SPECIFIC NUCLEAR UPTAKE OF 24,25-DIHYDROXYCHOLECALCIFEROL, A VITAMIN D_3 METABOLITE BIOLOGICALLY ACTIVE IN CARTILAGE

M. CORVOL†, A. ULMANN+ and M. GARABEDIAN*

*Hôpital des Enfants-Malades, †INSERM U. 30, +INSERM U. 90, *INSERM U. 30 et CNRS ER 126, 149, rue de Sèvres, 75730 Paris Cédex 15, France*

Received 10 June 1980

1. Introduction

Vitamin D_3 is metabolized in the course of two hydroxylations: the first, in the liver, yields 25-(OH)D_3; the second, in the kidney, takes place in positions 1α and 24 and produces 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. However, the kidney is probably not the sole production site for the latter sterol, since we demonstrated that cultured chondrocytes and cartilage tissue in vitro transform 25-(OH)D_3 into 24,25-$(OH)_2D_3$ [1]. The biological effect of this last dihydroxylated metabolite is not yet clear, but we showed earlier that in cultured chondrocytes, 24,25-$(OH)_2D_3$ stimulates both the synthesis and secretion of proteoglycans [2] as well as DNA polymerase activities [3]. Such stimulation was obtained with 24,25-$(OH)_2D_3$ at 10^{-12} M, suggesting that this metabolite is intrinsically active in chondrocytes at physiological concentrations. These results were the first in vitro demonstration that, at least in cartilage, 24,25-$(OH)_2D_3$ is an active vitamin D metabolite rather than a product of degradation. In that case, the action of 24,25-$(OH)_2D_3$ in chondrocytes might resemble that of other steroids in their target cells. Such cellular action involves temperature-dependent nuclear penetration of the sterol that precedes sterol-specific mRNA synthesis.

With this in mind, we looked for nuclear penetration of 24,25-$(OH)_2$[^{3}H]D_3 using the above cultured chondrocyte model.

Abbreviations: 25-(OH)D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 24,25-$(OH)_2$[^{3}H]D_3, tritiated 24,25-$(OH)_2D_3$; 25-(OH)[^{3}H]D_3, tritiated 25-(OH)D_3; FCS, fetal calf serum; GBS, Gey's buffer solution

2. Materials and methods

The general scheme of the experiments was as follows: chondrocyte cultures were started after enzyme digestion of 300 g rabbit growth plate cartilage. The tibiae were microdissected under sterile conditions and digested with 0.2% Trypsin (Worthington) and 0.2% clostridial collagenase (Boehringer) in GBS. Large culture flasks (75 cm^2) were inoculated with the chondrocyte suspension thus obtained (3.5×10^5 cells/flask) and cultured as in [4]. Each flask contained 14 ml Dulbecco's medium (Gibco-Flobio) supplemented with 10% FCS, 0.1 unit/ml penicillin and 0.1 g/ml streptomycin. Flasks were gassed with 10% CO_2 in air and maintained at 37°C for 19 days. The culture medium was changed 3 times a week.

On day 20 of culture, the chondrocytes stopped dividing. The medium was replaced with FCS-free Dulbecco's medium. Cells were divided into groups of 4 identical flasks each (80–100 μg DNA/flask). To each flask was added either 0.1% ethanol alone or 24,25-$(OH)_2D_3$ (a gift from Dr Uskokovic, Hoffman La Roche, Nutley, NJ), or 25-(OH)D_3 (a gift from Laboratoires Roussel, Paris). Flasks were placed at 37°C for an additional 20 h incubation prior to incubation with the tritiated sterols.

Cells were gently scraped off, pooled, centrifuged at $300 \times g$ for 3 min and resuspended in glass vials containing 4 ml Dulbecco's medium. Each group was incubated with 24,25-$(OH)_2$[^{3}H]D_3 or, in some experiments, with 25-(OH)[^{3}H]D_3. 25-(OH)[^{3}H]D_3 (90–100 Ci/mmol) was purchased from Amersham, (Versailles) and 24,25-$(OH)_2$[^{3}H]D_3 (45–50 Ci/mmol) was prepared from 25-(OH)[^{3}H]D_3 as in [1] or purchased from Amersham (Versailles).

The pure nuclear fractions were then prepared at

4°C as in [5]. Briefly, cells were centrifuged at 300 × *g*, rinsed 3 times with GBS and homogenized in TSM buffer containing 10 mM Tris–HCl (pH 7.4), 250 mM sucrose and 1 mM $MgCl_2$. The homogenates were centrifuged at 800 × *g* for 10 min. The resulting crude nuclear pellets were rinsed 4 times in TSM buffer, each rinse being followed by brief centrifugation (800 × *g* for 10 min). The final pellet was mixed with 10 ml 2 M sucrose solution and centrifuged at 50 000 × *g* for 60 min in a Sorvall RC 5 centrifuge. The resulting purified nuclear pellets were taken up in 1 ml TSM, rinsed and transferred to TSM containing Triton X-100 (1% final conc.), mixed and left for 10 min at 4°C. The nuclei were then collected and washed twice with Triton-free TSM, each wash being followed by 2 min centrifugation at 3200 × *g*. The purity of the preparation thus obtained was verified by phase contrast microscopy.

Purified nuclei were suspended in 1 ml methanol–chloroform solution (2:1, v/v) and left for 18 h at 4°C. They were then centrifuged at 3200 × *g* for 5 min. The supernatant was dried, and taken up with 5 ml Instafluor (Packard). Radioactivity was counted during 10–50 min with an SL 30 Intertechnique Scintillation Counter with 35% efficiency for tritium. DNA content of the pellet (20–40 μg DNA/nuclear extract) was assayed according to [6] with calf thymus DNA as standard.

In some experiments, 24,25-$(OH)_2$ [^{3}H]D_3 (100 pmol/mg nuclear DNA) was incubated with the cultured cells for 20 h. The radioactive pellet was then extracted from purified nuclei with 2 ml methanol and 1 ml chloroform. The chloroform phase was dried under nitrogen and redissolved in 150 μl ethanol. An aliquot was then counted and the rest of this extract was submitted to high-pressure liquid chromatography (Waters Associates, Milford, MA) as in [1]. An aliquot of each fraction of the effluent was evaporated, dissolved in 5 ml counting solution and counted. The radioactivity recovered in the form of 24,25-$(OH)_2D_3$ was evaluated as % of the radioactivity applied.

3. Results

After 20 h/incubation of 24,25-$(OH)_2$ [^{3}H]D_3 with cultured chondrocytes, 91% of the radioactivity extracted from purified nuclei is recovered in the form of 24,25-$(OH)_2D_3$ by high pressure liquid chromatography. 24,25-$(OH)_2$ [^{3}H]D_3 nuclear uptake is therefore expressed in fmol/mg DNA in the following experiments.

3.1. *Nuclear uptake of 24,25-$(OH)_2$[^{3}H]D_3 at 37°C or 4°C*

In a set of results for 6 separate experiments, cultured chondrocytes, some of which had been preincubated for 20 h at 37°C with unlabelled 24,25-$(OH)_2D_3$ (250 pmol/culture flask, i.e., 10 nmol/mg nuclear DNA) were incubated at 37°C for 30 min with concentrations of 24,25-$(OH)_2$ [^{3}H]D_3 ranging from 2.8 × 10^4 to 1.1 × 10^6 dpm/flask, i.e., 20–180 pmol/mg nuclear DNA. Uptake of 24,25-$(OH)_2$ [^{3}H]D_3 in purified nuclei (defined as total uptake) increased with the 24,25-$(OH)_2$ [^{3}H]D_3 concentration in the culture medium. For a given concentration, this uptake was 49–63% lower in preincubated cells. Since further metabolic transformation of 24,25-$(OH)_2D_3$ has not yet been demonstrated in cultured chondrocytes, the 24,25-$(OH)_2$ [^{3}H]D_3 is defined here as non-specific uptake, and the difference between total and non-specific uptake, as specific uptake, shown in fig.1. No such specific uptake was demonstrated when cells were incubated with 24,25-$(OH)_2$ [^{3}H]D_3 at 4°C for 30 min.

3.2. *Time-course of 24,25-$(OH)_2$[^{3}H]D_3 nuclear uptake*

Incubation of 24,25-$(OH)_2$ [^{3}H]D_3 (100 pmol/mg nuclear DNA) at 37°C was studied for various periods. Maximum specific 24,25-$(OH)_2$ [^{3}H]D_3 nuclear uptake was reached at 30 min and declined considerably by 240 min (fig.2). The incubation period chosen for all further experiments with 24,25-$(OH)_2$ [^{3}H]D_3 was therefore 30 min.

3.3. *Specificity of 24,25-$(OH)_2$[^{3}H]D_3 nuclear uptake*

The specificity of the tritiated 24,25-$(OH)_2$ [^{3}H]D_3 nuclear uptake was measured in cultured chondrocytes pre-incubated for 20 h in medium containing 10 nmol/mg DNA of one of the following: unlabelled 24,25-$(OH)_2D_3$, 25-(OH)D_3, 1,25-$(OH)_2D_3$, vitamin D_3, or progesterone, or sterol-free medium (control uptake). Chondrocytes were then incubated for 30 min at 37°C with 24,25-$(OH)_2$ [^{3}H]D_3 (350 pmol/mg nuclear DNA). Results are shown in table 1. Compared to control uptake, there was no apparent decrease in 24,25-$(OH)_2$ [^{3}H]D_3 in the cells preincubated with progesterone or vitamin D_3. Preincubation with 1,25-

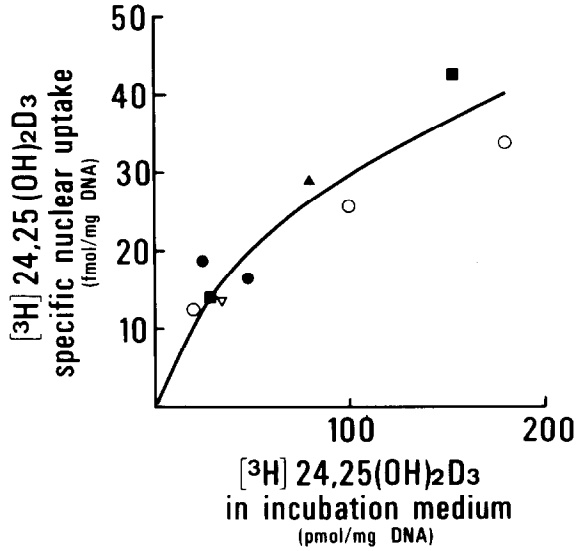

Fig.1. Specific nuclear uptake of 24,25-$(OH)_2$[^{3}H]D_3 in cultured chondrocytes incubated with increasing amounts of 24,25-$(OH)_2$[^{3}H]D_3. In this set of 6 separate expt, cultured chondrocytes, some of which had been preincubated for 20 h at 37°C with unlabelled 24,25-$(OH)_2D_3$ (250 pmol/ culture flask, i.e., 10 nmol/mg nuclear DNA), were incubated at 37°C for 30 min with 24,25-$(OH)_2$[^{3}H]D_3 at 2.8×10^4–1.1×10^6 dpm/flask (20–180 pmol/mg nuclear DNA). For a given concentration, specific uptake is the difference between total uptake measured in cells not-preincubated with unlabelled 24,25-$(OH)_2D_3$ and non-specific uptake measured in cells preincubated with unlabelled 24,25-$(OH)_2D_3$.

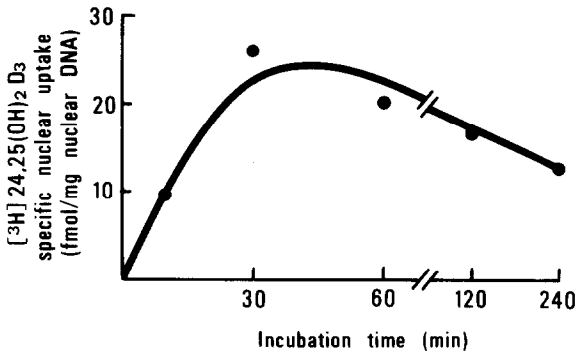

Fig.2. Specific nuclear uptake of 24,25-$(OH)_2$[^{3}H]D_3 in cultured chondrocytes incubated with this tritiated compound (100 pmol/mg nuclear DNA) during 10, 30, 60, 120 or 240 min at 37°C. For each time studied, specific nuclear uptake was calculated as explained in fig.1 from data obtained in cells preincubated or not with non-radioactive 24,25-$(OH)_2D_3$.

$(OH)_2D_3$ led to a 25% increase in 24,25-$(OH)_2$[^{3}H]D_3 nuclear uptake, a result which might be the cellular reflection of a synergistic effect exerted by 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$. Preincubation with 25-$(OH)D_3$ reduced 24,25-$(OH)_2D_3$ nuclear uptake by 25%.

3.4. *Nuclear uptake of 25-(OH)[^{3}H]D_3*

Cultured chondrocytes, whether or not preincubated with unlabelled 25-$(OH)D_3$ (250 nmol/ cultured flask), were further incubated with 25-(OH)[^{3}H]D_3 (50 or 500 pmol/mg nuclear DNA) for 30 min at 37°C. No specific nuclear uptake was demonstrated for 25-(OH)[^{3}H]D_3. The decrease in 24,25-$(OH)_2$[^{3}H]D_3 uptake in cells preincubated with 25-$(OH)D_3$ might therefore have been the result of the competition with the unlabelled 24,25-$(OH)_2D_3$ released by the transformation of 25-$(OH)D_3$ during preincubation [1].

Table 1
Specificity of 24,25-$(OH)_2$[^{3}H]D_3 nuclear uptake in purified nuclei from cultured rabbit chondrocytes

Unlabelled sterol in preincubation medium	24,25-$(OH)_2$[^{3}H]D_3 nuclear uptake (% of control)
None (control), n[a] = 10	100
24,25-$(OH)_2D_3$, n = 5	49–63 (54.5)[b]
25-$(OH)D_3$, n = 2	70–80
1,25-$(OH)_2D_3$, n = 3	98–160 (125)
Vitamin D_3, n = 1	104
Progesterone, n = 2	100–100

[a] n, number of experiments; [b] mean uptake is in parentheses

Chondrocytes were preincubated for 20 h at 37°C with one of the nonradioactive sterol (10 nmol/mg nuclear DNA), or with sterol-free medium (control). They were then incubated with 24,25-$(OH)_2$[^{3}H]D_3 for an additional 30 min at 37°C

3.5. *Reversibility of 24,25-$(OH)_2$[^{3}H]D_3 nuclear uptake*

We measured the reversibility of the tritiated 24,25-$(OH)_2$[^{3}H]D_3 nuclear uptake, using cultured chondrocytes incubated for 30 min at 37°C with 24,25-$(OH)_2$[^{3}H]D_3. Cells were then transferred either to a sterol-free medium or to a medium containing non-radioactive 24,25-$(OH)_2D_3$ (10 nmol/mg nuclear DNA) and incubated at 37°C for an additional 15 min. The 24,25-$(OH)_2D_3$ nuclear uptake was reversed by 44% when the chondrocytes were transferred to a medium containing unlabelled 24,25-$(OH)_2D_3$. When these cells were incubated in control sterol-free medium, uptake was only 24% reversible.

4. Discussion

Our results indicate that 24,25-$(OH)_2D_3$ acts in cultured rabbit chondrocytes in a similar way to steroid hormones in their target cells. Such a result therefore provides direct evidence for intrinsic action by 24,25-$(OH)_2D_3$ in these chondrocytes. However, 24,25-$(OH)_2D_3$ is not the only vitamin D metabolite active in cartilage in vitro, since we demonstrated earlier that 1,25-$(OH)_2D_3$ also increases sulfated proteoglycan synthesis [2] and DNA polymerase activities [3]. These chondrocytes therefore appear to be sensitive to both 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ at concentrations of the same order of magnitude. Such sensitivity raises the question of whether or not these sterols share a common cellular mechanism in cartilage tissue. Our results already support the hypothesis of an interrelated mechanism, since preincubation with 1,25-$(OH)_2D_3$ increased the nuclear uptake of 24,25-$(OH)_2D_3$.

Further experiments are necessary to clarify the interaction between 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$. For this purpose, the chondrocyte model seems very suitable, since it allows combined study of nuclear sterol binding and the resulting biological effects.

Acknowledgements

We thank Mrs M. F. Dumontier, Mrs L. Tsagris and Mrs M. Brami for excellent technical assistance. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (ATP 42-76-74) and from the Direction Générale de la Recherche Scientifique et Technique no. 79.7.1239.

References

[1] Garabédian, M., Bailly du Bois, M., Corvol, M. T., Pezant, E. and Balsan, S. (1978) Endocrinology 102, 1262–1268.
[2] Corvol, M. T., Dumontier, M. F., Garabédian, M. and Rappaport, R. (1978) Endocrinology 102, 1269–1274.
[3] Corvol, M. T., Dumontier, M. F., Ulmann, A., Garabédian, M. and Cournot-Witmer, G. (1979) in: Vitamin D basic research and its clinical application (Norman, A. W. et al. eds) pp. 419–424, de Gruyter, Berlin, New-York.
[4] Corvol, M. T., Dumontier, M. F. and Rappaport, R. (1975) Biomedicine 23, 103–107.
[5] Bachelet, M., Ulmann, A., Cloix, J. F. and Funck-Brentano, J. L. (1977) J. Ster. Biochem. 8, 1047–1049.
[6] Burton, K. (1956) Biochem. J. 62, 315–323.