Cortisol Inhibits the Synthesis of Insulin-like Growth Factor-binding Protein-5 in Bone Cell Cultures by Transcriptional Mechanisms*

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Bari Gabbitas‡, James M. Pash§, Anne M. Delany§, and Ernesto Canalis‡§

From the ‡ Departments of Research and Medicine, Saint Francis Hospital and Medical Center, Hartford, Connecticut 06105 and the § University of Connecticut School of Medicine, Farmington, Connecticut 06030

Glucocorticoids inhibit the synthesis of insulin-like growth factor-binding protein-5 (IGFBP-5) in osteoblasts, but the mechanisms involved are unknown. IGF-1 and IGF-II are among the most important local regulators of bone cell function, and their activity is modified by IGF-binding proteins (6–12). Although the exact function of IGF-binding proteins in bone is not known, IGFBP-5 is unique in that it consistently increases bone cell growth and enhances the anabolic actions of IGF-1 in bone (12). The regulation of IGFBP-5 synthesis in bone cells is complex, and it is often coordinated with the regulation of IGF-1 and the state of cell differentiation. Agents known to stimulate bone cell replication, such as transforming growth factor-β, fibroblast growth factor, and platelet-derived growth factor, inhibit IGF-1 and IGFBP-5 synthesis (13, 14). In contrast, agents that induce osteoblast cell differentiation, such as retinoic acid and IGF-1, stimulate IGFBP-5 synthesis in skeletal cells (11, 15).

Glucocorticoids are known to have complex effects on bone formation and resorption (16). Some of these effects are probably due to direct actions of glucocorticoids on specific genes expressed by the osteoblast, whereas others may be indirect (17). Glucocorticoids inhibit DNA and collagen synthesis in bone cultures and decrease the synthesis of IGF-I and selected IGF-binding proteins in osteoblasts (5, 16, 18). These effects may play a critical role in the actions of glucocorticoids in bone. Recent studies demonstrated that glucocorticoids inhibit IGFBP-5 mRNA levels in cultured human osteoblasts. However, the mechanism of this effect was not explored, and it could involve transcriptional and post-transcriptional processes (5). Since the mechanism of glucocorticoid action in bone has remained elusive, it is important to define possible levels of regulation of genes that appear essential to bone cell function.

This study was undertaken to examine the effects of cortisol on IGFBP-5 synthesis in cultures of osteoblast-enriched cells from fetal rat calvariae (Ob cells) and to determine the mechanism of action of cortisol on IGFBP-5 gene expression.

MATERIALS AND METHODS

Culture Technique—The culture method used has been described in detail previously (19). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal area (this project was approved by the Institutional Animal Care and Use Committee of Saint Francis Hospital and Medical Center). Cells were obtained by five sequential digestions of the parietal bone using bacterial collagenase (CLS II, Worthington). Cell populations harvested from the third to the fifth digestions were cultured as a pool and were previously shown to have osteoblastic characteristics. Ob cells were plated at a density of 8,000–12,000 cells/cm² and cultured in a humidified 5% CO₂ incubator at 37°C until reaching confluence (~50,000 cells/cm²). For the nuclear run-on experiments, first passage cultures were used. For transient transfections, subconfluent primary cultures were used. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids (Life Technologies, Inc.) and 10% fetal bovine serum (HyClone Laboratories, Logan, UT). At confluence (subconfluence for transfection experiments), the cells were rinsed and transferred to serum-free medium for 18–24 h, when they were again rinsed with serum-free medium and exposed to test or control medium in the absence of serum for 2–24 h. Cortisol (Sigma) was dissolved in ethanol and diluted 1:10,000 or greater in DMEM; cycloheximide (Sigma) was added directly to the medium. 5,6-Dichlorobenzimidazole riboside (DRB) (Sigma) was dissolved in absolute ethanol and diluted 1:200 in DMEM, and all experimental groups were exposed to an equal amount of ethanol. For RNA analysis, the cell layer was extracted with guanidine thiocyanate at the end of the incubation and stored at ~80°C. For the nuclear run-on assay, nuclei were isolated by Dounce homogenization. For protein analysis, the extracellular matrix was extracted and processed for Western blots.

Northern Blot Analysis—Total cellular RNA was isolated with guanidine thiocyanate, at acid pH, followed by phenol/chloroform (Sigma) extraction (20). RNA was precipitated with isopropl alcohol, resuspended, and reprecipitated with ethanol. The RNA recovered was quan-
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Fig. 1. Effect of the glucocorticoid cortisol at 1 μM on IGFBP-5 mRNA expression in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control (C) or cortisol (glucocorticoid (GC)-treated cultures was subjected to Northern blot analysis and hybridized with an IGFBP-5 cDNA. IGFBP-5 mRNA was visualized by autoradiography and is shown in the upper panels, while 18S rRNA is shown below. kb, kilobases.

tal conditions, to the membranes (30). Equal counts/minute of [32P]RNA from each sample were hybridized to cDNAs at 42°C for 72 h and washed in 1 × SSC at 65°C for 20 min. Hybridized cDNAs were visualized by autoradiography.

Deletion Constructions and Site-directed Mutagenesis—To determine changes in promoter activity, deletion constructs were made by digestion of the murine IGFBP-5 promoter (kindly provided by P. Rotwein, Washington University School of Medicine, St. Louis, MO) with restriction enzymes. Internal mutations were prepared by PCR from the smallest deletion construct, bp 70 to 120, using oligonucleotide primers containing the various mutations in the 5′ end. A 3′ truncation of the IGFBP-5 promoter was also prepared by PCR through the generation of a new restriction site at bp 22. Deletion constructs and PCR products were purified and cloned into the luciferase construct pGL2-Basic (24). All DNA products generated by PCR were sequenced to confirm mutations and to eliminate the possibility of unintended mutations or deletions.

Transient Transfections—Ob cells were cultured to 70% confluence and transiently transfected with IGFBP-5 deletion and mutation constructs by calcium phosphate-DNA coprecipitation as described (31). After 4 h, cells were exposed for 3 min to 10% glycerol. Ob cells were allowed to recover in serum-containing DMEM for 20 h, serum-deprived for 18 h, and exposed to control or test medium as described below and in the figure legends. Cells were washed with phosphate-buffered saline and harvested in reporter lysis buffer (Promega). To control for transfection efficiency, cells were cotransfected with a construct containing the β-galactosidase gene driven by the cytomegalovirus promoter (CMVGal, CLONTECH, Palo Alto, CA). Luciferase and β-galactosidase activities were measured using a luciferase assay kit (Promega) and a β-galactosidase assay using Galacton reagent (Tropix Inc., Bedford, MA), both in accordance with the manufacturer’s instructions. Data are expressed as means ± S.E. of luciferase activity corrected for β-galactosidase activity. Statistical differences were determined by analysis of variance and post hoc examination by Ryan-Einot-Gabriel-Welch F test (32, 33).

Western Blot Analysis—Extracellular matrix was prepared as described (34, 35). Briefly, Ob cells were rinsed in phosphate-buffered saline; cell membranes were removed with 0.5% Triton X-100 (Sigma), pH 7.4, and nuclei and cytoskeleton were removed by incubation with 25 mM ammonium acetate, pH 9.0, for 5 min. The extracellular matrix was rinsed with phosphate-buffered saline, extracted with Laemmli sample buffer containing 2% sodium dodecyl sulfate, and fractionated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blot analysis was performed using rabbit antiserum raised against native human IGFBP-5 (Upstate Biotechnology Inc., Lake Placid, NY) in 3% bovine serum albumin overnight. Blots were exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum, washed, and developed with horseradish peroxidase chemiluminescent detection reagent (DuPont NEN). The presence of IGFBP-5 was confirmed by autoradiography and is shown in the upper panels, while 18S rRNA is shown below. kb, kilobases.

RESULTS

Northern blot analysis of total RNA extracted from confluent cultures of Ob cells revealed a predominant IGFBP-5 transcript of 6.0 kilobases (Fig. 1). Continuous treatment of Ob cells with cortisol caused a time-dependent decrease in IGFBP-5 steady-
IGFBP was confirmed in previous studies in which the immunoreactive IGFBP-5 standard was used (Fig. 3). The identity of this protein was confirmed by the presence of a major form of immunoreactive IGFBP-5 mRNA in the extracellular matrix of untreated Ob cells determined by densitometry (Fig. 2). Western immunoblot analysis of the extracellular matrix of untreated Ob cell cultures revealed that treatment with cycloheximide for 24 h caused a 93% decrease in IGFBP-5 mRNA (Fig. 4). Because IGFBP-5 is found primarily in the extracellular matrix of Ob cell cultures and its expression in the medium is low, the detection of an inhibitory effect in the medium is impractical (14). To determine whether or not the effects observed on IGFBP-5 mRNA levels were dependent on protein synthesis, serum-deprived confluent cultures of Ob cells were treated with cortisol in the presence or absence of cycloheximide at 3.6 μM. In earlier experiments, cycloheximide at doses of 2 μM and higher was found to inhibit protein synthesis in Ob cell cultures by 80–85% (37). Northern blot analysis revealed that treatment with cycloheximide for 24 h caused a 93 ± 8% (n = 4) decrease in IGFBP-5 transcript levels, so further inhibitory effects of cortisol were difficult to detect (Fig. 4).

To examine whether or not the effect of cortisol on IGFBP-5 mRNA levels was due to changes in transcript stability, Ob cells were exposed to DMEM or cortisol for 60 min and then treated with the RNA polymerase II inhibitor DRB in the absence or presence of cortisol at 1 μM for 6, 16, or 24 h (24). The half-life of IGFBP-5 mRNA in transcriptionally arrested Ob cells was estimated at 18 h (Fig. 5). Slope analysis indicated no significant difference between control (slope = -0.0154, n = 11) and cortisol-treated (slope = -0.0194, n = 12) cultures (25). Treatment of Ob cell cultures with cortisol for 6 and 24 h decreased IGFBP-5 mRNA expression by 68 ± 8% (n = 3) and 23–34% (n = 2), respectively, as estimated by reverse transcription-PCR (Fig. 6). No signal of the hRNA product was detected in any of the samples tested when the reverse transcription step was omitted prior to the PCR, eliminating the possibility of DNA contamination. To confirm whether cortisol modified the transcription of the IGFBP-5 gene, nuclear run-on assays were performed on nuclei from Ob cells treated with 1 μM cortisol for 2, 6, and 24 h. Although the effect was small at 2 h, cortisol inhibited the rate of IGFBP-5 transcription by 29 ± 7% (n = 3) at 6 h and by 54 ± 4% (n = 3) at 24 h (Fig. 7). The ability of cortisol to regulate putative promoter regions of the IGFBP-5 gene in Ob cells was examined using transient transfections of luciferase constructs containing IGFBP-5 promoter sequences spanning bp −2695 to +120. Deletion constructs from bp −2695 to +120 to bp −70 to +120 (Fig. 8, A and B) showed a 35% decrease in IGFBP-5 promoter activity when...
treated with cortisol at 1 μM for 6 h (Fig. 8C). The reverse orientation of the largest construct, bp +120 to −2695, yielded little luciferase activity and no inhibition by cortisol. Site-directed mutations and a 3′-truncation of the bp −70 to +120 deletion construct were generated by PCR and used to further analyze the responsive regions of the IGFBP-5 promoter. A putative CAAT motif was mutated near the 5′-end, and a truncation from the 3′-end of the construct was made that eliminated a potential binding site for a nuclear factor for interleukin-6 expression (NFIL-6) (T(G/T)NNGNTT(G/T)) (Fig. 9, A and B). In addition, a region that contains a putative CCAAT/enhancer-binding protein α binding motif (T(T/G)NNG(T/C)AA(T/G)) was selected for mutation. In a representative experiment (n = 6), these mutated constructs each showed a 40–50% (p < 0.05) decrease in promoter activity in response to 1 μM cortisol for 6 h (Fig. 9C). In contrast, mutation of a consensus binding site for E-box proteins or c-Myb ((T(T/C)AC(G/T))G) abrogated the inhibitory effect of 1 μM cortisol on IGFBP-5 promoter activity.

**DISCUSSION**

Recent studies have demonstrated that cortisol decreases the synthesis of IGF-I and IGFBP-5 in skeletal cells, and this investigation was undertaken to determine the mechanism by which cortisol inhibits IGFBP-5 expression in calvaria-derived Ob cells. We demonstrated that cortisol decreases IGFBP-5 mRNA levels in Ob cells in a time- and dose-dependent manner. The basal expression of IGFBP-5 requires protein synthesis, and it was not possible to determine whether the effect of cortisol on IGFBP-5 was protein synthesis-dependent. Experiments in transcriptionally blocked Ob cells revealed that cortisol did not modify IGFBP-5 mRNA stability (24). This, in conjunction with a decrease in hnRNA levels and in rates of transcription, indicates that cortisol inhibits IGFBP-5 expression at the transcriptional level. Although cortisol inhibited both the levels of hnRNA and the rates of transcription, the effect on hnRNA was more pronounced after 6 h, whereas the effect on the rates of transcription was more evident after 24 h. Although changes in hnRNA frequently match changes in the rate of transcription, hnRNA levels also reflect RNA processing, which could account for the differences observed. Cortisol also inhibited the activity of murine IGFBP-5 promoter constructs driving a luciferase reporter gene in transiently transfected Ob cells. The elements responsible for the suppression of the glucocorticoid (GC) cortisol at 1 μM on IGFBP-5 promoter activity in transiently transfected Ob cells were transfected with pGL2-Basic containing the deletion constructs shown in B and exposed to DMEM (white bars) or cortisol (striped bars) for 6 h. Bars indicate luciferase units normalized to β-galactosidase (BGal) activity and represent means ± S.E. (n = 6). *, significantly different from control (p < 0.05).
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was shown that IGFBP-5 is degraded by calcium-dependent activity is another level of regulation by which cortisol could be secreted to the culture medium of Ob cells under the described conditions (14, 35). The amount of IGFBP-5 in this compartment is probably not involved in IGFBP-5 regulation by cortisol. Although neither c-Myb- nor E-box-binding sites have been identified on the IGFBP-5 promoter, it is possible that the inhibition of IGFBP-5 synthesis is mechanistically important to the actions of cortisol in bone. IGFBP-5 associated with the extracellular matrix of fibroblasts enhances IGF-I action on cell growth (35). This is also probably the case with osteoblasts since IGFBP-5 is known to enhance the effects of IGF-I on osteoblast cell replication, and the reduction of IGFBP-5 levels by cortisol in the extracellular matrix may be a mechanism by which cortisol decreases the skeletal effects of IGF-I. Although glucocorticoids have a number of actions on bone metabolism that are independent of their effects on the IGF-IGFBP axis, the inhibition of IGF-I and IGFBP-5 synthesis in osteoblasts may be relevant to the actions of cortisol on bone cell function.

In conclusion, this study demonstrates that cortisol inhibits IGFBP-5 mRNA and polypeptide levels in skeletal cells through mechanisms that involve diminished transcription. The gene elements responsible for this effect are located between bp −70 and +22 in the IGFBP-5 promoter, and E-box-binding proteins or c-Myb-related nuclear factors may be involved. The cortisol-reduced level of IGFBP-5 in the bone microenvironment may be relevant to its inhibitory actions on bone formation.

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**FIG. 9.** A, murine IGFBP-5 promoter from bp −70 to +120 showing enhancer elements, potential transcription factor-binding sites, and two sites for the start of transcription (indicated by arrows). B, wild-type (WT) IGFBP-5 promoter from bp −70 to +120 is shown above. The promoter has been reduced to constructs shown in boldface below. A 3′-truncation is represented at the bottom. C, effect of the glucocorticoid (GC) cortisol at 1 μM on IGFBP-5 promoter activity in transiently transfected Ob cells. Cultures were transfected with pGL2-Basic containing the constructs shown in B and exposed to DMEM (white bars) or cortisol (striped bars) for 6 h. A larger construct in reverse orientation, bp +120 to −2695, was used as a vector control (Rev). Bars indicate luciferase units normalized to β-galactosidase (βGal) activity and represent means ± S.E. (n = 6). * significantly different from control (p < 0.05). C/E box α, CAAAT/enhancer-binding protein α.
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