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Time- and Dose-Related Interactions between Glucocorticoid and Cyclic Adenosine 3′,5′-Monophosphate on CCAAT/Enhancer-Binding Protein-Dependent Insulin-Like Growth Factor I Expression by Osteoblasts*

THOMAS L. McCARTHY, CHANGHUA JI, YUN CHEN, KENNETH KIM, AND MICHAEL CENTRELLA

Department of Surgery, Plastic Surgery Section, Yale University School of Medicine, New Haven, Connecticut 06520

ABSTRACT

Glucocorticoid has complex effects on osteoblasts. Several of these changes appear to be related to steroid concentration, duration of exposure, or specific effects on growth factor expression or activity within bone. One important bone growth factor, insulin-like growth factor I (IGF-I), is induced in osteoblasts by hormones such as PGE_2 that increase intracellular cAMP levels. In this way, PGE_2 activates transcription factor CCAAT/enhancer-binding protein-δ (C/EBPδ) and enhances its binding to a specific control element found in exon 1 in the IGF-I gene. Our current studies show that preexposure to glucocorticoid enhanced C/EBPδ and C/EBPβ expression by osteoblasts and thereby potentiated IGF-I gene promoter activation in response to PGE_2. Importantly, this directly contrasts with inhibitory effects on IGF-I expression that result from sustained or pharmacologically high levels of glucocorticoid exposure. Consistent with the stimulatory effect of IGF-I on bone protein synthesis, pretreatment with glucocorticoid sensitized osteoblasts to PGE_2, and in this context significantly enhanced new collagen and noncollagen protein synthesis. Therefore, pharmacological levels of glucocorticoid may reduce IGF-I expression by osteoblasts and cause osteopenic disease, whereas physiological transient increases in glucocorticoid may permit or amplify the effectiveness of hormones that regulate skeletal tissue integrity. These events appear to converge on the important role of C/EBPδ and C/EBPβ on IGF-I expression by osteoblasts. (*Endocrinology* **141:** 127–137, 2000)

INSULIN-LIKE growth factor I (IGF-I) is synthesized by many tissues, including bone, where it is a key autocrine and paracrine regulator of osteoblast activity (1–3). PTH and PGE_2, which both increase bone resorption, also stimulate IGF-I synthesis by osteoblasts (4, 5). The effect of these hormones depends upon their ability to increase intracellular cAMP (6–8). Thus, the process of coupled bone remodeling may depend in part on new IGF-I synthesis by osteoblasts to replace previously resorbed bone (9).

With the availability of information and tools to examine the IGF-I gene promoter, it became possible to assess *cis*- and *trans*-acting molecular elements that account for the stimulatory effect of cAMP on IGF-I expression. In mammals the IGF-I gene is greater than 90 kb in length, and its expression originates from either of two transcriptional promoters (10). In liver, a primary source of circulating IGF-I, both gene promoters are used. In contrast, promoter 1 is the sole or dominant regulatory region of gene transcription in osteoblasts, as it is in other nonhepatic cell types (11). The cAMP responsive *cis*-acting region of the IGF-I gene, bp +202 to +209 (5′-CGCAATCG-3′), was initially termed HS3D by footprint analysis. This sequence occurs within a 5′-untranslated region (UTR) of exon 1 (7, 12) and appears to be the most evolutionarily conserved portion of the IGF-I gene, retaining 95% of sequence information between rat and chicken (13). More recent studies identified C/EBPδ as the principal cAMP-activated regulator of IGF-I gene transcription in osteoblasts, and that activated C/EBPδ binds specifically to HS3D to activate IGF-I gene transcription through promoter 1 (14). In the basal state, osteoblasts constitutively express C/EBPδ, and activation of preexisting C/EBPδ through a cAMP-dependent protein kinase A (PKA) pathway is sufficient to stimulate IGF-I gene expression (12).

In other tissues the C/EBPδ and C/EBPβ isoforms are thought to be integral components of the acute phase response, where they regulate gene expression during infection, inflammation, and other conditions that engender tissue remodeling (15–23). C/EBPs regulate the expression of interleukin-1β, tumor necrosis factor-α, and interleukin-6, which in bone can have important effects on osteoclast formation or activation. C/EBPs can therefore influence the expression of cytokines associated with both bone resorption as well as bone formation. Because IGF-I expression is stimulated by PTH and PGE_2, which also activate bone resorption, IGF-I may have an integral, if not essential, role as a coupling factor in the bone remodeling sequence (9). Con-

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Address all correspondence and requests for reprints to: Thomas L. McCarthy, Ph.D., Department of Surgery, Plastic Surgery Section, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208041, New Haven, Connecticut 06520-8041. E-mail: thomas.mccarthy@yale.edu.
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sequently, with the identification of C/EBPδ as the cAMP-dependent *trans*-activator of IGF-I expression in osteoblasts, it may also be relevant to consider C/EBPs as transcriptional coupling factors that coordinate bone remodeling in response to osteotropic hormones.

Cortisol and synthetic glucocorticoids such as dexamethasone (DEX) can have both permissive and suppressive effects on bone remodeling. These effects vary with dose and duration of treatment. For example, exposure to high doses of glucocorticoid or glucocorticoid exposure for extended periods produces clinically significant osteopenia *in vivo* (24, 25). In this regard, our studies and those from other laboratories showed that exposure to high doses of glucocorticoid suppresses new IGF-I gene expression by cultured osteoblasts (26–28). In contrast, intermittent exposure to glucocorticoid can enhance mineralized nodule formation by cultured osteoblasts and augment the anabolic response to PGs and PTH in cultured bone explants and isolated osteoblasts *in vitro* (29–33). The permissive effect of glucocorticoid on PG-activated osteoblasts also appears to include the IGF-I system, in part through changes in IGF-I expression and specific IGF-binding proteins (33, 34). However, molecular evidence to reconcile these opposing effects of glucocorticoid on IGF-I expression or activity has not been established. Therefore, our current studies were initiated to examine interactions between glucocorticoid and cAMP on IGF-I gene expression by osteoblasts and to assess whether they converge on changes in C/EBP expression or activity.

Materials and Methods

Cell cultures

Cells were prepared from parietal bones of 22-day-old Sprague Dawley rat fetuses (Charles River Laboratories, Inc., Raleigh, NC) using methods approved by the Yale animal care and use committee. Sutures were eliminated by dissection, and cells were released from parietal bones by five sequential 20-min collagenase digestions, as described previously (35, 36). Cells released during the last three digestions exhibit biochemical characteristics associated with differentiated osteoblasts, including high levels of PTH receptors and type I collagen synthesis, and a rise in osteocalcin expression in response to 1,25-dihydroxyvitamin D_3 (36, 37). Histochemical staining demonstrates that approximately 80% of the cells express alkaline phosphatase (McCarthy, T. L., and M. Centrella, unpublished data). By these criteria, differential sensitivity to transforming growth factor-β, bone morphogenetic protein-2, and various PGs and the ability to form mineralized nodules *in vitro* (38–42), osteoblast-enriched cultures are well distinguished from less differentiated periosteal cells. Cells pooled from the last three digestions were plated at 4800/cm^2 in DMEM containing 20 mM HEPES (pH 7.2), 100 μg/ml ascorbic acid, penicillin and streptomycin (Life Technologies, Inc., Grand Island, NY), and 10% FBS (Sigma, St. Louis, MO). All treatments were carried out in serum-free medium. DEX and PGE_2 (Sigma) were dissolved in 95% ethanol. Control treatments included the highest concentration of ethanol (vehicle) present in the treatment reagents. To assess IGF-I messenger RNA (mRNA) stability, cells were pretreated with vehicle or hormones and transcriptionally arrested with the RNA polymerase II inhibitor 5,6-dichloro-β-D-ribofuranosylbenzimidizole, and the amount of mRNA remaining at various times during the next 24 h was measured (11).

mRNA analysis

Confluent cultures (~50,000 cells/cm^2) were dissolved in 5 M guanidine monothiocyanate, 25 mM trisodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol and extracted with phenol-chloroform-isoamyl alcohol (75:25:1) with 0.2 M sodium acetate (43). Total RNA was precipitated, ethanol-washed, dried, and resuspended in diethyl pyrocarbonate-treated water. mRNA levels were assessed by ribonuclease (RNase) protection assay. Antisense ^{32}P-labeled RNA probes were prepared from cloned rat IGF-I, C/EBPδ, and C/EBPβ constructs. A genomic probe for rat IGF-I exon 3 that contained its upstream intron was used to detect IGF-I pre-mRNA (369 nucleotides) and mature mRNA (143 nucleotides; coding sequence) present in total RNA extracts, as previously described (11). Expression plasmids encoding C/EBPδ and C/EBPβ cloned into pcDNA3 were used to prepare [^{32}P]complementary RNA with a Maxiscript kit (Ambion, Inc., Austin, TX). An 18S ribosomal RNA probe was ^{32}P labeled with the T7 MEGAshortscript kit (Ambion, Inc.). The probes were gel-purified, and 8×10^4 cpm IGF-I, C/EBPδ, or C/EBPβ [^{32}P]complementary RNA probe were combined with 300 ng 18S probe at low specific activity (1×10^3 cpm, due to the high abundance of 18S transcripts) and 5 μg total cell RNA in hybridization buffer (80% formamide, 1 mM EDTA, 100 mM sodium citrate, and 300 mM sodium acetate, pH 6.4) at 45 C for 16 h. The samples were digested for 30 min at 37 C with RNase A (0.3 U) and RNase T1 (14 U), and then supplemented with 120 μg proteinase K and 0.5% SDS to inactivate RNases (11). Protected fragments (IGF-I pre-mRNA, 369 nt; IGF-I exon 3, 143 nt; C/EBPδ, 190 nt; C/EBPβ, 260 nt; 18S, 80 nt) were precipitated with 1 vol isopropyl alcohol, collected by centrifugation, resolved on a 5% denaturing polyacrylamide gel, and visualized by autoradiography with Amersham Pharmacia Biotech Hyperfilm (Arlington Heights, IL) and a DuPont Cronex intensifying screen (Wilmington, DE).

Plasmids

A rat IGF-I promoter 1-luciferase fusion construct termed IGF1711b/Luc, a recombinant construct of IGF1711b/Luc with specific mutations within HS3D ($HS3D_{mutAAA}$), a recombinant construct containing four tandem copies of the 19-bp wild-type HS3D sequence (5′AGAGCCT-G*CGCAATCG*AAA-3′) (italics indicate C/EBP element) termed 4X HS3D/Luc, and mutated 4X HS3D (4X $HS3D_{mut6}$, 5′AGAGCCTG*TAT-GATCG*AAA-3′) (*underline* indicates mutated bases) containing four tandem copies of a nonfunctional HS3D element have all been previously described (7, 12, 14). HS3D contains the cAMP response element of the rat IGF-I promoter, recently identified as a functional C/EBP-binding sequence (12, 14). $HS3D_{mut6}$ fails to bind C/EBPδ or C/EBPβ by electrophoretic mobility shift assay, and reporter construct 4X $HS3D_{mut6}$ is not activated by cAMP (12, 14). Rat C/EBPδ and C/EBPβ complementary DNA clones (provided by Dr. Peter Rotwein, Oregon Health Sciences University), were subcloned into eukaryotic expression vector pSV7d, as previously described (14, 44). Plasmid pMMTV-Luc, which contains promoter DNA derived from the mouse mammary tumor virus (MMTV) fused upstream of luciferase, was provided by Dr. Ronald Evans (The Salk Institute, La Jolla, CA). A C/EBPδ dominant negative (C/EBPδ DN) construct was created by a *Sac*II deletion of its activation domain. All plasmids were propagated in *Escherichia coli* strain DH5α with ampicillin selection and were prepared using a QIAGEN Midiprep Kit (Chatsworth, CA) and the manufacturer's recommended protocol.

Transfections

Promoter/luciferase reporter plasmids were transfected using Lipofectin (Life Technologies, Inc., Gaithersburg, MD) at 0.6–0.75 μg DNA/4.8-cm^2 culture well as previously reported (7, 12, 14). Transfection efficiency was assessed by cotransfection with a vector carrying the β-galactosidase gene under the control of the simian virus 40 promoter. Cultures at 50% confluence were rinsed in serum-free DMEM, transfected for 3 h, and then refed with DMEM containing 5% FBS. After 24–48 h, the cells were rinsed with serum-free medium and treated for the indicated time with vehicle (ethanol diluted 1:1,000 or greater), dexamethasone (1–100 nM), or PGE_2 (0.01–1 μM), as indicated. After treatment, cells were rinsed with PBS and lysed in 100 μl 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol (DTT), 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% Triton X-100 (cell lysis reagent, Promega Corp.). Lysates were collected, nuclei were pelleted at 12,000 × *g* for 5 min, and enzyme activity in supernatants was measured with commercial kits for luciferase (Promega Corp.) or β-galactosidase (Tropix). Data were corrected for transfection efficiency and protein content (45).

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Nuclear extracts

Osteoblast nuclear extracts were prepared as previously described (7, 12, 14, 46). Briefly, cell layers were rinsed twice with PBS at 4 C, and all subsequent steps were performed on ice or at 4 C. Cells were harvested, gently pelleted, and lysed in hypotonic buffer (10 mM HEPES, pH 7.4; 1.5 mM $MgCl_2$; 10 mM KCl; and 0.5 mM DTT) with phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride), protease inhibitors (0.5 mM phenylmethylsulfonylfluoride, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, and 2 μg/ml aprotinin; all from Sigma), and 1% Triton X-100. Nuclei were pelleted, and the cytoplasmic supernatant was collected. Nuclei were resuspended in hypertonic buffer containing 0.42 M NaCl, 0.2 mM Na_2EDTA, 25% glycerol, and the phosphatase and protease inhibitors indicated above. Soluble nuclear proteins released after 30-min incubation were collected by centrifugation at 12,000 $\times$ *g* for 5 min, and stored in aliquots at −75 C.

Electrophoretic mobility shift assay (EMSA)

EMSA assays were conducted as previously described (12, 14, 47). Briefly, radiolabeled double stranded probe was prepared by annealing complementary oligonucleotides, followed by fill-in of single stranded overhangs with deoxy (d)-CTP, dGTP, dTTP, and [α-^{32}P]dATP with the Klenow fragment of DNA polymerase I. Five to 10 μg nuclear protein were preincubated for 20 min on ice with 2 μg poly(dI:dC) with or without unlabeled specific or nonspecific competitor DNAs in 60 mM KCl, 25 mM HEPES (pH 7.6), 7.5% glycerol, 0.1 mM EDTA, 5 mM DTT, and 0.025% BSA. After the addition of 5×10^4 cpm DNA probe (0.1–0.2 ng) for 30 min on ice, samples were applied to a 5% nondenaturing polyacrylamide gel that had been preelectrophoresed for 30 min at 12.5 V/cm at 25 C in 45 mM Tris, 45 mM boric acid, and 1 mM EDTA and then run for 2.5 h at 12.5 V/cm. Dried gels were exposed to x-ray film at −75 C with an intensifying screen.

The probe containing the C/EBP-binding site that occurs in the IGF-I gene promoter, designated HS3D, used for gel shifts assays was 5′-GAGCAGATAGAGCCTGCGCAATCGAAATAAAGTC-3′ (the C/EBP half-site is *underlined*). A canonical Sp1 probe (5′-ATTCGA-TCGGGCGGGGCGAGC-3′; the Sp1 binding motif is *underlined*) was used as an internal standard for select gel shift studies. Oligonucleotides were produced by Life Technologies, Inc. For supershift analyses, nuclear extracts were incubated with 1 μl specific antisera (anti-C/EBPδ and anti-C/EBPβ antisera were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 C for 30 min before incubation with ^{32}P-labeled oligonucleotide.

Protein synthesis

To measure protein synthesis rates, cells were pulsed with 12.5 μCi/ml [2,3-^{3}H]proline (2.5 Ci/mmol; NEN Life Science Products, Boston, MA) for the last 2 h of culture. The cell layer was lysed by freeze-thawing, extracted in 0.5% Triton X-100 (Sigma), precipitated with 10% trichloroacetic acid, and chilled, and the acid-precipitated material was collected by centrifugation. Precipitates were acetone extracted, dried, resolubilized in 0.5 N acetic acid, and neutralized with NaOH. [^{3}H]Proline incorporation into collagen (collagenase-digestible) and noncollagen (collagenase-resistant) protein was measured using bacterial collagenase (Worthington Biochemical Corp., Freehold, NJ) free of nonspecific protease activity and is shown as the total amount of [^{3}H]proline incorporation into the cell layer in each culture (2). The percent collagen synthesis was calculated after correcting for the 5.4-fold greater relative abundance of proline in collagen *vs.* noncollagen protein.

Reagents

PGE_2, DEX, and cortisol were obtained from Sigma. Antisera to C/EBPδ and C/EBPβ were purchased from Santa Cruz Biotechnology, Inc.

Statistical analysis

Data were assessed by one-way ANOVA, with Kruskal-Wallis or Bonferonni methods for *post-hoc* analysis, using SigmaStat software (SPSS, Inc., Chicago, IL)

Results

Effects of glucocorticoid and PGE_2 on IGF-I mRNA

Our earlier studies revealed that glucocorticoid inhibits IGF-I mRNA and polypeptide expression by osteoblasts and suppresses the stimulatory effect of hormones such as PTH that increase intracellular cAMP (26). Consistent with this, when osteoblasts were treated for 16 h with 100 nM DEX and then supplemented for 6 h with 0.001–1 μM PGE_2, the stimulatory effect of PGE_2 on IGF-I pre-mRNA expression was significantly reduced; densitometry revealed a nearly 60% suppression of the maximally effective response to PGE_2 (Fig. 1A, compare *left* and *right panels*). In contrast, when cells were pretreated with DEX, and the hormone was removed before stimulation with a moderate dose of PGE_2 (0.1 μM), there was a synergistic, nearly 2-fold increase in PGE_2 effectiveness. This effect peaked at 1 nM DEX, a dose that alone had no detectable influence on IGF-I pre-mRNA expression (Fig. 1B). In agreement with our earlier studies (11), the half-life of IGF-I mRNA in PGE_2-treated cells was 15 h, without or with previous exposure to DEX, indicating no significant changes in IGF-I mRNA stability.

Requirement for the C/EBP binding sequence in the IGF-I gene

To address mechanisms by which these effects occurred, osteoblasts were transfected with the cAMP-responsive IGF-I promoter/reporter gene construct, IGF1711b/Luc. Cells were pretreated with DEX and then stimulated for 6 h with PGE_2. At the concentrations tested, DEX alone had no significant effect on IGF-I promoter activity even while it induced a nearly 10-fold increase in reporter gene expression driven by the glucocorticoid response elements in pMMTV-Luc (Fig. 2, *right panel*). In contrast, a 16-h preexposure to DEX dose-dependently increased the stimulatory effect of PGE_2 on reporter gene expression driven by IGF1711b/Luc by 4-fold (Fig. 2, *left panel*). Consistent with its effect in PGE_2-treated cells, DEX pretreatment enhanced the effect of forskolin on IGF-I promoter activity by 4.5 $\pm$ 0.1-fold, whereas the effect of a 6-h cotreatment of DEX and forskolin was essentially equivalent to that of treatment with forskolin alone (data not shown).

We earlier localized the cAMP-responsive element within the IGF-I gene to nucleotides +202 to +209 of exon 1, termed HS3D by footprint analysis, and identified it as a functional binding site for nuclear transcription factor C/EBP (7, 12, 14, 47). To assess whether this element accounted for the synergy between DEX and PGE_2 on IGF-I expression, we examined changes in reporter gene activity driven by four copies of HS3D fused to a minimal viral promoter in construct 4X HS3D/Luc (14, 47). Consistent with results from studies with construct IGF1711b/Luc, pretreatment with DEX dose dependently enhanced the stimulatory effect of PGE_2 on 4X HS3D/Luc (Fig. 2, *center panel*). In contrast, 6 h of cotreatment with DEX and PGE_2 failed to activate 4X HS3D/Luc more than the effect of PGE_2 alone (Fig. 3, *left*). Synergy between preexposure to DEX and subsequent treatment with PGE_2 was undetectable when the HS3D elements were mutated to eliminate C/EBP binding within the synthetic reporter construct, 4X $HS3D_{mut6}$ (Fig. 3, *center*) or when the HS3D mu-

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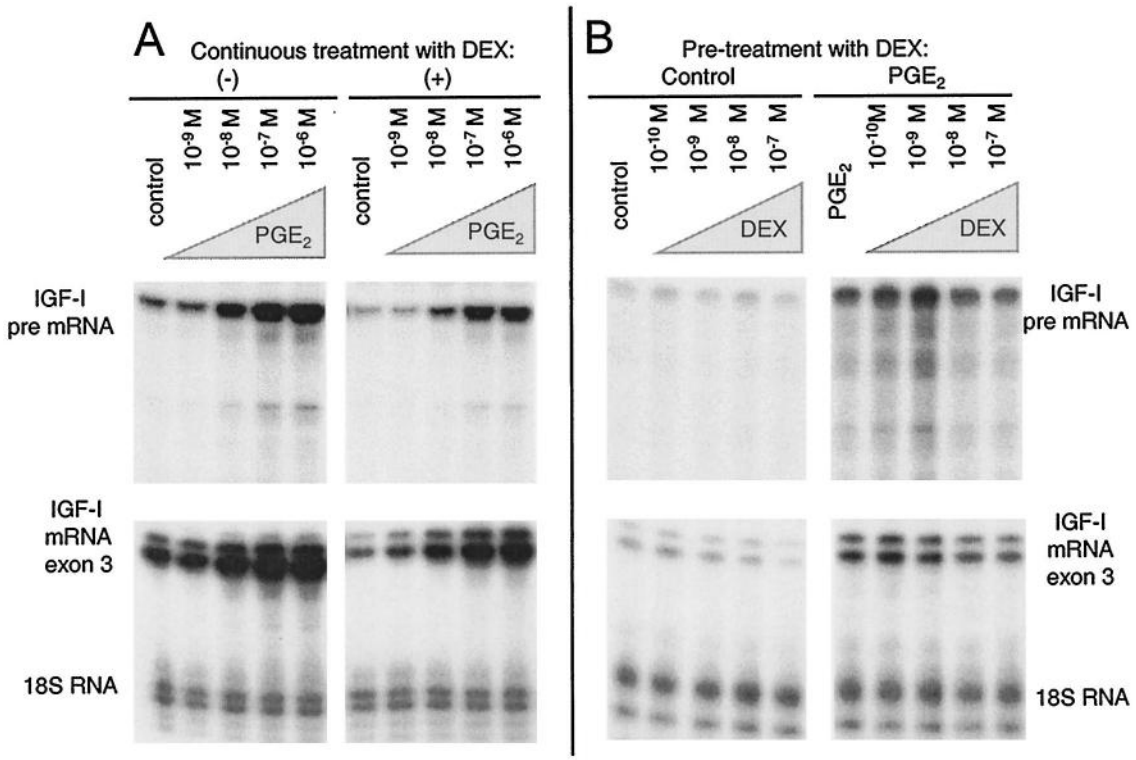

FIG. 1. Varied effects of glucocorticoid treatment on IGF-I mRNA. A (*Left panel*), Inhibitory effect of continuous high dose glucocorticoid treatment on IGF-I mRNA. Osteoblast cultures were treated for 16 h with vehicle or 100 nM DEX, and then treated for 6 h with control medium or the amounts of PGE_2 indicated. Where used, DEX remained in the cultures for the full treatment interval. Relative transcript levels were determined by RNase protection assay and densitometry. B (*Right panel*), Permissive effect of low dose glucocorticoid pretreatment on IGF-I mRNA. Osteoblast cultures were pretreated for 16 h with vehicle or the amounts of DEX indicated, and then treated for 6 h with control medium or 0.1 μM PGE_2. Where used, DEX was removed before PGE_2 treatment. Due to differences in relative transcript abundance, IGF-I pre-mRNA in the *top panels* were assessed after a longer exposure interval by comparison to mature IGF-I mRNA and the 18S ribosomal RNA internal control *in the lower panels*. Data represent the results of two or three independent experiments.

tation was within the native IGF-I promoter (data not shown). No effect was evident in cells transfected with the promoterless parental construct (Fig. 3, *right*). Therefore, DEX cotreatment did not enhance the effect of cAMP on activation of preexisting C/EBP (12, 14), but the C/EBP-binding site was essential for the synergistic effect of DEX pretreatment. Notably, however, pretreatment with DEX sensitized osteoblasts to previously ineffective (0.01 μM) or marginally effective (0.1 μM) concentrations of PGE_2 (Fig. 4, *left panel*). Again, no effect on IGF-I promoter activity was evident by cotreatment with DEX and PGE_2, even with lower amounts of PGE_2 treatment. Sensitization by DEX pretreatment also occurred with the C/EBP-dependent reporter construct 4X HS3D (Fig. 4, *right panel*).

Effects on C/EBP binding to DNA

EMSA was then used to determine the effect of DEX pretreatment on C/EBP binding, using ^{32}P-labeled HS3D oligonucleotide and nuclear extracts from control and hormone-treated osteoblasts. As in previous studies (12, 14), nuclear extract from untreated cells produced no detectable level of nuclear factor binding, whereas as little as 4 h of treatment with PGE_2 greatly enhanced DNA:protein complex formation. The two prominent complexes that formed were supershifted or eliminated by antiserum to C/EBPδ, whereas antiserum to C/EBPβ had a lesser effect (Fig. 5A, *first four lanes*). Nuclear extract from osteoblasts pretreated with DEX formed a low, but detectable, level of complex that was sensitive to anti-C/EBPδ antiserum (Fig. 5A, lanes 5–7). Pretreatment with DEX before stimulation with PGE_2 produced a 3-fold greater level of complex compared with that after treatment with PGE_2 alone, similar to the effects of these agents on gene promoter activity (shown above in Figs. 2–4). Nuclear factor derived from cells treated in this way reacted strongly with antiserum to both C/EBPδ (supershifted) and C/EBPβ (reduced binding; Fig. 5A, *last three lanes*). Complex formation with nuclear extract from cultures cotreated for 6 h with DEX and PGE_2 was not significantly different from that with extract from cells treated with PGE_2 alone (Fig. 5B, *center panel*). Furthermore, DEX and PGE_2, alone or in a pretreatment protocol, did not affect the binding of transcription factor Sp1 (Fig. 5C, *right panel*), suggesting selective effects by both PGE_2 and DEX on nuclear factor function.

Effects on C/EBPδ and C/EBPβ expression

Western immunoblots showed that C/EBPδ and C/EBPβ were both present in osteoblast-derived nuclear extracts. Earlier studies showed that PGE_2 rapidly increased the amount of C/EBP in nuclear extracts, at least in part through translocation from preexisting cytoplasmic pools (14). Pretreat-

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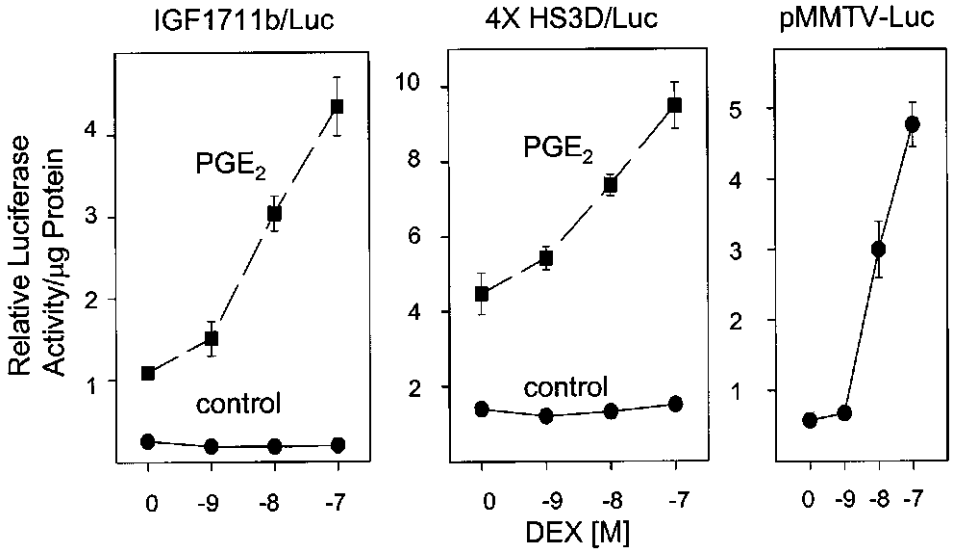

FIG. 2. Effect of glucocorticoid pretreatment on IGF-I promoter and C/EBP-dependent gene activation. *Left panel*, Osteoblast cultures were transfected with the IGF-I promoter construct IGF1711b/Luc and then treated for 16 h with vehicle or the amounts of DEX indicated. Cultures were subsequently treated for 6 h with control medium or with 1 μM PGE_2. In the *center panel* osteoblast cultures were transfected with 4X HS3D/Luc, containing four tandem copies of the C/EBP-sensitive element from the IGF-I gene promoter, then treated for 16 h with vehicle or the amounts of DEX indicated. Cultures were subsequently treated for 6 h with control medium or 1 μM PGE_2. Where used, DEX was removed before PGE_2 treatment, although similar results occurred when DEX remained in the medium during the short PGE_2 treatment interval. As a positive control for DEX treatment, parallel cultures were transfected with pMMTV-Luc and treated with the concentration of DEX indicated in the *right panel*. Data are the mean ± SEM and represent three independent experiments, with n = 3/experiment.

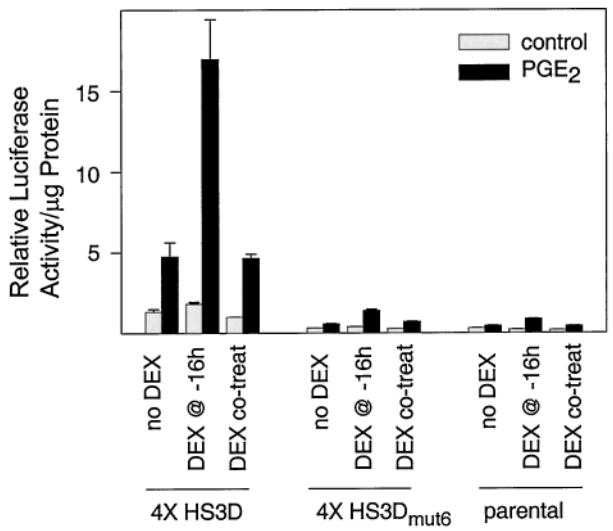

FIG. 3. Effect of glucocorticoid pretreatment or cotreatment on C/EBP-dependent gene expression. Osteoblasts were transfected with empty parental vector, 4X HS3D/Luc, or 4X $HS3D_{mut6}$, which contains mutations in all four of the tandem C/EBP binding sequences. For pretreatment studies, cells were treated for 16 h with vehicle or 100 nM DEX, and then treated for 6 h with control medium or 1 μM PGE_2. In these instances, DEX was removed before PGE_2 treatment, although similar results occurred when DEX remained in the medium during the short PGE_2 treatment interval. For cotreatment studies, cells were cotreated with DEX and PGE_2 for 6 h. Data are the mean ± SEM and represent two independent experiments, with n = 3/experiment.

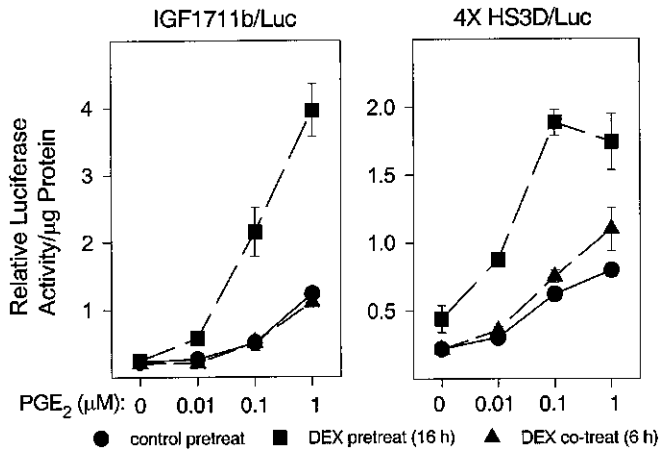

FIG. 4. Sensitizing effect of glucocorticoid on IGF-I gene promoter activity and C/EBP-dependent gene expression. Osteoblast cultures were transfected with either IGF-I promoter construct IGF1711b/Luc or 4X HS3D/Luc. For pretreatment studies, cells were treated for 16 h with vehicle or 100 nM DEX, and then treated for 6 h with control medium or 1 μM PGE_2. In these instances, DEX was removed before PGE_2 treatment, although similar results occurred when DEX remained in the medium during the short PGE_2 treatment interval. For cotreatment studies, cells were cotreated with DEX and PGE_2 for 6 h. Data are the mean ± SEM and represent two independent experiments, with n = 3/experiment.

ment with DEX enhanced the levels of both C/EBPδ and C/EBPβ in nuclear extracts. This effect appeared maximal by 16 h, as no further accumulation occurred after 32 h of DEX treatment. By densitometry, the combined effect of pretreatment with DEX and subsequent stimulation with PGE_2 was additive. In contrast, cotreatment with both hormones increased the level of each isoform of C/EBP to no more than that caused by stimulation with PGE_2 alone (Fig. 6).

Based on these results, we examined the effect of DEX on steady-state mRNA levels for C/EBPδ and C/EBPβ by RNase protection assay. Pretreatment with DEX caused

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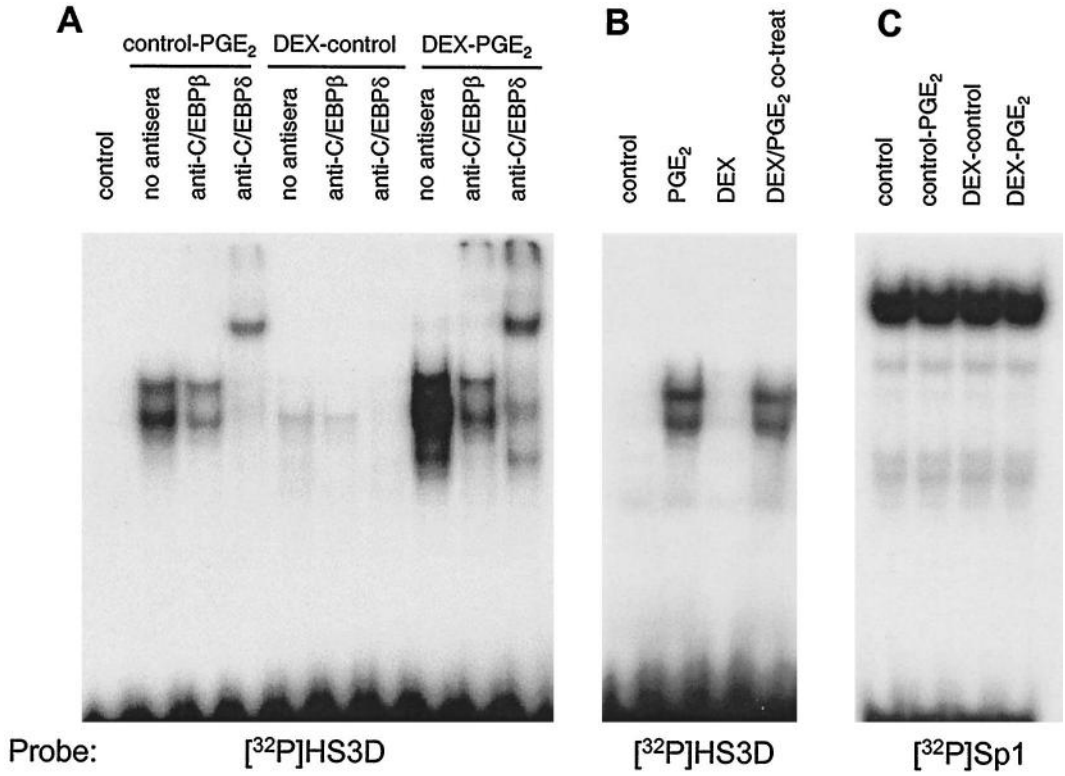

FIG. 5. Effect of glucocorticoid pretreatment on C/EBP-dependent EMSA. A, Osteoblast cultures were pretreated for 16 h with vehicle or 100 nM DEX, and then treated for 4 h with control medium or 1 μM PGE_2. Nuclear extracts were analyzed by EMSA with an oligonucleotide probe containing the HS3D (C/EBP)-binding sequence, without or with anti-C/EBPδ or anti-C/EBPβ antiserum, as indicated. B, Osteoblast cultures were treated with 100 nM DEX or 1 μM PGE_2, alone or in combination, for 4 h. C, Extracts used in A were tested with an independent oligonucleotide probe containing a consensus Sp1 binding sequence. Data are representative of three independent experiments.

significant and dose-dependent increases in C/EBPδ and C/EBPβ mRNAs. Densitometry indicated that at 100 nM DEX, there was a 3-fold increase in C/EBPδ mRNA and a 4.5-fold increase in C/EBPβ mRNA (Fig. 7). Therefore, the synergistic effects of DEX pretreatment and PGE_2 stimulation on IGF-I expression, IGF-I gene promoter activity, and C/EBP-dependent reporter gene expression were consistent with the ability of glucocorticoid to increase new C/EBPδ and C/EBPβ expression and their subsequent activation by PGE_2.

To examine the modulatory effect of DEX on C/EBP-dependent IGF-I promoter activation further, we created a C/EBPδ dominant negative (C/EBPδ DN) expression construct that lacked the *trans*-activation domain sequence, but retained dimerization and DNA-binding domains. C/EBPδ DN effectively suppressed both C/EBPδ- and C/EBPβ-dependent gene expression in COS-7 cells cotransfected with 4X HS3D/Luc (data not shown). With the IGF-I promoter-derived reporter construct, C/EBPδ DN dose dependently suppressed the effect of PGE_2 by up to 94% in both control and DEX-pretreated osteoblasts (Fig. 8). This effect could not be attributed to nonspecific squelching of reporter gene expression by C/EBPδ DN, because expression plasmid loading was corrected by complementation with parental vector. Therefore, C/EBPs appear to be essential components for PGE_2- and DEX-regulated IGF-I promoter activity.

Effects of glucocorticoid and PGE_2 on osteoblast protein synthesis

Finally, our earlier studies showed that an increase in IGF-I expression was responsible in part for the stimulatory effect of PTH or PGE_2 on matrix protein synthesis in intact bone explants (9, 33). Because preexposure to DEX enhanced the effect of PGE_2 on IGF-I expression, we examined the consequence of these effects on collagen and noncollagen protein synthesis in isolated osteoblasts. By itself, PGE_2 treatment had little effect on collagen synthesis, whereas it produced a small increase in noncollagen protein synthesis (Fig. 9, *closed circles*). In these experiments, pretreatment with DEX alone decreased new collagen synthesis by 70 ± 3% and noncollagen protein synthesis by 30 ± 4%. However, pretreatment with DEX caused a dose-dependent 2- to 2.5-fold increase in new collagen synthesis in PGE_2-treated cells that was maximal at 0.1 μM PGE_2 (Fig. 9, *left panel, closed squares*). Similarly, a 3-fold increase in noncollagen protein synthesis occurred under these same conditions (Fig. 9, *right panel, closed squares*).

Discussion

Glucocorticoids are widely used for treatment of asthma, rheumatoid arthritis, inflammatory bowel disease, autoimmune disease, and immunosuppression of organ transplant recipients. Circulating cortisol levels also increase with ag-

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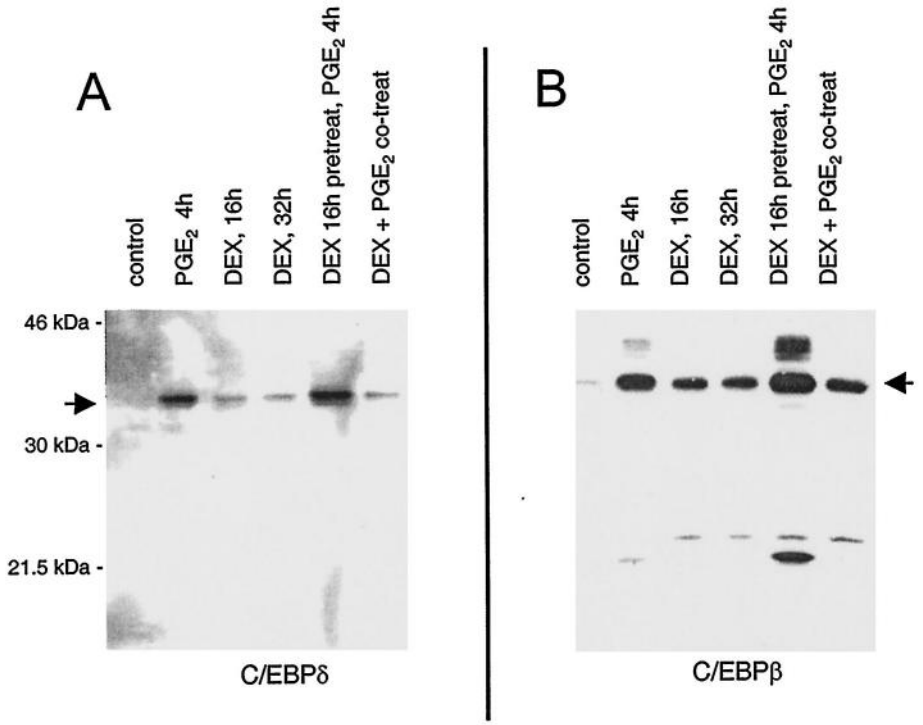

FIG. 6. Effect of glucocorticoid pretreatment on C/EBP by Western immunoblot analysis. Osteoblast cultures were pretreated for 16 h with vehicle or 100 nM DEX, and then treated for 4 h with control medium or 1 μM PGE_2. DEX was removed before PGE_2 treatment, although similar results occurred when DEX remained in the medium during the short PGE_2 treatment interval. Alternately, cultures were cotreated with DEX and PGE_2 for 4 h. Nuclear extracts were fractionated by SDS-PAGE, electroblotted, and probed with anti-C/EBPδ (A) or anti-C/EBPβ (B) antiserum, as indicated. *Arrowheads* indicate the immunoreactive bands consistent with the known mol wt of C/EBPδ or C/EBPβ. Results are representative of two or three independent experiments.

ing, vigorous exercise, traumatic injury, severe burns, invasive surgery, microgravity, or Cushing's disease (25, 48–52). Sustained high levels of glucocorticoids, from pharmacological or endogenous sources, are well known to inhibit skeletal growth and bone remodeling. Importantly, the risk of low trauma fractures increases in nearly one third of all patients receiving long-term glucocorticoid therapy (25).

IGF-I synthesis by osteoblasts is significantly enhanced by cAMP-inducing factors such as PTH and PGE_2 (4, 5), and many studies now substantiate that activation of cAMP-dependent PKA accounts for the anabolic effects of these hormones on bone (9, 53, 54). Activation of PKA increases C/EBPδ activation and binding at +202 to +209 bp within the 5′-UTR of exon 1 of the IGF-I gene (7, 12, 14, 47). This C/EBP-binding element appears evolutionarily conserved among fish, frogs, rats, and humans. Moreover, the entire 5′-UTR of exon 1 exhibits a remarkable 95% homology between rats and chickens (12, 13), suggesting its biological importance for IGF-I gene expression.

Our current studies show that preexposure to glucocorticoid increased the stimulatory effect of PGE_2 on IGF-I synthesis through an increase in new C/EBPδ and C/EBPβ expression. The effect of glucocorticoid on C/EBP expression alone was unable to produce large effects on DNA binding or IGF-I promoter activation. Rather, both C/EBP isoforms effectively formed DNA binding complexes and increased gene promoter activity only after treatment with PGE_2, demonstrating that the higher amount of C/EBPs induced in this way still required activation. A C/EBPδ DN expression construct virtually eliminated the effects of DEX and PGE_2, revealing a critical role for C/EBPs on regulation of IGF-I promoter activity. Furthermore, the effectiveness of PGE_2 at activating the IGF-I promoter occurred at a much lower concentration after glucocorticoid pretreatment, suggesting

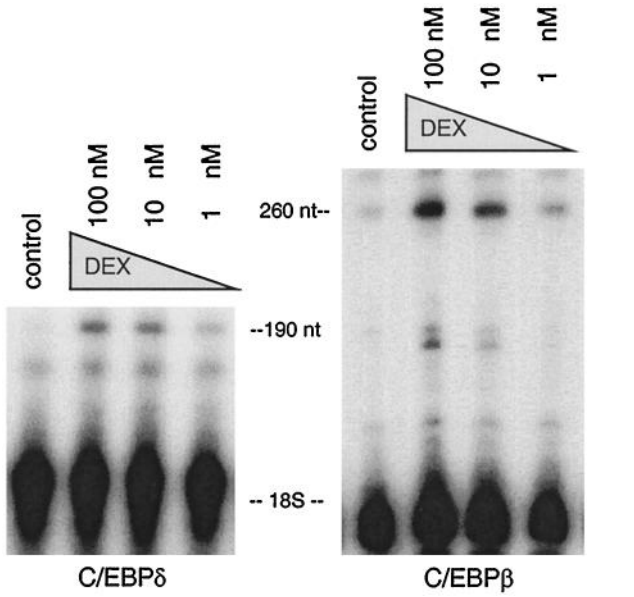

FIG. 7. Effects of glucocorticoid on C/EBPδ and C/EBPβ mRNAs. Osteoblast cultures were treated for 16 h with vehicle or with 1–100 nM DEX, and total RNA was assessed by RNase protection assay with probes specific for rat C/EBPδ (190 nucelotides) or C/EBPβ (260 nucleotides). An 18S ribosomal RNA probe (90 nucleotides) was used as an internal control. Subsequent autoradiography and densitometry were used to determine relative transcript levels. Results are representative of three independent experiments.

that the normal *in vivo* spikes in glucocorticoid release that occur throughout the day may have permissive effects on local or circulating osteotropic factors such as PGE_2 and PTH on IGF-I expression by osteoblasts. In contrast, high or sustained levels of glucocorticoid may mask these permissive

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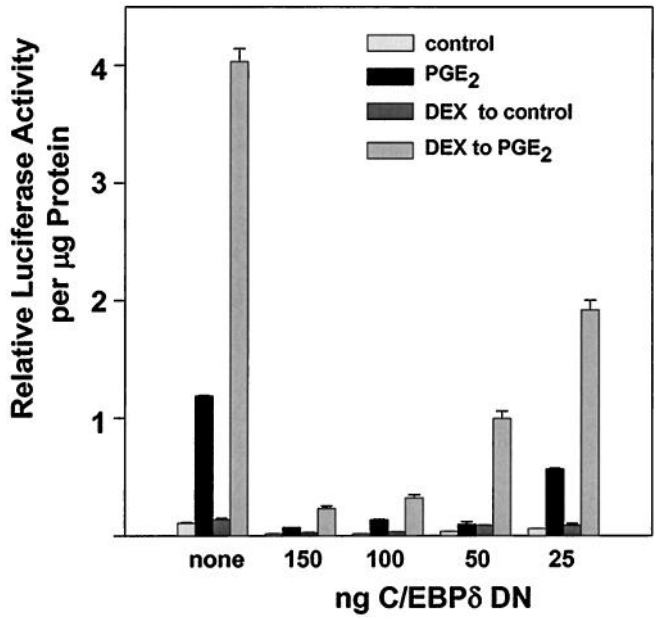

FIG. 8. Expression of a dominant-negative C/EBPδ suppressed the ability of DEX to potentiate PGE_2-activated IGF-I promoter function. Osteoblast cultures were transfected with the IGF-I promoter construct IGF1711b/Luc along with increasing amounts of the C/EBPδ dominant negative (DN) construct (complemented with parental vector to achieve an equal plasmid load). Cultures were subsequently treated for 16 h with vehicle or 100 nM DEX as indicated. Cultures were then treated for 6 h with control medium or 1 μM PGE_2. DEX was removed before PGE_2 treatment, although similar results occurred when DEX remained in the medium during the short PGE_2 treatment interval. Data are the mean ± SEM and represent two independent experiments, with n = 3/experiment.

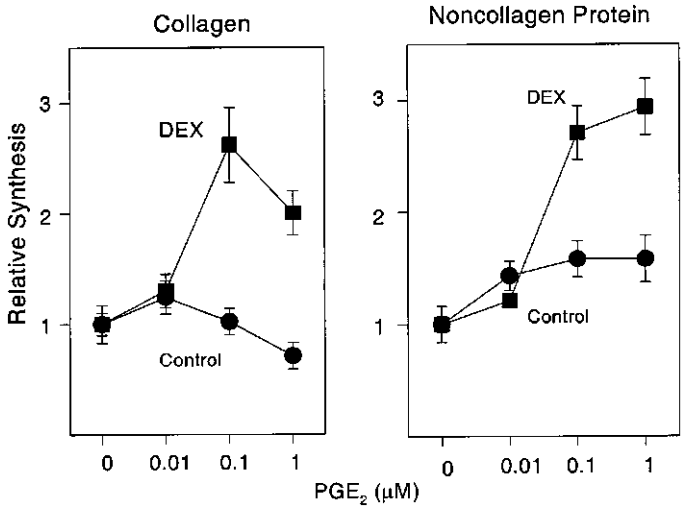

FIG. 9. Permissive effect of glucocorticoid pretreatment on bone cell protein synthesis in response to PGE_2. Osteoblast cultures were pretreated for 16 h with vehicle (*closed circles*) or 100 nM DEX (*closed squares*), treated for a total of 24 h with vehicle or the amounts of PGE_2 indicated, and pulse labeled with [^{3}H]proline for the last 2 h of culture to assess effects on collagen and noncollagen protein synthesis. DEX was removed before PGE_2 treatment. Consistent with earlier studies in bone, collagen (*left panel*) and noncollagen (*right panel*) protein syntheses were partially suppressed by pretreatment with glucocorticoid, as reported previously (33). The relative effects of PGE_2 are expressed by direct comparison to vehicle or DEX-pretreated control values, indicated as 1 on the *ordinates* of each graph. Data are the mean ± SEM and represent three independent experiments, with n = 4/experiment.

effects by suppressing new IGF-I expression (26). Preexposure to glucocorticoid also increased collagen and noncollagen protein synthesis by osteoblasts in response to PGE_2, consistent with changes previously observed in calvarial organ cultures (33), demonstrating a physiological consequence from these events.

Interestingly, unlike the IGF-I mRNA studies in Fig. 1, preexposure to a high dose of DEX increased the effect of PGE_2 on IGF-I promoter/reporter gene expression. This effect persisted regardless of whether DEX remained in the medium during a subsequent, shorter treatment period with PGE_2. In addition, in transfection studies, low doses of DEX only weakly activated the positive control reporter construct, pMMTV/Luc, whereas it potentiated cAMP-activated endogenous IGF-I gene expression. These findings may reflect different effects by glucocorticoid on native chromatin *vs.* episomal plasmid DNA, or differences between the half-lives of IGF-I mRNA and the rapidly turning over reporter enzyme luciferase. Moreover, our results suggest that the stimulatory effect of DEX on IGF-I promoter activity depends on an increase in C/EBP expression, which does not itself appear to enhance pMMTV/Luc expression directly. The weaknesses of some assay systems may therefore limit our understanding of results from individual studies, whereas information from several experimental approaches may better represent biological regulatory mechanisms.

C/EBPs regulate the expression of many cytokines and indirectly increase PG expression through an increase in cyclooxygenase-2 expression (55). Activation of C/EBP initiates a biochemical cascade associated with inflammation, wound healing, tissue remodeling, and control of cell proliferation and metabolism. In addition, expression of individual C/EBP isoforms varies with tissue differentiation (21, 56–60) and can be modulated by the endotoxin lipopolysaccharide, thermal injury, hypoxia, and inflammation itself (15–20, 22, 61, 62). Acquisition of the adipocyte phenotype by 3T3-L1 fibroblasts parallels a progressive variation in C/EBP isoform expression from C/EBPδ to C/EBPβ and finally to C/EBPα, where C/EBPα regulates terminal adipocyte differentiation. This process is initiated by several factors, including glucocorticoid and phosphodiesterase inhibitors that increase cAMP, whereas terminal adipocyte differentiation requires insulin or IGF-I (21, 58–60). Similarly, C/EBPδ is developmentally regulated in fetal lung, and its expression is further induced by cAMP and glucocorticoid (57). Because of the significant interacting effects that we noted among glucocorticoids, cAMP-inducing agents, and IGF-I on the control of bone cell function, a progression in C/EBP expression and activation also may be important for bone cell differentiation and skeletal integrity (1, 2, 6, 9, 26, 33).

Glucocorticoid-dependent alterations in hormone-induced adenylate cyclase activity or cAMP accumulation can occur in specific tissues or at specific times during development (29, 63–76). Whereas glucocorticoid diminishes PGE_2-induced cAMP in MC3T3-E1 preosteoblasts (70), it enhances the stimulatory effect of PTH on cAMP synthesis in highly differentiated ROS 17/2.8 osteoblastic cells, in part through

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an increase in PTH receptor expression (75). Notably, short term exposure to glucocorticoid *in vivo* enhances PTH-induced cAMP in perfused bones of rats or dogs, whereas chronic glucocorticoid treatment attenuates PTH activity (29, 63). Other interacting processes may also be involved. For example, glucocorticoid can reduce protein kinase C activity in rat liver parenchymal cells (77), and increased levels of cAMP and/or activation of protein kinase A can potentiate glucocorticoid-dependent gene expression by increasing glucocorticoid receptor affinity (78, 79) and consequently its catabolic effects on connective tissue after long term glucocorticoid exposure (80).

Based on our current results, a working model for the influence of glucocorticoid on IGF-I induction and its actions in osteoblasts is shown in Fig. 10. At physiological levels, glucocorticoid has a synergistic effect with cAMP-inducing hormones and transiently induces C/EBPβ and C/EBPδ expression in osteoblasts. When cAMP rises in response to hormone stimulation, higher endogenous levels of C/EBPs are quickly activated, translocate to the nucleus, and enhance IGF-I promoter activity. In addition, glucocorticoid may modulate PKA, which is stimulatory, and PKC, which suppresses IGF-I expression. Therefore, with transient glucocorticoid pretreatment, PGE_2 or PTH may more effectively activate adenylate cyclase and favor PKA-dependent activation of C/EBP. In contrast, high sustained levels of glucocorticoid may diminish these cooperative events by interfering with C/EBP function and ultimately suppress IGF-I expression. This effect may be C/EBP isoform selective and may result from a physical association between C/EBPs and the glucocorticoid receptor (81, 82; our unpublished observation). However, other findings not presented here indicate no apparent inhibition of C/EBP nuclear translocation by DEX. These and related studies are the subject of ongoing investigations. Finally, transient or sustained exposure to glucocorticoid may influence the biological effects of newly synthesized IGF-I through the downstream signaling events that occur in response to IGF-I itself.

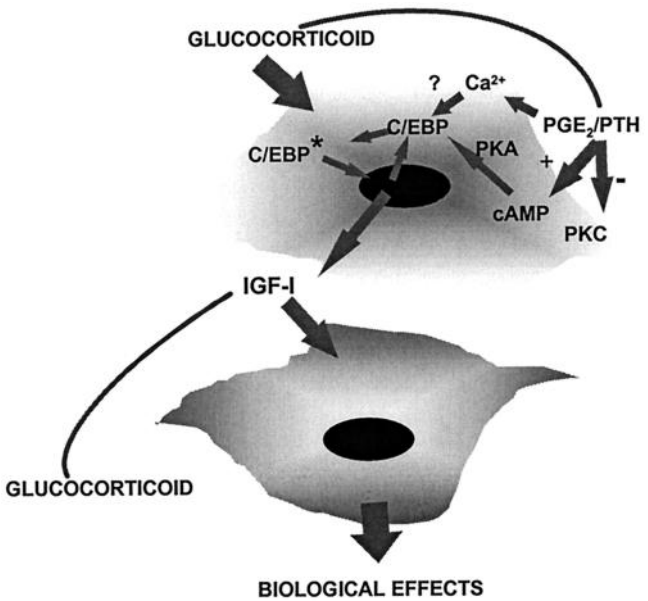

FIG. 10. Model of interactions among glucocorticoids and cAMP-inducing hormones on IGF-I gene expression and downstream biological activity in osteoblasts.

In conclusion, our current studies continue to reveal novel interactions between PKA-activating hormones and glucocorticoids and potential mechanisms for the permissive effects of glucocorticoids on IGF-I expression and protein synthesis by osteoblasts. Future studies will be necessary to define in more detail the complex molecular events that distinguish the permissive and suppressive effects of glucocorticoids on osteoblast function and skeletal integrity.

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References

1. **Hock JM, Centrella M, Canalis E** 1988 Insulin-like growth factor I has independent effects on bone matrix formation and cell replication. Endocrinology 122:254–260
2. **McCarthy TL, Centrella M, Canalis E** 1989 Regulatory effects of insulin-like growth factor I and II on bone collagen synthesis in rat calvarial cultures. Endocrinology 124:301–309
3. **Schmid C., Guler H-P, Rowe D, Froesh R** 1989 Insulin-like growth factor I regulates type I procollagen messenger ribonucleic acid steady state levels in bone of rats. Endocrinology 125:1575–1580
4. **McCarthy TL, Centrella M, Canalis E** 1989 Parathyroid hormone enhances the transcript and polypeptide levels of insulin-like growth factor I in osteoblast-enriched cultures from fetal rat bone. Endocrinology 124:1247–1253
5. **McCarthy TL, Centrella M, Raisz LG, Canalis E** 1991 Prostaglandin E_2 stimulates insulin-like growth factor I synthesis in osteoblast-enriched cultures from fetal rat bone. Endocrinology 128:2895–2900
6. **McCarthy TL, Centrella M, Canalis E** 1990 Cyclic AMP induces insulin-like growth factor I synthesis in osteoblast-enriched cultures. J Biol Chem 265:15353–15356
7. **McCarthy TL, Thomas MJ, Centrella M, Rotwein P** 1995 Regulation of insulin-like growth factor I transcription by cyclic adenosine 3′,5′-monophosphate (cAMP) in fetal rat bone cells through an element within exon 1: protein kinase A-dependent control without a consensus AMP response element. Endocrinology 136:3901–3908
8. **Vignery A, McCarthy TL** 1996 The neuropeptide calcitonin gene related peptide stimulates insulin-like growth factor I production by primary fetal rat osteoblasts. Bone 18:331–335
9. **Canalis E, Centrella M, McCarthy TL** 1989 Insulin-like growth factor I mediates selective effects of parathyroid hormone in bone cultures. J Clin Invest 83:60–65
10. **Hall LJ, Kajimoto Y, Bichell DP, Kim SW, James PL, Counts D, Nixon L, Tobin G, Rotwein P** 1992 Functional analysis of the rat insulin-like growth factor I gene and identification of an IGF-I gene promoter. DNA Cell Biol 11:301–313
11. **Bichell DP, Rotwein P, McCarthy TL** 1993 Prostaglandin E_2 rapidly stimulates insulin-like growth factor I gene expression in primary rat osteoblast cultures: evidence for transcriptional control. Endocrinology 133:1020–1028
12. **Thomas MJ, Umayahara Y, Shu H, Centrella M, Rotwein P, McCarthy TL** 1996 Identification of the cAMP response element that controls transcriptional activation of the IGF-I gene by prostaglandin E_2 in osteoblasts. J Biol Chem 271:21835–21841
13. **Mittanck DW, Kim S-W, Rotwein P** 1997 Essential promoter elements are located within the 5′ untranslated region of human insulin-like growth factor-I exon 1. Mol Cell Endocrinol 126:153–163
14. **Umayahara Y, Ji C, Centrella M, Rotwein P, McCarthy TL** 1997 CCAAT/enhancer binding protein δ activates insulin-like growth factor-I gene transcription in osteoblasts. J Biol Chem 272:31793–31800
15. **Akira S, Kishimoto T** 1992 IL-6 and NF-IL6 in acute-phase response and viral infection. Immunol Rev 127:25–50
16. **Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S** 1993 Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc Natl Acad Sci USA 90:10193–10197
17. **Zhang Y, Rom WN** 1993 Regulation of the interleukin-1β (IL-1β) gene by

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mycobacterial components and lipopolysaccharide is mediated by two nuclear factor-IL6 motifs. Mol Cell Biol 13:3831–3837
18. **Chang CJ, Shen BJ, Lee SC** 1995 Autoregulated induction of the acute-phase response transcription factor gene, agp/ebp (C/EBPβ). DNA Cell Biol 14:529–537
19. **Choi AM, Sylvester S, Otterbein L, Holbrook NJ** 1995 Molecular responses to hyperoxia *in vivo*: relationship to increased tolerance in aged rats. Am J Respir Cell Mol Biol 13:74–82
20. **Gilpin DA, Hsieh CC, Kuninger DT, Herndon DN, Papaconstantinou J** 1996 Effect of thermal injury on the expression of transcription factors that regulate acute phase response genes: the response of C/EBPα, C/EBPβ, and C/EBPδ to thermal injury. Surgery 119:674–683
21. **Lekstrom-Himes J, Xanthopoulos KG** 1998 Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem 273:28545–28548
22. **Wedel A, Ziegler-Heitbrock HWL** 1995 The C/EBP family of transcription factors. Immunobiology 193:171–185
23. **Poli V** 1998 The role of C/EBP isoforms in the control of inflammatory and native immunity function. J Biol Chem 273:29279–29282
24. **Hodgson SF** 1990 Corticosteroid-induced osteoporosis. Endocrinol Metab Clin. North Am 19:95–111
25. **Lukert BP** 1992 Glucocorticoid-induced osteoporosis. South Med J 85:2S48–2S51
26. **McCarthy TL, Centrella M, Canalis E** 1990 Cortisol inhibits the synthesis of insulin-like growth factor I in bone cell cultures. Endocrinology 126:1569–1575
27. **Delany AM, Canalis E** 1995 Transcriptional repression of insulin-like growth factor I by glucocorticoids in rat bone cells. Endocrinology 136:4776–4781
28. **Swolin D, Brantsing C, Matejka G, Ohlsson C** 1997 Cortisol decreases IGF-I mRNA levels in human osteoblast-like cells. J Endocrinol 149:397–403
29. **Sugimoto T, Fukase M, Tsutsumi M, Tsunenari T, Fujita T** 1986 Altered parathyroid hormone- or calcitonin-stimulated adenosine 3′,5′-monophosphate release by isolated perfused bone from glucocorticoid-treated rats. Calcif Tissue Int 38:163–169
30. **Bellows CG, Aubin JE, Heersche JN** 1987 Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells *in vitro*. Endocrinology 121:1985–1992
31. **Bellows CG, Heersche JN, Aubin JE** 1990 Determination of the capacity for proliferation and differentiation of osteoprogenitor cells in the presence and absence of dexamethasone. Dev Biol 140:132–138
32. **Wong MM, Rao LG, Ly H, Hamilton L, Tong J, Sturtridge W, McBroom R, Aubin JE, Murry TM** 1990 Long-term effects of physiologic concentrations of dexamethasone on human bone-derived cells. J Bone Miner Res 5:803–813
33. **Raisz LG, Fall PM, Gabitas BY, McCarthy TL, Kream BE, Canalis E** 1993 Effects of prostaglandin E_2 on bone formation in cultured fetal rat calvariae: role of insulin-like growth factor-I. Endocrinology 133:1504–1510
34. **Kream BE, Tetradis S, Lafrancis D, Fall PM, Feyen JH, Raisz LG** 1997 Modulation of the effects of glucocorticoids on collagen synthesis in fetal rat calvariae by insulin-like growth factor binding protein-2. J Bone Miner Res 12:889–895
35. **Centrella M, McCarthy TL, Canalis E** 1987 Mitogenesis in fetal rat bone cells simultaneously exposed to transforming growth factor β and other growth regulators. FASEB J 1:312–317
36. **McCarthy TL, Centrella M, Canalis E** 1988 Further biochemical and molecular characterization of primary rat parietal bone cell cultures. J Bone Miner Res 3:401–408
37. **Centrella M, Canalis E, McCarthy TL, Orloff JJ, Stewart AF, Insogna KL** 1989 Parathyroid hormone-related protein modulates the effects of transforming growth factor-β on deoxyribonucleic acid and collagen synthesis in fetal rat bone cells. Endocrinology 125:199–208
38. **Centrella M, Casinghino S, McCarthy TL** 1994 Differential actions of prostaglandins in separate cell populations from fetal rat bone. Endocrinology 135:1611–1620
39. **Centrella M, Kim J, Pham T, Casinghino S, Rosen V, Wozney J, McCarthy TL** 1995 Independent changes in type I and type II receptors for transforming growth factor β induced by bone morphogenetic protein-2 parallel expression of the osteoblast phenotype. Mol Cell Biol 15:3273–3281
40. **Centrella M, Casinghino S, Gundberg C, McCarthy TL, Wozney J, Rosen V** 1996 Changes in bone morphogenetic protein sensitivity relative to differentiation in fetal rat bone cell cultures. Ann NY Acad Sci 785:224–226
41. **Pockwinse SM, Wilming LG, Conlon DM, Stein GS, Lian JB** 1992 Expression of cell growth and bone specific genes at single cell resolution during development of bone tissue-like organization in primary osteoblast cultures. J Cell Biochem 49:310–323
42. **Hughes FJ, Collyer j, Stanfield M, Goodman SA** 1995 The effects of bone morphogenetic protein-2, -4, and -6 on differentiation of rat osteoblast cells *in vitro*. Endocrinology 136:2671–2677
43. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
44. **Truett MA, Blacher R, Burke RL, Caput D, Chu D, Dina D, Hartog K, Kuo CH, Msiarz FR, Merryweather JP, Najarian R, Pachl C, Potter SJ, Puma J, Quiroga M, Rall LB, Randolph A, Urdea MS, Valenzuela P, Dahl HH, Favalaro J, Hansen J, Nordfang O, Ezban M** 1985 Characterization of the polypeptide composition of human factor VIII:C and the nucleotide sequence and expression of the human kidney cDNA. DNA 4:333–349
45. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
46. **Lee KA, Bindereif A, Green MR** 1988 A small scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. Gene Anal. Technol 5:22–31
47. **Umayahara Y, Billiard J, Ji C, Centrella M, McCarthy TL, Rotwein P** 1999 CCAAT/enhancer binding protein δ is a critical regulator of insulin-like growth factor-I gene transcription in osteoblasts. J Biol Chem 274:10609–10617
48. **Di Padova F, Pozzi C, Tondre MJ, Tritapepe R** 1991 Selective and early increase in IL-1 inhibitors, IL-6 and cortisol after elective surgery. Clin Exp Immunol 85:137–142
49. **Klein GL, Wolf SE, Goodman WG, Phillips WA, Herndon DN** 1997 The management of acute bone loss in severe catabolism due to burn injury. Horm Res [Suppl 5] 48:83–87
50. **Leach CS, Cintron NM, Krauhs JM** 1991 Metabolic changes observed in astronauts. J Clin Pharmacol 31:921–927
51. **Van Cauter E, Leproult R, Kupfer DJ** 1996 Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. J Clin Endocrinol Metab 81:2468–2473
52. **Weight LM, Alexander D, Jacobs P** 1991 Strenuous exercise: analogous to the acute-phase response? Clin Sci 81:677–683
53. **Rixon RH, Whitfield JF, Gagnon L, Isaacs RJ, Maclean S, Chakravarthy B, Durkin JP, Neugebauer W, Ross V, Sung W, Willick GE** 1994 Parathyroid hormone fragments may stimulate bone growth in ovariectomized rats by activating adenylyl cyclase. J Bone Miner Res 9:1179–1189
54. **Sabatini M, Lesur C, Pacherie M, Pastoureau P, Kucharczyk N, Fauchere JL, Bonnet J** 1996 Effects of parathyroid hormone and agonists of the adenylylcyclase and protein kinase C pathways on bone cell proliferation. Bone 18:59–65
55. **Yamamoto K, Arakawa T, Ueneda M, Yamamoto S** 1995 Transcriptional roles of nuclear factor κB and nuclear factor-interleukin-6 in tumor necrosis factor α-dependent induction of cyclooxygenase-2 in MC3T3–E1 cells. J Biol Chem 270:31315–31320
56. **Birkenmeier EH, Gwynn B, Howard S, Jerry J, Gordon JI, Landschulz WI, McKnight SL** 1989 Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev 3:1146–1156
57. **Breed DR, Margraf LR, Alcorn JL, Mendelson CR** 1997 Transcription factor C/EBPδ in fetal lung: developmental regulation and effects of cyclic 3′,5′-monophosphate and glucocorticoids. Endocrinology 138:5527–5534
58. **Christy RJ, Ynag VW, Ntambi JM, Geiman DE, Landschulz WH, Friedman AD, Nakabeppu Y, Kelly TJ, Lane MD** 1989 Differentiation-induced gene expression in 3T3–L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. Genes Dev 3:1323–1335
59. **Darlington GJ, Ross SE, MacDougald oA** 1998 The role of C/EBP genes in adipocyte differentiation. J Biol Chem 273:30057–30060
60. **Yeh W-C, Cao Z, Classon M, McKnight SL** 1995 Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev 9:168–181
61. **Ramji DP, Vitelli A, Tronche F, Cortese R, Ciliberto G** 1993 The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBPδ/NF-IL-6β, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. Nucleic Acids Res 21:289–294
62. **Yan SF, Tritto I, Pinsky D, Liao H, Huang J, Brett J, May L, Stern D** 1995 Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. Central role of the binding site for nuclear factor-IL-6. J Biol Chem 270:11463–11471
63. **Korkor A, Martin KJ, Olgaard K, Bergfeld M, Teitelbaum S, Klahr S, Slatopolsky E** 1983 Altered adenosine 3′,5′-monophosphate release in response to parathyroid hormone by isolated perfused bone from glucocorticoid-treated dogs. Endocrinology 113:625–631
64. **Rodan SB, Fischer MK, Egan JJ, Epstein PM, Rodan GA** 1984 The effect of dexamethasone on parathyroid hormone stimulation of adenylate cyclase in ROS 17/2.8 cells. Endocrinology 115:951–958
65. **Catherwood BD** 1985 1,25-Dihydroxycholecalciferol and glucocoritcoid regulation of adenylate cyclase in an osteoblast-like cell line. J Biol Chem 260:736–743
66. **Rizzoli R, von Tscharner V, Fleisch H** 1986 Increase of adenylate cyclase catalytic-unit activity by dexamethasone in rat osteoblast-like cells. Biochem J 237:447–454
67. **Titus L, Rubin JE, Lorang MT, Catherwood BD** 1988 Glucocorticoids and 1,25-dihydorxyvitamin D_3 regulate parathyroid hormone stimulation of adenosine 3′,5′-monophosphate-dependent protein kinase in rat osteosarcoma cells. Endocrinology 123:1526–1534
68. **Titus L, Rubin JE, Nanes MS, Catherwood BD** 1989 Glucocorticoid and 1,25-dihydroxyvitamin D modulate the degree of adenosine 3′,5′-monophosphate-dependent protein kinase isoenzyme I and II activation by parathyroid hormone in rat osteosarcoma cells. Endocrinology 125:2806–2811
69. **Titus L, Jackson E, Nanes MS, Rubin JE, Catherwood BD** 1991 1,25-Dihy-

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dorxyvitamin D reduces parathyroid hormone receptor number in ROS 17/2.8 cells and prevents the glucocorticoid-induced increase in these receptors: relationship to adenylate cyclase activation. J. Bone Miner Res 6:631–637
70. **Kozawa O, Tokuda H, Kotoyori J, Oiso Y** 1993 Effects of glucocorticoid on signalling by prostaglandin E_2 in osteoblast-like cells. Prostaglandins Leukotrienes Essent Fatty Acids 49:867–872
71. **Roa LG, Wylie JN** 1993 Modulation of parathyroid hormone-sensitive adenylate cyclase in ROS 17/2.8 cells by dexamethasone, 1,25-dihydroxyvitamin D_3, and protein kinase C. Bone Miner 23:35–47
72. **Rude RK, Gruber HE, Oldham SB** 1993 Cortisone-induced osteoporosis: effects on bone adenylate cyclase. Miner Electorlyte Metab 19:71–77
73. **Slotkin TA, Lau C, McCook EC, Lappi SE, Seidler FJ** 1994 Glucocorticoids enhance intracellular signaling via adenylate cyclase at three distinct loci in the fetus: a mechanism for heterologous teratogenic sensitization? Toxicol Appl Pharmacol 127:64–75
74. **Sutherland MS, Rao LG, Muzaffar SA, Wylie JN, Wong MM, McBroom RJ, Murray TM** 1995 Age-related expression of osteoblast phenotypic markers in normal human osteoblasts cultured long-term in the presence of dexamethasone. Osteop Int 5:335–343
75. **Urena P, Iida-Klein A, Kong XF, Juppner H, Kronenberg HM, Abou-Samra AB, Segre GV** 1994 Regulation of parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acid by glucocoticoids and PTH in ROS 17/2.8 and OK cells. Endocrinology 134:451–456
76. **Nohutca RM, Somerman MJ, McCauley LK** 1995 Dexamethasone enhances the effects of parathyroid hormone on human periodontal ligment cells *in vitro*. Calcif Tissue Int 56:571–577
77. **Higuchi H, Katoh N, Miyamoto T, Uchida E, Yuasa A, Takahashi K** 1994 Dexamethasone-induced haptoglobin release by calf liver parenchymal cells. Am J Vet Res 55:1080–1085
78. **Haske T, Nakao M, Moudgil VK** 1994 Phosphorylation of immunopurified rat liver glucocorticoid receptor by the catalytic subunit of cAMP-dependent protein kinase. Mol Cell Biochem 132:163–171
79. **Moyer ML, Borror KC, Bona BJ, DeFranco DB, Nordeen SK** 1993 Modulation of cell signaling pathways can enhance or impair glucocorticoid-induced gene expression without altering the state of receptor phosphorylation. J Biol Chem 268:22933–22940
80. **Sato A, Sheppard KE, Fullerton MJ, Funder JW** 1996 cAMP modulates glucocorticoid-induced protein accumulation and glucocorticoid receptor in cardiomyocytes. Am J Physiol 271:E827–E833
81. **Gotoh T, Chowdhury S, Takiguchi M, Mori M** 1997 The glucocorticoid-response gene cascade. Activation of the rat arginase gene through induction of C/EBPβ. J Biol Chem 272:3694–3698
82. **Boruk M, Savory JG, Hache RJ** 1998 AF-2-dependent potentiation of CCAAT/enhancer binding protein beta-mediated transcriptional activation by glucocorticoid receptor. Mol Endocrinol 12:1749–1763

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