Cortisol inhibits hepatocyte growth factor/scatter factor expression and induces c-met transcripts in osteoblasts

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Bianquaert, Frederic, Renata C. Pereira, and Ernesto Canalis. Cortisol inhibits hepatocyte growth factor/scatter factor expression and induces c-met transcripts in osteoblasts. Am. J. Physiol. Endocrinol. Metab. 278: E509–E515, 2000.—Hepatocyte growth factor/scatter factor (HGF/SF) is expressed by osteoblasts and has important effects on repair and bone remodeling. Because glucocorticoids regulate these two functions, we tested the effects of cortisol on the expression of HGF/SF and c-met, the protooncogene encoding the HGF/SF receptor, in cultures of osteoblast-enriched cells from 22-day fetal rat calvariae (Ob cells). Cortisol decreased HGF/SF mRNA levels and diminished the induction of HGF/SF transcripts by fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor BB (PDGF BB). Cortisol also decreased FGF-2 and PDGF BB-induced HGF/SF mRNA and polypeptide levels in MC3T3 cells. In contrast, cortisol enhanced the expression of c-met transcripts in Ob cells. Cortisol did not modify the half-life of HGF/SF or of c-met mRNA in transcriptionally arrested cells, and it increased the rate of transcription of c-met. In conclusion, cortisol decreases HGF/SF transcripts in Ob cells and enhances c-met expression transcriptionally. The effects of cortisol on HGF/SF could be relevant to its inhibitory actions on bone formation and repair.

skeletal tissue; glucocorticoids; wound healing; fractures; growth factors

GLUCOCORTICOIDS HAVE MARKED EFFECTS on the skeleton, and prolonged exposure to excessive amounts of these corticosteroids results in osteoporosis (8, 13). Glucocorticoids have complex actions on bone formation and resorption, although their inhibitory effects on bone formation appear central to the bone loss observed after glucocorticoid excess (8, 13). Glucocorticoids decrease the pool of available osteoblasts and cause osteoblast apoptosis (44). In addition, they have direct actions on specific genes expressed by the osteoblast and regulate the synthesis and activity of locally produced growth factors (8, 13). For example, glucocorticoids have opposite effects to those of insulin-like growth factor (IGF)-1 on bone formation and inhibit the transcription of the growth factor in osteoblasts (12, 24). This would suggest a possible role of IGF-I and other growth factors in mediating selected actions of glucocorticoids in bone.

Hepatocyte growth factor/scatter factor (HGF/SF) is a polypeptide composed of a 69-kDa α-chain with four krinkle domains and a 34-kDa β-chain with a serum protease-like sequence linked by disulfide bonds (31, 38). HGF/SF stimulates mitogenesis in hepatic and extrahepatic cells, enhances angiogenesis, and plays a role in repair in liver and kidney, and possibly in other tissues (26, 33, 36, 38, 42). HGF/SF signals via the product of the protooncogene c-met, a tyrosine kinase-activated receptor (5, 32). HGF/SF and c-met are expressed by mesenchymal cells, osteoblasts, and osteoclasts (4, 21). HGF/SF is mitogenic for cells of the osteoblastic and osteoclastic lineage, and its synthesis by the osteoblast is enhanced by growth factors with a role in wound and fracture repair (4, 21). Therefore, it was postulated to have a function in bone remodeling and repair (21).

Glucocorticoids not only cause a decrease in bone formation, but they also alter wound and possibly fracture healing (3). Although this may be the result of direct actions of glucocorticoids on cellular events at the wound or fracture site, it may involve alterations in the production or activity of locally produced factors, such as HGF/SF. In an initial effort to explore a possible role of HGF/SF as a mediator of glucocorticoid action in bone, in the present study we examined the effects of cortisol on the expression of HGF/SF and c-met in cultures of osteoblast-enriched cells from 22-day fetal rat calvariae (Ob cells).

MATERIALS AND METHODS

Culture technique. The culture method used was described in detail previously (29). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal area. The 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 by use of bacterial collagenase (CLS II, Worthington Biochemical, 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 (26). 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). Cells were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with nonessential amino acids, 20 mM HEPES, and 10% fetal bovine serum (FBS; Summit, Biotechnology, Fort Collins, CO) and were grown to confluence. MC3T3 cells, an osteoblastic cell line created by Sudo et al. (39) and derived from fetal mouse calvaria, were cultured in α-MEM (Life
E510 CORTISOL INHIBITS HGF/SF EXPRESSION

Northern blot analysis. Total cellular RNA was isolated using an RNeasy kit and 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. The RNA samples were processed for hybridization using a Gene Screen Plus by slot blotting according to the manufacturer’s directions (Du Pont). Murine 18S cDNA was used to estimate uniformity of counts applied to the membrane. Equal counts per 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 30 min. Hybridized cDNAs were visualized by autoradiography. Nuclear run-on assays were done twice.

RESULTS

Northern blot analysis of total RNA extracted from Ob cells revealed HGF/SF transcripts of 6.3, 3.7, and 3.1 kb (Fig. 1). There was a time-dependent increase in HGF/SF mRNA levels in serum-deprived confluent Ob cells cultured over a 2- to 48-h period. This increase was noted after 24 and 48 h, and it was prevented by cortisol at 1 µM so that cortisol decreased HGF/SF mRNA levels from a respective 24-h control value of 1.00 to a value of 0.6 ± 0.05 (SE; n = 13), and from a respective 48-h cortisol value of 1.00 to a value of 0.3 ± 0.04 (n = 3), as determined by densitometry (Fig. 1). The inhibitory effect of cortisol on HGF/SF mRNA was dose dependent, and continuous treatment of Ob cells with cortisol at 100 nM and 1 µM for 24 h decreased HGF/SF transcripts from a control value of 1.00 to values of 0.7 ± 0.04 (SE; n = 7) and 0.6 ± 0.05 (n = 13) (Fig. 2).

Cortisol inhibited control and growth factor-induced expression of HGF/SF. Confirming previous observations in MCT3 cells, FGF-2 at 2 nM and PDGF BB at 3.3 nM for 24 h increased HGF/SF mRNA levels in Ob cells, and cortisol at 1 µM for 24 h decreased the induction of HGF/SF mRNA levels by the two growth factors (Fig. 3) (4). The constitutive expression of HGF/SF in serum-
deprived MC3T3 cells is minimal; therefore, FGF-2 and PDGF BB tend to cause a more pronounced relative stimulatory effect on HGF/SF in MC3T3 than in Ob cells compared with control untreated cultures (Figs. 3 and 4). The stimulatory effect of FGF-2 at 2 nM and PDGF BB at 3.3 nM in MC3T3 cells also was opposed by cortisol at 1 µM for 24 h (Fig. 4).

The levels of immunoreactive HGF/SF in control untreated and cortisol-treated Ob and MC3T3 cells were below the limit of detection of the assay, which is 0.4 ng/ml. Neither FGF-2 nor PDGF BB caused a detectable increase in immunoreactive HGF/SF in Ob cells, so that the inhibitory effect of cortisol on immunoreactive HGF/SF could not be tested in these cells. In contrast, FGF-2 and PDGF BB increased HGF/SF polypeptide levels in MC3T3 cells treated for 48 h, and the effect was opposed by cortisol (Table 1).

Northern blot analysis of total RNA extracted from confluent cultures of Ob cells revealed a predominant c-met transcript of 8.6 kb (Fig. 5). Continuous treatment of Ob cells with cortisol caused a time-dependent increase in c-met steady-state mRNA levels. The effect was first consistently observed after 6 h of exposure to cortisol at 1 µM and was sustained for 48 h. Treatment with cortisol increased c-met mRNA levels by 2.5 ± 0.3 (SE; n = 6–9), 2.5 ± 0.2, and 2.5 ± 0.1 multiples of increase after 6, 24, and 48 h, respectively, as determined by densitometry (Fig. 5). The effect of cortisol was dose dependent, and continuous treatment of Ob cells with cortisol for 24 h at 10 nM, 100 nM, and 1 µM increased c-met transcripts by 2.0 ± 0.4 (SE; n = 4), 3.3 ± 0.9, and 3.6 ± 0.6 multiples of increase, respectively (Fig. 6).

Fig. 1. Effect of glucocorticoid (GC) cortisol at 1 µM on hepatocyte growth factor/scatter factor (HGF/SF) 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 an α-32P-labeled HGF/SF cDNA. Blot was stripped and rehybridized with labeled murine 18S cDNA. HGF/SF mRNA was visualized by autoradiography and is shown above; 18S mRNA is shown below.

Fig. 2. Effect of GC cortisol at 0.01–1 µM on HGF/SF mRNA expression in cultures of Ob cells treated for 24 h. Total RNA from control (0) or cortisol (GC)-treated cultures was subjected to Northern blot analysis and hybridized with an α-32P-labeled HGF/SF cDNA. Blot was stripped and rehybridized with labeled murine 18S cDNA. HGF/SF mRNA was visualized by autoradiography and is shown above; 18S mRNA is shown below.

Fig. 3. Effect of fibroblast growth factor-2 (FGF-2) at 2 nM and platelet-derived growth factor BB (PDGF BB) at 3.3 nM, in the absence (−) and in the presence (+) of GC cortisol at 1 µM, on HGF/SF mRNA expression in cultures of Ob cells treated for 24 h. Total RNA from control, PDGF BB, FGF-2, and cortisol (GC)-treated cultures was subjected to Northern blot analysis and hybridized with an α-32P-labeled HGF/SF cDNA. Blot was stripped and rehybridized with labeled murine 18S cDNA. HGF/SF mRNA was visualized by autoradiography and is shown above; 18S mRNA is shown below.

Fig. 4. Effect of FGF-2 (FGF) at 2 nM and PDGF BB (BB) at 3.3 nM in absence (−) and in presence (+) of GC cortisol at 1 µM on HGF/SF mRNA expression in cultures of MC3T3 cells treated for 24 h. Total RNA from control, FGF-2, PDGF BB, and cortisol (GC)-treated cultures was subjected to Northern blot analysis and hybridized with an α-32P-labeled HGF/SF cDNA. Blot was stripped and rehybridized with labeled murine 18S cDNA. HGF/SF mRNA was visualized by autoradiography and is shown above; 18S mRNA is shown below.
To determine possible mechanisms involved in the regulation of HGF/SF and c-met by glucocorticoids, we examined whether or not the effects were protein synthesis dependent, and whether they occurred at the transcriptional or posttranscriptional level. To determine whether the effect of cortisol was dependent on protein synthesis, Ob cells were treated with cortisol at 1 µM for 24 h in the presence or absence of cycloheximide at 3.6 µM, a dose previously shown to block protein synthesis in osteoblasts (11). Densitometric analysis revealed that, in an experiment in which cortisol decreased HGF/SF mRNA levels from control values of 1.00 to 0.66 ± 0.1 (SE; n = 5), cycloheximide increased HGF/SF mRNA levels to 1.7 ± 0.2 in the absence, and to 2.0 ± 0.2 in the presence of cortisol. Consequently, the inhibitory effect of cortisol could not be detected in the presence of cycloheximide, although the results are difficult to interpret because of the accumulation of HGF/SF mRNA in cycloheximide-treated cells. This accumulation or superinduction of transcripts in the presence of protein synthesis inhibitors is usually attributed to the inhibition of RNA-degrading enzymes (1, 6). The effect of cortisol on c-met mRNA levels appeared to be independent of de novo protein synthesis, because treatment with cycloheximide increased c-met mRNA levels and enhanced the stimulatory effect of cortisol (Fig. 7). To determine whether cortisol decreased HGF/SF or increased c-met mRNA levels by changing transcript stability, cultures of Ob cells were exposed to cortisol at 1 µM for 1–4 h and then treated with the RNA polymerase II inhibitor DRB for 30 min to 18 h (45). About 75% of Ob cells are viable in the presence of DRB for 24 h, as determined by trypan blue exclusion (Canalis, unpublished observations). The half-lives of both HGF/SF and c-met mRNA in transcriptionally arrested osteoblasts were ~3–4 h.

Table 1. Effect of FGF-2 and PDGF BB in the presence and absence of cortisol on HGF/ SF levels in cultures of MC3T3 cells

<table>
<thead>
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<th>HGF/SF (ng/ml)</th>
<th>HGF/SF (ng/mg protein)</th>
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<tr>
<td>Control</td>
<td>&lt;0.4</td>
<td>&lt;0.4</td>
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<tr>
<td>Cortisol</td>
<td>&lt;0.4</td>
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<tr>
<td>FGF-2</td>
<td>3.4 ± 0.2</td>
<td>9.4 ± 0.7</td>
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<tr>
<td>FGF-2 + cortisol</td>
<td>0.8 ± 0.1</td>
<td>2.1 ± 0.1</td>
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<tr>
<td>PDGF BB</td>
<td>0.8 ± 0.1</td>
<td>1.6 ± 0.1</td>
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<td>PDGF BB + cortisol</td>
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Confluent MC3T3 cell cultures were serum deprived and exposed to control medium, cortisol at 1 µM, fibroblast growth factor-2 (FGF-2) at 2 nM, and platelet-derived growth factor BB (PDGF BB) at 3.3 nM, singly and in combination for 48 h. Conditioned medium was collected for the last 24 h of the incubation (24- to 48-h treatment period), and hepatocyte growth factor/scatter factor (HGF/SF) was determined by enzyme immunoassay. Values are means ± SE for 4 independent determinations.

To determine possible mechanisms involved in the regulation of HGF/SF and c-met by glucocorticoids, we examined whether or not the effects were protein synthesis dependent, and whether they occurred at the transcriptional or posttranscriptional level. To determine whether the effect of cortisol was dependent on protein synthesis, Ob cells were treated with cortisol at 1 µM for 24 h in the presence or absence of cycloheximide at 3.6 µM, a dose previously shown to block protein synthesis in osteoblasts (11). Densitometric analysis revealed that, in an experiment in which cortisol decreased HGF/SF mRNA levels from control values of 1.00 to 0.6 ± 0.1 (SE; n = 3), cycloheximide increased HGF/SF mRNA levels to 1.7 ± 0.2 in the absence, and to 2.0 ± 0.2 in the presence of cortisol. Consequently, the inhibitory effect of cortisol could not be detected in the presence of cycloheximide, although the results are difficult to interpret because of the accumulation of HGF/SF mRNA in cycloheximide-treated cells. This accumulation or superinduction of transcripts in the presence of protein synthesis inhibitors is usually attributed to the inhibition of RNA-degrading enzymes (1, 6). The effect of cortisol on c-met mRNA levels appeared to be independent of de novo protein synthesis, because treatment with cycloheximide increased c-met mRNA levels and enhanced the stimulatory effect of cortisol (Fig. 7). To determine whether cortisol decreased HGF/SF or increased c-met mRNA levels by changing transcript stability, cultures of Ob cells were exposed to cortisol at 1 µM for 1–4 h and then treated with the RNA polymerase II inhibitor DRB for 30 min to 18 h (45). About 75% of Ob cells are viable in the presence of DRB for 24 h, as determined by trypan blue exclusion (Canalis, unpublished observations). The half-lives of both HGF/SF and c-met mRNA in transcriptionally arrested osteoblasts were ~3–4 h,
and these were not significantly altered by cortisol (Fig. 8). To determine the effect of cortisol on the rate of transcription of the HGF/SF and c-met genes, nuclear run-on assays were performed. Nuclei isolated from Ob cells exposed to control medium or cortisol at 1 µM for 2, 6, or 24 h revealed that cortisol did not cause a detectable change in the rate of HGF/SF transcription but increased the rate of c-met transcription by about twofold (Fig. 9).

**DISCUSSION**

Recent studies have shown that glucocorticoids have significant effects on the number and function of osteoblasts, acting through a variety of mechanisms (8, 13, 44). The present investigation was undertaken to determine whether cortisol regulates the expression of HGF/SF and c-met in osteoblasts. There was a time-related increase in HGF/SF mRNA levels in serum-deprived Ob cell cultures, and this increase was probably due to an accumulation of endogenous growth factors, such as FGF and PDGF, which are synthesized by skeletal cells and can enhance HGF/SF synthesis in osteoblasts (4, 10, 35). Cortisol prevented the time-related increase, causing a relative decrease in HGF/SF mRNA levels in osteoblasts. Cortisol also decreased the induction of HGF/SF transcripts by FGF-2 and PDGF BB in Ob and MC3T3 cells.

Cycloheximide superinduced HGF/SF transcripts in the presence and absence of cortisol, suggesting the inhibition of HGF/SF mRNA-degrading enzymes, which could be induced by cortisol and could be responsible for the decrease in HGF/SF mRNA caused by this steroid (1, 6). Glucocorticoids have been found to induce cytosolic proteins in osteoblasts, which are responsible for changes in the stability of other transcripts, such as those of collagenase 3 (15). Cytosolic proteins are known to bind to AU-rich elements in the RNA, and these sequences often modulate mRNA stability of other genes (20, 43). Although cortisol may regulate RNA-binding proteins in osteoblasts, it is not known whether or not they bind to AU-rich regions of HGF/SF RNA and whether or not they play a role in the inhibitory effect of cortisol on HGF/SF mRNA expression. Furthermore, experiments in transcriptionally blocked Ob cells, by use of the RNA polymerase II inhibitor DRB, revealed that cortisol did not destabilize HGF/SF transcripts (45). It is possible that cortisol destabilizes HGF/SF mRNA, but the effect was not detectable under conditions of transcriptional arrest, which may have suppressed the expression of genes coding for proteins required to regulate HGF/SF transcript stability. Our data are not conclusive, because it was not possible to demonstrate a decrease in the rate of HGF/SF transcription. This could be due to lack of a transcriptional effect or lack of sufficient sensitivity for the detection of an inhibitory effect. Similar difficulties
were encountered to prove a transcriptional effect of FGF-2 on HGF/SF expression in osteoblasts and of various cytokines in fibroblasts (4, 41).

The levels of immunoreactive HGF/SF in control and growth factor-induced Ob cells were below the limit of detection with use of currently available assays, so that we could not demonstrate a decrease in HGF/SF levels by cortisol in Ob cells. However, detectable levels of HGF/SF were achieved in MC3T3 cells after induction with FGF-2 and PDGF BB. HGF/SF levels in MC3T3 cells were suppressed by cortisol, revealing that this steroid has the capability to reduce HGF/SF synthesis in osteoblasts. It is not clear why HGF/SF levels can be induced to a greater extent in MC3T3 than in Ob cells, but differences in the level of growth factor expression between Ob and MC3T3 cells are not uncommon (19).

In contrast to the inhibitory effects on HGF/SF expression, cortisol caused a time- and dose-dependent increase in c-met mRNA levels in Ob cells. Cycloheximide superinduced c-met transcripts and had an additive effect to that of cortisol, suggesting the presence of c-met mRNA-degrading enzymes in Ob cell cultures. The effect of cortisol on c-met occurred by transcriptional mechanisms, because cortisol caused no change in the half-life of the transcript in transcriptionally arrested cells and increased the rate of transcription.

In our study, the effects of cortisol on HGF/SF and c-met expression were observed at doses that modify other parameters of metabolic function in Ob cells, suggesting that the effect is physiologically relevant. Glucocorticoids have complex effects on bone remodeling and have a major impact on bone formation. The inhibitory actions of glucocorticoids on bone formation are secondary to a decrease in bone cell replication, to a decrease in bone collagen synthesis, and to an increase in collagenase 3 expression (7, 14, 15). In addition, some of the actions of glucocorticoids are due to modifications in the synthesis of growth factors produced by skeletal cells or alterations in receptor binding or binding proteins (8, 12, 18, 34). The decrease in HGF/SF expression by cortisol may explain selected actions of glucocorticoids in bone, and it may be particularly relevant to the impaired healing of tissues exposed to glucocorticoids.

FGF-2 and PDGF BB stimulate the replication of cells of the osteoblastic lineage and have been implicated in wound and fracture repair; the two growth factors induced HGF/SF expression, an effect attenuated by glucocorticoids (9, 16, 23, 28, 30). This, in conjunction with the known effect of HGF/SF in tissue repair, would suggest a role for HGF/SF in bone repair, which can be opposed by glucocorticoids (26, 38). This effect may serve, in part, to explain the inhibitory actions of glucocorticoids on wound and fracture healing. The induction of c-met by glucocorticoids may be a compensatory mechanism to maintain HGF/SF function in bone. Whereas the induction of c-met by glucocorticoids seems unique to osteoblasts, the decrease in HGF/SF production also occurs in bone marrow stromal cells and fibroblasts (27, 40).

In conclusion, cortisol decreases the synthesis of HGF/SF and increases c-met expression in osteoblasts. These effects may play a role in the inhibitory actions of glucocorticoids on bone formation and repair.

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CORTISOL INHIBITS HGF/SF EXPRESSION


