CLINICAL STUDY

Prednisolone reduces the ability of serum to activate the IGF1 receptor *in vitro* without affecting circulating total or free IGF1

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Abstract

Objective: End-point bioassays based on thymidine or sulfate incorporation have demonstrated that glucocorticoid (GC) treatment inhibits serum IGF1 action, but the mechanism is unknown as serum IGF1 concentrations have been reported to either increase or remain unchanged.

Aim: To investigate whether GC treatment affects the ability of serum to activate the IGF1 receptor (IGF1R) *in vitro* (i.e. bioactive IGF1), using a specific cell-based IGF1 kinase receptor activation assay.

Subjects and methods: Twenty children with stable asthma (age 7.7–13.8 years) treated for 1 week with 5 mg prednisolone in a randomized, double-blind, placebo-controlled crossover study. Non-fasting serum samples were collected in the afternoon after each 7-day period and assayed for bioactive IGF1, free IGF1, total IGFs, IGF-binding proteins (IGFBPs), and insulin.

Results: Prednisolone treatment reduced IGF1 bioactivity by 12.6% from 2.22 ± 0.18 to 1.94 ± 0.15 $\mu\text{g/l}$ ($P=0.01$) compared with placebo. In contrast, no changes were observed for ($\mu\text{g/l}$; placebo vs prednisolone) total IGF1 (215 ± 27 vs 212 ± 24), free IGF1 (1.50 ± 0.16 vs 1.43 ± 0.17), total IGF2 (815 ± 26 vs 800 ± 31), IGFBP3 (3140 ± 101 vs 3107 ± 95), IGFBP2 (238 ± 21 vs 220 ± 19), IGFBP1 (32 ± 6 vs 42 ± 10), or IGFBP1-bound IGF1 (24 ± 5 vs 26 ± 7). Insulin remained unchanged as did IGFBP levels as estimated by western ligand blotting. Prednisolone had no direct effects on IGF1R phosphorylation.

Conclusions: Our study gives evidence that GC treatment induces a circulating substance that is able to inhibit IGF1R activation *in vitro* without affecting circulating free or total IGF1. This may be one of the mechanisms by which GC inhibits IGF1 action *in vivo*. However, the nature of this circulating substance remains to be identified.

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Introduction

Long-term administration of exogenous glucocorticoid (GC) accelerates protein breakdown and inhibits protein synthesis in a variety of tissues, effects that may ultimately result in skeletal muscle atrophy, osteoporosis, and impaired linear growth (1, 2, 3). The catabolic effects of GC appear to be mediated at least in part through modulation of the biological activity of insulin-like growth factor I (IGF1) locally as well as systemically (4, 5). At the level of the growth plate, experimental studies have demonstrated that GC administration down-regulates the mRNA expression of IGF1 and the IGF1 receptor (IGF1R) and suppresses IGF1-induced DNA synthesis (5). When it comes to endocrine IGF1, numerous clinical studies have demonstrated that

administration of GC reduces the circulating IGF1 bioactivity within <12 h as estimated by the ability of serum to stimulate incorporation of radiolabeled sulfate or thymidine in cultured cartilage (6, 7, 8). However, the mechanisms by which GC may alter IGF1 action remain unclear as serum IGF1, as measured by immunoassay, remains unaltered or even increases following exposure to GC (6, 7, 9, 10). Consequently, attention has been focused on GC-induced changes in the IGF-binding proteins (IGFBPs), which are able to inhibit IGF1-mediated effects *in vivo*. However, no clear picture has emerged. Some IGFBPs are suppressed (IGFBP1 and IGFBP2), others are increased (IGFBP3 and IGFBP6) or remain unaffected (IGFBP4 and IGFBP5), and overall, the molar ratio between IGF1 and its most predominant binding protein, IGFBP3,

remains unchanged or increases in favor of IGF1, a reaction that, if anything, would tend to increase free, unbound IGF1, and consequently the bioactivity of IGF1 (7, 11, 12, 13). Accordingly, the ability of GC to inhibit IGF1 action appears to be unrelated to changes in the immunoreactive serum concentrations of the IGF1R. This is supported by previous gel chromatography studies by Unterman & Phillips (6), who showed that GC administration increased the presence of circulating IGF1 inhibitors with a molecular weight of ~ 12 – 20 kDa. However, the authors never pursued the identity of these IGF1 inhibitors, which may have represented low molecular IGF1R fragments induced by proteolysis.

The output readings of the early end-point bioassays were based on events (i.e. thymidine or sulfate uptake) being several steps downstream of the initial activation of the IGF1R, and consequently, the assays yielded relatively unspecific signals (14). Nevertheless, these assays have unambiguously demonstrated that GC treatment suppresses the ability of serum IGF1 to stimulate thymidine and sulfate incorporation in cultured cells, despite unchanged or even increased serum IGF1 concentrations (6, 7). However, end-point bioassays are unsuitable when it comes to identifying the intracellular level at which IGF1 signal transduction becomes inhibited, and consequently, the underlying mechanism has remained unknown. Therefore, we found it of interest to study the impact of GC treatment on the very first step of IGF1 action, i.e. IGF1R phosphorylation. Furthermore, we aimed to identify the IGF1 inhibitors originally described several years ago (6). To this end, we performed a randomized, double-blind, placebo-controlled crossover study comparing the *in vitro* bioactivity of IGF1 in serum from children after 7 days of prednisolone vs placebo.

Materials and methods

Subjects

The study included 20 children (17 boys and three girls) with a mean age of 10.4 (range: 7.7–13.8) years. Three boys were Tanner stage 3, the other children were prepubertal. Apart from mild asthma, all children were healthy. The mean height was 144 (range: 112–175) cm, mean weight 37.2 (range: 19.4–59.5) kg, and mean BMI 17.5 (range: 13.7–21.2) kg/m². All children were diagnosed with asthma and had been treated with inhaled GCs according to the recommended guidelines for at least 1 year before the study.

Study design

The study was performed as a randomized, double-blind, placebo-controlled crossover trial, comparing oral prednisolone (5 mg/day) vs placebo. Ten children were randomized to receive prednisolone first, then placebo, and the other half was randomized to receive treatment

in the opposite order. Treatment periods as well as run-in and washout periods lasted for 1 week. All tablets were taken at 1900 ± 1 h. Ten tablets were delivered to each child before each treatment period; the remaining tablets were returned after the treatment periods and counted for assessment of compliance. Compliance $< 80\%$ would lead to withdrawal from the study. During run-in and washout periods, the children took no medication. No drugs except the study medication and rescue-inhaled terbutaline (Bricanyl Turbuhaler, 0.5 mg/inhalation) were allowed during the study. Acute illness requiring bed rest, hospitalization, or medication other than study medication would lead to exclusion from the trial. Data on the effect of oral prednisolone on changes in skin (15) and lower leg growth rate (16) have been published previously.

Ethics

The study was conducted in accordance with the international guidelines issued by The European Commission in 1990 and the Declaration of Helsinki and approved by the Local Ethics Committee. Informed consent was obtained from all children and their families.

Immunoassays

Bioactive IGF1 was determined by our in-house KIRA assay as described previously (17) and recently discussed (18). In brief, cells transfected with the human *IGF1R* gene were cultured in 48-well microtiter plates, 24 h with serum followed by 24 h without serum. After 48 h of culture, the cells were stimulated with serial dilutions of recombinant human IGF1 (rhIGF1; Austral Biologicals, San Ramon, CA, USA; range: 0.3–10 μ g/l) or by serum-diluted 1:10 in Krebs–Ringer Buffer. All samples were pre-incubated for 15 min at 37 °C before they were transferred to the microtiter wells containing the IGF1R-transfected cells. After incubation for 15 min at 37 °C, all samples were quickly removed, and the cells lysed. Afterwards, the crude cell lysates were transferred to 96-well microtiter plates pre-coated with an antibody directed against the extracellular domain of the IGF1R. After an overnight incubation, samples were removed and replaced by assay buffer containing an europium-labeled monoclonal phosphotyrosine antibody (PY20, Sigma–Aldrich). The captured antibody was finally detected by time-resolved fluorometry. The assay readings corresponding to unknown serum samples were compared with a serial dilution of rhIGF1, which enabled us to express the level of bioactive IGF1 in unknown samples in microgram per litre of IGF1 equivalents. Owing to limited sample volumes, we were only able to measure bioactive IGF1 in 19 subjects.

Immunoreactive serum levels of IGF1 and IGF2 were assayed in acid-ethanol extracted serum, using

validated in-house time-resolved immunofluorometric assays as described previously (19). The two assays are very sensitive, and this allows serum extracts to be assayed in a final dilution of 1:1000, hereby making the assays very robust to interference from IGFBPs still present in the extracts (20). The same IGF1 calibrator was used in the bioassay and the immunoassay.

IGFBP1, IGFBP1-complexed IGF1 and IGFBP2 were determined by validated in-house assays as described previously (21, 22), whereas IGFBP3 and insulin were determined by commercial assays from DiaSource (IRMA; BioSource, Inc., Nivelles, Belgium) and Dako (insulin ELISA kit K6249; Glostrup, Denmark) respectively, in accordance with the recommendations outlined in the kit inserts. In addition, IGFBP levels were examined by western ligand blotting (WLB), performed as originally described by Hossenlopp *et al.* (23) using iodinated IGF1 (24). Corresponding samples of serum following placebo and prednisolone were analyzed in neighboring lanes on the same gel and scanned by laser densitometer and expressed in pixel densities (absorbency units (AU)) per mm².

All samples from an individual were assayed within the same assay run. The within and between assay coefficient of variation of the in-house assays averaged 7 and 15% for bioactive IGF1 (17), 5 and 10% for IGF1 and IGF2 (19), 5 and 15% for IGFBP1-complexed IGF1 (21), and 5 and 12% for IGFBP1 and IGFBP2 (22).

Two-dimensional gel electrophoresis

Serum samples were dissolved in lysis buffer consisting of 9 M urea, 2% dithiothreitol (DTT), 2% Triton X-100, and 2% IPG buffer. Horizontal isoelectric focusing was performed using a non-linear pH3-10NL IPG strip and rehydrated for 20 h at room temperature with a rehydration buffer (8 M urea, 2% CHAPS, 2% IPG-buffer, and 0.3% DTT). The first dimension was carried out on a Multiphor II Electrophoresis unit at 500 V for 5 h and at 3500 V for 14.5 h. Before the second dimension, the IPG strip was equilibrated twice with an equilibration buffer (0.05 M Tris-base, 6 M urea, 26% glycerol, and 1% SDS) and transferred to a polyacrylamide gel. The second dimension separation was run vertically at 50 V for 19 h (25, 26).

Silver staining

Visualization was achieved by silver staining, suitable for quantitative protein analysis. Briefly, gels were fixed overnight in a fixation solution (50% ethanol, 12% acetic acid, and 0.5% formaldehyde), then washed three times for 20 min in 35% ethanol, pre-treated for 1 min in 0.2% Na₂S₂O₃, and rinsed in water three times for 2–3 min. Staining of gels with silver nitrate was performed for 20 min, after which they were again rinsed twice with water. Gels were developed using a developer solution (6% Na₂CO₃, 0.0185% formaldehyde,

0.0004% Na₂S₂O₃, and 5 H₂O) for ~3 min. Development was finally stopped in a stop solution (50% methanol and 12% acetic acid). The 2-D gels were dried in cellophane sheets and sealed in plastic bags (27).

Image analysis

Silver-stained gels were scanned using the FUJIFILM LAS-4000 info. Gray-scaled images of the gels were transported as TIFF files into the PDQuest Software analysis program (Bio-Rad, Copenhagen, Denmark). Protein spots were automatically defined and adjusted to the background. The quality of each protein spot was critically evaluated, in order to ensure that all relevant protein spots were identified. The pixel intensity of each protein spot was translated to a proportional protein volume, which was normalized to the total density of the gel. Finally, re-analysis was manually done on all matched spots (26).

In vitro studies

To investigate the possible interference of GC in the cell-based bioassay, serial dilutions of cortisol, prednisolone, and prednisone (all from Sigma–Aldrich; products H4001, P6004, and P6254), containing 0, 0.1, 1, 10, and 100 mg/l, were co-incubated in the bioassay with an equal volume of sample containing Krebs–Ringer buffer, Krebs–Ringer buffer added 2.5 µg/l rhIGF1, or serum diluted 1:5 in Krebs–Ringer buffer. The GC-containing samples were incubated similar to the serum samples from the study subjects, i.e. the final dilution of serum was 1:10 and all samples were equilibrated for 15 min at 37 °C before they were incubated for 15 min with the cells.

Statistical analysis

Data obtained after prednisolone and placebo were compared by paired tests, either Student's paired *t*-test, or the Wilcoxon paired rank sum test, as appropriate. If the difference was significant, the possibility of a carryover effect was tested by comparing changes in subjects receiving treatments in the order placebo–prednisolone vs subjects receiving the opposite treatment order, i.e. prednisolone–placebo. The comparison was made by Student's unpaired *t*-test or the Mann–Whitney unpaired rank sum test as appropriate. Correlations were performed using Spearman's regression analysis. All data are given as mean ± s.e.m.; *P* values <0.05 were considered as statistically significant.

Results

The primary outcome of our investigation, bioactive IGF1, decreased significantly following 7 days of prednisolone compared with placebo, 1.94 ± 0.15 vs

Figure 1 The individual changes in serum bioactive IGF1 after 7 days of placebo or prednisolone (5 mg daily). Altogether, 19 paired samples were available. In 13 subjects, prednisolone lowered bioactive IGF1 levels (solid lines), whereas in six subjects levels increased (dashed lines). The overall reduction was significant, as indicated.

$2.22 \pm 0.18 \mu\text{g/l}$ ($P=0.01$), corresponding to a relative reduction averaging 12.6%. When looking at the individual changes, 13 subjects demonstrated numerically lower levels of bioactive IGF1, whereas six subjects showed numerically higher levels following prednisolone (Fig. 1). Irrespective of treatment order, prednisolone resulted in numerically lower levels of bioactive IGF1, i.e. from 1.95 ± 0.21 to $1.71 \pm 0.13 \mu\text{g/l}$ ($n=10$; $P=0.10$) in those receiving placebo as the first treatment, and from 2.47 ± 0.27 to $2.15 \pm 0.22 \mu\text{g/l}$ ($n=9$; $P<0.07$) in those receiving prednisolone as the first treatment. Finally, there was no statistical significance when comparing change in bioactive IGF1 between the two treatment orders ($P=0.70$), indicating that a carryover effect had not to be considered.

In contrast to the significant reduction in bioactive IGF1, serum-free IGF1 remained unaffected by

prednisolone treatment, and the same was true for immunoreactive IGF levels (Table 1). Furthermore, no changes in the levels of the IGFBPs during prednisolone treatment were observed: immunoreactive levels of IGFBP1 to IGFBP3 remained unchanged and the same was true for the binary complex of IGF1 and IGFBP1. Similarly, when employing WLB using ^{125}I -labeled IGF1, the densities of the different IGF-binding bands remained unaffected by prednisolone (Table 1).

We examined all serum samples by two-dimensional gel electrophoresis (2-D PAGE), focusing on changes within the 10–30 kDa molecular weight range and the isoelectric focusing range from pI 3 to 10, but we were unable to identify any significant changes (data not shown).

Spearman's correlation analysis was used to study the association between bioactive IGF1 and the other IGF-related measurements (Table 2). In the placebo situation, bioactive IGF1 correlated positively with free IGF1, total IGF1 and IGFBP3, whereas IGFBP1, IGFBP2, and the dimeric complex of IGF1 and IGFBP1 correlated inversely with bioactive IGF1. Following 7 days of prednisolone, the above-mentioned correlations were generally weakened, and IGFBP2 and IGFBP3 were no longer correlated with bioactive IGF1.

To rule out any effect of GC *per se* on IGF1R phosphorylation, the cells were stimulated for 15 min with serial dilutions of prednisolone, prednisone and cortisol respectively, co-incubated with buffer, rhIGF1, or serum. However, neither of the selected GCs was able to affect IGF1R phosphorylation *in vitro* (only data from the prednisolone experiment have been shown; Fig. 2).

In an earlier publication based on the same study sample, it was possible to demonstrate by knemometry that 1 week of prednisolone treatment reduced lower leg growth rate in 17 of 20 children ($P<0.01$), from 0.58 mm/week during placebo to -0.23 mm/week during prednisolone treatment (16). The question

Table 1 IGF-related parameters after 7 days of placebo or prednisolone treatment (5 mg/day) in 20 children aged 10.4 years (range 7.7–13.8 years). Data are mean $\pm$ S.E.M. Two different IGFBP1 measurements are stated: total IGFBP1 was measured by immunoassay measuring all phospho forms (22) and the complex composed by IGF1 and IGFBP1 was measured by a specific assay (21).

Treatment variable	Placebo	Prednisolone	P value of the paired test
Total IGF1 ($\mu\text{g/l}$)	215 ± 27	212 ± 24	0.72
Total IGF2 ($\mu\text{g/l}$)	815 ± 26	800 ± 31	0.11
Free IGF1 ($\mu\text{g/l}$)	1.50 ± 0.16	1.43 ± 0.17	0.26
IGFBP1 ($\mu\text{g/l}$)	32 ± 6	42 ± 10	0.87
IGFBP1:IGF1 ($\mu\text{g/l}$)	24 ± 5	26 ± 7	0.75
IGFBP3 ($\mu\text{g/l}$)	3140 ± 101	3107 ± 95	0.47
IGFBP2 ($\mu\text{g/l}$)	238 ± 21	220 ± 19	0.24
Insulin (pmol/l)	122 ± 28	126 ± 17	0.89
WLB; 38–42 kDa band (AU/mm^2)	209 ± 46	298 ± 58	0.26
WLB; 32 kDa band (AU/mm^2)	60 ± 10	60 ± 9	1.00
WLB; 28 kDa band (AU/mm^2)	22 ± 6	22 ± 4	0.65
WLB; 24 kDa band (AU/mm^2)	14 ± 3	20 ± 4	0.21
WLB; sum of all bands (AU/mm^2)	306 ± 59	401 ± 67	0.29

WLB, western ligand blotting.

Table 2 Spearman's rank sum correlations between bioactive IGF1 and the other IGF-related parameters after 7 days of either placebo or prednisolone treatment (5 mg/day). Owing to lack of serum, bioactive IGF1 was only measured in 19 of 20 children.

Spearman's correlations	Placebo	Prednisolone
IGF1 bioavailability vs		
Free IGF1	$r=0.82; P<0.0001$	$r=0.73; P<0.0002$
Total IGF1	$r=0.65; P<0.003$	$r=0.57; P<0.02$
Total IGF2	NS	NS
IGFBP3	$r=0.66; P<0.003$	NS
IGFBP2	$r=-0.55; P<0.02$	NS
IGFBP1	$r=-0.57; P<0.02$	$r=-0.51; P<0.03$
Dimeric IGF1:IGFBP1	$r=-0.48; P<0.04$	$r=-0.48; P<0.04$
Insulin	NS	NS

therefore remained whether bioactive IGF1 correlated with these growth rates. However, no significant correlations were observed when comparing changes in bioactive IGF1 and in lower leg growth rate (data not shown).

Discussion

This study investigated the impact of GC treatment on the very first step of IGF1 action, i.e. IGF1R phosphorylation *in vitro*. Serum samples were obtained from healthy children, treated for 1 week with 5 mg prednisolone daily and placebo respectively using a randomized, double-blind, placebo-controlled crossover study design. The ability of serum to phosphorylate the IGF1R *in vitro* was determined by a highly specific and sensitive cell-based IGF1 KIRA assay. Using this approach, we were able to demonstrate that GC treatment within 7 days induced a circulating substance that inhibited the ability of serum to phosphorylate the IGF1R *in vitro*. Of note, this IGF1R inhibition was not caused by GC *per se*, and it appeared to occur without any detectable changes in serum IGF or IGFBP concentrations. Thus, our study may have identified one possible mechanism by which GC is able to inhibit IGF1-mediated actions *in vivo*.

Generally, clinical conditions regarded as catabolic are characterized by low serum IGF1 concentrations and/or increased serum IGFBP concentrations. This is true for patients with anorexia nervosa (28, 29), end-stage renal failure (30), and poorly controlled type 1 diabetes (31), whereas GC treatment makes a notable exception. Despite its catabolic effects, GC excess is generally not accompanied by a suppression of the circulating IGF system (6, 7, 12, 32). With the present data in mind, it appears that the ability of GC to inhibit serum IGF1 action is mediated through an inhibition of IGF1R phosphorylation rather than through changes in the circulating concentrations of free IGF1, total IGFs, or insulin. However, it has to be acknowledged that

serum-free IGF2 was not assessed, and theoretically, changes in this parameter could be of importance. On the other hand, in the bioassay, the cross-reactivity of IGF2 with the IGF1R was only 12% (17), and the ability of serum to phosphorylate the IGF1R *in vitro* (i.e. bioactive IGF1) showed no relationship with serum total IGF2, neither after placebo nor after prednisolone. Therefore, IGF2 seems an unlikely candidate to explain our observation.

The present data ruled out a direct effect of GC on IGF1R phosphorylation *in vitro*. Therefore, our data support the hypothesis that GC treatment induces a blood-borne substance that is able to inhibit the very first step of IGF1 action, i.e. IGF1R phosphorylation, independent of circulating IGF. This notion is in fact in agreement with early data from Unterman & Phillips (6). In 1985, they demonstrated that in normal subjects, the ability of serum to stimulate labeled sulfate uptake in costal cartilage from hypophysectomized rats was reduced by close to 50% 6 h after the intake of 60 mg prednisone, despite no detectable changes in serum IGF1 levels as measured by RIA.

To elucidate the mechanism by which prednisolone may inhibit IGF1R phosphorylation *in vitro*, we determined immunoreactive levels of ultrafiltered free IGF1, IGFBP1, IGFBP2, IGFBP3, as well as IGFBP1 complexed IGF1, and we also assessed the ability of the IGFBPs to bind 125 I-IGF1 by WLB. However, serum-free IGF1 remained unchanged by prednisolone, and the same was true for the IGFBPs, hereby indicating that

Figure 2 The *in vitro* bioactivity of serum (open triangles), rhIGF1 (open squares), and buffer (closed squares) incubated with increasing concentrations of prednisolone. After co-incubation, samples were assayed in the bioassay based on IGF1R-transfected cells. Each data point represents the mean of a triplicate determination. The error bars did not exceed the size of the symbols and are therefore omitted.

the ability of prednisolone to inhibit IGF1R phosphorylation is not mediated through changes in the IGFBPs. It should, nevertheless, be recognized that our data do not allow any firm conclusions in this regard. Based on a comparison between serum levels of free IGF1 and bioactive IGF1, it appears that the majority of the signal obtained by the bioassay originates from IGFs liberated from the IGFBPs (i.e. dissociable IGF) during incubation of serum with the IGF1R-transfected cells (18). Thus, the possibility exists that prednisolone affects the concentration of dissociable IGF1 without affecting free IGF1 levels, for instance by changing the distribution of IGF1 among the IGFBPs. In this context, Baxter *et al.* (33) previously showed that prednisolone treatment of a patient with tumor hypoglycemia caused a shift in serum IGFBP3 from the 40 to the 150 kDa complex, which may render IGF1 less accessible to the IGF1R. Secondly, there is the issue of IGFBP proteases. In 1985, when Unterman & Phillips (6) by size-exclusion gel chromatography were able to demonstrate that prednisolone induced the presence of IGF1 inhibitors with an estimated molecular size ranging from 12 to 20 kDa, the importance of IGFBP proteolysis was yet to be discovered. However, the molecular range fits with that of the IGFBP fragments. In this context, Lalou *et al.* (34) demonstrated that proteolysis of IGFBP3 may yield fragments that possess no IGF1 binding activity but nevertheless retain the ability to inhibit IGF1 action *in vitro*. Whether this mechanism is operational following prednisolone treatment is, however, uncertain as prednisolone failed to induce IGFBP3 proteolysis in healthy adults (32). However, it could be speculated that prednisolone is able to induce inhibitory fragments from other IGFBPs than IGFBP3. Thirdly, having the molecular precision of size-exclusion gel chromatography in mind, one has to consider the possibility that the 12–20 kDa fraction may have contained some intact 24 kDa IGFBP4, which is the smallest of the IGFBPs (35). Intact IGFBP4 has been demonstrated to inhibit IGF1 action, in contrast to the two IGFBP4 fragments generated after proteolytic cleavage by pregnancy-associated plasma protein-A (24). However, we only assessed IGFBP4 by WLB, which may have been insensitive to detect changes in serum IGFBP4 levels. On the other hand, a significant increase in IGFBP4 should translate into a reduction in serum-free IGF1 and this was not observed. Thus, this possibility seems less likely.

As CG appeared to inhibit IGF1R activation by a blood-borne substance, serum was investigated by 2-D gel chromatography, but we were unable to identify any candidate proteins (data not shown). Thus, we can only speculate on the possible mechanisms by which GC inhibits IGF1R phosphorylation. However, IGF1R phosphorylation is regulated not only by ligand stimulation but also by intracellular phosphatases. One candidate demonstrated to affect IGF1-stimulated IGF1R phosphorylation is the α V β 3 integrin receptor, which is a

transmembrane protein essential for regulation of cell growth and function. The integrin α V β 3 receptor has multiple ligands (36), and numerous studies have demonstrated that ligand activation of the α V β 3 integrin receptor augments IGF1R phosphorylation, whereas inhibition of the α V β 3 integrin receptor decreases IGF1R phosphorylation (37). The cross talk between the α V β 3 integrin receptor and the IGF1R appears to be mediated via the Src-homology domain-2 containing protein tyrosine phosphatase 2 (SHP-2), a cytoplasmatic enzyme (38). A recent *in vitro* study in human smooth muscle cells demonstrated that inhibition of the α V β 3 integrin receptor reduced IGF1-mediated IGF1R phosphorylation and that this appeared to be mediated via SHP-2 (39). Another recent *in vitro* study demonstrated that prolactin enhanced IGF1R phosphorylation by decreasing the association of SHP-2 with the IGF1R (40). As the impact of SHP-2 on IGF1R phosphorylation is detectable within minutes (39, 40), we speculate that GC mediates its effects through changes in SHP-2. However, this remains to be proven.

The present dose of prednisolone had a measurable impact on lower leg growth rate as assessed by knemometry (16). However, it should be emphasized that the demonstrated growth inhibition represents the combined effect of prednisolone on bone growth, knee and ankle joint ligaments, and tendons as well as shrinking of soft tissue (cutis, subcutis, and cartilage) over the knee and under the heel, and consequently, knemometry yields a composite measurement (41). This may explain why we were unable to demonstrate the existence of an association between the growth change and the *in vitro* IGF1 bioactivity of serum. Alternatively, GC may exert direct effects on the local IGF system within the growth plate, independently of its impact of the circulating IGF1 bioactivity. This possibility is supported by studies demonstrating that in cultured chondrocytes, GC is able to reduce the gene expression of IGF1, as well as the proliferative potential of IGF1, apparently via the PI3 kinase pathway (42, 43). In any case, our data suggest that measurement of the *in vitro* IGF1 bioactivity may serve as an indicator of the local growth inhibiting effect of GC. However, it remains to be investigated whether the effects of GC on the IGF1 action in serum and locally in the tissues are related.

Our study has some limitations that need to be considered. First, it remains to be proven that the GC-induced reduction in the ability of serum to phosphorylate the IGF1R *in vitro* translates into a similar reduction in bioactive IGF1 within the tissue, i.e. *in vivo*. Secondly, the assays that we used to assess changes in the IGFBPs have their limitations. WLB is semiquantitative by nature and does not allow detection of subtle changes in intact and fragmented IGFBPs. The immunoassays, on the other hand, enable a relatively precise quantification of the individual IGFBP levels, but

they often lack the ability to discriminate between intact and fragmented IGFBPs.

In conclusion, in this randomized, double-blind, placebo-controlled, crossover study, we have demonstrated that 1 week of treatment with 5 mg prednisolone daily was able to reduce the ability of serum to phosphorylate the IGF1R *in vitro* without any concomitant changes in serum IGF concentrations. Furthermore, we excluded an effect of prednisolone *per se*. Thus, our study has identified one possible mechanism by which GC may inhibit IGF1-mediated actions *in vivo*. However, our study yielded no definitive information as to whether this circulating substance is related to the IGFBPs or represents an IGFBP-independent mechanism.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Schakman O, Gilson H, Kalista S & Thissen JP. Mechanisms of muscle atrophy induced by glucocorticoids. *Hormone Research* 2009 **72** (Suppl 1) 36–41. (doi:10.1159/000229762)
- Olney RC. Mechanisms of impaired growth: effect of steroids on bone and cartilage. *Hormone Research* 2009 **72** (Suppl 1) 30–35. (doi:10.1159/000229761)
- Mauras N. Can growth hormone counteract the catabolic effects of steroids? *Hormone Research* 2009 **72** (Suppl 1) 48–54. (doi:10.1159/000229764)
- Mehls O, Himmele R, Homme M, Kiepe D & Klaus G. The interaction of glucocorticoids with the growth hormone–insulin-like growth factor axis and its effects on growth plate chondrocytes and bone cells. *Journal of Pediatric Endocrinology & Metabolism* 2001 **14** (Suppl 6) 1475–1482.
- van der Eerden BC, Karperien M & Wit JM. Systemic and local regulation of the growth plate. *Endocrine Reviews* 2003 **24** 782–801. (doi:10.1210/er.2002-0033)
- Unterman TG & Phillips LS. Glucocorticoid effects on somatomedins and somatomedin inhibitors. *Journal of Clinical Endocrinology and Metabolism* 1985 **61** 618–626. (doi:10.1210/jcem-61-4-618)
- Miell JP, Taylor AM, Jones J, Holly JM, Gaillard RC, Pralong FP, Ross RJ & Blum WF. The effects of dexamethasone treatment on immunoreactive and bioactive insulin-like growth factors (IGFs) and IGF-binding proteins in normal male volunteers. *Journal of Endocrinology* 1993 **136** 525–533. (doi:10.1677/joe.0.1360525)
- Elders MJ, Wingfield BS, McNatt ML, Clarke JS & Hughes ER. Glucocorticoid therapy in children. Effect on somatomedin secretion. *American Journal of Diseases of Children* 1975 **129** 1393–1396.
- Skjærbaek C, Frystyk J, Grøfte T, Flyvbjerg A, Lewitt MS, Baxter RC & Ørskov H. Serum free insulin-like growth factor-I is dose-dependently decreased by methylprednisolone and related to body weight changes in rats. *Growth Hormone & IGF Research* 1999 **9** 74–80. (doi:10.1054/ghir.1999.0090)
- Juul A. Serum levels of insulin-like growth factor I and its binding proteins in health and disease. *Growth Hormone & IGF Research* 2003 **13** 113–170. (doi:10.1016/S1096-6374(03)00038-8)
- Van Doorn J, Cornelissen AJ & Buul-Offers SC. Plasma levels of insulin-like growth factor binding protein-4 (IGFBP-4) under normal and pathological conditions. *Clinical Endocrinology* 2001 **54** 655–664. (doi:10.1046/j.1365-2265.2001.01248.x)
- Eivindson M, Grønbaek H, Flyvbjerg A, Frystyk J, Zimmermann-Nielsen E & Dahlerup JF. The insulin-like growth factor (IGF)-system in active ulcerative colitis and Crohn's disease: relations to disease activity and corticosteroid treatment. *Growth Hormone & IGF Research* 2007 **17** 33–40. (doi:10.1016/j.ghir.2006.10.003)
- Hickson RC, Oehler DT, Byerly RJ & Unterman TG. Protective effect of glutamine from glucocorticoid-induced muscle atrophy occurs without alterations in circulating insulin-like growth factor (IGF)-I and IGF-binding protein levels. *Proceedings of the Society for Experimental Biology and Medicine* 1997 **216** 65–71.
- Sadick MD, Intintoli A, Quarmby V, McCoy A, Canova-Davis E & Ling V. Kinase receptor activation (KIRA): a rapid and accurate alternative to end-point bioassays. *Journal of Pharmaceutical and Biomedical Analysis* 1999 **19** 883–891. (doi:10.1016/S0731-7085(98)00144-7)
- Schou AJ, Heuck C & Wolthers OD. Differential effects of short-term prednisolone treatment on peripheral and abdominal subcutaneous thickness in children assessed by ultrasound. *Steroids* 2003 **68** 525–531. (doi:10.1016/S0039-128X(03)00086-2)
- Schou AJ, Heuck C & Wolthers OD. Ultrasound of skin in prednisolone-induced short-term growth suppression. *Journal of Pediatric Endocrinology & Metabolism* 2003 **16** 973–980.
- Chen JW, Ledet T, Ørskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *American Journal of Physiology. Endocrinology and Metabolism* 2003 **284** E1149–E1155.
- Frystyk J. Quantification of the GH/IGF-axis components: lessons from human studies. *Domestic Animal Endocrinology* 2012 **43** 186–197. (doi:10.1016/j.domaniend.2011.11.005)
- Frystyk J, Dinesen B & Ørskov H. Non-competitive time-resolved immunofluorometric assays for determination of human insulin-like growth factor I and II. *Growth Regulation* 1995 **5** 169–176.
- Ivarsen P, Chen JW, Tietze I, Christiansen JS, Flyvbjerg A & Frystyk J. Marked reductions in bioactive insulin-like growth factor I (IGF-I) during hemodialysis. *Growth Hormone & IGF Research* 2010 **20** 156–161. (doi:10.1016/j.ghir.2009.12.001)
- Frystyk J, Højlund K, Rasmussen KN, Jørgensen SP, Wildner-Christensen M & Ørskov H. Development and clinical evaluation of a novel immunoassay for the binary complex of insulin-like growth factor-I (IGF-I) and IGF-binding protein-1 in human serum. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 260–266. (doi:10.1210/jc.87.1.260)
- Krassas GE, Pontikides N, Kaltsas T, Dumas A, Frystyk J, Chen JW & Flyvbjerg A. Free and total insulin-like growth factor (IGF)-I, -II, and IGF binding protein-1, -2, and -3 serum levels in patients with active thyroid eye disease. *Journal of Clinical Endocrinology and Metabolism* 2003 **88** 132–135. (doi:10.1210/jc.2002-021349)
- Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S & Binoux M. Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies. *Analytical Biochemistry* 1986 **154** 138–143. (doi:10.1016/0003-2697(86)90507-5)
- Jeyaratnaganathan N, Grønbaek H, Holland-Fischer P, Espelund U, Chen JW, Flyvbjerg A, Vilstrup H & Frystyk J. Ascites from patients with alcoholic liver cirrhosis contains higher IGF-I bioactivity than serum. *Clinical Endocrinology* 2010 **72** 625–632. (doi:10.1111/j.1365-2265.2009.03707.x)

- 25 Mandal N, Heegaard S, Prause JU, Honore B & Vorum H. Ocular proteomics with emphasis on two-dimensional gel electrophoresis and mass spectrometry. *Biological Procedures Online* 2009 **12** 56–88. (doi:10.1007/s12575-009-9019-7)
- 26 Østergaard L, Honore B, Thorsen LB, Baandrup J, Eskildsen-Helmond Y, Laursen BE, Vorum H, Mulvany MJ & Simonsen U. Pulmonary pressure reduction attenuates expression of proteins identified by lung proteomic profiling in pulmonary hypertensive rats. *Proteomics* 2011 **11** 4492–4502. (doi:10.1002/pmic.201100171)
- 27 Mortz E, Krogh TN, Vorum H & Gorg A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Proteomics* 2001 **1** 1359–1363. (doi:10.1002/1615-9861(200111)1:11<1359::AID-PROT1359>3.0.CO;2-Q)
- 28 Argente J, Caballo N, Barrios V, Munoz MT, Pozo J, Chowen JA, Morande G & Hernandez M. Multiple endocrine abnormalities of the growth hormone and insulin-like growth factor axis in patients with anorexia nervosa: effect of short- and long-term weight recuperation. *Journal of Clinical Endocrinology and Metabolism* 1997 **82** 2084–2092. (doi:10.1210/jc.82.7.2084)
- 29 Støving RK, Chen JW, Glintborg D, Brixen K, Flyvbjerg A, Horder K & Frystyk J. Bioactive insulin-like growth factor (IGF) I and IGF-binding protein-1 in anorexia nervosa. *Journal of Clinical Endocrinology and Metabolism* 2007 **92** 2323–2329. (doi:10.1210/jc.2006-1926)
- 30 Frystyk J, Ivarsen P, Skjærbaek C, Flyvbjerg A, Pedersen EB & Ørskov H. Serum free insulin-like growth factor I correlates with clearance in patients with chronic renal failure. *Kidney International* 1999 **56** 2076–2084. (doi:10.1046/j.1523-1755.1999.00798.x)
- 31 Bereket A, Lang CH, Blethen SL, Ng LC & Wilson TA. Insulin treatment normalizes reduced free insulin-like growth factor-I concentrations in diabetic children. *Clinical Endocrinology* 1996 **45** 321–326. (doi:10.1046/j.1365-2265.1996.7760786.x)
- 32 Dall R, Wolthers T, Flyvbjerg A, Grøfte T, Christiansen JS & Jørgensen JO. No influence of prednisolone on IGFBP-3 proteolysis in healthy young men. *Hormone Research* 2001 **55** 95–101. (doi:10.1159/000049977)
- 33 Baxter RC, Holman SR, Corbould A, Stranks S, Ho PJ & Braund W. Regulation of the insulin-like growth factors and their binding proteins by glucocorticoid and growth hormone in nonislet cell tumor hypoglycemia. *Journal of Clinical Endocrinology and Metabolism* 1995 **80** 2700–2708. (doi:10.1210/jc.80.9.2700)
- 34 Lalou C, Lassarre C & Binoux M. A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. *Endocrinology* 1996 **137** 3206–3212. (doi:10.1210/en.137.8.3206)
- 35 Zhou R, Diehl D, Hoefflich A, Lahm H & Wolf E. IGF-binding protein-4: biochemical characteristics and functional consequences. *Journal of Endocrinology* 2003 **178** 177–193. (doi:10.1677/joe.0.1780177)
- 36 Cox D, Brennan M & Moran N. Integrins as therapeutic targets: lessons and opportunities. *Nature Reviews. Drug Discovery* 2010 **9** 804–820. (doi:10.1038/nrd3266)
- 37 Clemmons DR & Maile LA. Minireview: integral membrane proteins that function coordinately with the insulin-like growth factor I receptor to regulate intracellular signaling. *Endocrinology* 2003 **144** 1664–1670. (doi:10.1210/en.2002-221102)
- 38 Matozaki T, Murata Y, Saito Y, Okazawa H & Ohnishi H. Protein tyrosine phosphatase SHP-2: a proto-oncogene product that promotes Ras activation. *Cancer Science* 2009 **100** 1786–1793. (doi:10.1111/j.1349-7006.2009.01257.x)
- 39 Kuemmerle JF. Occupation of α -V- β -3-integrin by endogenous ligands modulates IGF-I receptor activation and proliferation of human intestinal smooth muscle. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 2006 **290** G1194–G1202. (doi:10.1152/ajpgi.00345.2005)
- 40 Carver KC, Piazza TM & Schuler LA. Prolactin enhances insulin-like growth factor I receptor phosphorylation by decreasing its association with the tyrosine phosphatase SHP-2 in MCF-7 breast cancer cells. *Journal of Biological Chemistry* 2010 **285** 8003–8012. (doi:10.1074/jbc.M109.066480)
- 41 Wolthers OD. Methodological aspects of short-term knemometry in the assessment of exogenous glucocorticoid-induced growth suppression in children. *Annals of Human Biology* 1997 **24** 539–546. (doi:10.1080/03014469700005302)
- 42 Fernandez-Cancio M, Esteban C, Carrascosa A, Toran N, Andaluz P & Audi L. IGF-I and not IGF-II expression is regulated by glucocorticoids in human fetal epiphyseal chondrocytes. *Growth Hormone & IGF Research* 2008 **18** 497–505. (doi:10.1016/j.ghir.2008.04.004)
- 43 Macrae VE, Ahmed SF, Mushtaq T & Farquharson C. IGF-I signalling in bone growth: inhibitory actions of dexamethasone and IL-1 β . *Growth Hormone & IGF Research* 2007 **17** 435–439. (doi:10.1016/j.ghir.2007.05.002)

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