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Cortisol Enhances the Expression of mac25/Insulin-Like Growth Factor-Binding Protein-Related Protein-1 in Cultured Osteoblasts*

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ABSTRACT

Glucocorticoids inhibit the synthesis of insulin-like growth factor I (IGF-I) and regulate the expression of IGF-binding proteins (IGFBPs) in osteoblast cultures. IGFBP-related protein-1 (IGFBP-rP1), the product of the mac25 gene, binds IGF-I, IGF-II, and insulin, and we postulated that glucocorticoids regulate IGFBP-rP1 synthesis in osteoblasts. We tested the expression of mac25/IGFBP-rP1 in cultures of osteoblast-enriched cells from 22-day-old fetal rat calvariae (Ob cells). Cortisol treatment at 10 nM to 1 μM for 24–48 h caused a time- and dose-dependent increase in mac25/IGFBP-rP1 messenger RNA (mRNA) levels in Ob cells. Cycloheximide at 3.6 μM did not alter mac25/IGFBP-rP1 transcripts in control or cortisol-treated cells. Cortisol did not modify the decay of mac25/IGFBP-rP1 mRNA in transcriptionally arrested Ob cells and increased the rate of IGFBP-rP1 transcription as determined by nuclear run-on assays. Retinoic acid also increased mac25/IGFBP-rP1 mRNA levels, but 17β-estradiol, testosterone, 5α-dihydrotestosterone, progesterone, and 1,25-dihydroxyvitamin D_3 did not. In conclusion, cortisol stimulates mac25/IGFBP-rP1 expression in Ob cells by transcriptional mechanisms. As IGFBP-rP1 binds and possibly modifies the effects of IGFs and insulin, its increased expression could be relevant to the inhibitory actions of cortisol in bone. (*Endocrinology* **140:** 228–232, 1999)

RECENT studies have shown that, in addition to the six characterized insulin-like growth factor-binding proteins (IGFBP), the protein encoded by the mac25 gene shares biochemical and functional properties with the IGFBPs and has been termed IGFBP-7 and, more recently, IGFBP-related protein-1 (IGFBP-rP1) (1, 2). The product of the mac25 gene has 40–45% similarity and 20–25% amino acid sequence identity with IGFBPs (1–5). In the amino-terminus, it has 11 of 12 cysteine clusters, which are characteristic of IGFBPs, although it lacks 6 cysteine clusters in the carboxyl-terminus (1, 3). Like IGFBPs, the protein encoded by mac25 binds IGF-I and IGF-II, although its affinity for IGFs is low (1). In contrast to more classic IGFBPs, the product of mac25 binds insulin with high affinity and inhibits insulin-stimulated phosphorylation of its receptor (6). This suggests that it acts primarily as an insulin-binding protein.

The protein encoded by mac25 has a molecular mass of 31,000 and is identical to the proteins termed tumor adhesion factor and prostacyclin-stimulating factor (7–9). The protein encoded by mac25 stimulates cell adhesion and PG synthesis, and its functions on cell growth are diverse and not well characterized (5, 7, 8). In BALB/c3T3 mouse fibroblasts, it stimulates cell growth and enhances the mitogenic effects of IGF-I, whereas in selected osteosarcoma cells it inhibits cell growth (7, 10). The mac25 gene is expressed in a variety of normal and malignant cells of epithelial and mesenchymal origin, although its expression by skeletal cells has not been reported (1, 3, 4). mac25 expression is increased in senescent human mammary epithelial cells and after exposure to retinoic acid, suggesting that it may play a role in cell growth or differentiation (4).

Glucocorticoids have complex effects on bone formation and resorption (11, 12). Some of these are probably due to direct actions of glucocorticoids on specific genes expressed by the osteoblast, whereas others may be indirect and mediated by locally produced growth factors (11, 12). IGF-I and IGF-II have important stimulatory effects on bone formation, and glucocorticoids inhibit the expression of IGF-I, although they have inconsistent effects on IGF-II synthesis in osteoblasts (13, 14). Skeletal cells express the six classic IGFBPs, and selected effects of glucocorticoids in bone appear mediated by their actions on IGFBP expression (15–17). For instance, glucocorticoids inhibit the synthesis of IGFBP-5, a binding protein that may enhance the effects of IGF-I on bone cell function, and increase the expression of IGFBP-6, a binding protein that selectively blocks the effects of IGF-II on osteoblasts (18, 19). Glucocorticoids, like retinoic acid, enhance the differentiation of cells of the osteoblastic lineage (20–23). In view of the known actions of glucocorticoids on the IGF-IGFBP axis and on the possible relationship between mac25 gene expression and differentiation, we postulated mac25/IGFBP-rP1 gene regulation by glucocorticoids in osteoblasts.

The present studies were undertaken to examine the effects of cortisol on mac25 or IGFBP-rP1 gene expression in

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cultures of osteoblast-enriched cells from 22-day-old fetal rat calvariae (Ob cells) and compare them to the actions of other steroids with known effects on bone cell function.

Materials and Methods

Culture technique

The culture method used was described in detail previously (24). 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 Biochemical Corp., Freehold, NJ). Cell populations harvested from the third to the fifth digestions were cultured as a pool and were previously shown to have osteoblastic characteristics (24). Ob cells were plated at a density of 8,000–12,000 cells/cm^2 and cultured in a humidified 5% CO_2 incubator at 37 C until reaching confluence (~50,000 cells/cm^2). Cells were cultured in DMEM supplemented with nonessential amino acids and 10% FBS (both from Summit Biotechnology, Fort Collins, CO). Ob cells were grown to confluence, transferred to serum-free medium for 20–24 h, and exposed to test or control medium in the absence of serum for 2–48 h, as indicated in the text and legends. In 48-h treated cultures, the medium was replaced after 24 h with fresh control or test solutions. For nuclear run-on assays, Ob cells were grown to subconfluence, trypsinized, replated, and grown to confluence, when they were serum deprived and exposed to test or control solutions for 6–48 h. Cortisol, retinoic acid, 17β-estradiol, testosterone, 5α-dihydrotestosterone, progesterone (all from Sigma Chemical Co., St. Louis, MO), and 1,25-dihydroxyvitamin D_3 (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) were dissolved in ethanol and diluted 1:1,000 or greater in DMEM. Cycloheximide and 5,6-dichlorobenzimidazole riboside (DRB; both from Sigma Chemical Co.) were dissolved in absolute ethanol and diluted 1:1,000 and 1:200, respectively, in DMEM. Control and experimental cultures were exposed to equal amounts of ethanol. For RNA analysis, the cell layer was extracted with guanidine thiocyanate at the end of the incubation and stored at −70 C. For nuclear run-on assays, nuclei were isolated by Dounce homogenization (Kontes Co., Vineland, NJ).

Northern blot analysis

Total cellular RNA was isolated using an RNeasy kit following the manufacturer's instructions (Qiagen, Chatsworth, CA). The RNA recovered was quantitated by spectrometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA, documenting equal RNA loading of the various experimental samples. The RNA was then blotted onto GeneScreen Plus charged nylon (DuPont, Wilmington, DE) and the uniformity of transfer was documented by revisualization of ribosomal RNA. A 1.1-kb *Eco*RI/*Xho*I restriction fragment of the mouse mac25/IGFBP-rP1 complementary DNA (cDNA; provided by M. Kato, Ibaraki, Japan) was purified by agarose gel electrophoresis (10). mac25/IGFBP-rP1 cDNA was labeled with [α-^{32}P]deoxy-CTP and [α-^{32}P]deoxy-ATP (50 μCi of each; SA, 3000 Ci/mmol; DuPont) using the random hexanucleotide-primed, second strand synthesis method (25). Hybridizations were carried out at 42 C for 16–72 h, and posthybridization washes were performed at 65 C in 0.2 × SSC (saline-sodium citrate) for 30 min. The blots were stripped and rehybridized with a 752-bp *Bam*HI/*Sph*I restriction fragment of the murine 18S cDNA (American Type Culture Collection, Manassas, VA) at 42 C for 16–72 h, and posthybridization washes were performed at 65 C in 0.1 × SSC. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak Co., Rochester, NY) employing Cronex Lightning Plus intensifying screens (DuPont). Relative hybridization levels were determined by densitometry. The Northern analyses shown are representative of three or more cultures.

Nuclear run-on assay

To examine changes in the rate of transcription, nuclei were isolated by Dounce homogenization in a Tris buffer containing 0.5% Nonidet P-40. Nascent transcripts were labeled by incubation of nuclei in a reaction buffer containing 500 μM of each adenosine, cytidine, and guanosine triphosphate; 150 U RNasin (Promega Corp., Madison, WI); and 250 μCi [α-^{32}P]UTP (3000 Ci/mM; DuPont) (26). RNA was isolated by treatment with deoxyribonuclease I and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Linearized plasmid pBluescript SK$^+$ DNA containing about 1 μg of the mac25/IGFBP-rP1 cDNA used for Northern blotting was immobilized onto GeneScreen Plus by slot blotting according to the manufacturer's directions (DuPont). The plasmid vector pGL3-Basic (Promega Corp.) was used as a control for nonspecific hybridization, and 18S RNA cDNA was used to estimate loading of the radiolabeled RNA. Equal counts per min of [^{32}P]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.

Statistical methods

Values are expressed as the mean ± SEM. Slopes to determine messenger RNA (mRNA) decay were analyzed by the method of Sokal and Rohlf (27).

Results

Northern blot analysis of total RNA extracted from confluent cultures of Ob cells revealed a predominant mac25/IGFBP-rP1 transcript of 1.1 kb (Fig. 1). Continuous treatment of Ob cells with cortisol caused a time-dependent increase in mac25/IGFBP-rP1 steady state mRNA levels. The effect was first observed after 24 h of exposure to cortisol at 1 μM, and treatment with cortisol increased mac25/IGFBP-rP1 mRNA levels by (mean ± SEM; n = 6–9) 1.9 ± 0.2-fold after 24 h and by 2.8 ± 0.4-fold after 48 h as determined by densitometry (Fig. 1). The effect of cortisol was dose dependent. Continuous treatment of Ob cells with cortisol for 48 h at 10 nM, 100 nM, and 1 μM increased mac25/IGFBP-rP1 transcripts by (mean ± SEM; n = 3) 1.9 ± 0.1-, 2.1 ± 0.2-, and 2.7 ± 0.3-fold, respectively (Fig. 2).

To determine whether the effects observed on mac25/IGFBP-rP1 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% (28). Northern blot analysis revealed that treatment with cycloheximide for 24 h (not shown) or 48 h (Fig. 3) did not prevent and modestly

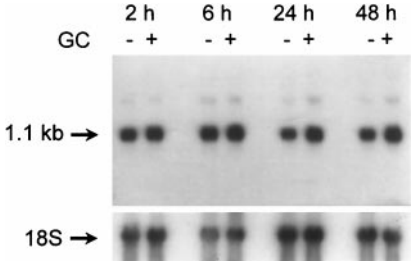

FIG. 1. Effect of the glucocorticoid (GC) cortisol at 1 μM on mac25/IGFBP-rP1 mRNA expression in cultures of Ob cells treated for 2, 6, 24, or 48 h. Total RNA from control (−) or cortisol-treated (+) cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-rP1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-rP1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

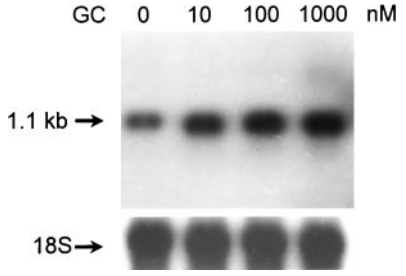

FIG. 2. Effect of the glucocorticoid (GC) cortisol (10–1000 nM) on mac25/IGFBP-rP1 mRNA expression in cultures of Ob cells treated for 48 h. Total RNA from control (0) or cortisol-treated cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-rP1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-rP1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

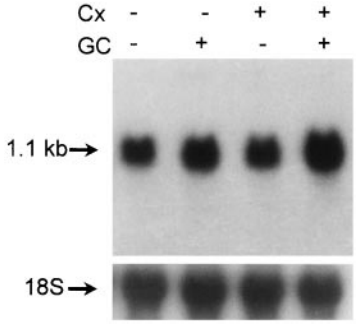

FIG. 3. Effect of the glucocorticoid (GC) cortisol at 1 μM in the presence (+) or absence (−) of cycloheximide (Cx) at 3.6 μM on mac25/IGFBP-rP1 mRNA expression in cultures of Ob cells treated for 48 h. Total RNA from control (−) or treated (+) cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-rP1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-rP1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

enhanced the stimulatory effect of cortisol on mac25/IGFBP-rP1 mRNA levels.

To examine whether the effect of cortisol on mac25/IGFBP-rP1 mRNA levels was due to changes in transcript stability, confluent cultures of Ob cells were exposed to DMEM or cortisol for 4 h, and then treated with the RNA polymerase II inhibitor DRB in the absence or presence of cortisol at 1 μM for 6–24 h (29). About 75% of Ob cells are viable in the presence of DRB for 24 h, but cell viability is impaired after exposure to DRB for longer periods of time, as determined by trypan blue exclusion (Canalis, E., unpublished observations). After 24 h of DRB exposure, a 20% decay in mac25/IGFBP-rP1 mRNA was detected, and the change was not different in control and cortisol-treated samples (Fig. 4). The half-life of mac25/IGFBP-rP1 mRNA in transcriptionally arrested Ob cells was estimated to be over 48 h, by extrapolation of the values obtained in the first 24 h. To confirm whether cortisol modified the transcription of the mac25/IGFBP-rP1 gene, a nuclear run-on assay was performed on nuclei from Ob cells treated for 48 h. This assay demonstrated that cortisol increased the rate of mac25/IGFBP-rP1 transcription by 3-fold (Fig. 5).

Similarly to cortisol, retinoic acid caused a time- and dose-dependent increase in mac25/IGFBP-rP1 mRNA levels in Ob cells. The effect appeared after 24 h and was sustained for 48 h (Fig. 6). After 24 h, retinoic acid at 10 nM, 100 nM, and 1 μM increased mac25/IGFBP-rP1 transcripts by (mean ± SEM; n = 3) 1.8 ± 0.2-, 2.3 ± 0.2-, and 3.5 ± 0.2-fold, respectively (Fig. 7). The induction of mac25/IGFBP-rP1 was selective to cortisol and retinoic acid, and other steroids, such as 17β-estradiol, testosterone, 5α-dihydrotestosterone, progesterone, and 1,25-dihydroxyvitamin D_3, did not modify the expression of mac25/IGFBP-rP1 mRNA in Ob cells (Fig. 8).

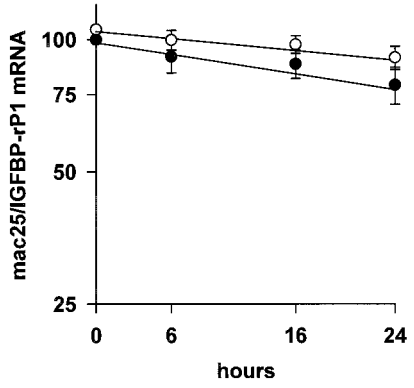

FIG. 4. Effect of the glucocorticoid (GC) cortisol at 1 μM on mac25/IGFBP-rP1 mRNA decay in transcriptionally blocked Ob cells. Cultures were exposed to control medium (●) or treated with cortisol (○) 4 h before and 6, 16, or 24 h after the addition of DRB. RNA was subjected to Northern blot analysis, hybridized with α-^{32}P-labeled mac25/IGFBP-rP1 cDNA, visualized by autoradiography, and quantitated by densitometry. Ethidium bromide staining of ribosomal RNA was used to check uniform loading of the gels and transfer. Values are the mean ± SEM for eight cultures. Values were obtained by densitometric scanning and are presented as a percentage of mac25/IGFBP-rP1 mRNA levels relative to the time of DRB addition. Slopes were analyzed by the method of Sokal and Rohlf and were not found to be statistically different.

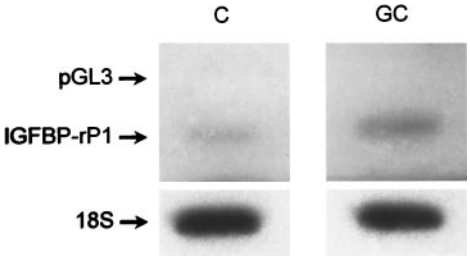

FIG. 5. Effect of the glucocorticoid (GC) cortisol at 1 μM on the rate of mac25/IGFBP-rP1 transcription in cultures of Ob cells treated for 48 h. Nascent transcripts from control (C) or cortisol-treated (GC) cultures were labeled *in vitro* with [α-^{32}P]UTP, and the labeled RNA was hybridized to immobilized cDNA for mac25/IGFBP-rP1. 18S RNA cDNA was used to demonstrate loading, and pGL3-Basic vector DNA was used as a control for nonspecific hybridization.

Discussion

Recent studies have shown that cortisol has significant effects on the IGF-IGFBP axis in osteoblasts and fibroblasts, but it has not been reported to alter the expression of IGFBP-7 or IGFBP-rP1 (11, 14, 17–19). The present investigation was undertaken to determine whether osteoblasts express the mac25/IGFBP-rP1 gene and to examine its regulation by glucocorticoids. We demonstrated that cortisol increases

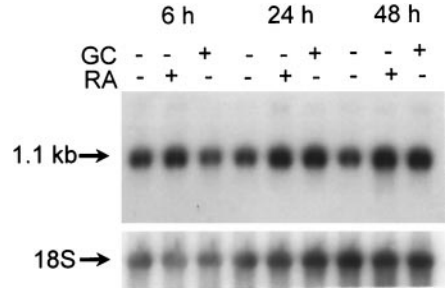

FIG. 6. Effects of retinoic acid (RA) and the glucocorticoid (GC) cortisol, both at 1 μM, on mac25/IGFBP-rP1 mRNA expression in cultures of Ob cells treated for 6, 24, or 48 h. Total RNA from control (−) or treated (+) cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-rP1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-rP1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

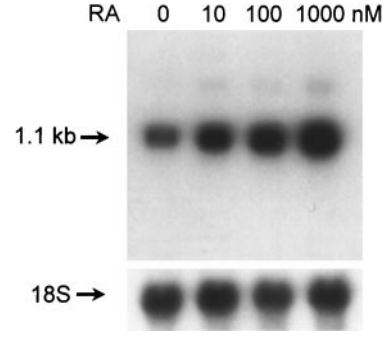

FIG. 7. Effect of retinoic acid (RA) at 10–1000 nM on mac25/IGFBP-rP1 mRNA expression in cultures of Ob cells treated for 24 h. Total RNA from control (0) or RA-treated cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-rP1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-rP1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

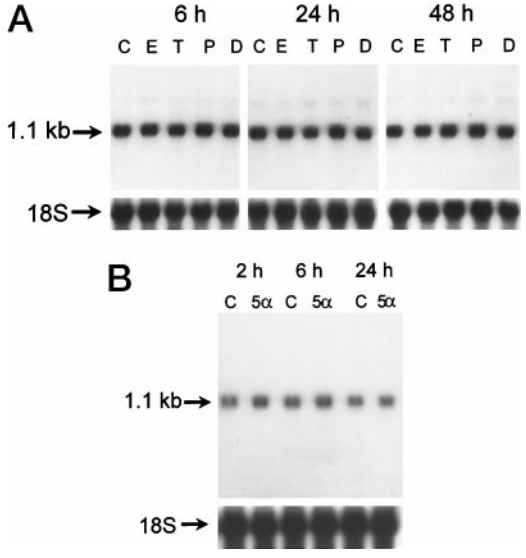

FIG. 8. Effects of 17β-estradiol (E), testosterone (T), progesterone (P), and 1,25-dihydroxyvitamin D_3 (D), all at 100 nM, for 6, 24, or 48 h (a) and of 5α-dihydrotestosterone at 10 nM for 2, 6, or 24 h (b) on mac25/IGFBP-rP1 mRNA expression in cultures of Ob cells. Total RNA from control (C) or treated cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-rP1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-rP1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

mac25/IGFBP-rP1 mRNA levels in Ob cells in a time- and dose-dependent manner, and that the effect does not require *de novo* protein synthesis. In fact, the effect of cortisol was enhanced modestly by cycloheximide, possibly due to an inhibitory effect on the synthesis of RNA-degrading enzymes. Experiments in transcriptionally blocked Ob cells, using the RNA polymerase II inhibitor DRB, revealed that cortisol did not modify mac25/IGFBP-rP1 mRNA stability (29). This, in conjunction with an increase in the rate of transcription, indicates that cortisol stimulates mac25/IGFBP-rP1 expression at the transcriptional, not at the post-transcriptional level.

In our study, the effects of cortisol on mac25/IGFBP-rP1 synthesis were observed at doses that modify other parameters of metabolic function in Ob cells, suggesting that the stimulation of IGFBP-rP1 synthesis by cortisol is physiologically relevant. IGF-I and IGF-II are abundant in skeletal tissue, and studies in mice with targeted IGF-I and IGF-II gene disruption and studies of transgenic mice overexpressing IGF-I and IGF-II have documented their relevance to skeletal growth (30–32). As IGFBP-rP1 has the potential to bind IGFs and to modify their actions on cell growth, its induction by cortisol could be a mechanism to regulate the actions of IGFs in bone (1). IGFBP-rP1 binds insulin with higher affinity than IGFs, suggesting that its increased expression is more likely to modify insulin actions in bone (16). However, it is important to note that in the present studies we did not demonstrate a change in protein levels. An attempt at detecting IGFBP-rP1 in rat Ob cell cultures was made using antibodies and assays to the human protein, but it was unsuccessful, possibly due to the lack of cross-reactivity of the antibody with rodent IGFBP-rP1 (9) (Pereira, R., *et al.*, unpublished observations).

Glucocorticoids have complex effects on bone remodeling and have a major impact on the IGF-IGFBP axis; their effects on bone formation are the opposite of those of IGF-I, IGF-II, and insulin (11–13, 33–35). This suggests that some of the effects of glucocorticoids in bone could be mediated by changes in the synthesis or activity of IGF-I, IGF-II, and insulin. Glucocorticoids decrease the synthesis of IGF-I and IGFBP-5, a binding protein that can stimulate bone cell growth, and increase IGFBP-6 expression, a binding protein that selectively binds IGF-II (2, 14, 18, 19). Consequently, although acting through different mechanisms, chronic exposure of the skeletal tissue to glucocorticoids may result in a significant depletion of both IGF-I and IGF-II. This may result in a decrease in the replication and differentiated function of osteoblastic cells and a decrease in bone formation. The increase in mac25/IGFBP-rP1 expression may explain additional actions of glucocorticoids in bone. Since insulin increases bone collagen synthesis and glucocorticoids are inhibitory, it is possible that an increase in mac25/IGFBP-rP1 could bind insulin and prevent some of its actions in bone. The experimental BB rat with spontaneous diabetes displays decreased bone formation and osteoporosis, and patients

with insulin-dependent, but not with insulin-independent, diabetes have an increased incidence of osteoporosis (36–39). These observations indicate the relevance of insulin to bone homeostasis *in vivo*. An increase in IGFBP-rP1 secondary to glucocorticoid excess could have a detrimental effect on bone formation by reducing the insulin available to the skeletal tissue.

The exact function of mac25/IGFBP-rP1 has not been established. There is evidence to suggest that mac25/IGFBP-rP1 plays a role in the differentiation of certain cells, as it inhibits the growth of osteosarcoma cells and induces capillary vessel formation (5, 10). A role in differentiation is further supported by the induction of mac25/IGFBP-rP1 by retinoic acid (4). Since glucocorticoids induce the differentiation of cells of the osteoblastic lineage, their stimulatory effects on mac25/IGFBP-rP1 could also play a role in this process (20, 21, 40). However, it is important to note that other peptides, such as bone morphogenetic protein-6, have been shown to play a central role mediating the glucocorticoid effects on osteoblastic cell differentiation (22).

In conclusion, the present studies demonstrate that cortisol stimulates mac25/IGFBP-rP1 transcripts in skeletal cells through mechanisms that involve increased transcription. An increased level of IGFBP-rP1 in the bone microenvironment may bind IGFs and insulin and be relevant to the inhibitory actions of cortisol on bone formation.

Acknowledgments

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