Skeletal Muscle and the Maintenance of Vitamin D Status

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Abstract: Vitamin D, unlike the micronutrients, vitamins A, E and K, is largely obtained, not from food, but by the action of solar UV light on its precursor, 7-dehydrocholesterol, in skin. With the decline in UV light intensity in winter, most skin production of vitamin D occurs in summer. Because no defined storage organ or tissue has been found for vitamin D, it has been assumed that adequate vitamin D status in winter can only be maintained by oral supplementation. Skeletal muscle cells have now been shown to incorporate the vitamin D-binding protein (DBP) from blood into the cell cytoplasm where it binds to cytoplasmic actin. This intracellular DBP provides an array of specific binding sites for 25-hydroxyvitamin D (25(OH)D) which diffuses into the cell from the extracellular fluid. When intracellular DBP undergoes proteolytic breakdown, the bound 25(OH)D is then released and diffuses back into blood. This uptake and release of 25(OH)D by muscle, accounts for the very long half-life of this metabolite in the circulation. As 25(OH)D concentration in blood declines in winter, its cycling in and out of muscle cells appears to be upregulated. Parathyroid hormone is the most likely factor enhancing the repeated cycling of 25(OH)D between skeletal muscle and blood. This mechanism appears to have evolved to maintain adequate vitamin D status in winter.

Key words: vitamin D; muscle; parathyroid hormone; vitamin D-binding protein
The concentration in blood serum or plasma of 25-hydroxyvitamin D [25(OH)D], the most plentiful vitamin D metabolite, has become established as the definitive indicator of vitamin D status [1,2]. When the concentration falls below a generally agreed level, (usually 50 nmol/L), vitamin D status is said to be insufficient or deficient, in the same way that status is defined for other small molecules derived from the environment, such as vitamins A, E and K. However, there are three substantial differences between this measurement of vitamin D status and those of these fat-soluble micronutrients. The first is that 25(OH)D concentration in blood varies with season. Because it is derived from vitamin D produced in skin by the action of solar ultraviolet radiation on 7-dehydrocholesterol, the 25(OH)D levels rise during the months of summer and fall during winter, particularly in those people who live far from the equator [3,4]. Are the lower values in winter really an indication of deficiency or insufficiency of vitamin D, if this is a universal feature of populations?

The second difference with vitamins A, E and K, is that there is no apparent storage organ or tissue for vitamin D or 25(OH)D. Although vitamin D is found in adipose tissue, suggesting that this is a storage site [5,6], it can only be released when stored fatty acids are mobilized to supply energy [7,8,9]. Thus, sequestered vitamin D in adipocytes cannot be a functional store, ready to be transported to the liver and converted to 25(OH)D, when circulating levels of this metabolite decline.

The third unique feature is that 25(OH)D has a very long residence time in blood. The half-life is very variable between 15 and 50 days [10] with a mean value in a recent study of 89 days [11]. In contrast, other hormonal steroids in blood, including the vitamin D hormone, 1,25-dihydroxyvitamin D (1,25(OH)₂D), are cleared in minutes or hours after entering the circulation [12,13]. An explanation for this long residence time of 25(OH)D is not readily apparent, particularly because the vitamin D binding protein (DBP) in blood, to which 25(OH)D is tightly bound, has a comparatively short half-life of only 1-3 days and is continuously being replenished from synthesis and secretion by the liver [14,15]. Therefore, for 25(OH)D to be retained in blood for such a long time, it either has to transfer from one DBP molecule to
another in the circulation or else it repeatedly passes to and from some extravascular site, binding to a new DBP molecule with each cycle. The DBP concentration in blood is in vast excess to that of 25(OH)D with only 1-5% of the protein molecules having a 25(OH)D molecule bound to the single, high affinity, specific binding site [16].

The discovery that maternal 25(OH)D in rats was transported across the placenta and accumulated in the skeletal muscle of fetuses, suggested that skeletal muscle might have a functional role in conserving this vitamin D metabolite [17]. Although the concentration of 25(OH)D in muscle of sheep and cattle is only about 0.1-0.3 µg/100g [18], muscle represents 30-38% of body mass in humans [19] and thus total 25(OH)D in total skeletal muscle could be comparable to that in the circulation (Table 1).

Table 1. Comparison of total body 25(OH)D in blood plasma and skeletal muscle

<table>
<thead>
<tr>
<th></th>
<th>Blood Plasma Volume (L)</th>
<th>Muscle Mass (kg)</th>
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<tbody>
<tr>
<td>Total for 70 kg human</td>
<td>2.7 – 4.3 L¹</td>
<td>21 - 26.6 kg²</td>
</tr>
<tr>
<td>25(OH)D concentration</td>
<td>20 µg/L¹</td>
<td>1 – 3 µg/kg³</td>
</tr>
<tr>
<td>Total body 25(OH)D</td>
<td>54 – 86 µg</td>
<td>21 – 80 µg</td>
</tr>
</tbody>
</table>

¹Reference [20]  
²Assuming body mass of 70 kg [19]  
³Minimum adequate status 25(OH)D concentration  
⁴Assuming human muscle has similar 25(OH)D concentrations to sheep and cattle [18]

In addition, studies in adolescent children [21] found that plasma 25(OH)D concentration was positively correlated with total body lean mass, the main component of which is skeletal muscle. Furthermore, there are now several published findings of a positive relationship between the intensity of physical exercise and the concentration of 25(OH)D in blood [e.g. 22,23], particularly in winter when there would be little exposure to solar UV light during outdoor exercise [21]. Muscle biopsies from sheep grazing outdoors in winter showed a significantly higher concentration of 25(OH)D than biopsies from sheep outdoors at the end of summer (Table 2).
Table 2. Plasma and muscle concentrations of 25(OH)D in outdoor grazing sheep at latitude 33.9° S. Mean values ± SEM.

<table>
<thead>
<tr>
<th>Season</th>
<th>Plasma 25(OH)D3</th>
<th>Muscle 25(OH)D</th>
</tr>
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<tbody>
<tr>
<td>End of summer values (n = 5)</td>
<td>10.67 ± 1.65 ng/ml</td>
<td>0.27 ± 0.04 µg/100g tissue</td>
</tr>
<tr>
<td>End of winter values (n = 5)</td>
<td>5.36 ± 0.71 ng/ml*</td>
<td>0.47 ± 0.12 µg/100g tissue*</td>
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</table>

*significantly different from summer values by t-test p<0.05

The key discovery that pointed to a role for muscle in maintaining vitamin D status came from studying the properties of muscle cells in vitro [24]. The cell membrane was found to contain the proteins megalin and cubilin. These proteins, like those in the renal tubule cells, transport DBP from the extracellular fluid into the cell cytoplasm. Some of the internalized DBP could be bound to actin in actomyosin, via its specific actin-binding site, but much of the remainder is bound to actin dispersed throughout the cytoplasm. Both megalin and DBP have also been found in human muscle biopsies [25]

In vivo experiments with 131I-labelled DBP in rabbits, revealed that DBP had a short residence time in skeletal muscle and soon underwent proteolysis [14]. DBP is a member of the albuminoid family of proteins. It is synthesized and secreted by the liver, with similar characteristics to blood albumin [26]. Because albumin in the extracellular fluid in skeletal muscle is only about one third the concentration in plasma [27], the extracellular DBP concentration will likewise be about 1.5 to 2 µmol/L compared to the blood concentration of 5-6 µmol/L [28]. Thus, much of the 25(OH)D in the extracellular fluid will have dissociated from the low concentration of DBP and would be able to diffuse into muscle cells where it would bind to the internalized DBP in the cell cytoplasm. In comparison with control cells such as osteoblasts, 25(OH)D is retained within myocytes for considerably longer (Fig. 1). When cytoplasmic DBP undergoes proteolysis, the bound 25(OH)D is released and would diffuse from the myocyte and return to the circulation, once again being bound to the plentiful DBP. This repeated uptake and release of 25(OH)D by the total mass of skeletal muscle cells accounts for the apparent long residence time of 25(OH)D in blood plasma.
Figure 1.

Fig 1. Retention of tritiated 25(OH)D3 in C2 myotubes, C2 myoblasts and MG63 osteoblasts.
Cells were incubated for 16 h with 25-[26,27-3H]hydroxyvitamin D3 (Perkin Elmer, MA, USA) in DMEM supplemented with serum replacement (Sigma-Aldrich, MO, USA) followed by 3x washes with ice cold PBS. At this point, 0 h, or 4 h later, cells were harvested, lysed and assayed for protein by bicinchoninic acid assay (Thermo-Scientific, IL, USA) or radioactivity counted by liquid scintillation counting (see [24]). Data as means ± SEM (n = 3 wells/group).

An alternative explanation for the long half-life of 25(OH)D in blood could be that there is continuous entry of newly synthesized 25(OH)D, perhaps from parent vitamin D trapped in adipose tissue. This possible steady input could be replacing a steady loss of 25(OH)D from blood, and thus account for an apparently long half-life. However, the long residence time in blood of tritium-labelled 25(OH)D in both mice [24] and humans [10] demonstrates that it is persistence of the same molecules in blood that is the real explanation. This phenomenon can only be explained by recycling of 25(OH)D from some extravascular region and skeletal muscle is the only candidate for that region.

Nevertheless, experiments investigating the uptake and retention of 25(OH)D in cultured muscle cells in vitro, have shown that raising the concentration of DBP in the culture medium blocks the uptake of 25(OH)D into the cells (Table 3). The higher the concentration of DBP, the lower the concentration of unbound 25(OH)D. This observation fits with the interpretation that it is unbound 25(OH)D that enters muscle cells, rather than that which might be carried in,
bound to DBP, via the megalin/cubilin protein internalization process. The fact that only 1-5% of
DBP in the circulation is transporting a specifically bound 25(OH)D molecule, also indicates that
transport on DBP cannot be the mechanism for the intracellular accumulation of 25(OH)D.

Table 3. Uptake of tritium-labelled 25(OH)D$_3$ by differentiated myotubes after 4 h in the
presence of varying concentrations of DBP.

<table>
<thead>
<tr>
<th>DBP concentration</th>
<th>0</th>
<th>1nM</th>
<th>10nM</th>
<th>100nM</th>
</tr>
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<tbody>
<tr>
<td>$[^3]$H]25(OH)D cpm/mg cell protein</td>
<td>1284±147</td>
<td>1233±67</td>
<td>778±76*</td>
<td>402±20*</td>
</tr>
</tbody>
</table>

* significantly different from 1nM DBP p<0.001

The accumulation of tritium-labelled 25(OH)D over 16 hours by cultures of differentiated
mouse skeletal myotube cells (the differentiated cell type derived from myoblasts in culture,
equivalent to myocytes in vivo), compared to the very low uptake by undifferentiated
myoblasts and osteoblasts [29], demonstrated that some internal 25(OH)D specific binding sites
were present in the myotubes and not in the other two control cell types (Fig. 2). As the culture
medium during the time of these incubations did not contain DBP the specific binding inside
myotubes would have been to DBP that had been internalized from the culture medium while
the myotubes were differentiating from myoblasts. When myotubes were cultured with
fluorescently labelled DBP for up to 24 h, and then observed by confocal microscopy, the
fluorescent protein was clearly seen within the cells (Fig. 3). Some of the fluorescence was in
linear streaks along the long axis of the myotubes, suggesting that some of the labelled DBP
was bound to actin in the actomyosin contractile elements. But the fluorescent pattern was
also distributed generally within the cell, indicating that much of the DBP was bound to the
abundant actin, known to be distributed throughout the myotube cytoplasm (30).
Figure 2.

**Fig 2. Time dependent $^3$H-25(OH)D uptake in C2 myotubes and myoblasts and MG63 osteoblasts.** Cells were incubated for 4 or 16 h with [26,27-$^3$H]25-hydroxyvitamin D$_3$ in DMEM supplemented with serum replacement followed by 3x washes with ice cold PBS. At each time point, cells were harvested, lysed and assayed for protein by bicinchoninic acid assay or radioactivity counted by liquid scintillation counting (see [24]). Data shown as means ± SEM (n = 3 wells/group)

It now appears that the megalin/cubilin protein internalization mechanism in myocytes is not limited to DBP because other extracellular proteins, such as albumin, have also been demonstrated to be incorporated into myotube cells in culture by this mechanism (unpublished results). The function of uptake of other extracellular proteins by skeletal muscle cells in vivo is a matter of speculation. Because skeletal muscle has a high rate of protein turnover, particularly when undergoing regular physical exercise [31] the internalized proteins, after proteolysis, could be supplying essential amino acids for protein resynthesis. It is therefore conceivable that because DBP in myotube cultures is capable of retaining 25(OH)D for many hours (Fig. 1) [24], that the DBP bound to cytoplasmic actin, is protected from the proteolysis that other internalized proteins undergo. Nevertheless, when the cytoplasmic DBP is eventually broken down, the retained 25(OH)D would be released and could then diffuse out of the cell and back into the circulation.
Figure 3. The uptake of Alexafluor488-labeled DBP into primary muscle fibers. Myofibers were isolated from the flexor digitorum brevis of euthanized Balb/c mice and incubated in quadruplicate wells on poly-L-lysine-coated glass coverslips. Fibers were incubated for 0, 4, 12 or 24 hours with 1 µM of Alexa Fluor488-DBP (labelling kit from Molecular Probes) in DMEM supplemented with 20% serum replacement. Incubations were set up to finish at the same time. Cells were then fixed in paraformaldehyde for 20 minutes. Fluorescent images were captured with a Zeiss LSM 510 Meta Spectral confocal microscope, using optimized saturation settings for the 24-hour time point. The fluorescence intensity can be seen increasing from A) 0 hours, B) 4 hours, C) 12 hours and D) 24 hours.

Because the concentration of 25(OH)D in skeletal muscle of sheep (Table 2) was significantly higher in winter than in summer, while the plasma concentration of 25(OH)D was decreasing, it is likely that some endocrine factor was modifying the storage capacity of muscle according to changes in vitamin D status. The circulating levels of at least two hormones rise in winter and decline in summer. These are thyroid stimulating hormone (TSH) [32] and parathyroid hormone [32].
There is no information to link TSH or thyroid hormone with changes in the uptake or release of 25(OH)D by skeletal muscle. However, the small increase in PTH concentration in blood plasma when 25(OH)D levels fall to 50-60 nM/L [33] make it a candidate as a regulator of muscle uptake and release of 25(OH)D.

Receptors for PTH have been demonstrated on mouse muscle fibers and on differentiated myotubes in culture, but receptors are not found on undifferentiated myoblast cells [34]. Incubation of myotubes with low concentrations of PTH (0.1-1 pM) for 3 hours, diminished the subsequent uptake of $^3$H-25(OH)D over 16 hours. Conversely, the release back into the medium of $^3$H-25(OH)D, already accumulated by myotubes, was enhanced by addition of low concentrations of PTH to the incubation medium [34]. Although these results demonstrate an effect of PTH on the transport of 25(OH)D into and out of myotubes, they paradoxically suggest that an increased exposure of muscle cells to PTH would decrease their ability to conserve 25(OH)D. The rise in PTH concentration in blood as the 25(OH)D levels fall in winter suggests that if PTH is the hormone regulating muscle uptake and release of 25(OH)D, then that enhanced uptake and delayed release of $^3$H-25(OH)D should have been seen with cells in vitro. However, in those experiments [34], the incubation medium with added PTH did not contain any DBP. Hence the action of PTH in those studies was compatible with the hypothesis that its effect on muscle cells would be to enhance both the breakdown of cytoplasmic DBP and perhaps its rate of uptake from extracellular fluid. The effect in vivo of enhanced DBP uptake and breakdown in muscle would be to increase the rate of cycling of 25(OH)D by muscle cells and thus prolong the residence time of 25(OH)D in the circulation. In the disease state of hyperparathyroidism, the greatly elevated concentration of PTH in blood, results in increased loss of 25(OH)D, mediated by the increased renal production of $1,25(OH)_2$D which activates the hepatic uptake and destruction of 25(OH)D [10]. In contrast, the very small increase in PTH in blood with the seasonal decline in 25(OH)D concentration, does not provoke increased secretion of $1,25(OH)_2$D from the kidney. Nevertheless, that concentration of PTH observed with the seasonal fall in vitamin D status has been shown to activate the muscle PTH receptor on myocytes in vitro [34].
Conclusion

This active cycling of 25(OH)D into and out of skeletal muscle cells explains the long residence time of 25(OH)D in blood. Perhaps then, if ways could be found to optimize this process some of the diseases linked epidemiologically with low vitamin D status could be minimized. Low circulating levels of vitamins A, E and K can be related directly to the specific pathology in their function caused by deficiency of these micronutrients. Because vitamin D has been classified as a similar micronutrient, attempts have been made to obtain evidence for a direct link between low concentrations of 25(OH)D in blood and a range of diseases such as diabetes, and various cardiovascular and oncology diseases, where there are epidemiological suggestions that low vitamin D status is a causative factor in their etiology. Although the level of 25(OH)D in blood may indicate the adequacy of vitamin D supply to meet requirements for production of 1,25(OH)_{2}D, it is not a direct indicator of the function of that hormone. Because the many regulatory functions of 1,25(OH)_{2}D also involve other endocrine and gene expression variables, an epidemiological link between low vitamin D status and a disease process is not necessarily a cause and effect relationship. This is well illustrated by the difficulty of demonstrating health benefits of vitamin D supplementation in many clinical trials [35]. Nevertheless, if ways could be found of optimizing the efficiency of the muscle conservation mechanism for 25(OH)D, perhaps by a pharmacological agent or some exercise regime, that would ensure that vitamin D status would also be optimized by a process that has evolved to adapt to seasonal changes in vitamin D supply.

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