# RETINOL AND RETINOIC ACID MODULATE THE METABOLISM OF 25-HYDROXYVITAMIN $D_3$ IN KIDNEY CELL CULTURE

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## 1. Introduction

Vitamins A and D play important roles in the normal development of the skeleton [1–3]. Although vitamin A and its metabolite retinoic acid are thought to act directly at the bone level, they could also influence bone metabolism indirectly through an action on vitamin $D_3$ metabolism. $1,25(OH)_2D_3$, the most active hormonal form known of vitamin $D_3$, as well as $24,25(OH)_2D_3$ is formed in the renal proximal tubule through hydroxylation of $25(OH)D_3$ [4,5]. In vivo, the production of $1,25(OH)_2D_3$ is tightly regulated by a complex multifactorial control system [6–8]. The possibility of an influence of vitamin A on $25(OH)D_3$-hydroxylation is suggested by findings showing that the renal proximal tubule is also a target for vitamin A which is taken up and probably also metabolized at this site, retinoic acid being one of its metabolites [9,10]. Retinol and retinoic acid may modulate the 11 $\beta$-hydroxylase of corticosteroids [11], which is an enzyme similar to the renal $25(OH)D_3$-1-hydroxylase [7,12]. Cell culture systems represent a useful tool to study the modulation of renal $25(OH)D_3$ metabolism in vitro [13–17]. Here, we demonstrate that retinol and retinoic acid can modify the metabolism of $25(OH)D_3$ in primary chick kidney cell culture, suggesting that these agents might play a role in the modulation of the renal $25(OH)D_3$-metabolism in vivo.

## 2. Materials and methods

Primary chick kidney cell cultures were produced and the $25(OH)D_3$-hydroxylases assayed as in [16]. Briefly, kidneys of 10-day-old chicks were digested with collagenase and hyaluronidase. $10^6$ Cells were plated in Petri dishes, 3.5 cm diam., in 1.5 ml minimum essential medium (MEM) containing antibiotics and 10% fetal calf serum. The medium contained 1.2 mM $Ca^{2+}$. To create conditions similar to those present in vitamin D-replete birds, the cells were cultured in the presence of $25(OH)D_3$ in the medium [16]; 2 × 200 pmol $25(OH)D_3$ were added 24 h and 48 h after plating. Confluence was attained after 3 days. $25(OH)D_3$, retinol, retinyl acetate, retinoic acid, actinomycin D and cycloheximide were added in 7.5–15 $\mu$l ethanol. Control dishes received ethanol alone. $^3$H-Labelled $25(OH)D_3$-metabolism was investigated by incubating the cultures for 30 min with 100 pmol $25(OH)[^3H]D_3$ added to 1 ml MEM containing no fetal calf serum, $25(OH)D_3$, retinol or retinoic acid. Tritiated vitamin $D_3$ metabolites were extracted into chloroform/methanol and analyzed by high-pressure liquid chromatography (HPLC). 80 ± 3% (mean ± SD) of the radioactivity applied to the HPLC column was recovered in the chromatograms of 99 extracts. There were no significant differences in recovery between groups of the same experiment. Results are expressed as the percentage of the radioactivity recovered from the HPLC. DNA was measured as in [18]. Synthetic $25(OH)D_3$ was kindly donated by Hoffmann-La Roche (Basle). Retinol, retinoic acid, retinyl acetate, were from Sigma (St Louis MO). Actinomycin D was from Calbiochem (San Diego CA), cycloheximide from Fluka (Buchs), Eagle's essential medium and antibiotics from Seromed GmbH (Munich), fetal calf serum from North American Biologicals (Miami FA). Collagenase (type I) and hyalu-

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ronidase (type I) were from Sigma. Organic solvents and all other chemicals were analytical grade.

## 3. Results

As shown in fig.1, retinol and retinoic acid added for 48 h to primary chick kidney cell cultures produced a dose-related stimulation of the 25(OH)$D_3$-1-hydroxylase activity, retinoic acid being ~50-times more potent. Maximum effects were observed with $10^{-7}$ M retinoic acid and with $10^{-6}$ M retinol. The decrease of the 1-hydroxylase with the highest dose of retinol might have been due to a toxic effect on the cells. The effects of the compounds on the 25(OH)$D_3$-24-hydroxylase were not consistent. In fig.1 this enzyme was not significantly altered at any dose-level of retinol or retinoic acid. In other experiments, however, e.g., that of table 1 the 24-hydroxylase activity was significantly decreased. Table 1 demonstrates that the stimulatory effect of retinoic acid on the 1-hydroxylase was not due to an increased cell number, since the compound did not increase the DNA content of the cultures.

To assess the time-course of the effects of retinol and retinoic acid on the 25(OH)$D_3$-metabolism, kidney cell cultures were incubated with the agents for shorter time intervals. Table 2 shows that an 18 h incubation of cultures with retinoic acid led only to a small increase of the 1-hydroxylase whereas retinol had no effect, even at $10^{-6}$ M, a level which was highly effective at 48 h. Interestingly, at 18 h, the 24-hydroxylase activity was increased by $10^{-6}$ M retinol or retinoic acid. When the incubation time was reduced

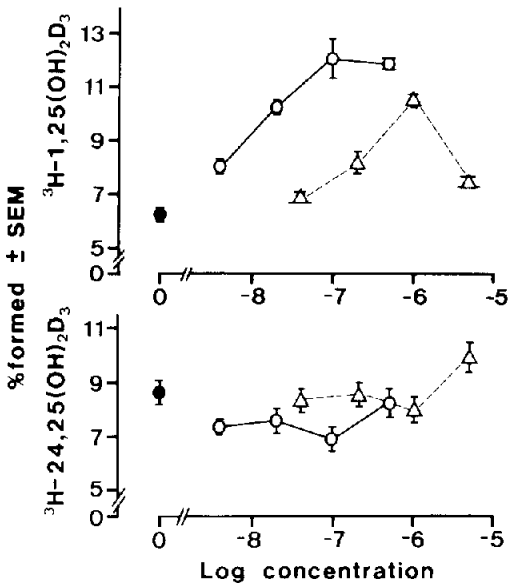


Fig.1. Chronic effect (48 h) of retinol (△——△) and of retinoic acid (○——○) on the metabolism of 25(OH)[$^3$H]$D_3$ in primary chick kidney cell culture. Cells were cultured and the 25(OH)$D_3$-hydroxylases assayed as in section 2. Retinol and retinoic acid were added 24 h and 48 h after plating. 25(OH)[$^3$H]$D_3$-metabolism was investigated 48 h after the first addition of the agents. Values are means ± SEM, $n = 8$ for controls, $n = 4$ for treated. The effect of retinol on 1,25(OH)$_2$[$^3$H]$D_3$ production was statistically significant at $2 \times 10^{-7}$ M ($p < 0.05$) and $10^{-6}$ M ($p < 0.001$), the effect of retinoic acid at $4 \times 10^{-9}$ M ($p < 0.01$) and at all other concentrations ($p < 0.001$). Statistical significance was not reached for any of the 24,25(OH)$_2$[$^3$H]$D_3$ values. Statistical analysis was by Student's $t$-test.

Table 1
Chronic effects (48 h) of retinoic acid on the metabolism of $^3$H-labelled 25(OH)$D_3$ and on DNA content in kidney cell culture

| Retinoic acid ($10^{-6}$ M) | DNA (μg/dish) | Percent conversion to | |
|---|---|---|---|
| | | 24,25(OH)$_2$[$^3$H]$D_3$ | 1,25(OH)$_2$[$^3$H]$D_3$ |
| – | 2.35 ± 0.03 (5)[a] | 7.0 ± 0.4 (4) | 6.6 ± 0.3 (4) |
| + | 2.22 ± 0.04 (6) | 5.0 ± 0.3 (4)[b] | 11.8 ± 0.2 (4)[c] |

[a] Number of replicates in parentheses

Values are means ± SEM; [b] $p < 0.001$; [c] $p < 0.001$ *vs* control by Student's $t$-test

Experimental protocol as in fig.1. The compounds were added at 24 h and again at 48 h after plating. 25(OH)$D_3$-hydroxylases were assayed and parallel cultures were prepared for DNA determination 48 h after the first addition of retinoic acid

Table 2
Effects of retinol and of retinoic acid added for 18 h on the metabolism of $^3$H-labelled 25(OH)$D_3$ in kidney cell culture

| Agent | Conc. (M) | $n$ | Percent conversion to | |
|---|---|---|---|---|
| | | | 24,25$(OH)_2$[$^3$H]$D_3$ | 1,25$(OH)_2$[$^3$H]$D_3$ |
| – | – | 4 | 4.6 ± 0.2 | 2.8 ± 0.1 |
| Retinoic acid | $10^{-7}$ | 4 | 5.2 ± 0.2 | 3.5 ± 0.1[a] |
| | $10^{-6}$ | 4 | 5.6 ± 0.2[a] | 3.4 ± 0.1[a] |
| Retinol | $10^{-7}$ | 4 | 4.9 ± 0.3 | 2.8 ± 0.1 |
| | $10^{-6}$ | 4 | 5.4 ± 0.1[a] | 2.7 ± 0.2 |

Values are means ± SEM; [a] $p < 0.05$ *vs* control by Student's *t*-test

Cells were cultured as in section 2. Retinol and retinoic acid were added 2 days after plating; 18 h later the 25(OH)$D_3$-hydroxylases were assayed

to 6 h, retinol and retinoic acid produced a dose-related stimulation of the 24-hydroxylase and a decrease of the 1-hydroxylase (table 3). No effect of either retinol or retinoic acid on 25(OH)$D_3$-metabolism was observed after a 1 h incubation.

## 4. Discussion

From our results it appears that the stimulatory effect of retinol and of retinoic acid on the 1-hydroxylase is preceded by a stimulation of the 24-hydroxylase. The initial decrease of the 1-hydroxylase observed with both retinol and retinoic acid might be due to an inhibitory effect on enzyme already present, or to a decreased synthesis or an increased breakdown of enzyme components. Alternatively, however, it might be due to a decreased availability of the substrate to the 1-hydroxylase as a consequence of the increased 24-hydroxylation. Indeed changes in the accessibility of 25(OH)$D_3$ to the hydroxylating enzymes have been suggested to play a role in the regulation of the renal 25(OH)$D_3$-hydroxylases [7]. The chronic increase of the 1-hydroxylase activity might then be responsible

Table 3
Short-term effect (6 h) of retinol and of retinoic acid on the metabolism of $^3$H-labelled 25(OH)$D_3$ in primary chick kidney cell culture

| Agent | Conc. (M) | $n$ | Percent conversion to | |
|---|---|---|---|---|
| | | | 24,25$(OH)_2$[$^3$H]$D_3$ | 1,25$(OH)_2$[$^3$H]$D_3$ |
| Retinol | 0 | 4 | 4.1 ± 0.4 | 3.4 ± 0.1 |
| | $10^{-7}$ | 4 | 4.7 ± 0.4 | 3.5 ± 0.1 |
| | $10^{-6}$ | 4 | 5.5 ± 0.4 | 3.1 ± 0.2 |
| | $10^{-5}$ | 4 | 5.5 ± 0.1[a] | 1.5 ± 0.1[c] |
| Retinoic acid | 0 | 4 | 3.4 ± 0.2 | 4.1 ± 0.1 |
| | $10^{-7}$ | 4 | 4.9 ± 0.3[b] | 3.6 ± 0.1[b] |
| | $10^{-6}$ | 4 | 5.8 ± 0.3[c] | 3.4 ± 0.1[c] |
| | $10^{-5}$ | 4 | 7.1 ± 0.3[c] | 2.6 ± 0.1[c] |

Values are means ± SEM; [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ by Student's *t*-test *vs* respective controls

Cells were cultured as in section 2. Retinol and retinoic acid were added 3 days after plating; 6 h later the 25(OH)$D_3$-hydroxylases were assayed as in fig.1

for the decrease of the apparent 24-hydroxylase activity to levels observed in control cultures at 48 h incubation with retinol or retinoic acid by decreasing the availability of substrate to the 24-hydroxylase.

The apparent greater potency of retinoic acid than of retinol to elicit a chronic stimulation of the 1-hydroxylase is interesting, since it is consistent with the hypothesis that retinoic acid might be one of the active metabolites of retinol [3]. It should be noted, however, that the difference in potency observed here might be due to a difference in binding of the compounds to the retinol-binding protein present in the serum-containing medium.

Although several factors such as PTH, vitamin $D_3$ metabolites, steroid hormone, growth hormone, prolactin, insulin and prostaglandins have been shown to influence the renal 25(OH)$D_3$-hydroxylase, the physiological regulation of this enzyme is not yet fully understood [6–8]. The question arises whether vitamin A might have a function in the modulation of the renal 25(OH)$D_3$-metabolism in vivo. Retinol itself is unlikely to be a modulator, since a regulation of its plasma concentration has not been demonstrated. Plasma retinol has been found to be rather constant as long as liver stores of the vitamin are sufficient in rats [19]. Retinoic acid, however, might influence the 1-hydroxylase. The kidney has been shown to metabolize retinol to retinoic acid [9]. It is therefore tempting to speculate that retinoic acid produced locally in the kidney might modulate the renal metabolism of 25(OH)$D_3$. Further work is needed to investigate this possibility.

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