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Regulation of 1,25(OH)$_2$Vitamin D$_3$ Receptor Content in Cultured LLC-PK$_1$ Kidney Cells Limits Hormonal Responsiveness+

Margaret Hirst and David Feldman*

Departments of Physiology and Medicine
Stanford University School of Medicine
Stanford, CA 94305

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Receptor content in cultured kidney (LLC-PK$_1$) cells was found to be modulated following the introduction of a culture medium change, declining to 40% of control values at 18 h. Scatchard analysis indicated that the reduced 1,25(OH)$_2$-[^{3}H]D$_3$ nuclear binding we detected was due to decreased abundance of receptors (3811 vs 1619 sites/cell) with no change in the K_d (0.4-0.5 nM). Cells with reduced receptors exhibited diminished ability to respond to 1,25(OH)$_2$D$_3$ as measured by induction of 25(OH)vitaminD-24-hydroxylase activity. There was a close coupling between decreased receptor levels and diminished hormone responsiveness. The data suggest the absence of "spare" receptors and that receptor abundance is a limiting factor in cell responsiveness to 1,25(OH)$_2$D$_3$.

We have previously demonstrated the presence of specific receptors for 1,25-(OH)$_2$D$_3$ in the LLC-PK$_1$ pig kidney cell line (1). In addition, 1,25(OH)$_2$D$_3$ was shown to induce 25(OH)D$_3$-24-hydroxylase activity and evidence was provided to suggest that this action was receptor mediated. In the current study we examined the phenomenon of 1,25(OH)$_2$D$_3$ receptor regulation in these cells and investigated alterations in cellular functional responsiveness attendant upon changes in receptor content using 24-hydroxylase activity as a measure of responsiveness. 1,25(OH)$_2$D$_3$ receptor content of cultured LLC-PK$_1$ cells was found to be modulated by the introduction of fresh culture medium. Although we do not yet understand the specific basis of how this maneuver leads to receptor regulation, the finding nevertheless provides an excellent system to examine the relationship of 1,25(OH)$_2$D$_3$ receptor abundance and the magnitude of the biological response exerted by 1,25(OH)$_2$D$_3$.

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* To whom reprint requests should be addressed.

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METHODS

Materials: 1α,25-Dihydroxy(23,24[^{3}H])vitamin D_3 (60-110 Ci/mMol) and 25 hydroxy (26,27)-methyl [^{3}H])vitamin D_3 (18-22 Ci/mMol) were obtained from Amersham Corp. (Arlington Heights, IL). Crystalline $1,25(OH)_2D_3$, $25(OH)D_3$ and $24,25(OH)_2D_3$ were gifts from Dr. M Uskokovic of Hoffmann-La Roche Company (Nutley, NJ). Serum and culture media were purchased from GIBCO (Grand Island, NY).

Cells and cell culture conditions: LLC-PK_1 cells (American Type Tissue Collection, passage #118) were utilized from passage 140-175. Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% newborn calf serum as described previously (1). At confluence, cells were split 1:6 (day 1) and the medium was changed on day 2 and again on day 5 and cells were assayed on day 8. Where indicated, there was an additional medium change introduced on day 7 which was studied for its ability to modulate the $1,25(OH)_2D_3$ receptor.

Preparation of cell extracts and $1,25(OH)_2$[^{3}H]D_3 binding assay: For single point binding assays, conditions were employed which were previously shown to be valid estimates of total receptor content in high speed supernatants (2). Cells were scraped from the flask in phosphate buffered saline (PBS), resuspended in a hypertonic buffer (300 mM KCl, 10 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol and 10 mM Na_2MoO_4 pH 7.4) and disrupted by sonication. High speed supernatants (204,000 x g for 30 min at 4°C) were obtained and 200 µl aliquots (1-2.5 mg protein/ml) were incubated with $1,25(OH)_2$[^{3}H]D_3 (1.3 nM) at 0°C for 3 hrs. Parallel samples contained 250-fold excess radioinert $1,25(OH)_2D_3$ to correct for non-specific binding which was usually less than 10%. Protein bound $1,25(OH)_2$-[3H]D_3 was separated from free hormone by the hydroxylapatite method as previously described (3). Protein concentration was measured by the coomassie dye binding technique (4). Nuclear binding was assessed by a modification of the method of Eil et al (5). Cells were removed from the flask by trypsinization and resuspended in MEM plus 25 mM hepes buffer and 1% newborn calf serum (pH 7.4). The cell number was adjusted to 7.5 x 10^6 cells/ml and 200 µl aliquots were incubated at 37°C in a shaking waterbath with various concentrations of 1,25-$(OH)_2$[3H]D_3. Identical samples additionally received 250-fold excess radioinert $1,25(OH)_2D_3$ to determine non-specific binding. After 45 min of incubation, cells were chilled and washed twice with cold PBS containing 1 mg/ml bovine serum albumin. To prepare nuclei, cells were vortexed in a buffer containing 250 mM sucrose, 20 mM Tris, 1.1 mM $MgCl_2$ and 0.5% Triton X-100. The cells were centrifuged and the nuclear pellets were washed two more times with the same buffer. Nuclear bound $1,25(OH)_2$[^{3}H]D_3 was extracted with 0.5 ml 100% ethanol and radioactivity determined.

Enzyme Assay: $25(OH)D_3$-24- hydroxylase activity was measured by a modification of the method of Chandler et al (6) as previously described (7). Growth medium was replaced with DMEM with no added serum. Monolayers were incubated with $1,25(OH)_2D_3$ for a one hour induction pulse. At the end of six hours, cells were harvested and resuspended in MEM plus 1% newborn calf serum. 2 x 10^6 cells were incubated with 5 x 10^{-7}M 25(OH)[3H]D_3 substrate for 30 min to allow production of dihydroxylated metabolites. The assay was terminated by addition of methanol:-chloroform (2:1 v/v). Steroids were extracted (8) and analyzed on a Varian HPLC equipped with a 30 cm x 4 mm MicroPak SI-10 Silica column. The mobile phase, hexane/isopropanol (90:10 v/v), was run at a flow rate of 2 ml/min. Hydroxylation of substrate to $24,25(OH)_2$[3H]D_3 was identified by comigration with authentic $24,25(OH)_2D_3$, which exhibited a retention time of 5.5 min.

RESULTS

In routine experiments assessing LLC-PK_1 receptors, it was noted that the interval between assay and the prior medium change was a critical factor in determining the number of $1,25(OH)_2D_3$ receptors present. Cells receiving a medium

change the night prior to the receptor assay reproducibly exhibited low levels of receptor while those cells not exposed to this additional medium change typically exhibited 2-3 fold higher levels of receptor. A representative time course experiment examining this phenomenon is shown in Fig. 1. All cells were assayed concurrently at zero time. Cells not exposed to a medium change (designated 0 time after medium change) had 50 fmol of $1,25(OH)_2[^3H]D_3$ binding per mg protein while cells treated with a medium change 18 h previously exhibited only 20 fmol/mg protein. Note that this effect was biphasic and within 4 hours of a medium change cells transiently exhibited a rise in receptor levels to ~ 90 fmol/mg protein. Because of the transient nature of the receptor rise, in this paper we have focused our attention on the receptor decline.

The experiment shown in Fig. 1 assessed total receptors extracted by 0.3 M KCl in single point assays with 1.3 nM $1,25(OH)_2[^3H]D_3$. If this receptor regulation were expected to be significant in terms of responsiveness of the cells, we reasoned that accumulation of receptors in the nuclear compartment was most criti-

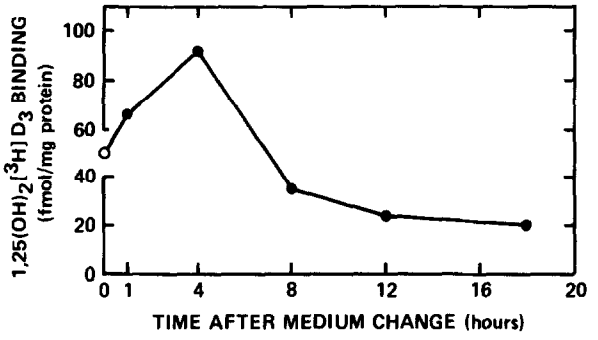

FIGURE 1. Time course of receptor modulation following a culture medium change. LLC-PK_1 cells were grown as monolayers in DMEM supplemented with 10% serum. On day 7 and 8 of culture, the medium was changed sequentially in flasks at the indicated number of hours prior to assay on day 8. All flasks were assayed for receptors at the same time and compared to cells with no medium change designated "0 time". Receptors were measured by incubation of high speed supernatants of 0.3 M KCl extracts with a saturating concentration (1.3 nM) of 1,25 $(OH)_2[^3H]D_3$ for 3 h at 0°C. Bound $1,25(OH)_2[^3H]D_3$ was measured by hydroxylapatite and a correction was made for non-specific binding.

cal. Also, to determine whether this change was the result of a decline in number of binding sites or a decrease in affinity for $1,25(OH)_2[3H]D_3$, or both, a Scatchard analysis of the binding data was essential. We therefore performed a full dose-response binding study and specifically measured nuclear binding sites. Fig. 2A shows the saturation plot and Fig. 2B the Scatchard analysis of the data. It is apparent that cells exposed to a medium-change show a substantial decrease in the number of nuclear binding sites compared to no medium-change control cells; the mean +/-S.E. of 3 experiments shows 3811 +/- 285 vs 1619 +/- 334 sites/cell. There was no change in the apparent K_d of the receptor for $1,25(OH)_2[3H]D_3$; 0.42 +/- 0.047 vs 0.42 +/- 0.045 nM.

We next addressed the question of whether the cells with down-regulated receptors exhibited normal or decreased responsiveness to $1,25(OH)_2D_3$. The functional response we examined was $1,25(OH)_2D_3$ induction of 24-hydroxylase activity.

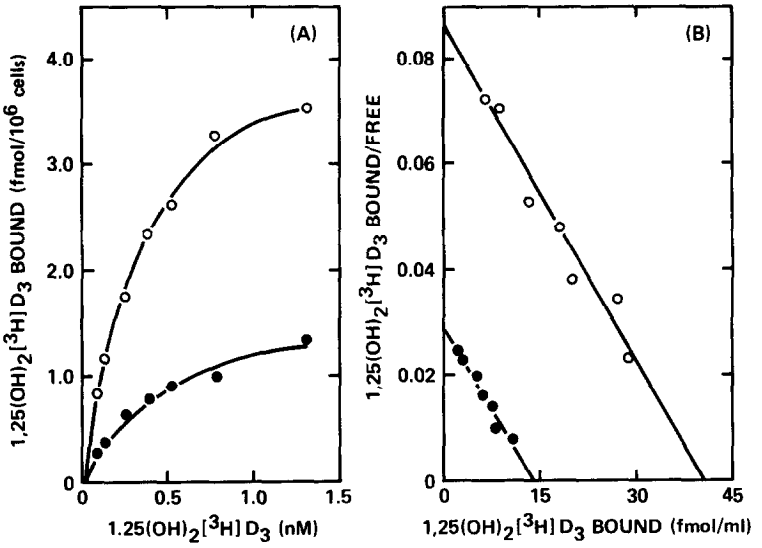

FIGURE 2. Analysis of nuclear receptor binding. Intact cells with no medium change (○) or 18 h after a medium change (●) were incubated with varying concentrations of $1,25(OH)_2[^3H]D_3$ (0.09 - 1.3 nM) ± 250-fold radioinert $1,25(OH)_2D_3$ for 45 min at 37°C. Nuclei were prepared by treatment of cells with a buffer containing 0.5% Triton X-100. After washing, nuclear counts were extracted with 100% ethanol. Panel A: isotherm; Panel B: Scatchard plot. Values for control vs medium change cells: K_d, 0.48 vs 0.49 nM; N_{max}, 3190 vs 1072 sites/cell.

As shown in Fig. 3, the cells with low-receptor content induced only a fraction of the enzyme activity that was elicited in control cells. This was true even at maximal $1,25(OH)_2D_3$ inducing concentrations. Note that basal activity was equivalently low in both cell types. When receptor number was decreased ~ 60% (18 h after medium change), functional responsiveness to $1,25(OH)_2D_3$ was decreased a roughly equivalent amount, ~ 75%. The decreased activity was due to decreased V_{max} of enzyme and not due to a change in the K_m of enzyme for substrate (Lineweaver-Burk analysis, data not shown) suggesting a change in the amount of enzyme induced. Furthermore, the decrease in enzyme activity could be accounted for by increased recovery of unaltered substrate. This indicates that the diminished activity was not due to a shift in metabolism to other products. The ED_{50} to induce 24-hydroxylase activity for the medium-changed cells (low receptor) was shifted to the right (1 nM) compared to control cells (0.4 nM) suggesting a

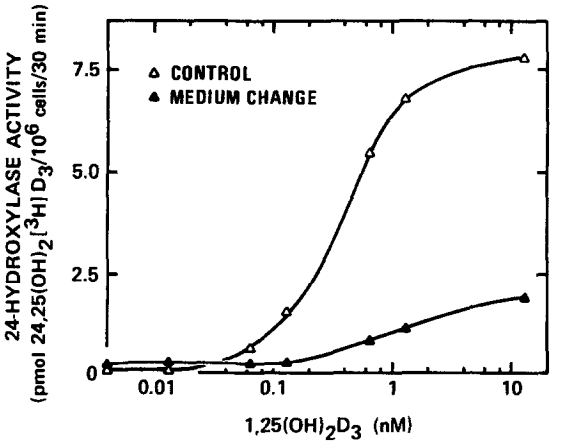

FIGURE 3. Comparison of $1,25(OH)_2D_3$ induction of 24-hydroxylase activity in control and cells exposed to a change in culture medium. Control cells (Δ) and cells exposed to a medium change 18 h earlier (▲) were incubated with varying concentrations of $1,25(OH)_2D_3$ for a 1 h induction pulse in serum-free medium. At the end of a 6 h treatment period, 24-hydroxylase activity was assessed. Cells were incubated with $25(OH)[^3H]D_3$ substrate for 30 min and metabolites were extracted in methanol:chloroform (2:1, v/v). Production of $24,25(OH)_2[^3H]D_3$ was quantitated in an isocratic HPLC system using hexane:isopropanol (90:10, v/v) as the mobile phase.

slight decrease in sensitivity to $1,25(OH)_2D_3$ in addition to a decreased maximal response.

DISCUSSION

The data presented in this paper indicate that $1,25(OH)_2[3H]D_3$ binding levels in LLC-PK_1 cells are modulated by culture conditions and can be manipulated by the timing of medium change (Fig. 1). We assume this change represents an alteration in functional number of receptors since a comparison of Scatchard analyses of $1,25(OH)_2D_3$ nuclear binding (Fig. 2) indicated that there was a single class of binding sites and the affinity of hormone for receptor was equivalent in both high and low binding states. The decreased receptor content following medium change was of similar magnitude whether measured as total binding or as nuclear binding and whether expressed on the basis of protein or cell number. These findings support our presumption that we are dealing with a different abundance of the same receptor.

We have determined that similar receptor alterations are achieved when the medium change employs charcoal treated-serum (data not shown) indicating that receptor modulation is not induced by vitamin D metabolites or other steroid hormones in the serum. The biphasic nature of the receptor modulation after medium change (Fig. 1) suggests that new cell growth or cell cycle effects (due to partial synchronization with addition of new media) may be involved. Both growth and cell cycle have previously been correlated with steroid receptor changes (9,10). The specific factor modulating receptor levels is not known, and it is not clear whether the change in receptor abundance is due to altered rates of receptor synthesis, degradation or both.

The reduced 24-hydroxylase response to $1,25(OH)_2D_3$ in medium-changed cells (Fig. 3) suggests that receptor levels are critical determinants of functional activity. We interpret the findings to indicate that a decrease in receptor abundance comparably decreases the ability of the cell to be induced by $1,25(OH)_2D_3$. In addition, although receptor affinity is apparently unchanged, the low receptor cells exhibit a slight decrease in sensitivity to $1,25(OH)_2D_3$. The ED_{50} for the control cells is $\sim$ 0.4 nM, which is in close agreement with the K_d of nuclear bind-

ing. On the other hand, the ED_{50} for the low-receptor cells is ~ 1 nM, shifted to the right compared to the ED_{50} for the control cells (~ 0.4 nM) as well as the K_d for nuclear binding. The mechanism for this effect is unknown but may represent a post-receptor change.

The concordance of binding and bioactivity suggests that $1,25(OH)_2D_3$ induction of 24-hydroxylase activity is a receptor mediated event. At least for this function, in this cell, there do not appear to be "spare" receptors. The decrease in receptors below control levels reduced the ability of the cell to respond to hormone in a tightly coupled relationship except for the minor decrease in sensitivity mentioned above. This is in agreement with recent findings that cultured fibroblasts from patients with vitamin D resistant rickets lack both functional $1,25(OH)_2D_3$ receptors and the ability to respond to $1,25(OH)_2D_3$ (7). It remains to be seen how functional responsiveness will be altered, if at all, when receptors are up-regulated above control levels.

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