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1,25-Dihydroxycholecalciferol (calcitriol) modifies uptake and release of 25-hydroxycholecalciferol in skeletal muscle cells in culture

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ABSTRACT

The major circulating metabolite of vitamin D_3, 25-hydroxycholecalciferol [25(OH)D], has a remarkably long half-life in blood for a (seco)steroid. Data from our studies and others are consistent with the hypothesis that there is a role for skeletal muscle in the maintenance of vitamin D status. Muscle cells internalise vitamin D-binding protein (DBP) from the circulation by means of a megalin/cubilin plasma membrane transport mechanism. The internalised DBP molecules then bind to actin and thus provide an intracellular array of high affinity binding sites for its specific ligand, 25(OH)D. There is evidence that the residence time for DBP in muscle cells is short and that it undergoes proteolytic degradation, releasing bound 25(OH)D. The processes of internalisation of DBP and its intracellular residence time, bound to actin, appear to be regulated. To explore whether 1,25-dihydroxycholecalciferol (calcitriol) has any effect on this process, cell cultures of myotubes and primary skeletal muscle fibers were incubated in a medium containing 10^{-10} M calcitriol but with no added DBP. After 3 h pre-incubation with calcitriol, the net uptake of 25(OH)D by these calcitriol-treated cells over a further 4 h was significantly greater than that in vehicle-treated control cells. This was accompanied by a significant increase in intracellular DBP protein. However, after 16 h of pre-incubation with calcitriol, the muscle cells showed a significantly depressed ability to accumulate 25(OH)D compared to control cells over a further 4 or 16 hours. These effects of pre-incubation with calcitriol were abolished in fibers from VDR-knockout mice. The effect was also abolished by the addition of 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), which inhibits chloride channel opening. Incubation of C2 myotubes with calcitriol also significantly reduced retention of previously accumulated 25(OH)D after 4 or 8 h. It is concluded from these in vitro studies that calcitriol can modify the DBP-dependent uptake and release of 25(OH)D by skeletal muscle cells in a manner that suggests some inducible change in the function of these cells.

1. Introduction

Our knowledge about the role of vitamin D continues to expand beyond its classical roles in calcium and phosphate homeostasis. The concentration of 25-hydroxycholecalciferol (25(OH)D) in blood is usually taken as an index of vitamin D status. However, it gives no indication of the total body pool size of 25(OH)D.

There is now evidence that skeletal muscle cells contain a mobile pool of 25(OH)D which accumulates from and returns to the extracellular environment. We have previously shown in cultures of mature muscle cells that 25(OH)D, is taken up and retained in the cells by binding to vitamin D binding protein (DBP), which had been internalized via membrane megalin and then binds to actin in the cytoplasm [1]. We postulated that if the capacity to hold 25(OH)D out of the circulation in skeletal muscle were high, when vitamin D status was falling in winter, it would be protected from wasteful uptake and destruction in the liver. This would increase the residence time of circulating 25(OH)D and thus would maintain adequate status during the months when vitamin D supply was low. If this muscle retention mechanism for 25(OH)D were important in increasing its half-life in blood, the process may be regulated by calciotropic hormones, such as parathyroid hormone and 1,25-dihydroxyvitamin D_3 (calcitriol). We have

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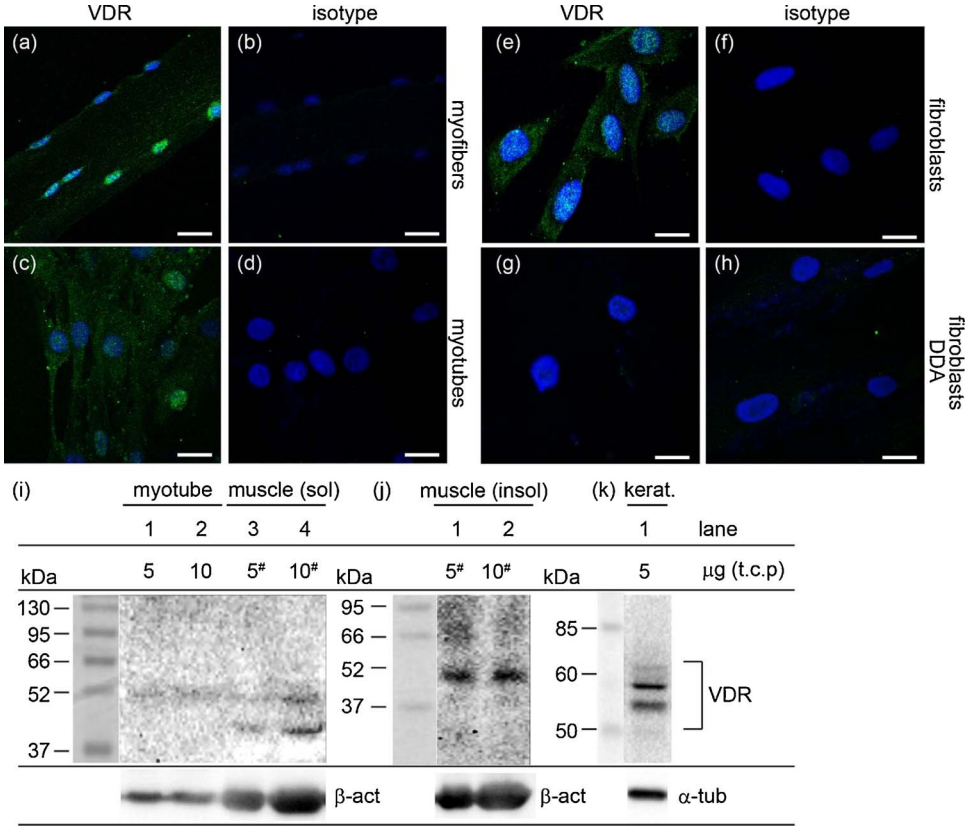

Fig. 1. Detection of VDR in muscle and skin cells by immunofluorescence and western blot. Using VDR-D6 monoclonal antibody, VDR was detected by IF within (a) muscle fibers of young WT mice, (c) differentiated C2 myotubes, (g) skin fibroblasts, but not detected in (g) DDA fibroblasts lacking the VDR. (b, d, f, h) Isotypes for all cell types. Scale bar represents 10 µm. Western blot detection of VDR with D6 monoclonal antibody from 5 or 10 µg total cellular protein (t.c.p) of (i) differentiated C2 myotubes (myotube) and the soluble fraction of muscle tissue (sol), (j) the insoluble fraction of muscle tissue (insol), and (k) primary human keratinocytes (kerat) as a positive control for VDR protein expression. α-tubulin (α-tub) and β-actin (β-act) are shown as loading control. # protein concentration of extracted muscle tissue was not quantified and instead volume of extract (µL) is shown.

shown that net muscle uptake or release of 25(OH)D is indeed modulated by parathyroid hormone [2] and have reported a short term effect of calcitriol on muscle uptake of 25(OH)D over short incubation periods [3].

In the current study, we tested whether longer incubations with calcitriol would have similar or different effects on 25(OH)D uptake or release. 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was used as an inhibitor of chloride channel opening, which has been used to investigate non-genomic actions of calcitriol [4].

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma Aldrich (MO, USA) unless otherwise indicated. Calcitriol was purchased from Cayman, USA, dissolved in spectroscopic grade ethanol and then diluted in the differentiation medium to concentrations of 10^{-10}, 10^{-9}, and 10^{-8}M. 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) was purchased from Sigma Aldrich (MO, USA) and dissolved in dimethyl sulfoxide (DMSO). The vitamin D receptor antibody, clone D6, used for immunohistochemistry and western blots and the DBP antibody, clone H300, were from Santa Cruz (TX, USA). Serum replacement 2 (SR), used as a serum alternative that did not contain DBP, was from Sigma Aldrich.

All murine studies were performed in compliance with the guidelines of the National Health and Medical Research Council of Australia and approved by the Animal Ethics Committees of the University of Sydney and the Garvan Institute. Adult female BALB/c mice were obtained from Animal Research Resources, Western Australia, Australia. Male wild-type and vitamin D receptor knock-out (VDRKO) C57BL/6 mice were from a strain originally developed by Li et al. [5].

2.2. Cell culture

Differentiated C2 myotubes were prepared from 80% confluent C2 myoblasts and plated as previously described [1]. Primary muscle fibers were isolated from the flexor digitorum brevis (FDB) muscle of euthanized mice and cultured in 24-well plates as previously described [1]. In brief, freshly isolated muscle fibers were placed in cell culture wells and immediately treated with calcitriol or vehicle, prior to 25(OH)D uptake studies, as described below. Cells were incubated at 37 °C under 5% CO_2 in air.

2.3. Tritium-labelled 25(OH)D uptake in C2 murine cells or primary fibers from mice

Differentiated C2 myotubes or primary flexor digitorum brevis (FDB) muscle fibers from Balb/C mice [1] or, where indicated, C57BL/6 wild type or VDRKO mice [3] were pre-incubated with the relevant vehicle or various treatment media, outlined below, in differentiating medium in a humidified incubator at 37° C in an atmosphere of 5% CO_2 in air. Uptake studies were conducted by incubation of cells in 250 µL of culture medium containing [26,27 ^{3}H] 25-hydroxyvitamin D_3 (purchased from PerkinElmer; specific activity 162.6 Ci/mmol) at a concentration of 40 nCi/mL in Dulbeccos Modified Eagles Medium (DMEM) supplemented with various agents, as listed below, and 20% serum replacement 2 (SR) for the times indicated. C2 myotubes or muscle fibers were washed and lysed for determination of radioactivity and protein analysis, as previously described [1]. Radioactivity was measured by scintillation counting and the results were expressed as counts per minute (cpm) per mg protein (BCA assay) for the cell lines, or cpm per fiber for the primary FDB myofibers counted in each well. For these studies, the cells were pre-incubated with vehicle or calcitriol for short (3 h) or long (16 h) time periods and then incubated for a further short (4 h) or long (16 h) period in the presence of calcitriol plus labelled 25(OH)D, before washing and lysis, as shown in schematic