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The effect of parathyroid hormone on the uptake and retention of 25-hydroxyvitamin D in skeletal muscle cells

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ABSTRACT

Data from our studies, and those of others, support the proposal that there is a role for skeletal muscle in the maintenance of vitamin D status. We demonstrated that skeletal muscle is able to internalise extracellular vitamin D binding protein, which then binds to actin in the cytoplasm, to provide high affinity binding sites which accumulate 25-hydroxyvitamin D_3 (25(OH)D_3) [1]. This study investigated the concentration- and time-dependent effects of parathyroid hormone (PTH) on the capacity of muscle cells to take up and release ^{3}H-25(OH)D_3. Uptake and retention studies for ^{3}H-25(OH)D_3 were carried out with C2C12 cells differentiated into myotubes and with primary mouse muscle fibers as described [1]. The presence of PTH receptors on mouse muscle fibers was demonstrated by immunohistochemistry and PTH receptors were detected in differentiated myotubes, but not myoblasts, and on muscle fibers by Western blot. Addition of low concentrations of vitamin D binding protein to the incubation media did not alter uptake of 25(OH)D_3. Pre-incubation of C2 myotubes or primary mouse muscle fibers with PTH (0.1 to 100 pM) for 3 h resulted in a concentration-dependent decrease in 25(OH)D_3 uptake after 4 or 16 h. These effects were significant at 0.1 or 1 pM PTH ($p < 0.001$) and plateaued at 10 pM, with 25(OH)D_3 uptake reduced by over 60% ($p < 0.001$) in both cell types. In C2 myotubes, retention of 25(OH)D_3 was decreased after addition of PTH (0.1 to 100 pM) in a concentration-dependent manner by up to 80% ($p < 0.001$) compared to non-PTH treated-C2 myotubes. These data show that muscle uptake and retention of 25(OH)D_3 are modulated by PTH, a physiological regulator of mineral homeostasis, but the cell culture model may not be a comprehensive reflection of vitamin D homeostatic mechanisms in whole animals.

1. Introduction

There are major differences in vitamin D status between individuals living in the same region at the end of winter that are difficult to explain from input alone [2]. Our previous studies and those of others are consistent with the proposal that skeletal muscle has a role in maintaining the long residence time of 25(OH)D_3 in blood. There is a positive relationship between the intensity of physical exercise in young [3–7] middle-aged [6,8–15] and elderly people [7,16–19], and the level of 25(OH)D_3 in blood. This effect of exercise is independent of the input of vitamin D because exercise indoors, away from solar UV light, was associated with higher 25(OH)D_3 levels in blood than in a control group of more sedentary people [12,19]. We have previously reported that in adolescent children, vitamin D status is positively correlated with total body lean mass and negatively correlated with adipose tissue mass [20–22]. The main component of lean mass is skeletal muscle. Previous studies have also shown that the vitamin D content of fetal and neonatal rats is in the form of 25(OH)D_3 and this is mainly located in skeletal muscle [23]. 25-Hydroxyvitamin D is quantitatively transferred from the maternal circulation across the placenta into the fetuses. Rat milk contains negligible quantities of vitamin D or 25(OH)D [23], so the 25(OH)D retained in skeletal muscle of fetal rats acts as a reserve to meet requirements for vitamin D function in early neonatal life.

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More recently, our research has revealed a previously unknown mechanism in skeletal muscle cells that allows vitamin D binding protein (DBP) to be accumulated from the extracellular fluid, bound tightly to actin in the cytoplasm, thus providing an intracellular array of high affinity binding sites for 25(OH)D. Extracellular 25(OH)D then accumulates on these intracellular DBP sites before being released back into the extracellular environment [1]. We also reported that the net uptake of labelled 25(OH)D_3 is modulated by the hormone, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), in a vitamin D receptor dependent manner [24]. Since parathyroid hormone (PTH) concentrations are also affected by vitamin D status, we wondered whether PTH might alter the ability of myotubes or primary muscle fibers to take up and release 25(OH)D_3 as well as the mechanisms involved.

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma Aldrich (Castle Hill, Australia) unless otherwise indicated. Human Parathyroid hormone (PTH 1–34) was purchased from Auspep, Australia. PTH was dissolved in PBS, and PTH solutions at the following concentrations (0.1 pM, 1 pM, 10 pM and 100 pM) were prepared in differentiating medium.

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 BALB/c mice and cultured in 24-well plates as previously described [1]. Cells were incubated at 37 °C, under 5% CO_2. All murine and sheep studies were performed in compliance with the guidelines of the National Health and Medical Research Council of Australia and approved by the Animal Ethics Committee of the University of Sydney. Adult female BALB/c mice were obtained from Animal Research Resources, Western Australia, Australia.

2.3. Immunohistochemistry

Primary FDB myofibers were cultured on coverslips, fixed, permeabilized and incubated as described in Abboud et al., [1,25]. Primary antibodies used were rabbit polyclonal antisera to PTH receptor (PTH/PTHrP-R 200 ug/mL) obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, California) was diluted in 0.25% (wt/vol) bovine serum albumin (BSA) in PBS. Primary antibody incubation was at room temperature for 40 min, followed by a secondary goat anti-rabbit IgG antibody conjugated to Alexo Fluor 488 (2.6 ug/mL, Molecular Probes, Eugene, Oregon) diluted in 1% (w/v) BSA in PBS for 20 min.

Method controls were incubated with an isotype control IgG instead of the primary antibody. Coverslips were mounted on slides using UltraCruz mounting medium with 4′,6′-diamino-2-phenylindole (DAPI; Santa Cruz Biotechnology). Fluorescence microscopy was undertaken on a Zeiss LSM 510 Meta spectral confocal microscope (Carl Zeiss, Jena, Germany).

2.4. Western blots

Cells: Sub-confluent C2C12 or myotube cells were permitted to lyse in 50 mM Tris pH 7.5, 150 mM NaCl, 1% (v/v) TX-100, 0.5% (w/v) SDS, 0.1% (w/v) sodium deoxycholate with protease inhibitors for 10 min on ice. To obtain the soluble fraction, cell lysates were scraped, briefly sonicated (Branson Ultrasonics, CT, USA) to reduce viscosity, and spun at 10,000 × *g* for 10 min at 4 °C. Total protein concentration of the soluble fractions were measured by BCA assay (Thermo Fisher Scientific, MA, USA).

Tissue: Snap frozen mouse muscle tissue was homogenized, in the above mentioned lysis buffer, in a Tissue Lyser LT (Qiagen, MD, USA) for 3 rounds of 2 min (50 Hz). Homogenized tissue was spun at 10,000 × *g* for 10 min at 4 °C. The supernatant represented the soluble fraction, and the pellet represented the insoluble fraction. Relatively small amounts of PTH-R were detected in the soluble fraction (not shown). The insoluble fraction from homogenization was re-suspended in 100 mM Tris–HCl pH 6.8, 8% (w/v) SDS, 20% (v/v) glycerol, 0.15 M DTT and heated at 95 °C for 15 min. After cooling to room temperature, the solution was centrifuged at 10,000 × *g* for 10 min at room temperature and the supernatant represented the re-solubilized fraction where PTH-R was readily detected.

Western blot: Cell and tissue extractions were subject to SDS-PAGE electrophoresis under reducing conditions followed by western blotting to PVDF membranes. PTH-R was detected with rabbit polyclonal H-125 (Santa Cruz Biotechnology, TX, USA) and alpha-tubulin was detected with mouse monoclonal B-7 (Santa Cruz Biotechnology). Anti-rabbit and anti-mouse Ig-HRP antibodies (Cell Signalling technology, DA, USA) were used at 0.1 μg/mL followed by chemiluminescent detection (ChemiDoc, Bio-Rad, CA, USA) with a luminol based HRP substrate (Santa Cruz Biotechnology).

2.5. Tritium-labelled 25(OH)D_3 uptake in C2 murine cells or primary fibers from mice

Differentiated C2 myotubes or primary flexor digitorum brevis muscle fibers from Balb/C mice [1,25] were pre-incubated with the relevant vehicle or parathyroid hormone (PTH) (0.1 pM 1 pM, 10 pM and 100 pM) for 3 h in differentiating medium in a humidified incubator at 37° C with 5% CO_2. Uptake studies were conducted by incubation of cells in 250 uL containing 25-[26,27 ^{3}H] hydroxyvitamin D_3 (purchased from PerkinElmer; specific activity 162.6 Ci/mmol) at a concentration of 40 nCi/mL in DMEM supplemented with various agents, as listed below, and 20% serum replacement 1 (Sigma Aldrich, St Louis, MO) for the times indicated. C2 myotubes or muscle fibers were washed and lysed for determination of radioactivity and protein analysis, as described in [1] [25]. Radioactivity was measured by scintillation counting and the results were expressed as counts per minute (cpm) per milligram of protein for the cell lines, or cpm per fiber for the primary FDB myofibers counted in each well. Several previous experiments established that labelled 25(OH)D_3 is recovered largely unchanged during these incubations. Muscle containing known quantities of 25(OH)D_3 was incubated overnight at 25 °C or 50 g muscle samples injected in multiple locations with 25(OH)D_3 dissolved in small volumes of methanol, were incubated at 25 °C for 24 h. In both these circumstances, recovery of 25(OH)D_3 determined by HPLC analysis was repeatedly above 90% [26].

2.6. Effect of added DBP

C2 differentiated myotubes were incubated in standard incubation medium containing serum replacement. Vitamin D binding protein was added at the indicated concentrations for 5 min before the addition of labelled 25(OH)D_3.

2.7. Effect of PTH on tritium-labelled 25(OH)D_3 retention in C2 myotubes

Differentiated C2 myotubes were incubated with [^{3}H]-25(OH)D_3 at a concentration of 40 nCi/mL in DMEM supplemented with 20% serum replacement 1 for 16 h, after which the medium was