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RETINOIC ACID MODULATION OF 1,25(OH)$_2$ VITAMIN D$_3$ RECEPTORS AND BIORESPONSE IN BONE CELLS: SPECIES DIFFERENCES BETWEEN RAT AND MOUSE

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Retinoic acid (RA) caused a reduction in the level of 1,25(OH)$_2$D$_3$ receptors to 1/3 of control in rat osteoblast-like cells (ROB) while increasing the receptor level to 3-fold the control in mouse osteoblast-like cells (MOB). Scatchard analysis of receptor binding indicated that there was no change in affinity for 1,25(OH)$_2$D$_3$. The changes in receptor levels required time to develop and were dose-dependent. RA also modulated the ability of cells to respond to 1,25(OH)$_2$D$_3$ as measured by the induction of the enzyme 25(OH)D$_3$-24 hydroxylase. Induction of enzyme activity by 1,25(OH)$_2$D$_3$ closely paralleled receptor level established by RA pretreatment. In MOB, the up-regulation of the receptor occurred despite the action of RA to inhibit DNA, RNA and protein synthesis. However, RA stimulation of 1,25(OH)$_2$D$_3$ receptor levels was blocked by the addition of cycloheximide or actinomycin D, indicating that the up-regulation required protein and RNA synthesis. The opposite effect of RA on mouse and rat cells suggests that important species-dependent factors modulate the action of retinoids on mammalian cells.

Vitamin A (retinol) and its natural and synthetic analogues (retinoids) have been found to play an essential role in visual processes, reproduction and the normal growth and differentiation of a number of tissues including bone (1,2). Direct effects of retinol on bone and cartilage were first demonstrated in organ culture by its ability to stimulate bone resorption (3-5). Recently, retinol was also found to inhibit collagen synthesis in embryonic-chick and neonatal-murine calvariae (6) indicating a direct action of vitamin A on osteoblastic bone forming cells.

The actions of vitamin A to stimulate bone resorption and inhibit collagen synthesis are similar to the effects of 1,25(OH)$_2$D$_3$, the active vitamin D metabolite, a hormone essential for maintaining calcium homeostasis (7). A possible inter-relationship between these two bone-acting agent was recently uncovered by the discovery of Petkovich et al (8) who demonstrated that retinoic acid (RA), a potent analogue of vitamin A, increased the binding capacity of 1,25(OH)$_2$D$_3$ receptors in rat osteogenic sarcoma (ROS) cells (8). The present studies are aimed at further delineating the effects of RA to modulate 1,25(OH)$_2$D$_3$ receptors in normal bone cells and to correlate the changes in the 1,25(OH)$_2$D$_3$ receptor

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levels with bioresponses following hormone treatments of cells. We have examined both rat osteoblast-like cells (ROB) and mouse osteoblast-like cells (MOB) and found opposite effects of RA on $1,25(OH)_2D_3$ receptor binding and biological function apparently based on species difference. Of interest, the response in normal rat OB cells was opposite to that reported for malignant rat ROS cells (8).

MATERIALS AND METHODS

Cell culture. Rat and mouse OB cells were obtained from 19-20 day fetal rats and 2-5 day newborn mice by sequential collagenase digestion as previously described (9,10). Cells were plated in primary culture at a density of 2 x $10^4/cm^2$ in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum, and maintained in 50% MEM - 50% bone fluid medium (11) after the first day in culture. For $1,25(OH)_2D_3$ receptor assays, cells were treated at late log growth phase with or without varying concentrations of RA (13-cis isomer, a gift from Dr. Milan Uskokovic, Hoffmann LaRoche, Nutley, NJ.) for 24 h. For 24-hydroylase assay, cultures were induced with 13 nM of 1,25 $(OH)_2D_3$ for an additional 20 h. Both RA and $1,25(OH)_2D_3$ were made as stock solutions with 100% ethanol. The final concentrations of ethanol in culture medium did not exceed 0.5%. Control cultures contained the same amount of ethanol as vehicle.

$1,25(OH)_2D_3$ receptor assay. The binding of receptor to [^{3}H] 1,25 $(OH)_2D_3$ was performed at 0 - 4°C as previously described (9,10). Cultures were washed extensively with ice-cold phosphate-buffered-saline (PBS) and sonicated in hypertonic buffer (KTED, 0.3M KCl, 10mM Tris, 1mM EDTA, 5mM dithiothreitol). After 30 min centrifugation at 207,000 x g, the supernatants were incubated with varying concentrations of [^{3}H] 1,25 $(OH)_2D_3$ (85-100 Ci/mmol, Amersham, Arlington Heights, IL) for 16-20 h. Bound [^{3}H]$1,25(OH)_2D_3$ was separated from free steroid by hydroxylapatite (Bio-Rad) and the bound radioactivity was extracted with ethanol. Specific binding was obtained by subtracting the radioactivity of non-specific binding obtained from parallel samples containing additional 250 fold unlabeled $1,25(OH)_2D_3$.

$25(OH)D_3$ - 24-hydroxylase asssay. After removal of monolayers from culture flasks with collagenase, cell suspensions were incubated with 0.5 μ M [^{3}H]-$25(OH)D_3$ (14-20 Ci/mol, Amersham, Arlington Heights, IL) at 37°C for 30 min in MEM containing 1% calf serum and 10mM HEPES. Cells and medium were then extracted with methanol/chloroform according to the method of Bligh and Dyer (12). The extracts were dried under N_2, brought up in a small volume, and aliquots layered on TLC-strips in a solvent system containing 1:1 of ethyl acetate and methylene chloride to separate [^{3}H] $24,25(OH)_2D_3$ from other [^{3}H] vitamin D metabolites as described by Veith et al. (13). We found that the results of this TLC method were in excellent agreement with the HPLC method we previously described (14). The TLC method had the advantage of allowing simultaneous assay of multiple samples from each treatment in a single experiment.

RESULTS AND DISCUSSION

Species-dependent alteration of $1,25(OH)_2D_3$ receptors by RA. In ROB and MOB, we found that the effects of RA to modulate the $1,25(OH)_2D_3$ receptor were opposite. Following 24 h of treatment, RA down-regulated the $1,25(OH)_2D_3$ receptor in ROB and up-regulated the receptor in MOB. Saturation binding assays were carried out in KTED extracts of control (vehicle) and RA (13 μM) treated cultures by incubating with varying

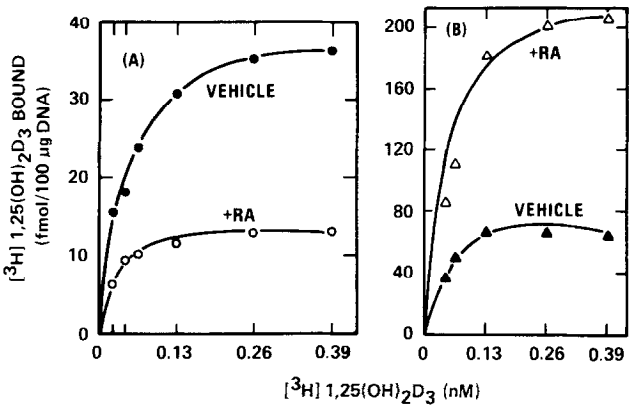

Fig. 1. [^{3}H]1,25(OH)$_2$D$_3$ binding isotherms in (A) Rat and (B) Mouse OB Cells. Cultures were treated ± RA and the KTED extracts of cell pellets were incubated with varying concentrations of [^{3}H]1,25(OH)$_2$D$_3$ at 0°C for 16 h. Bound and free steroids were separated by hydroxylapatite. Specific binding was obtained by subtracting the non-specific binding in samples containing 250-fold excess unlabeled 1,25(OH)$_2$D$_3$ from total binding.

concentrations of [^{3}H] 1,25(OH)$_2$D$_3$. Fig. 1 (A) illustrates the results of such experiments performed in ROB. It shows the binding was substantially less in RA treated cultures compared with vehicle-treated cultures, although both of the binding curves saturated at about the same concentrations of [^{3}H] 1,25(OH)$_2$D$_3$. Scatchard analysis of the data indicates that RA treatment led to a reduction in the maximal binding capacity (N_{max}) to about one-third of control values. There was no significant change in the affinity (K_d) of the receptor for 1,25(OH)$_2$D$_3$ (Table I). Fig. 1(B) illustrates the results of similar experiments conducted in MOB. As opposed to the results from ROB, RA treatment dramatically increased the level of 1,25(OH)$_2$D$_3$ receptors over the level in vehicle-treated

Table I

Effects of RA on 1,25(OH)$_2$D$_3$ Receptors in Rat and Mouse Osteoblasts

	ROB		MOB	
	-RA	+RA	-RA	+RA
N_{max} (fmol/100µg DNA)	45.7 ± 9.6	15.3 ± 2.5	81.7 ± 2.3	235.7 ± 7.9
K_d (10^{-10}M)	0.26 ± 0.04	0.21 ± 0.01	0.37 ± 0.03	0.40 ± 0.06

Scatchard analyses were performed on data from multiple experiments as described in Fig. 1. N_{max} = maximal number of binding sites. K_d = apparent dissociation constant. Values are mean ± range of 2 (ROB) or mean ± SE of 3 experiments (MOB).

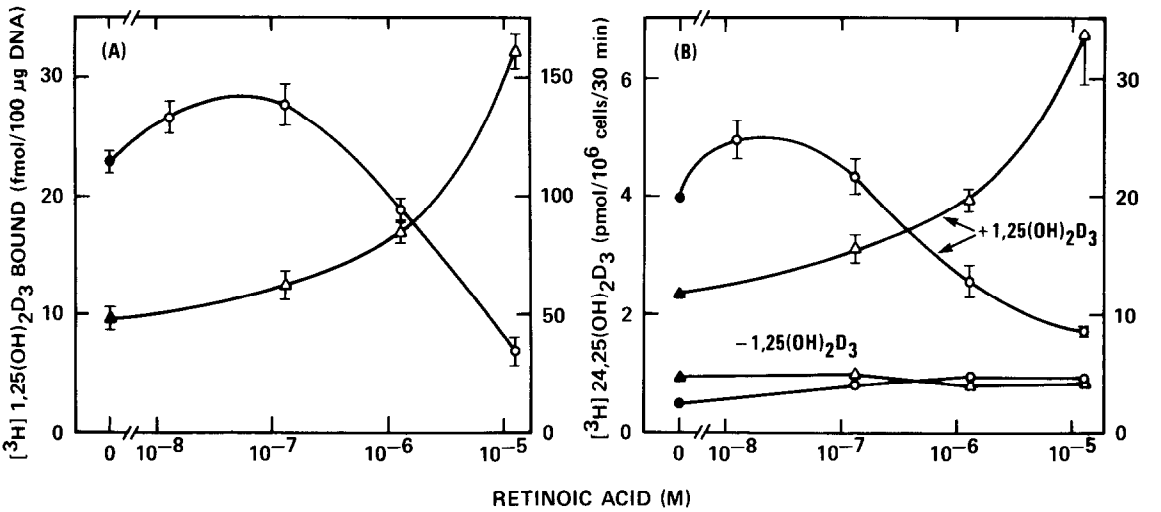

Fig. 2. Effects of RA on $1,25(OH)_2D_3$ receptor and bioresponse. (A) $1,25(OH)_2D_3$ receptors. [^{3}H] $1,25(OH)_2D_3$ (1.3nM) binding was assayed after 24 h of RA treatment. (B) 24-hydroxylase activity. Enzyme activity was assayed after 24 h RA treatment followed by 20 h induction with 13 nM $1,25(OH)_2D_3$. The left ordinates indicate the values for ROB (● o) and the right ordinates depicts the values for MOB (▲ Δ).

cultures. Scatchard analysis of the data shows that RA treatment caused a 2-fold increase in receptors compared to vehicle-treated cultures while the K_d was not changed significantly following RA treatment (Table I). It should be noted that addition of RA directly to cytosol extracts has no effect on receptor binding.

Modulation of $1,25\ (OH)_2D_3$ receptor by RA is dose-dependent. To further examine the effect of RA in rodent bone cells, cultures were treated with varying concentrations of RA and the level of $1,25(OH)_2D_3$ receptors assayed. There were dose-dependent changes in both ROB and MOB by RA treatment as shown in Fig 2(A). In ROB, the levels of $1,25(OH)_2D_3$ receptors were slightly increased at low RA concentrations (0.013 and 0.13 µM). This was followed by substantial decreases at the higher concentrations of 1.3 and 13 µM. In contrast, MOB responded to RA with a dose-dependent increase in the level of $1,25(OH)_2D_3$ receptors. The increase was consistent between 0.13 µM and 13 µM. For both MOB and ROB the effects of RA to modulate $1,25(OH)_2D_3$ receptors did not appear to reach a plateau at 13 µM RA. We did not study higher concentrations.

Correlation of cell responsiveness to $1,25(OH)_2D_3$ with changes in $1,25(OH)_2D_3$ receptor levels. In order to determine whether modulation of $1,25(OH)_2D_3$ receptor levels by RA resulted in changes in the capacity of $1,25(OH)_2D_3$ to mediate a bioresponse, we assessed the ability of $1,25(OH)_2D_3$ to induce the enzyme $25(OH)D_3$-24-hydroxylase (24-

Table II

Effect of Inhibitors of Translation and Transcription on the Ability of RA to Up-regulate of $1,25(OH)_2D_3$ Receptors in Mouse Osteoblasts

Treatment	Receptor level		RNA synthesis		Protein Synthesis	
	-RA	+RA	-RA	+RA	-RA	+RA
Vehicle	100	423±41**	100	86±2*	100	69±2**
Cycloheximide	80±16	146±19*	80±7*	65±3**	49±6**	54±1**
Actinomycin D	137±23	137±30	50±5**	32±3**	17±3**	14±2**

MOB were treated with or without RA for 24 h. Inhibitors were added 2 h prior to RA treatments. $1,25(OH)_2D_3$ receptor levels were measured with 1.3 nM [^{3}H]$1,25(OH)_2D_3$. RNA and protein synthesis were assayed in parallel cultures with [^{3}H]uridine (1 μ Ci/ml) and [^{14}C] leucine (0.5 μ Ci/ml) incorporations into TCA insoluble fractions. Values are expressed as % of control level treated with vehicle only. The control value for receptor level is 38.9±2 fmol/100 μ g DNA; the values for RNA and protein synthesis are 163±3 dpm/mg protein/h and 40±2 dpm/mg protein/h respectively.

Significantly different from control values at *$p<0.05$, **$p<0.001$.

hydroxylase). This enzyme has been found to be induced by $1,25(OH)_2D_3$ in all $1,25(OH)_2D_3$ target tissues studied thus far (14-16). ROB and MOB were pretreated with varying concentrations of RA for 24 h prior to the addition of 13 nM $1,25(OH)_2D_3$ for enzyme induction. The results illustrated in Fig. 2(B) clearly indicate that the induction of enzyme activity by $1,25(OH)_2D_3$ was modulated by RA and closely paralleled the changes in receptor levels. In ROB, RA caused a dose-dependent reduction of $1,25(OH)_2D_3$ induced enzyme activity. In contrast, increasing concentrations of RA potentiated $1,25(OH)_2D_3$ induced enzyme activity in MOB. RA alone, in the absence of $1,25(OH)_2D_3$, did not alter the basal levels of enzyme activity for either cell type.

Cellular mechanisms for the RA effect on $1,25(OH)_2D_3$ receptors. We also found that RA inhibited DNA, RNA and protein synthesis in ROB and MOB. In MOB, RNA synthesis was inhibited ↶ 15%, protein synthesis was inhibited ↶ 30% (Table II), and DNA synthesis was inhibited ↶ 40%. It is particularly interesting that RA stimulates an increase in the number of $1,25(OH)_2D_3$ receptors in MOB despite inhibiting DNA synthesis since we previously showed that inhibition of DNA synthesis is associated with decreased receptor levels in MOB (17). To further explore the mechanism of the RA effect to increase $1,25(OH)_2D_3$ receptors in MOB, we additionally treated the cells with cycloheximide and actinomycin D, inhibitors

of protein and RNA synthesis, respectively. The results are illustrated in Table II. In the absence of RA, cycloheximide (1.0 μ g/ml) and actinomycin D (0.05 μ g/ml) caused only minimal changes in the basal level of $1,25(OH)_2D_3$ receptors. When the inhibitors were added in addition to RA, the increase in receptor levels normally seen with RA treatment was blocked. The results indicate that both protein and RNA synthesis are required for the augmentation of $1,25(OH)_2D_3$ receptor levels by RA in MOB. Although RA causes a general inhibition of protein and RNA synthesis, it specifically increases the receptor by either selectively stimulating the synthesis of receptor protein or by reducing the level of enzyme(s) involved in the degradation of the receptor.

The present studies indicate that RA is a potent modulator of $1,25(OH)_2D_3$ receptors in ROB and MOB. Furthermore, the alterations in receptor levels resulted in corresponding changes in the ability of $1,25(OH)_2D_3$ to mediate a bioresponse as measured by the induction of 24-hydroxylase activity. The regulation of $1,25(OH)_2D_3$ receptors in these cells are diametrically opposite to the action of glucocorticoids which up-regulate $1,25(OH)_2D_3$ receptors in ROB (9) and down-regulate the receptors in MOB (10). The species difference in the RA effect on $1,25(OH)_2D_3$ receptors is not due to the age of the animal (fetal rats vs. newborn mice), since we found that OB cells from newborn rats responded the same as cells from fetal rats (data not shown). In contrast to the down-regulation we have demonstrated in normal OB cells from rat, RA induced $1,25(OH)_2D_3$ receptor up-regulation in malignant rat ROS cells (8). Present studies suggest that there are important species-dependent factors which modulate the actions of retinoids on mammalian cells and that within the same species normal and malignant cells respond differently. Further investigation will be necessary to elucidate the mechanism for these phenomena.

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