Cortisol increases growth hormone-receptor expression in human osteoblast-like cells

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Abstract

It is well known that high levels of glucocorticoids cause osteoporosis and that physiologic levels of growth hormone (GH) are required for normal bone remodeling. It has been suggested that glucocorticoids regulate GH-responses via the regulation of GH-receptor expression. The aim of the present study was to investigate whether cortisol plays a role in the regulation of GH-receptor expression in cultured human osteoblasts.

The effect of serum starvation and cortisol on GH-receptor expression was tested in human osteoblast (hOB)-like cells. Serum starvation for 24 h resulted in an increase in GH-receptor mRNA levels (90 ± 1% over control culture). Cortisol increased GH-receptor mRNA levels in a dose-dependent manner with a maximal effect at 10^{-6} M. The stimulating effect of cortisol on GH-receptor mRNA levels was time-dependent, reaching a peak 12 h after the addition of cortisol (126 ± 29% over control culture) and remaining up to 12 h later. The increase in GH-receptor mRNA levels was accompanied by an increase in ^{125}I-GH binding which reached a maximum at 24 h (196 ± 87% over control culture).

In conclusion, glucocorticoids increase GH-receptor expression in hOB-like cells. Further studies are needed to clarify whether glucocorticoid-induced regulation of the GH-receptor is important in human bone physiology.

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Introduction

Bone metabolism is regulated by a balance between bone resorption, caused by osteoclasts, and bone formation caused by osteoblasts. Clinical and experimental studies have demonstrated that growth hormone (GH) is important in normal bone remodeling. Patients with GH-deficiency have a decreased bone mineral density (Rosén *et al.* 1993) which can be reversed by long-term treatment with GH (Saggese *et al.* 1993, Rosén *et al.* 1994). Furthermore, GH enhances bone turnover in young healthy male volunteers as well as in osteopenic post-menopausal women (Brixen *et al.* 1990, 1995). The mechanism for the stimulating effect of GH on the amount of bone is not fully understood. We and others have demonstrated that human osteoblast (hOB)-like cells express functional GH-receptors and that GH exerts an anabolic effect on the osteoblasts (Kassem *et al.* 1993, Morel *et al.* 1993, Nilsson *et al.* 1995). This indicates a direct stimulating effect of GH on bone formation.

Experimental studies have demonstrated that high concentrations of glucocorticoids result in a decrease in bone cell replication and collagen synthesis in calvarial explants (Canalis 1983). The mechanism for this inhibitory effect is not fully understood. Children with growth failure due to chronic glucocorticoid therapy have normal GH levels indicating the possibility of some degree of end organ insensitivity to GH (Robinson *et al.* 1995). It has therefore been suggested that glucocorticoids influence bone metabolism via a modulation of different components of the GH/insulin-like growth factor-I (IGF-I) system. Glucocorticoids decrease IGF-I expression in rat (McCarthy *et al.* 1990) and human osteoblasts (Swolin *et al.* 1996). Furthermore, several IGF-binding proteins (IGFBPs) which are known to modulate the bioactivity of IGF-I, are regulated by glucocorticoids. IGFBPs -3, -4 and -5 are decreased (Okazaki *et al.* 1994) while IGFBP-6 (Gabbitas & Canalis 1996) is increased by glucocorticoids in osteoblast cultures.

Glucocorticoids may also interact with the GH/IGF-I axis via a regulation of GH-receptor expression. Conflicting *in vivo* as well as *in vitro* results have been presented regarding the effects of glucocorticoids on GH-receptor expression. Glucocorticoids increase GH-receptor mRNA levels in the liver and in the growth plate of rabbits (Heinrichs *et al.* 1994) while they decrease GH-receptor mRNA levels in rat liver (Gabrielsson *et al.* 1995, Bennett *et al.* 1996). Glucocorticoids also increase the GH-binding

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capacity of cultured rat osteosarcoma cells (Salles *et al.* 1994), rat pancreatic islet cells (Moldrup *et al.* 1993) and rat hepatocytes (Niimi *et al.* 1991). In contrast, King & Carter-Su (1995) demonstrated that glucocorticoids decrease GH binding to a mouse fibroblast cell line.

The interaction of GH and glucocorticoids in the regulation of human bone metabolism has not yet been fully investigated. The present study was therefore undertaken to investigate the effects of glucocorticoids on GH-receptor expression in hOB-like cells.

Materials and Methods

Cell culture

Cultured hOB-cells were isolated from trabecular bone by orthopedic surgery as previously described (MacDonald *et al.* 1984, Nilsson *et al.* 1995). The bone explants were minced into small bonechips, washed extensively in Dulbecco's modified Eagle's medium (DMEM)/F12 1:1 (Gibco, Paisley, Scotland, UK) and then transferred into either 75 or 162 cm^2 culture flasks (Costar, Cambridge, MA, USA). Cells were cultured in DMEM/F12, 1:1 with 10% v/v fetal calf serum (FCS; Gibco), fungizone (500 μg/l; Gibco), gentamycin sulfate (50 mg/l; Sigma, St Louis, MO, USA), L-glutamine (2 mmol/l; Gibco), L-ascorbic acid (100 mg/l; Merck, KGaA, Darmstadt, Germany), in a humidified 5% CO_2:95% O_2 atmosphere at 37 °C. Before the addition of hormone, cells were starved for various hours in DMEM (without phenol red (Gibco)) with 0·5% dextran-coated charcoal–stripped FCS (kindly provided by M Slootweg, The Netherlands). Cortisol (hydrocortisone, H–0135, Lot 44H9402 +41H9409+115H4629) was first diluted in 99·5% ethanol and then further diluted in DMEM (without phenol red (Gibco)). The study was approved by the Ethical Committee of Sahlgrenska University Hospital in Göteborg.

Preparation of nucleic acid

Total nucleic acids (TNA) were prepared according to the protocol described by Durnam & Palmiter (1983). RNA was isolated according to the method described by Chomczynski & Sacchi (1987).

GH-receptor probe

A 500 bp fragment of exon 10 of the human GH (hGH)-receptor gene (Nilsson *et al.* 1995) was used for the measurement of GH-receptor mRNA levels in hOB-like cells. ^{35}S-labeled hGH-receptor antisense RNA probe was generated with Sp6 polymerase from an EcoRI linearized PGEM–7Z(+) plasmid. The hGH-receptor sense RNA probe was generated with T7 polymerase from a BamHI linearized plasmid.

RNase protection solution hybridization assay

Total nucleic acids (TNA) were prepared by homogenizing harvested cells with a polytrone in a buffer containing 1% (w/v) SDS, 20 mM Tris–HCl (pH 7·5) and 4 mM EDTA. The homogenized cells were digested by an overnight proteinase-K treatment and total nucleic acids were prepared by subsequent phenol–chloroform extraction, according to the method described by Durnam & Palmiter (1983). The RNase protection solution hybridization assay was carried out according to the protocol described by Mathews *et al.* (1986). Protected RNA–RNA hybrids were precipitated with trichloroacetic acid, collected on glass fiber filters and counted in a scintillation counter. The signal was compared with a standard curve obtained by hybridization to known amounts of GH-receptor mRNA. The intra-assay coefficient of variation for the hGH-receptor assays was less than 10% in the range of 50–2500 amol RNA standard. The results were correlated to the DNA content measured according to the method of Labarca & Paigen (1980).

RNase protection assay

For the RNase protection assay followed by gel separation (RPA II kit, Ambion, Austin, TX, USA), 25 μg total cellular RNA were hybridized at 45 °C overnight with an ^{35}S-labeled hGH-receptor riboprobe and then digested with RNase. The RNA–RNA hybrids were precipitated, re-suspended and separated on a 6% TBE–urea gel (Novex, San Diego, CA, USA). Signals from protected fragments were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Binding studies

^{125}I-hGH (I–125–013, Batch: XS 4208, XS 4325, Euro-Diagnostica, Malmö, Sweden) was repurified by gel filtration on a Sephadex G75 column equilibrated and eluted with phosphate buffer (0·05 mol/l) and 1% (w/v) BSA (Sigma). The cells were starved in 24-well dishes (Costar) for 10 to 12 h before the addition of cortisol, and were then preincubated at room temperature for 1 h with PBS supplemented with 1% (w/v) BSA. The liquid was discarded and labeled tracer added (100 000 c.p.m./ml) in PBS supplemented with 0·1% (w/v) BSA, incubated for 4 h at 24 °C. The incubation was terminated by washing the cells three times in ice cold PBS at 4 °C. Cells were solubilized in 2 × 125 μl NaOH (1 M) and counted in a gammacounter (LKB 1277 Gammamaster, Sweden). All calculations were carried out in quadruplicate.

Statistical procedure

Values are given as means ± S.E.M. Statistical evaluations of the data were carried out using Student's *t*-test for unpaired and paired samples.

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Table 1 hGH-receptor mRNA levels after different times of starvation in serum-free medium. Cells were cultured as described in Materials and Methods. Levels are expressed as fg/μg DNA. Three different experiments were performed. Experiments were performed using cells from one patient. Values from the three different patients (male, 8 years old and females, 51 and 71 years old) were then pooled and are given as means ± S.E.M. vs control cells (cells grown in 10% FCS)

	hGH-receptor mRNA (fg/μg DNA)
Starvation time (h)	
0	70·0 ± 14·5
10	104·7 ± 25·2
24	133·0 ± 0·6*

*P<0·05 compared with 0 h starvation time.

Results

No obvious correlation between GH-receptor mRNA levels and age or sex was found in hOB-cell preparations from different patients (13 males between 0·5 and 75 years of age and 12 females between 12 and 86 years of age; data not shown). Serum starvation of hOB-cells resulted in an increase in GH-receptor mRNA levels (Table 1). The effect on GH-receptor mRNA levels of some substances, previously shown to increase GH-receptor expression in cultured cells from rodents, were tested in hOB-like cells. Cortisol (10^{-6} M) increased GH-receptor mRNA levels by 85·5% and in a dose-dependent manner, while no significant effect was observed with retinoic acid (10^{-6} M) or GH (5–100 ng/ml; Fig. 1). The maximum effect of cortisol was found at 10^{-6} M (Figs 2 and 3). The stimulating effect of cortisol on GH-receptor mRNA levels was time-dependent, reaching a peak 12 h after the addition of cortisol (control, 81 ± 5 fg/μg DNA, 12 h, 183 ± 22 fg/μg DNA) and remaining up to 12 h later (Fig. 4). To investigate the specificity of the GH-receptor probe used in the RNase-protection solution hybridization assay, RNase-protected probe fragments were analyzed on denaturing polyacrylamide gels. A protected fragment of expected size (500 bp) was detected in the mRNA from hOB-cells (Fig. 3).

Cortisol (10^{-6} M) was tested in binding studies using ^{125}I-GH. The cortisol-induced increase in GH-receptor mRNA levels was accompanied by an increase in ^{125}I-GH binding in a time-dependent manner. Cortisol-induced ^{125}I-GH binding appeared after 14 h, reaching a maximum at 24 h (196 ± 87% over control) and remaining up to 17 h later (Fig. 5).

Discussion

Cushing (1932) showed that increased levels of glucocorticoids cause osteoporosis and increase the risk of bone fractures. Osteoblasts express receptors for glucocorticoids and high levels of glucocorticoids decrease the proliferation

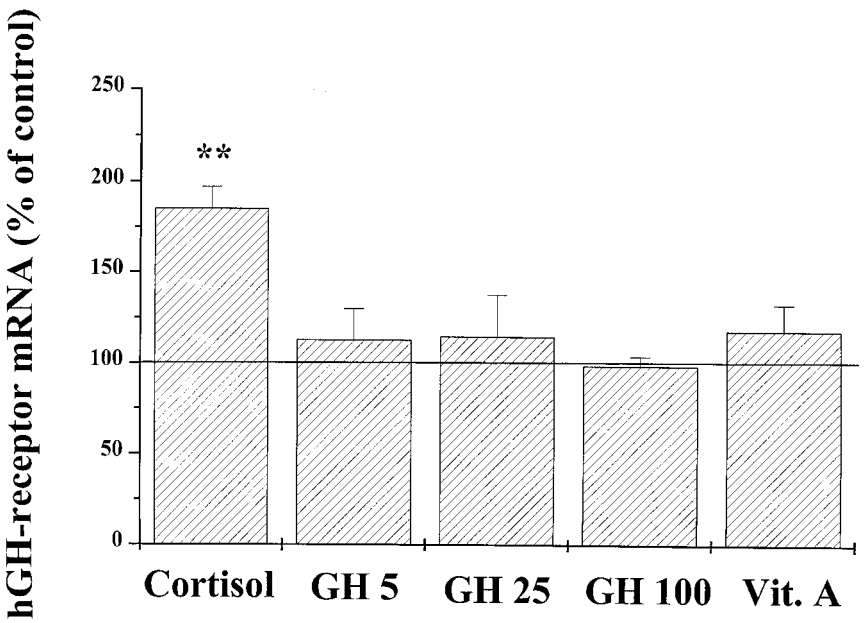

Figure 1 The effect of cortisol (1 μM), GH (5, 25 and 100 ng/ml) and retinoic acid (vitamin A) (1 μM) on GH-receptor mRNA levels in hOB-cells. Cells were cultured as described in Materials and Methods, starved for 30 h and stimulated with cortisol, GH or retinoic acid 16 h before cell harvest. Levels of GH-receptor mRNA are expressed as % of control (horizontal line at 100%). Values are means ± S.E.M. of four pooled patients. **P<0·01 vs control.

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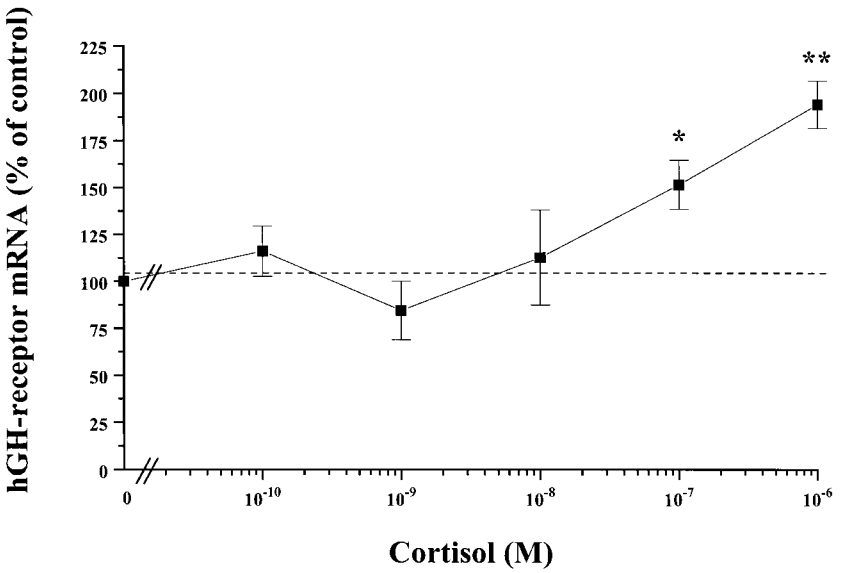

Figure 2 The dose–response effect of cortisol on GH-receptor mRNA levels in cultured hOB-cells. Cells were cultured as described in Materials and Methods, starved for 30 h and stimulated with different doses of cortisol 16 h before cell harvest. Levels of GH-receptor mRNA are expressed as % of control. Values are means ± S.E.M. of five pooled patients (males, 18, 29 and 38 years old and females, 12 and 68 years old). *P<0·05 vs control, **P<0·01 vs control.

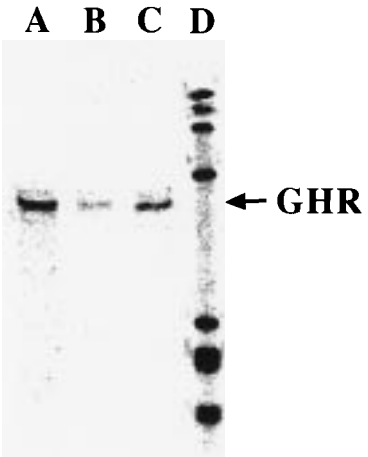

Figure 3 RNase-protected hGH-receptor (GHR) mRNA (500 bp) from hOB-cells (female, 15 years old), was analyzed by polyacrylamide gel electrophoresis. Cells were cultured as described in Materials and Methods, starved for 24 h and stimulated with cortisol (10^{-6} M) 16 h before cell harvest. (A) Standard 0·003 ng; (B) unstimulated hOB-cells; (C) cortisol-stimulated hOB-cells; (D) Hae III ladder, for size detection.

and expression of differentiation markers in cultures of osteoblasts (Canalis & Avioli 1992, Jonsson *et al.* 1993, Delany *et al.* 1995), demonstrating the direct effect of glucocorticoids on osteoblasts (Chen & Feldman 1979). The present study investigated whether glucocorticoids interact with GH-receptor expression in hOB-like cells.

Our results showing that serum starvation increases GH-receptor expression in hOB-like cells, are similar to those previously demonstrated in rat epiphyseal chondrocytes (Nilsson *et al.* 1990) and in osteosarcoma cells (Salles *et al.* 1994). GH and retinoic acid increase GH-receptor mRNA levels in cultured mouse and rat cells (Nilsson *et al.* 1990, Ohlsson *et al.* 1993, Slootweg *et al.* 1996). In contrast, in the present study using hOB-like cells, no significant effect of GH or retinoic acid was seen on GH-receptor mRNA levels, suggesting that regulation of the GH-receptor transcript may be species specific. It is also possible that the lack of any significant effect of GH on GH-receptor mRNA levels may be due to hOB-cells expressing fewer GH-receptors than in rat epiphyseal chondrocytes (Nilsson *et al.* 1990, 1995), or that primary hOB-cells are a heterogenous population of cells and/or due to a variation in patient samples.

We have, for the first time, demonstrated the effects of glucocorticoids on GH-receptor expression in human cells. Similar to *in vivo* results on the GH-receptor mRNA levels in the liver and growth plate of rabbits (Heinrichs *et al.* 1994), an increase in the GH-receptor mRNA levels was seen in hOB-like cells after treatment with glucocorticoids. This finding is in contrast to earlier results *in vivo* in rats in which glucocorticoids decreased the GH-receptor mRNA levels (Gabrielsson *et al.* 1995), indicating a species

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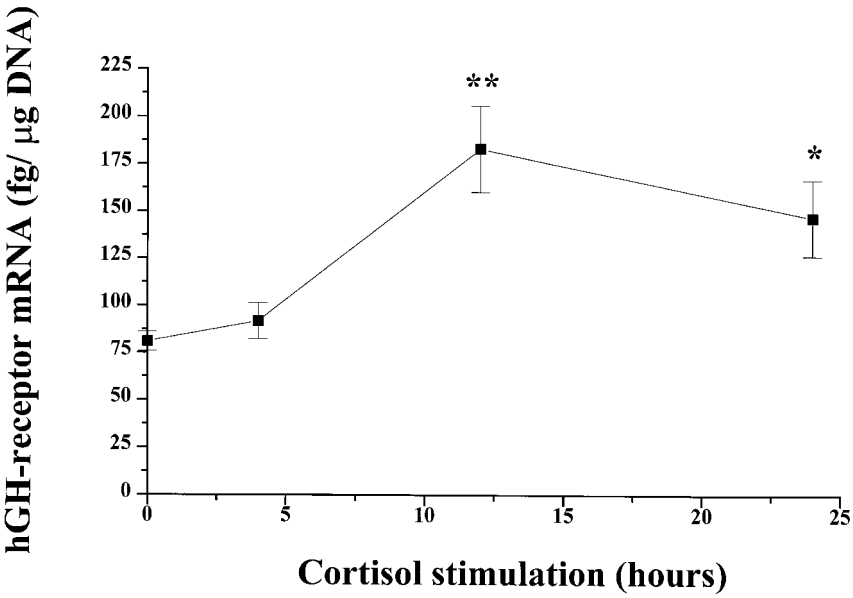

Figure 4 The time-dependent effect of cortisol on GH-receptor mRNA levels. Cells were cultured as described in Methods, starved for 30 h and stimulated with cortisol (10^{-6} M) at various times before cell harvest. Values are expressed as fg/μg DNA, means ± S.E.M. from four pooled patients (females, 12 and 29 years old; males, 41 and 55 years old). *P<0·05, **P<0·01.

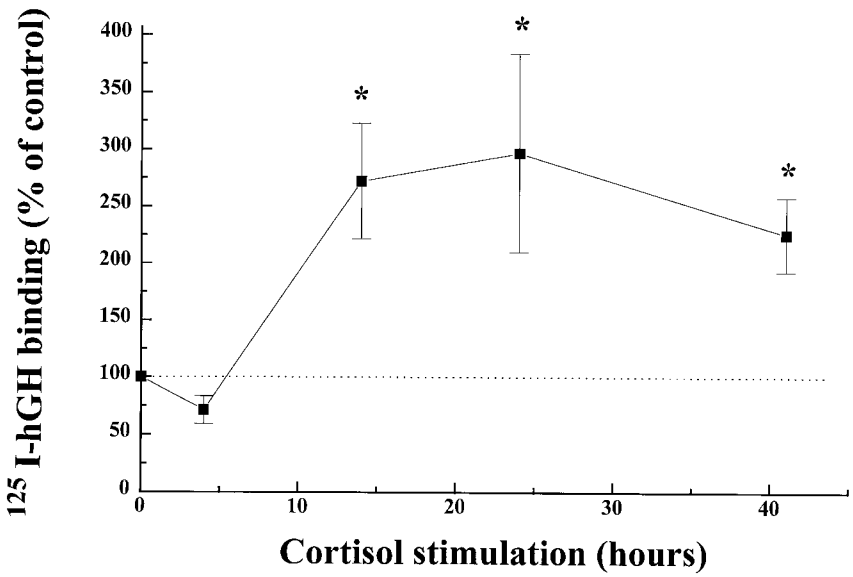

Figure 5 The time-dependent effect of cortisol (10^{-6} M) on ^{125}I-hGH binding on hOB-cells. The time of starvation was 53 h. Values are expressed as % of control ± S.E.M. from four pooled patients (females, 11 and 47 years old; males, 26 and 29 years old). *P<0·05 vs untreated cells.

difference in the regulation of GH-receptor expression. This species difference in the expression and regulation of the GH-receptor and its soluble extracellular part (GHBP) is supported by the finding that GHBP in rabbits and humans is synthesized via a proteolytic cleavage of the extracellular part of the GH-receptor, while in rodents it is synthesized via alternative splicing from a separate mRNA transcript.

We and others have previously shown that glucocorticoids decrease IGF-I expression in osteoblasts.

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Furthermore, IGF-I down-regulates the GH-receptor expression in a local negative feedback loop (Slootweg *et al.* 1995, Leung *et al.* 1996). Therefore, the cortisol-induced increase in GH-receptor expression may be due to a decrease in IGF-I expression leading to a reduced negative feedback on GH-receptor expression.

The conflicting effects of glucocorticoids on the GH-receptor mRNA levels may also be explained by the tissue-specific effects of glucocorticoids on GH-receptor expression. This possibility is supported by the finding that the first exon in the GH-receptor transcript is untranslated (5′UTR) and that there are several alternative exon 1 variants, which may be more-or-less tissue specific (Robinson *et al.* 1995). Thus, the different GH-receptor transcripts may be regulated in different ways. The hGH-receptor probe, used in the present study, recognizes a translated part of the GH-receptor mRNA corresponding to the intracellular part of the GH-receptor. This sequence of the GH-receptor is found in all known variants of mRNAs coding for the hGH-receptor. It is therefore interesting to see if glucocorticoids regulate GH-receptor expression in other human cells.

Whereas conflicting results have been presented regarding the effect of glucocorticoids on GH-receptor expression, more consistent results have been presented regarding the inhibitory effects of glucocorticoids on GH-induced cell proliferation and the activation of different components of the GH-receptor signaling cascade (Salles *et al.* 1994, King & Carter-Su 1995). Salles *et al.* (1994) found that cortisol increased GH-binding and reduced GH-induced cell proliferation in rat osteosarcoma cells. In contrast, King & Carter-Su (1995) found reduced GH-binding and decreased early events in GH-signaling in glucocorticoid-treated mouse fibroblasts. It has been suggested that the contrasting effects of glucocorticoids on GH-binding may be explained by methodological differences in the conditions of the GH-binding (King & Carter-Su 1995). In the study using rat osteosarcoma cells (Salles *et al.* 1994), binding studies were carried out with ^{125}I-GH at room temperature for 24 h, while in the study using mouse fibroblasts, binding was performed overnight at 4 °C (King & Carter-Su 1995). A prolonged binding study at room temperature may lead to the internalization of some amount of tracer, making it difficult to distinguish whether binding to cell surface receptors or receptor turnover are being measured. In the present study, binding studies were performed with short duration (4 h) in order to minimize the amount of internalized tracer. However, binding studies were carried out at room temperature since GH-binding to human osteoblasts is difficult to measure at 4 °C. In summary, the conflicting results of the effect of glucocorticoids on GH-receptor mRNA levels and ^{125}I-GH-binding in different studies may be explained by species and tissue differences and/or different methodologies.

It is generally believed that high levels of glucocorticoids cause osteoporosis and GH-insensitivity. Furthermore, GH exerts anabolic effects on osteoblasts (Slootweg *et al.* 1988, Nilsson *et al.* 1995). Thus, our finding that cortisol increases GH-receptor expression in osteoblasts could appear contradictory. However, cortisol may block GH effects at a post-receptor level, resulting in a secondary increase in GH-receptor expression. This hypothesis is supported by the finding that in rat osteosarcoma cells glucocorticoids increase GH-binding while GH-effects are decreased (Salles *et al.* 1994). Alternatively, it is possible that short-term stimulation with glucocorticoids *in vitro* is not an optimal model for studying the mechanism of action for glucocorticoid-induced GH-insensitivity *in vivo*.

In conclusion, glucocorticoids increase GH-receptor expression in hOB-like cells. Further studies are needed to clarify whether glucocorticoid-induced regulation of the GH-receptor is important in human bone physiology.

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