAT5 (signal transducers and activators of transcription 5) leading to the expression of IGF-1 and IGFALS. The aim of this study was to compare the GH signaling pathway in newborns and prepubertal boys.

Subjects and methods: We determined the GHR protein content and the effect of stimulation with recombinant human GH (rhGH; 200 ng/mL) on JAK2 and STAT5 phosphorylation in skin fibroblast cultures obtained from newborns and prepubertal boys. The transcript levels of IGFALS and IGF-I, were also studied and compared after 16 h or 24 h of stimulation with GH in both study groups.

Results: Newborn infants showed less GHR protein than the prepubertal boys. After rhGH stimulation, JAK2 and STAT5 phosphorylation was absent in skin fibroblasts from newborns, but was clearly detectable in prepubertal boys. After 16 h of treatment with rhGH, IGFALS and IGF-I transcript levels increased in the prepubertal boys when compared to baseline. In newborns, however, we did not observe a response after 16 and 24 h of rhGH stimulation.

Conclusion: The significant attenuation of the GH signaling pathway observed in fibroblasts from newborn boys appears to be related to a reduction in GHR content and lack of phosphorylation of JAK2 and STAT5 in response to rhGH. This might impair STAT5 dimer formation, leading to a reduction in the transcript levels of IGFALS and IGF-I during the newborn period.

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1. Introduction

Growth in humans is characterized by a number of features, including a high rate of fetal growth, relative growth deceleration following birth, a prolonged phase of stable growth during childhood, and an adolescent growth spurt. Although many factors contribute to linear growth during infancy, childhood and adolescence, studies to date indicate that IGF-I production is highly dependent on growth hormone (GH) during postnatal life, but largely independent of GH during fetal life.

Published human growth curves document that a newborn triples its weight and increases its length by approximately 50% during the first year of life [1,2]. This high rate of gain in weight and height during the first year of life is not observed at any other stage of postnatal life. It is interesting to note, however, that GH does not appear to play a leading role regarding the promotion of growth during the first few months of life [3]. The relationship between the patterns of growth following birth and the circulating concentrations of the main two hormones of the somatotropic axis, GH and IGF-I, is quite interesting. During this period of accelerated growth, the serum concentrations of GH are relatively high, but the serum concentrations of IGF-I are quite low [4].

The intracellular GH pathway requires GH binding to its transmembrane receptor (GHR), a cytokine receptor that lacks intrinsic kinase activity and exists as a pre-assembled non-functional dimer [5,6]. This is followed by conformational changes leading to stabilization of the dimer and induction of signal transduction through the recruitment and activation of Janus kinase 2 (JAK2) [6,7]. This kinase phosphorylates itself and GHR on multiple intracellular tyrosine residues, thus providing docking sites for signaling mediators. Among these, the most important are signal transducers and activators of transcription (STAT), particularly STAT5b. Activated by phosphorylation, these cytoplasmic factors dimerize and translocate to the nucleus, where they bind to GH-responsive elements (GHRE) in DNA and activate transcription of target genes, including *IGF1*, *IGFBP3* and *IGFALS* [8,9,10].

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To date, very few studies have evaluated the GH signaling pathway in newborns at the molecular level, *i.e.* the content of GHR, the main intracellular mediators, JAK2 and STAT5, as well as the expression of the final effectors, *IGF-I* and *IGFALS*. The aim of our study was to evaluate several components of the GH signal transduction pathway in fibroblasts obtained from newborns and compare this, with previously reported data obtained from prepubertal boys. The results of our study show a lower GHR content, a lack of phosphorylation of JAK2 and STAT5, and a reduction in *IGFALS* and *IGF-I* transcript levels in fibroblasts from newborns compared to prepubertal boys.

2. Subjects and methods

2.1. Study subjects

The study population consisted of two groups of children: i) newborn boys (8.6 ± 0.6 days of age) with normal length and weight and ii) prepubertal boys (6.3 ± 0.4 years of age) with normal height and weight for gender and age. These subjects underwent elective surgery for an unrelated condition (mostly circumcision in newborns, and hernia repair in the older boys), and were recruited at the San Borja-Arriarán Hospital in Santiago, Chile. The study was approved by the Ethics Committee of the University of Chile, and informed consent was obtained from the parents of all the children who participated in this study.

2.2. Chemicals

Recombinant human growth hormone (rhGH) was a gift from Dr. A. F. Parlow (National hormone and peptide program, NIH, USA). Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L of glucose, Dulbecco's phosphate buffered solution (DPBS), penicillin/streptomycin, fungizone and TRIzol reagent were purchased from Invitrogen (Grand Island, NY, USA). Heat inactivated fetal calf serum (FCS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Prestained molecular mass standard proteins and GeneRuler 1 kb DNA Ladder were obtained from Fermentas (Burlington, Ontario, Canada), and all the other chemicals were purchased from Sigma (St. Louis, Mo, USA), unless stated otherwise.

2.3. Cell cultures

The HEPG2 cells were obtained from the American Type Culture Collection (ATCC) and were grown in high glucose DMEM and 1.5 g/L of sodium bicarbonate, supplemented with 10% FCS in 5% CO_2 at 37 °C. These cells were used as a positive control for the different assays. Primary fibroblast cultures were established from skin-biopsy specimens obtained from all children. The tissue (~1 mm^3) was cultured in DMEM supplemented with 10% FCS, 10 U/mL penicillin/streptomycin and 0.25 μg/mL fungizone at 37 °C in a 5% CO_2 atmosphere. Fibroblast cultures were used between the third and sixth passages, in order to avoid the influence of plasma factors and senescent changes over the cellular response.

2.4. Protein extraction

Skin fibroblasts and HEPG2 cells seeded on 100-mm dishes at 80% confluence were washed with DPBS, serum deprived for 48 h and treated with 200 ng/mL rhGH. A time course study was performed to analyze JAK2 and STAT5 phosphorylation (basal, 15, 30 and 60 min). Protein extracts were prepared as described previously [11]. The protein content was determined by the Bio-Rad Bradford (Richmond, CA, USA) assay. Soluble fractions from fibroblasts were heated at 95 °C with 0.33 vol of SDS-PAGE sample buffer.

2.5. 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) and anti-phospho-JAK2 $Tyr^{1007/1008}$ 1:1000 purchased from Cell Signaling Technology (Danvers, MA, USA); anti-STAT5a/b 1:800 and anti-GHR 1:800 purchased from Abcam (Cambridge, MA, USA), anti-phospho-STAT5 $Tyr^{694/699}$ 1:1000 from Santa Cruz (Dallas, Texas, USA); anti-β-actin 1:100,000 obtained from Sigma-Aldrich (St. Louis, MO, USA) and anti-TFIIB 1:400 purchased from BD Transduction Laboratories (Franklin Lakes, NJ, 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 visualized using the Pierce® ECL Western Blotting Substrate Kit (Thermo Scientific, Rockford, IL, USA), blots were captured by exposure using the Discovery 10gD Ultralum with Ultraquant software (6.0.0344 version). The OD of the protein bands relative to the respective total content, was quantified by the Image J 1.38x (NIH, USA). For reblotting, membranes were stripped by incubation for 2 min at room temperature with Re-blot Mild Solution (Chemicon, Temecula, CA, USA). Blots were washed, reblotted and immunolabeled as described above. Immunoreactive proteins were visualized, blots were captured and the OD of the protein bands relative to the respective total content, was quantified by the Image J 1.38x (NIH, USA).

2.6. Total RNA extraction and reverse transcription from skin fibroblast cultures

Skin fibroblasts seeded on 100-mm dishes at 80% confluence were washed with DPBS and serum deprived for 48 h. Two days after deprivation, the cells were treated with or without 200 ng/mL rhGH for 16 or 24 h. Total RNA was extracted with TRIzol reagent from treated skin fibroblast cell cultures and skin fibroblast cultures under basal conditions. Reverse transcription of 3 μg of total RNA was performed in 20 μL reactions containing 1 μL/reaction of Impron-II reverse transcriptase (Promega, Madison, WI, USA), 50 mM Tris–HCl (pH 8.3, 25 °C), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each deoxyribonucleotide triphosphate, and 0.5 μg of random primers (Invitrogen, Grand Island, NY, USA). Reactions were conducted at 39 °C for 60 min.

2.7. Analysis of IGF-I and IGFALS in skin fibroblasts by reverse transcription-PCR

Two microliters of the reverse transcription reaction was used for PCR amplification in a 20 μL reaction containing 2.0 mM $MgCl_2$, 0.5 mM each deoxyribonucleotide triphosphate, 2.5 U of GoTaq DNA polymerase (Promega, Madison, WI, USA), 10 mM Tris–HCl (pH 8.5, 25 °C), 50 mM KCl, 0.1% Triton X-100, and 25 pmol of each primer.

The PCR program for *IGF-1* consisted of an initial cycle of denaturation (10 min, 95 °C), followed by 34 cycles of denaturation (15 s, 95 °C), annealing (1 min, 61.2 °C), and extension (1 min, 72 °C). *IGF-1* primers: 5′-TGTGCCTGCTCACCTTCA-3′ (forward) and 5′-GGACAGAG CGAGCTGACTT-3′ (reverse) yield a 255-bp product.

The PCR program for *IGFALS* consisted of an initial cycle of denaturation (2 min, 95 °C), followed by 35 cycles of denaturation (20 s, 95 °C), annealing (50 s, 58.8 °C), and extension (50 s, 72 °C). *IGFALS* primers were described by Suwanichkul et al. [12] which yield a 300-bp product.

For normalization purposes β-actin cDNA was measured using primers previously described [13] and yield a 256-bp product. DNA fragments in PCR products were separated on 2% agarose gels, followed by red gel staining to visualize DNA fragments. The signal intensity of DNA fragments in PCR products was quantified by using ImageJ 1.43x (NIH, Bethesda, MD, USA).

2.8. Real-time RT qPCR

Primer sequences for real-time PCR were: GHR 5′-GTGATGCTTTTTCT GGAAGTGA-3´ (forward) and 5´-TCAGGGCATTCTTTCCATTC-3′ (reverse) [14]; for normalization purposes GADPH was also measured using the primers 5′-AGCCGCATCTTCTTTTGC-3′ (forward) and 5′-AATGAAGGGGTCATTGATGG-3′ (reverse) [15].

Quantization of GHR mRNA expression was determined by quantitative real-time PCR (StepOne Plus Real-Time PCR System; Applied Biosystems) and normalization of the data was carried out using GADPH as a housekeeping control.

The reaction volume for each PCR contained 5 μL of Maxima SYBR Green qPCR Master Mix (2×) [Thermo Scientific K0229], 0.1 μM of GHR or 0.6 μM of GADPH of each specific human primer and 1 μL of diluted cDNA (1:5) used as template. All reactions were performed in triplicate in a final volume of 10 μL. The cycling parameters and the annealing temperature were previously described [14]. Each primer pair was analyzed for amplification efficiency by using 6 serial 2-fold cDNA dilutions and has an amplification efficiency greater than 90%. PCR products were also analyzed by agarose gel electrophoresis to verify specificity.

2.9. Statistical analyses

Results are expressed as mean ± SEM. The SDS for weight and height for our subjects were based on the standards from the National Center for Health Statistics (NCHS). 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 16 h or 24 h, with and without rhGH, were compared for each subject by the Kruskal–Wallis test. Statistical analysis was performed with the GraphPad Prism program version 5.00, and a p-value less than 0.05 was considered significant.

3. Results

3.1. Clinical characteristics of the study subjects

The 12 newborns had a mean age of 8.6 ± 0.6 days, a mean length of −0.24 ± 0.2 SDS, and a mean weight of −0.06 ± 0.33 SDS. The 12 prepubertal boys had a mean age of 6.3 ± 0.4 years, a mean height of −0.52 ± 0.15 SDS, and a mean weight of −0.05 ± 0.18 SDS.

3.2. GH receptor protein content and expression in newborns and prepubertal boys

The GH receptor (Fig. 1A; left panel, arrow) was detectable in the fibroblasts obtained from both study groups under basal conditions. However, newborn infants showed 36% less GHR protein content (0.99 ± 0.21) when compared to prepubertal boys (1.55 ± 0.21; $p < 0.05$, Fig. 1A, right panel). To corroborate our results, cDNA obtained

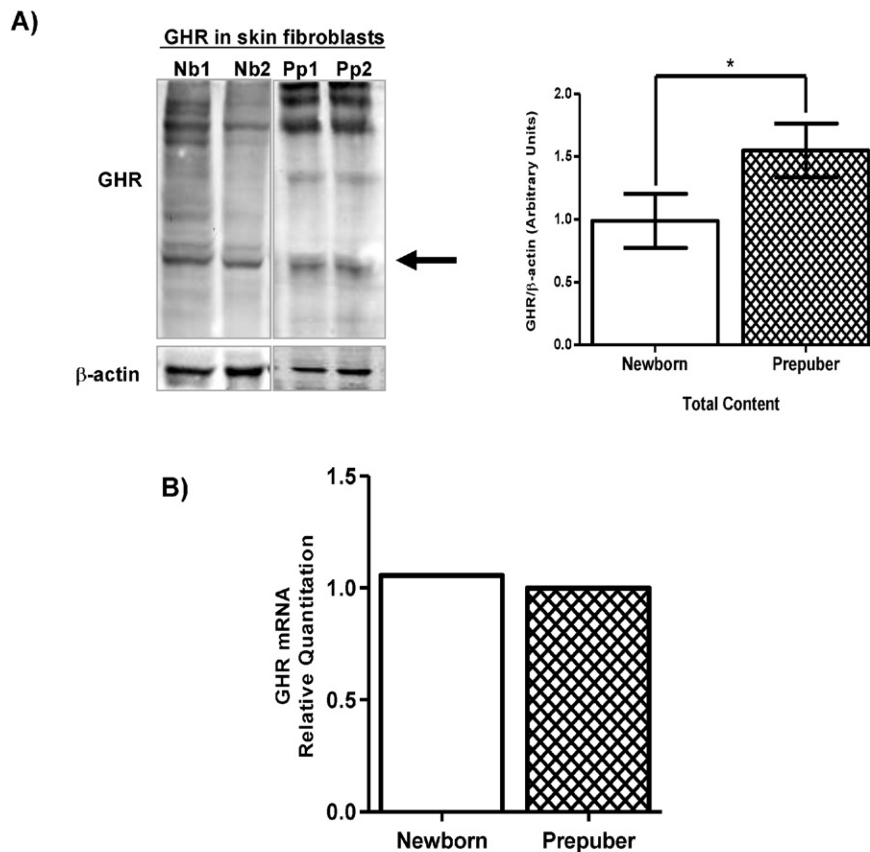

Fig. 1. (A) Protein content of GHR in newborn infants and in prepubertal boys under basal conditions. Representative Western immunoblots raised against GHR and β-actin are shown. Quantitated GHR protein content in newborn infants and prepubertal boys. Values are expressed as mean ± SEM. *$p < 0.05$. Nb = newborn; Pp = prepuber. (B) GHR mRNA expression in skin fibroblasts. Total RNA was extracted and mRNA expression was analyzed in triplicate by real-time PCR. The relative RNA level was calculated by efficiency percent method where GADPH was used as endogenous control. Control prepubertal fibroblasts were used as a calibrator sample.

from newborn and prepubertal samples was randomly chosen for qPCR analysis. The mRNA of GHR (Fig. 1B) was expressed in newborn and prepubertal skin fibroblasts, but no significant differences were detected between both groups of boys.

3.3. JAK2 phosphorylation in newborns and prepubertal boys

We subsequently compared the protein content and time-course phosphorylation of JAK2 in both study groups. As shown in Fig. 2A, JAK2 was detectable in newborns and the protein content was similar in both groups of subjects under basal conditions.

As previously reported by our group, JAK2 activation in prepubertal boys increased after 15 min of stimulation with rhGH compared to basal levels [11]. Based on these data, we investigated how JAK2 responds to GH stimulation in newborns under the same culture conditions. The time-course activation of JAK2 following stimulation with rhGH revealed that the fibroblasts from newborns did not exhibit any activity at all the times studied (Fig. 2B). Specifically, after 15 min of rhGH stimulation, when an increase in JAK2 phosphorylation is observed in the prepubertal boys (Fig. 2C, dashed bars) [11], phosphorylation levels were similar to those observed under basal conditions in the newborn group (Fig. 2C, white bars).

3.4. STAT5 phosphorylation in the non-nuclear fraction of newborns and prepubertal boys

Subsequently, we compared the protein content of STAT5 in both groups of children under basal conditions. The results showed that newborns have higher basal STAT5 protein content when compared to prepubertal boys (Fig. 3B). As we previously described in prepubertal boys, STAT5 phosphorylation in the non-nuclear fraction increases within 15 min, peaks after 30 min and returns to basal levels after

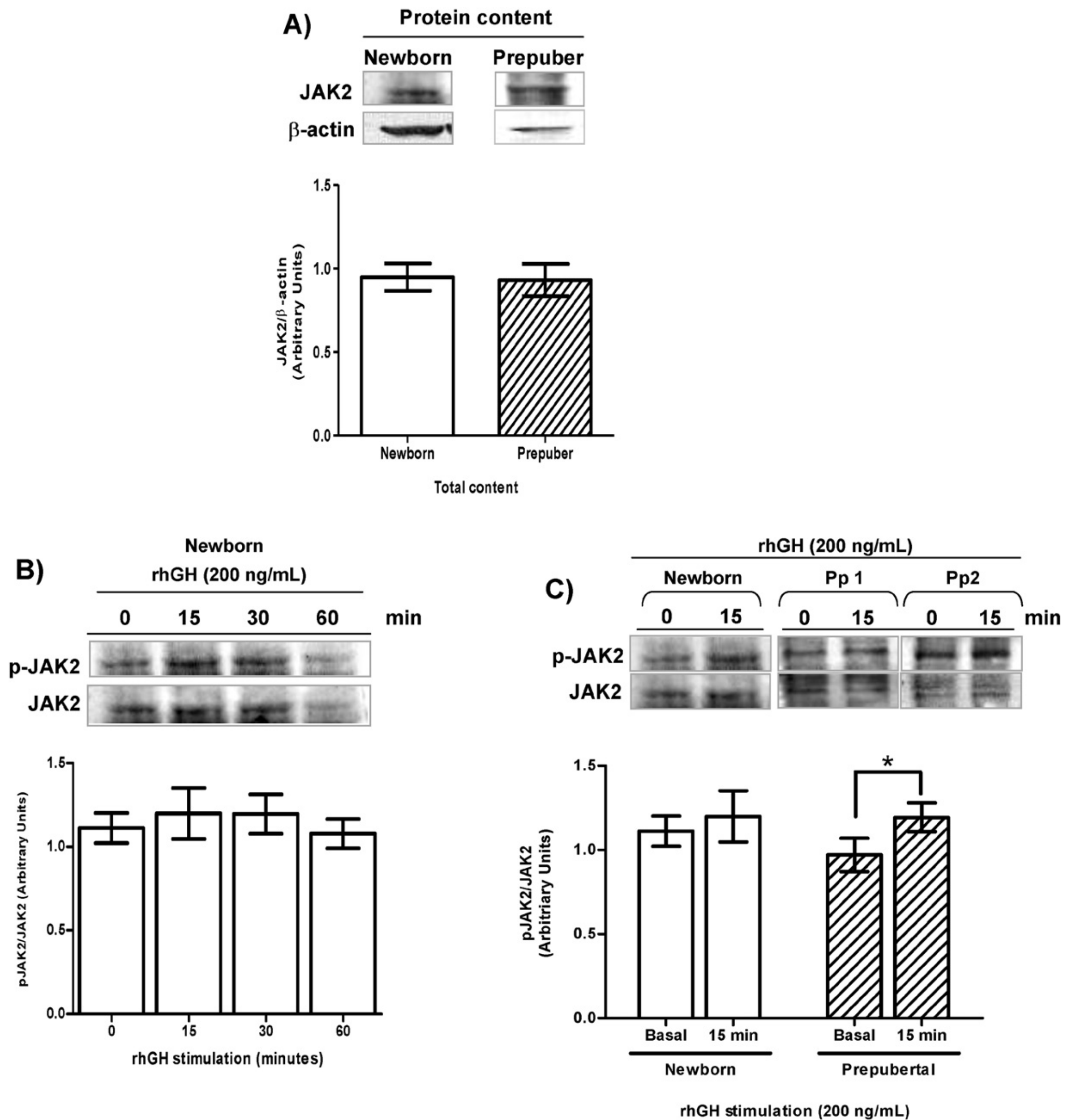

Fig. 2. JAK2 activation in skin fibroblasts after rhGH stimuli in newborn infants. JAK2 activation in prepubertal boys was previously reported by Ocaranza et al. [11]. Representative Western immunoblots raised against JAK2 and β-actin are shown. (A) Quantitated JAK2 protein content in newborn infants and prepubertal boys. (B) Time-course phosphorylation of JAK2 in newborn infants. (C) Phosphorylation levels of JAK2 after rhGH stimulation (Pp = prepuber). Values are expressed as mean ± SEM. *$p < 0.05$.

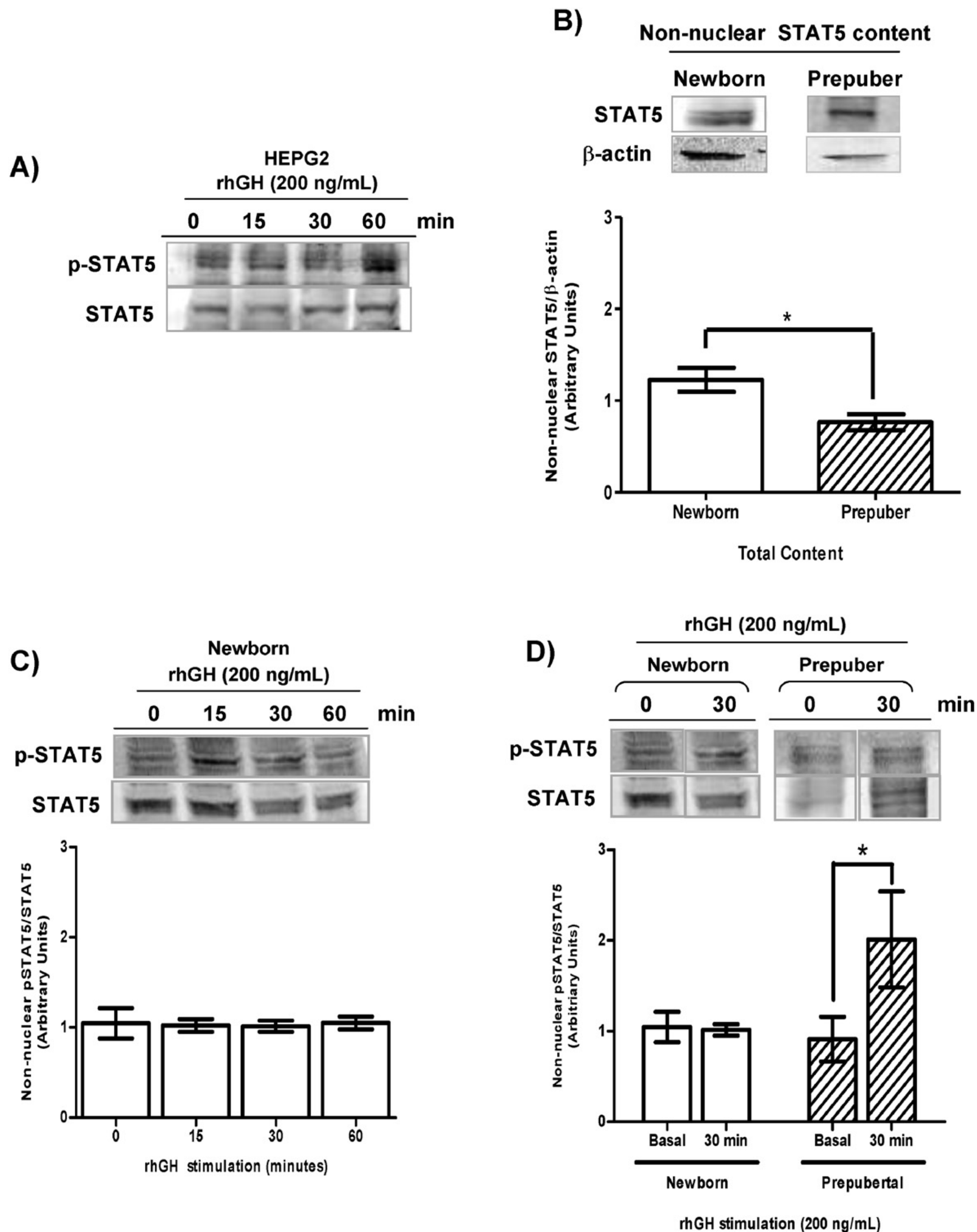

Fig. 3. STAT5 phosphorylation in skin fibroblasts after rhGH stimuli in newborn infants. Representative Western immunoblots raised against pSTAT5, STAT5 and β-actin in the non-nuclear fraction are shown. (A) Time-course phosphorylation of STAT5 in HEPG2 cells, used as positive control. (B) Quantitated STAT5 protein content in newborn infants and prepubertal boys. (C) Time-course phosphorylation of STAT5 in newborn infants. (D) Phosphorylation levels of STAT5 after rhGH stimulation. Prepubertal data was previously reported [16]. Values are expressed as mean ± SEM. *$p < 0.05$.

60 min [16]. The time-course study in newborns, however, showed that STAT5 phosphorylation remained similar to basal conditions at all times studied (Fig. 3C).

Considering that the maximum STAT5 phosphorylation increase in the prepubertal group of boys is observed after 30 min of rhGH stimulation (Fig. 3D, dashed bars) [16], we compared the response between groups at that time point. We did not observe an increase in STAT5 phosphorylation in the fibroblasts from newborns at 30 min compared to its basal level (Fig. 3D, white bars). As mentioned, however, newborns have higher STAT5 protein content under basal conditions (Fig. 3B).

3.5. Nuclear STAT5 phosphorylation in newborns and prepubertal boys

Considering that STAT5 phosphorylation is necessary for its dimerization and translocation to the nucleus, we evaluated the protein content and phosphorylation of STAT5 in this cellular compartment.

Our results show that the protein content of STAT5 in the nucleus was detectable in both study groups, and was similar under basal conditions (Fig. 4B). A time-course study for STAT5 in prepubertal boys that was previously reported in the nuclear fraction [16] showed an increase in phospho-STAT5 levels at 15 min of GH-treatment, peaked after 30 min of rhGH stimulation and returned to basal levels after 60 min. However, a time-course study for nuclear STAT5 phosphorylation in newborns (Fig. 4C) showed that the ratio phospho-STAT5/STAT5 was similar, both in the absence or presence of rhGH at all times studied. In addition, an increase in phospho-STAT5 was observed in prepubertal boys, but was absent in newborns after 30 min of rhGH stimulation (Fig. 4D).

3.6. *IGFALS* gene expression in newborns and prepubertal boys

In order to assess the activity of this phosphorylated transcription factor, we determined the induction of the STAT5-mediated *IGFALS* gene after stimulation with rhGH. As shown in Fig. 5A, basal expression of *IGFALS* was similar in both groups of subjects. The expression of *IGFALS* in the prepubertal boys (Fig. 5B, dashed bars), showed a GH-induced activation for *IGFALS* above basal levels after 16 h of stimulation. However, this increase in *IGFALS* gene expression was not observed in the newborns (Fig. 5B, white bars), under the same cell culture conditions.

3.7. *IGF-1* gene expression in newborns and prepubertal boys

Basal expression of *IGF-I* was higher in the newborns compared to the prepubertal boys (Fig. 6A). After 16 h of stimulation with rhGH, point at which *IGFALS* transcript levels increased in the prepubertal boys (Fig. 5B), the transcription levels for *IGF-I* also increased in this group of subjects (Fig. 6B, dashed bars). In the newborns, however, the transcription levels for *IGF-I* were similar, both in the presence or absence of rhGH (Fig. 6B, white bars).

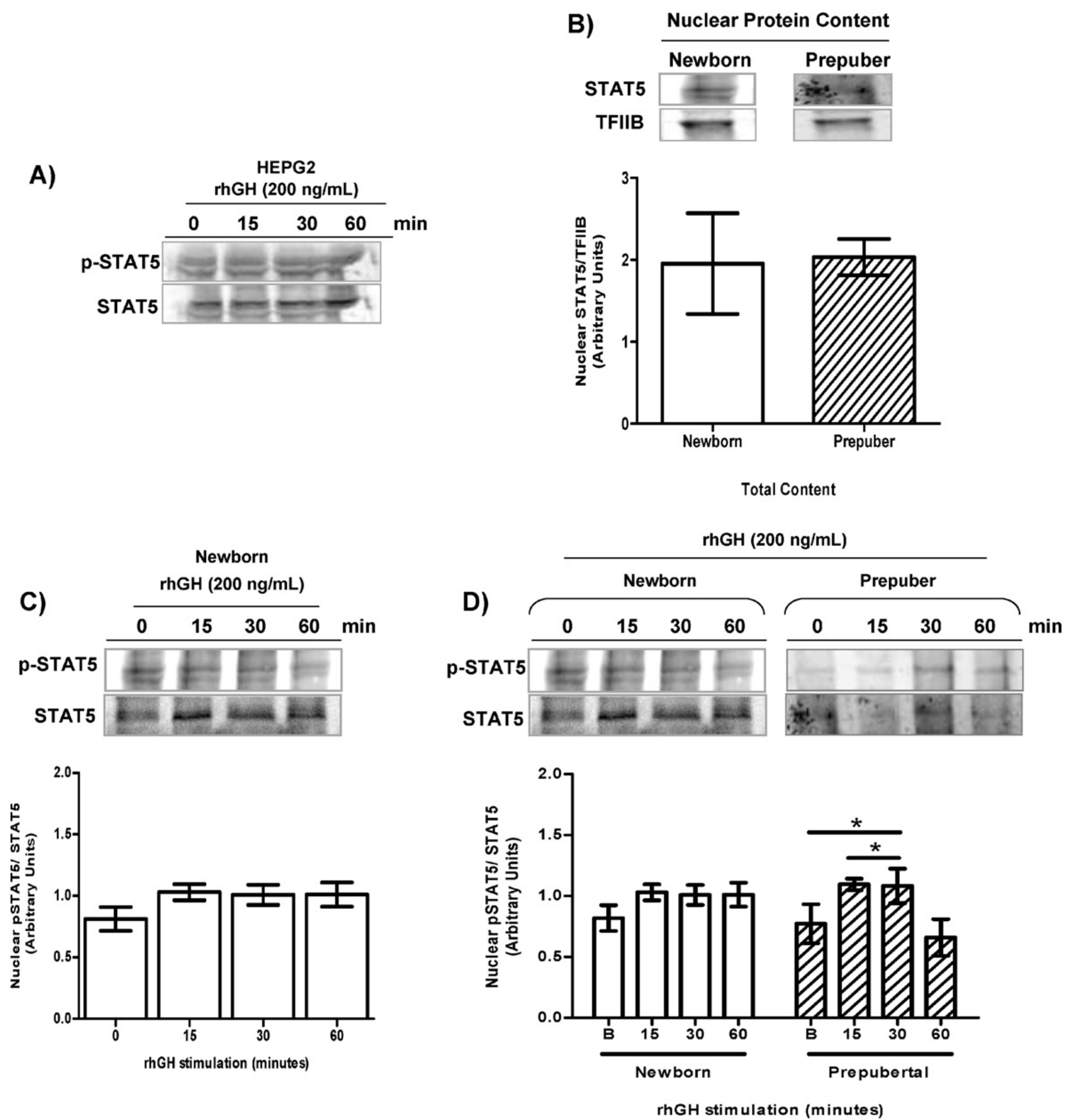

Fig. 4. Nuclear phospho-STAT5 in skin fibroblasts after rhGH stimuli in newborn infants. Representative Western immunoblots raised against pSTAT5, STAT5 and TFIIB in the nuclear fraction are shown. (A) Time-course phosphorylation of nuclear STAT5 in HEPG2 cells, used as positive control. (B) Quantitated nuclear STAT5 protein content in newborn and prepubertal boys. (C) Time-course phosphorylation of nuclear STAT5 in newborn infants. (D) Phosphorylation levels of nuclear STAT5 after rhGH stimulation. Prepubertal data was previously reported [16].Values are expressed as mean ± SEM. *$p < 0.05$.

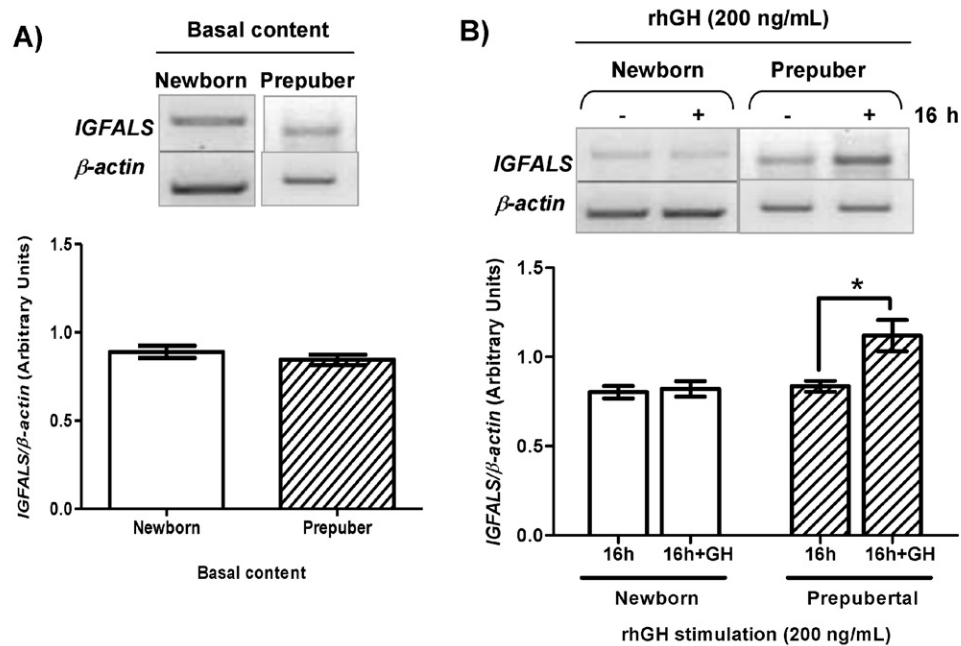

Fig. 5. Transcript levels of IGFALS in skin fibroblasts under basal conditions and after 16 h of rhGH stimuli in newborn infants and prepubertal boys. Representative RT-PCRs for newborn and prepubertal boys are shown. Bands were quantified and normalized to β-actin. (A) Basal content of transcript levels of IGFALS in both groups of boys. (B) IGFALS transcript levels after 16 h of rhGH treatment. Values are expressed as mean ± SEM. *$p < 0.05$.

Considering that we did not observe an increase in *IGF-I* transcript levels in newborns after 16 h of rhGH stimulation (Fig. 6B), we studied these transcript levels after 24 h of rhGH stimulation. In the newborns the transcription levels for *IGF-I* were similar, both in the presence or absence of rhGH (Fig. 6C).

4. Discussion

Postnatal growth in humans exhibits a period of rapid growth [2] following birth, which appears to be independent of GH [1]. Subsequently, there is a GH-dependent phase of growth during childhood, which culminates with the growth spurt observed during puberty. The precise timing of the transition to the GH-dependent phase growth is not well defined, but it appears to represent a change from the autocrine/paracrine control of linear growth during fetal life, to a predominantly endocrine regulation of linear growth after birth [17].

The limited GH influence on somatic growth during the fetal and early postnatal stages of growth has been attributed to an immature GHR during this period [18]. Information to date indicates that GH does not fully stimulate hepatic IGF-I production until some time after birth. This observation is based upon the relatively low serum concentration of IGF-I observed during this period, at a time when serum concentrations of GH are quite high [4]. This transition period ultimately leads to an increase in the serum concentrations of IGF-I, which are associated with a decrease in the serum concentrations of GH [19,20]. The progressive decline in circulating GH concentrations is associated with a parallel rise in GH-binding proteins (GH-BP) and IGF-I levels [21]. This process agrees with the conclusion of a previous study by Massa and coworkers [22] who established that low levels of GH-BP are present in the human fetus throughout the third trimester of pregnancy, and that these levels increase with gestational age. It has been suggested that the circulating levels of GH-BP reflect the abundance of cellular GHR. These findings may help to explain the relationship between the high concentrations of serum hGH, and the low concentrations of IGF-1 and GH-BP observed during late gestation and early postnatal life. Thus, we postulate that these changes may be associated with variations in GH sensitivity, possibly related to changes in the intracellular growth hormone signaling pathway, where mediators of GH signal transduction such as JAK2/STAT5, play an important role.

To gain a better insight into this process, we evaluated the response of JAK2 and STAT5 to rhGH stimulation in skin fibroblast cultures obtained from newborns, which were compared to fibroblasts obtained from prepubertal boys. We used fibroblasts from prepubertal boys as controls, because the GH signaling pathway is well established at this stage of life [23,24]. HEPG2 cells were also used as a reference for the study of GH signaling components. It's worth highlighting that this study included only boys, because it was easier to obtain skin fibroblasts from boys during elective surgery, which consisted exclusively of circumcision in normal newborns. This represents a potential limitation of this study, since it is not known whether GH sensitivity may differ in boys and girls during infancy and childhood.

The use of fibroblasts as an *in vitro* model for GH signaling has some advantages, since it maintains its intrinsic characteristics after being removed from its natural *in vivo* environment [23]. Fibroblast cultures have been used to study GH signaling in children with short stature caused by GH insensitivity (GHI) [24,25], and in children with idiopathic short stature [13,26,27]. It has been demonstrated that IGFBP-3 (mRNA and content) increases in fibroblasts from normal children when they are stimulated with rhGH, when compared to fibroblasts obtained from children with GHI [25]. The JAK/STAT signaling pathway is rapidly activated in these cell cultures, when they are stimulated by rhGH at a concentration of 200 ng/mL, and STAT1 and STAT5 appear to bind DNA [24]. Local IGF-I production in these cells has also been reported [28], but the expression of GHR mRNA and protein is relatively low [23,29] when compared to hepatic tissue. However, since GHR cellular expression and protein levels are low in newborns, and the GHR may be immature during early infancy, these may represent limitations of the fibroblast cell model during this period of life.

To date, very few studies regarding GHR in human newborns have been published. In 2001, Goodyer and coworkers [18] analyzed 2 fibroblast samples designated as “early postnatal cells” (4 and 8 months), whereas a second group included samples from infants, prepubertal and pubertal subjects, with ages ranging between 1.5 and 19 years. These authors suggested that the human GHR (hGHR) appears to be immature during the first few months of postnatal life. The results of

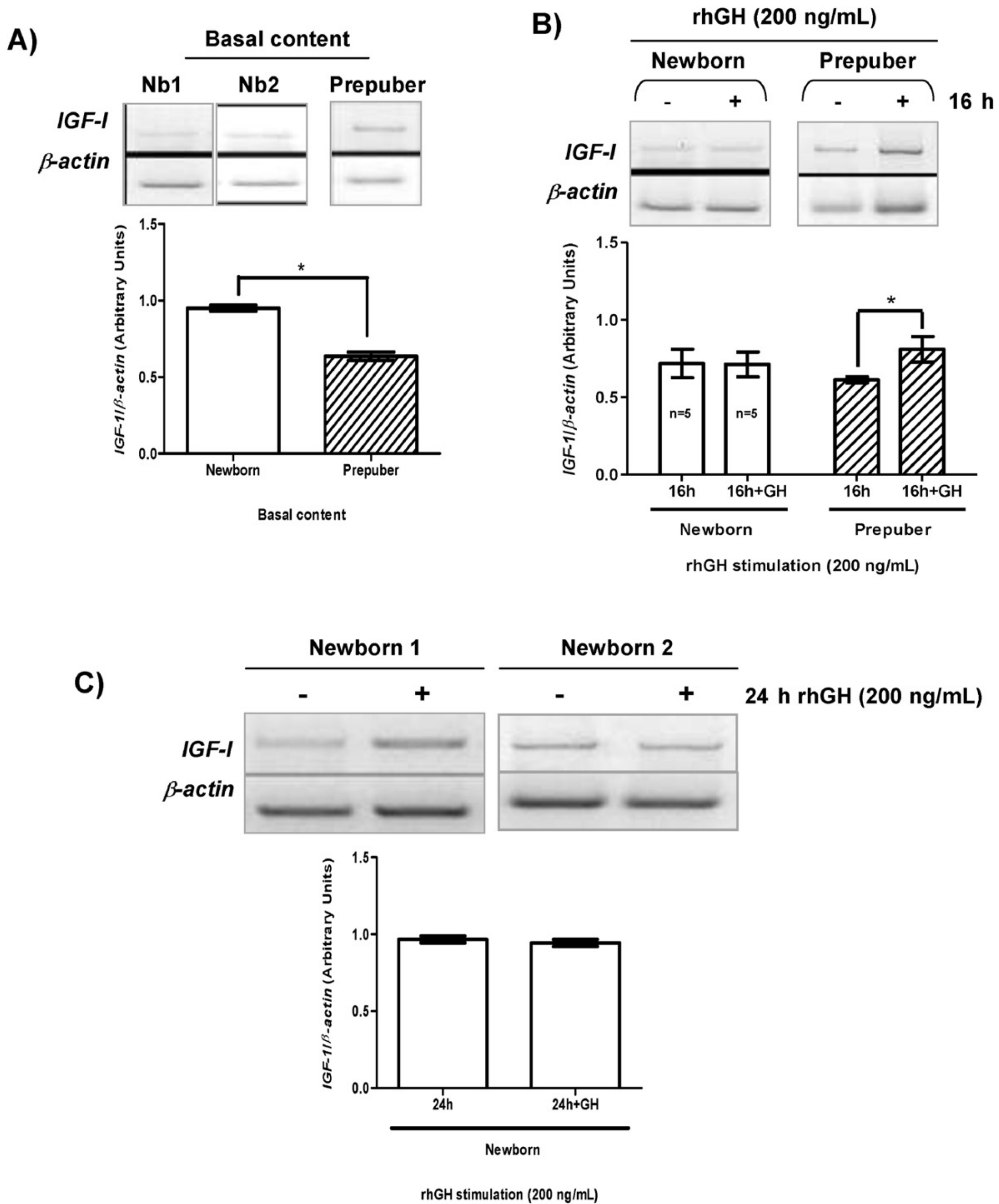

Fig. 6. Transcript levels of *IGF-I* in skin fibroblasts under basal conditions and after rhGH stimuli in newborn infants and prepubertal boys. Representative RT-PCRs for newborn infants and prepubertal boys are shown. Bands were quantified and normalized to β-actin. (A) Basal content of *IGF-I* transcript levels in both group of boys. (B) *IGF-I* transcript levels after 16 h of rhGH treatment in both group of boys. (C) IGF-I transcript levels in newborn boys after 24 h of rhGH treatment. Values are expressed as mean ± SEM. *p < 0.05.

our study, which were obtained in normal newborns at 8 days of life, showed less GHR protein content in these fibroblasts. This lower GHR content, possibly in combination with immaturity of the receptor [18], may contribute to the attenuated cellular GH sensitivity observed during this period. In this study, we evaluated the content of the receptor with an antibody that recognizes the intracellular domain, which is similar for the 3 different isoforms of GHR described to date. These isoforms, *i.e.* full length, exon 3-deficient and the truncated isoform, have been detected in human fetal tissues as early as the 8th week of fetal life [30,31]. We did not determine, however, which GHR isoform or combination of isoforms predominates during the newborn period or during the prepubertal stage of life. It is conceivable that variations in GHR isoforms may help to explain differences in GH/GHR interactions, or in the stimulation of positive mediators for GH, such as STAT5. To our knowledge, protein content and GHR expression have not been quantified in newborns; even though previous research has detected but not quantified the protein content of GHR and mRNA in human skin fibroblasts [32,33]. We analyzed by qPCR a randomly chosen sample from each group of subjects. Fibroblast skin samples from both newborns and prepubertal boys, expressed similar levels of *GHR*. It is conceivable, however, that the protein content may be different in these subjects, but this hypothesis was not investigated in this study.

JAK2 is essential for GH signal transduction and its phosphorylation allows the recruitment of a variety of signaling proteins [34]. Our results

showed that the basal content of this protein was similar in newborns and prepubertal boys, but the JAK2 phosphorylation pattern was very different. As previously reported [11] in healthy prepubertal boys, a peak in JAK2 activation is observed within 30 min of rhGH stimulation, but this increase was not observed in fibroblasts from newborns.

As reported in prepubertal boys [16], an increase in STAT5 phosphorylation was observed reaching maximum phosphorylation after 30 min of rhGH stimulation. We did not observe, however, STAT5 phosphorylation in the non-nuclear fraction of fibroblasts obtained from newborns. These data are concordant with the absence of JAK2 phosphorylation after stimulation with rhGH, although the newborns apparently had an increased basal content of this protein compared to prepubertal boys. The phosphorylated STAT5 translocates to the nucleus, where it acts as a transcription factor for specific genes [8,12,35]. In the nucleus, STAT5 protein content under basal conditions was similar in both study groups. In the newborns, however, stimulation with rhGH did not produce an increase in phospho-STAT5 levels. The absence of phospho-STAT5 in the nucleus of fibroblasts from newborns, indicates that the transcript levels of the final effectors of the GH signaling pathway may differ from that observed in prepubertal boys.

This possibility was studied by determining the transcript levels of two key components of the IGF-I binding complex, IGFALS and IGF-I. The IGFALS proteins are crucial for maintaining the circulating IGF/IGFBP system. Its main role is to extend the half-life of IGF-I by protecting the ternary complex from degradation [36]. The absence of ALS is believed to result in rapid clearance of IGF-I and IGFBP-3. In addition, IGF-I is the main effector of the somatotropic axis, which mediates GH action on cartilage and bone growth [37,38]. This peptide mediates many of the actions of GH and has anabolic, mitogenic, and antiapoptotic effects on a variety of cell types [39,40].

In our study, we observed a lack of response for IGF-1 and IGFALS after 16 h of stimulation with rhGH in the fibroblasts obtained from newborns. These data are concordant with the described lack of JAK2 and STAT5 activation observed in this group of subjects. In prepubertal boys, however, IGFALS transcript levels increased after 16 h of stimulation with rhGH. In order to further study this process, we assessed IGF-I transcript levels 16 h after rhGH stimulation in both study groups. An increase in IGF-I and IGFALS transcript levels was observed in the fibroblasts from prepubertal boys, but we did not observe a similar increase in the fibroblasts from the newborns.

In conclusion, we have investigated the intracellular GH signaling pathway in fibroblasts obtained from newborns and prepubertal boys. Our findings suggest that there is a physiological stage of attenuated GH-sensitivity during the newborn period, which may explain the relatively low serum concentrations of IGF-1 and high serum concentrations of GH observed during this period. This process may be similar to that observed for testosterone and aldosterone, which are quite elevated during the newborn period, but that appear to elicit a diminished biological response during this period of life [41,42].

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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