Hydrocortisone and Deflazacort Induce Different Effects on Vitamin D Receptor Level Increase Produced by 1,25-Dihydroxyvitamin D3 in Rat Osteoblast-like UMR-106 Cells

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ABSTRACT. Objective. The homologous upregulation produced by 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] on vitamin D receptor (VDR) levels, and the effects produced by the heterologous agents hydrocortisone or deflazacort, alone or in conjunction with this vitamin D metabolite, were studied in rat osteoblastic UMR-106 osteosarcoma cells.

Methods. VDR were determined by binding analysis (B$_{max}$ and dissociation constant). VDR mRNA expression levels were measured by Northern blot analysis.

Results. Incubation with 10 nM 1,25(OH)$_2$D$_3$ produced a significant increase in B$_{max}$ with respect to ethanol-treated cells (100.2 ± 13.2 vs 11.4 ± 4.8 fmol $^3$H-1,25(OH)$_2$D$_3$ bound/mg protein) together with a significant increase in VDR mRNA expression (483 ± 170% vs 100%). The addition of 10 nM hydrocortisone to 1,25(OH)$_2$D$_3$ produced a significant decrease in B$_{max}$ (from 100.2 ± 13.2 to 44 ± 5.6), with mRNA levels similar to those of basal conditions (116 ± 25% vs 100%). However, the addition of 10 nM deflazacort did not reduce the activation in B$_{max}$ produced by 1,25(OH)$_2$D$_3$ (92.4 ± 16 vs 100.2 ± 13.2), maintaining the increase in mRNA levels (430 ± 10% vs 483 ± 170%). If 10 nM hydrocortisone or 10 nM deflazacort was added to UMR-106 cells without 1,25(OH)$_2$D$_3$, a similar increase was observed in B$_{max}$ with respect to basal conditions (20.4 ± 1.3 or 20.9 ± 1.6 vs 11.4 ± 4.8 in control cells), but hydrocortisone did not produce any significant variation in mRNA VDR levels, while deflazacort itself produced an increase in VDR mRNA expression.

Conclusion. Our findings of different actions produced by hydrocortisone and deflazacort on the increase of VDR levels produced by 1,25(OH)$_2$D$_3$ could explain some of the different actions produced by both antiinflammatory medications on bone metabolism. (J Rheumatol 2004;31:167–72)

Key Indexing Terms: CORTISOL DEFLAZACORT 1,25-DIHYDROXYVITAMIN D VITAMIN D RECEPTOR VITAMIN D RECEPTOR mRNA

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] is a fundamental key in bone metabolism. This metabolite interacts with osteoblast vitamin D receptors (VDR) to produce its genomic actions$^{1,2}$. Thus, regulation of VDR concentrations is very important in bone homeostasis.

It has been shown that 1,25(OH)$_2$D$_3$ and other vitamin D metabolites upregulate VDR concentrations (homologous regulation)$^3$. Heterologous regulation of VDR has also been described with other compounds such as glucocorticoids$^{3,4}$.

It is known that glucocorticoids, like cortisol, produce undesirable collateral effects on bone$^5,6$. To reduce the side effects of these potent drugs, synthetic derivatives have been developed that maintain a high antiinflammatory/immunosuppressive potency but with a less detrimental effect on the maintenance of bone mass. One of these compounds is deflazacort, a synthetic glucocorticoid (an oxazoline derivative of prednisolone) with antiinflammatory and antimune activity. It has been observed that this compound produces fewer undesirable effects on bone metabolism than those produced by hydrocortisone$^7,10$. However, the molecular mechanisms that could explain the different behaviors of these glucocorticoids are not fully understood.

Several investigators have studied the effects of deflazacort$^{11}$ and cortisol$^{12}$ in cultures of intact rat calvariae and of other glucocorticoids in human bone-derived cells$^{13,14}$. However, the direct actions of deflazacort on VDR levels and VDR mRNA expression in osteoblast-like cells are not fully known and they have not been compared with those of cortisol.

We investigated the homologous upregulation produced
by 1,25(OH)_{2}D_{3} on VDR levels, and we examined and compared the effects produced by the heterologous agents hydrocortisone and deflazacort, alone or in conjunction with the vitamin D metabolite, on this regulation. This study was performed in rat UMR-106 osteosarcoma cells, an osteoblastic phenotype line. VDR levels in basal conditions and under stimulation by the different agents were determined by binding analysis, and VDR mRNA expression was also measured.

MATERIALS AND METHODS

Materials. ^{3}H-1,25(OH)_{2}D_{3} (120 Ci/mmol) and 32P-dCTP (3000 Ci/mmol) were obtained from Amersham UK (Buckinghamshire, UK). Deflazacort was kindly supplied by Dr. H.W. Böhme and Dr. H. Guaglianone (Hoechst Marion Roussel, Bridgewater, NJ, USA). 1,25(OH)_{2}D_{3}, 10 nM was a gift from Dr. A. Kaiser and Dr. U. Fischer (Hoffman-La Roche, Basel, Switzerland). Actinomycin D and hydrocortisone were purchased from Sigma-Aldrich, Steinheim, Germany. The clone containing a 1784 bp fragment of the rat vitamin D receptor (VDR) cDNA cloned into pUC18 vector was generously provided by Dr. H.M. Darwish and Dr. H.F. DeLuca (University of Wisconsin, Madison, WI, USA). Ultraspec™^{32}P RNA isolation kit and random-primed DNA labeling kit were obtained from Biotecx (Houston, TX, USA) and Boeringher-Mannheim (Mannheim, Germany), respectively. X-Omat AR films were obtained from Kodak. All other chemicals were purchased from Sigma and were of analytical quality.

Cell culture. UMR-106 cells were purchased from the European Collection of Cell Cultures (Porton Down Salisbury, UK) and were seeded at 30,000 cells/cm² in plastic cell culture flasks. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37°C under 5% CO₂, 95% air. The culture medium was changed every 2 days. Cells reached confluence in 7–8 days. All experiments were carried out in confluent cultures.

VDR binding assay. Twenty hours before the experiment, confluent cells were cultured in a serum-free medium with 0.5% bovine serum albumin (BSA). On the day of the experiment, cells were incubated for 4 h in serum-free medium with 0.1% BSA plus vehicle (ethanol) or the corresponding compounds: 10 nM 1,25(OH)_{2}D_{3}, 10 nM hydrocortisone, 10 nM deflazacort, or 10 nM 1,25(OH)_{2}D_{3} plus 10 nM hydrocortisone or 10 nM deflazacort. All culture flasks, including controls, had a final ethanol concentration of 0.05%. At the end of incubation, the culture medium was removed and the cells were further incubated in DMEM plus 10% FBS for 2 h. At this time, cells were harvested and washed with ice-cold PBS and cell pellets were resuspended in hypertonic buffer (500 mM KCl, 50 mM K_{2}HPO_{4}, 1 mM EDTA, 5 mM DTT, pH 7.4) containing aprotinin (50 µg/ml), and sonicated on ice with four 15 s cycles interrupted by 15 s pauses. The sonicate was centrifuged at 105,000 g for 60 min at 4°C and the supernatant was collected for VDR binding assay. Protein concentration in the supernatant was measured by the Bradford method, using BSA as a standard. Duplicate (250 µl) aliquots of supernatant extract were incubated at 4°C for 16 h with different amounts of ^{3}H-1,25(OH)_{2}D_{3} from 0.005 to 0.080 pmol with or without 0.1 nmol of unlabeled 1,25(OH)_{2}D_{3} to determine total (Bₜ) and nonspecific binding (NSB), respectively. Bound and free fractions were separated by charcoal adsorption and after centrifugation the radioactivity was determined in the supernatant. The curve of specific binding (Bₜ) was determined by subtracting the curve of nonspecific from that of total binding. Bₜ was calculated from curve of specific binding and was expressed as fmol of bound ^{3}H-1,25(OH)_{2}D_{3} per mg cytosol protein. Apparent dissociation constant (K_{d}) was determined by nonlinear regression fit, from values of specific binding.

RNA isolation and Northern blot analysis. Twenty hours before the experiment, confluent cells were cultured in serum-free medium with 0.5% BSA.

On the day of the experiment, cells were incubated 4 h in serum-free medium with 0.1% BSA plus vehicle (ethanol) or the corresponding compounds: 10 nM 1,25(OH)_{2}D_{3}, 10 nM hydrocortisone, 10 nM deflazacort, and 10 nM 1,25(OH)_{2}D_{3} plus 10 nM hydrocortisone or 10 nM deflazacort. We selected this time according to previous results of VDR mRNA isolation in this cellular line. After incubation, RNA was isolated by Ultraspec™ RNA kit. The purity and concentration of RNA were measured spectrophotometrically at 260 and 280 nm. Fifteen micrograms of total RNA from each group were split up on 1% agarose gels with 0.66 M formaldehyde in MOPS buffer. The RNA was then transferred to nitrocellulose membranes (Gene Screen filters) by capillary action at room temperature. Membranes with bound RNA were prehybridized at 42°C for 6 h in a solution of 50% formamide, 1% sodium dodecyl sulfate (SDS), 1 × Denhardt’s, 5 × SSC (1 × SSC = 150 mM NaCl and 15 mM Na citrate, pH 7.0), 20 nM NaH_{2}PO_{4} and 1 µg/ml herring sperm DNA. Then hybridization was performed for 16–18 h at 42°C in fresh prehybridization solution with ^{32}P-labeled cDNA probe for the rat VDR (this probe was previously obtained by growing in JM109 strain of Escherichia coli and isolated by digestion with Eco RI) and 20% dextran sulfate. Filters were washed once with 2 × SSC and 0.1% SDS for 30 min at room temperature, once with 1 × SSC and 0.1% SDS for 30 min at 55°C, and twice with 0.1 × SSC and 0.1% SDS for 15 min at 55°C, and exposed to x-ray films. The signals on the autoradiograms were quantified with a densitometric scanner (Personal densitometer 50340). Data were expressed as the ratio of VDR mRNA to 28S rRNA.

Actinomycin D effects, at different times, on VDR mRNA. Twenty hours before the experiment, confluent cells were cultured in a serum-free medium with 0.5% BSA. On the day of experiment, cells were incubated 4 h in serum-free medium with 0.1% BSA plus vehicle (ethanol) or the corresponding compounds (10 nM 1,25(OH)_{2}D_{3}, 10 nM hydrocortisone, or 10 nM deflazacort). Actinomycin D (1 µg/ml) was added at different times in the 4 h incubation period: at 0, 90, 180, and 240 minutes. After the incubation, RNA was isolated and analyzed as described. Statistics. Statistical evaluations were made by nonparametric Mann-Whitney test. Significance was ascribed at p < 0.05. For the binding analysis, the best-fit line was determined by nonlinear regression (GraphPad Prism 2.0 computer program).

RESULTS

Effects of 10 nM 1,25(OH)_{2}D_{3}, 10 nM hydrocortisone, 10 nM deflazacort, or 10 nM 1,25(OH)_{2}D_{3} plus 10 nM hydrocortisone or 10 nM deflazacort on ^{3}H-1,25(OH)_{2}D_{3} binding to VDR. Figure 1 shows a representative experiment of binding of ^{3}H-1,25(OH)_{2}D_{3} to VDR in the supernatant of the extract of UMR cells in ethanol-treated cells in basal conditions. Total binding (Bₜ), nonspecific binding (NSB), and specific binding (Bₜ) are represented (the latter is the result of the difference between Bₜ and NSB). Bₜ shows a typical saturation curve at 60 pM hormonal concentration.

The effects of 10 nM 1,25(OH)_{2}D_{3}, 10 nM hydrocortisone, 10 nM deflazacort, or 10 nM 1,25(OH)_{2}D_{3} plus 10 nM hydrocortisone or 10 nM deflazacort on ^{3}H-1,25(OH)_{2}D_{3} binding to VDR in UMR-106 cell cultures are shown in Table 1. After 4 h of incubation with 10 nM 1,25(OH)_{2}D_{3}, there was a significant increase in Bₜ from 11.4 ± 4.8 fmol/mg protein in control cells (ethanol-treated cells) to 100.2 ± 13.2 fmol/mg protein in cells treated with 1,25(OH)_{2}D_{3} (homologous upregulation). The addition of 10 nM hydrocortisone in conjunction with 10 nM 1,25(OH)_{2}D_{3} produced a significant decrease in the binding
with respect to that observed after 1,25(OH)₂D₃ treatment, from 100 to 44 fmol ³H-1,25(OH)₂D₃ bound/mg protein, although a significant increase was observed with respect to Bmax in ethanol-treated cells. However, 10 nM deflazacort did not significantly reduce the activation produced by 1,25(OH)₂D₃, as the value of Bmax was 92.4 ± 16 fmol ³H-1,25(OH)₂D₃ bound/mg protein. The figure illustrates data from a representative experiment.

Table 1. Effect of 1,25 (OH)₂D₃ plus hydrocortisone, 1,25 (OH)₂D₃ plus deflazacort, hydrocortisone, or deflazacort on ³H-1,25 (OH)₂D₃ binding to VDR in rat UMR-106 cells. Values represent the mean ± SD of 4 different experiments. UMR-106 cells were incubated 4 h with 10 nM 1,25 (OH)₂D₃, 10 nM hydrocortisone, 10 nM deflazacort, 10 nM 1,25 (OH)₂D₃ plus 10 nM hydrocortisone, or 10 nM 1,25 (OH)₂D₃ plus 10 nM deflazacort. Binding studies and Northern blot analysis were carried out. Bmax values were calculated from the corresponding saturation curves.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bmax ³H-1,25 (OH)₂D₃, fmol/mg protein</th>
<th>Kd, pM ³H-1,25 (OH)₂D₃, pM</th>
<th>VDR mRNA/28S, % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.4 ± 4.8</td>
<td>18 ± 8</td>
<td>100%</td>
</tr>
<tr>
<td>10 nM 1,25 (OH)₂D₃</td>
<td>100.2 ± 12.1*</td>
<td>24 ± 7</td>
<td>483 ± 170*</td>
</tr>
<tr>
<td>10 nM hydrocortisone</td>
<td>20.4 ± 1.3*</td>
<td>29 ± 11</td>
<td>108 ± 33</td>
</tr>
<tr>
<td>10 nM deflazacort</td>
<td>20.9 ± 1.6*</td>
<td>28 ± 9</td>
<td>191 ± 50*</td>
</tr>
<tr>
<td>10 nM 1,25 (OH)₂D₃ + 10 nM hydrocortisone</td>
<td>44.0 ± 5.6*</td>
<td>29 ± 10</td>
<td>116 ± 25</td>
</tr>
<tr>
<td>10 nM 1,25 (OH)₂D₃ + 10 nM deflazacort</td>
<td>92.4 ± 16.0*</td>
<td>33 ± 13</td>
<td>430 ± 150*</td>
</tr>
</tbody>
</table>

*p < 0.05 vs control conditions; *p < 0.05 vs 10 nM 1,25 (OH)₂D₃, Mann-Whitney test. # Control: ethanol vehicle. Data of VDR mRNA levels expressed as ratio of the amount of VDR mRNA to that of 28S mRNA (% control).
control) (Table 2). These results prove that the observed increase in VDR mRNA produced by 1,25(OH)₂D₃ or deflazacort resulted from an increased transcription of the vitamin D receptor gene between 90 and 180 min into the period of incubation of UMR-106 cells. However, no variations in VDR mRNA levels in cells treated with hydrocortisone and actinomycin D at different times were observed.

**DISCUSSION**

As vitamin D receptor plays a central role in mediating 1,25(OH)₂D₃ function, factors that regulate its expression in target tissues are of obvious importance in mineral metabolism. We investigated the possible differences between the mechanism of action of hydrocortisone and deflazacort on osteoblasts, through the regulation of VDR concentrations, differences that could justify the secondary effects produced by these 2 antiinflammatories on bone. It is known that the...
synthetic antiinflammatory deflazacort presents fewer collateral effects on bone than hydrocortisone, although the molecular mechanisms involved in these differences are not clear.

Previous in vitro studies indicated that glucocorticoids produce an increase or a decrease in VDR concentration in osteoblasts and other cells or tissues. Canalis and Avioli observed that cortisol and deflazacort had similar inhibitory effects on bone DNA and collagen synthesis in studies performed in vitro. Other in vivo studies showed that the treatment with deflazacort results in a lesser decrease than prednisone in vertebral bone mineral density and serum osteocalcin levels. According to these investigations, deflazacort acts on some aspects of mineral metabolism in a way similar to natural glucocorticoids, and in a different manner on other variables.

The main finding of our study is that deflazacort does not inhibit the increase in VDR levels produced by 1,25(OH)2D3 in UMR-106 rat osteosarcoma cells, while hydrocortisone results in a significant decrease (about 55%) of that stimulation. The increase in VDR levels produced by 1,25(OH)2D3 was combined with a concomitant increase in VDR mRNA with respect to untreated cells. When deflazacort with 1,25(OH)2D3 was added to the culture medium, there was no significant variation in VDR mRNA levels compared to the addition of 1,25(OH)2D3 alone. However, hydrocortisone totally suppressed the VDR mRNA increase produced by 1,25(OH)2D3, despite the increase found in VDR levels when 1,25(OH)2D3 and hydrocortisone were simultaneously added to osteoblastic cells with respect to the experiments with untreated cells (44 ± 5.6 fmol vs 11.4 ± 4.8 fmol).

If both glucocorticoids were added to the culture medium without 1,25(OH)2D3, a similar increase was observed in VDR levels, but there was a difference between them with respect to the increase produced in VDR mRNA: the hydrocortisone did not produce any significant variation with respect to the increase produced by 1,25(OH)2D3, despite the increase found in VDR levels when 1,25(OH)2D3 and hydrocortisone were simultaneously added to osteoblastic cells with respect to the experiments with untreated cells (44 ± 5.6 fmol vs 11.4 ± 4.8 fmol). The results following the addition of actinomycin D at different times showed that the increase in VDR binding in UMR-106 cells in response to 1,25(OH)2D3 was due to an increased transcription of the vitamin D receptor gene in these cells, although a simultaneous decrease in VDR mRNA degradation cannot be excluded. This increase in VDR mRNA levels is produced and visualized between 90 and 180 min of the incubation period, a fast regulation of VDR levels.

Our results were obtained in a rat osteosarcoma cell line. Although these are not human cells, osteoblast-like osteosarcoma cells have been widely used by other investigators in the study of bone physiology and the results extrapolated to the human model.

Our study illustrates the different actions produced by hydrocortisone and deflazacort on the increase of VDR levels produced by 1,25(OH)2D3. These different actions could explain some of the different effects produced by the 2 antiinflammatories on bone metabolism, although it is not known what would be the effect on bone metabolism of an increase or decrease in the number of VDR. Recently, Issa et al. reported that different p160 coactivators, the glucocorticoid receptor-interacting protein-1 and the receptor-associated coactivator-3, are involved in modulating tissue-specific functions of the VDR, but only in the presence of 1,25(OH)2D3.

REFERENCES