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Vitamin D and muscle wasting

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Abstract
Muscle wasting is caused by vitamin D insufficiency in both humans and animals. Sprague Dawley male rats were used to generate a model of vitamin D deficiency in rats. When compared to controls, the deficient group had higher proteasomal enzyme activity, E2 Ubiquitin conjugating enzyme expression, and ubiquitin conjugates. Lysosomal and calpain activities, on the other hand, were unaffected. When compared to control muscle, the deficient muscle had a lower Type II fibre area, a sign of atrophy in the muscle. In D-deficient muscle, proteasomal subunit and muscle atrophy marker genes were up-regulated, while myogenic genes were down-regulated. Increased muscle protein breakdown has been associated to vitamin D deficiency. It seems that the main pathway for vitamin D deficiency-induced muscle protein breakdown is the ubiquitin proteasome pathway, and calcium supplementation alone in the absence of vitamin D only partially reverses the alterations. It does not appear that the lysosomal or calpain proteolytic mechanisms are at play.

Keywords: Vitamin D, muscle wasting, diabetes, atrophy

Introduction
The classical activities of vitamin D are known to include bone growth and maintenance, calcium and phosphate balance, and immune activity (DeLuca 2004) [8]. Its biologically active hormonal form, 1,25-dihydroxyvitamin D [1,25(OH)2D3], functions by attaching to the nuclear receptor superfamily member vitamin D receptor (VDR) (Reichel *et al*., 1989) [28]. The conventional target organs of vitamin D are the gut, kidney, bone, and parathyroid glands (DeLuca, 2004) [8]. There has been growing evidence over the past few decades that vitamin D is essential for the health of many different body tissues, including skeletal muscle (Reichel *et al*., 1989) [28]. Clinical observations of vitamin D deficiency-induced muscular weakness in osteomalacia in adults and hypotonia in babies led to the first link between vitamin D and muscle function (Prineas *et al*., 1965; Schott and Wills, 1976) [10, 29]. There is a relationship between vitamin D and muscle, according to reports of a reversible myopathy associated with severe vitamin D insufficiency (Boland, 1986) [13]. Elderly participants who received low-dose vitamin D had a lower incidence of falls and hip fractures (Sato *et al*., 2005) [13]. Adults with vitamin D insufficiency and proximal myopathy had a prevalence of type II muscle fibre atrophy, according to histologic analysis of muscle biopsy specimens (Sato *et al*., 2005: Pfeifer 2009) [13, 14]. Further proof of vitamin D's direct function in muscle tissue was provided by the discovery of the VDR in muscle cells (Simpson *et al*.,1985: Boland *et al*. 1985) [15-2, 4]. A significant side effect of several diseases, including diabetes, cancer, uremia, and heart failure, is muscle atrophy (Lecker *et al*., 1999) [5] (Sato *et al*., 2005, Snijder *et al*., 2006: Dhesi *et al*., 2004) [13, 17, 16].

Methodology
Chemicals
All reagents, unless otherwise stated, were bought from Sigma-Aldrich. Santa Cruz Biotechnology provided the anti-Ub antibody and HRP-conjugated secondary antibodies, while Boston Biochem provided the anti-E2-Ub conjugating enzyme antibody. The ECL chemiluminescent substrate was provided by Pierce Chemical Co. All of the chemicals involved in proteasomes were provided by Enzo Life Sciences.

Muscle protein turnover may be measured
As muscle neither produces nor degrades tyrosine and therefore does not accumulate inside the intracellular pool of muscle, the rate of tyrosine released into the media (TPD) was examined in the mixed fibre epitrochlearis muscle. In order to prevent the utilisation of amino acids released during protein breakdown, preincubation of epitrochlearis muscles with cycloheximide (0.5 mM) in a Kreb's Ringer bicarbonate buffer containing 10 mM glucose was used.

This method was used to determine the absolute rate of protein breakdown. After a 2-hour preincubation period at 37°C, the muscles were moved to a fresh Kreb's Ringer bicarbonate buffer (Waalkes and Udenfriend, 1957) [22]. Tyrosine release was quantified fluorometrically in order to gauge the rate of protein degradation after peptides and proteins were eliminated using trichloroacetic acid (final concentration 10%). The conclusion can be summarised as follows in terms of tyrosine released nanomoles per hour per gramme of muscle. Total protein synthesis was calculated using the amount of [14C] tyrosine that muscles absorbed (Wang *et al*., 2009) [23].

Tyrosine concentrations of 0.5 mmol/L were achieved by preincubating the epitrochlearis muscles with 0.5 mM cold tyrosine for 30 minutes at 37°C in DMEM (3 mL) with 95 percent O2 and 5 percent CO2. After that, the muscles were blotted and placed in new DMEM that contained [14C] tyrosine (0.05 Ci/mL) for two hours at 37 °C. The muscle homogenate was dissolved in 10 percent trichloroacetic acid for 60 minutes at 60 degrees Celsius, and the pellet was then centrifuged four times in the same solution to eliminate any unincorporated [14C] tyrosine. Tyrosine incorporation into protein was measured by liquid scintillation counting after the resultant solution was dissolved in 0.3 N NaOH. muscle is measured in picomoles per hour.

Proteasomal enzyme activities in muscle

The chymotrypsin, trypsin, and caspase like activities of extracts of Gastrocnemius (GM) and Soleus (SOL) muscles were evaluated using substrates (final concentration of 50M) specific to the three enzymes as reported by (Beyet *et al*., 1998) [24]. Leucine arginine glutamate receptor (TLR), leucine leucine glycine glutamate receptor, and chromophore L activity were all measured using succinyl coumarin (AMC) (CpL). A final concentration of 10 M of the 26S proteasome inhibitor clasto-lactacystin was used. Using a multi-mode reader for proteolytic activity, excitation and emission of the AMC group were detected at 380 nm (excitation) and 460 nm (emission) (Spectramax-MS; Molecular Devices). The standard curve was produced using Free AMC. The difference between the two conditions' peptidase activity was identified, and the results were expressed in nanomoles/min/mg protein. Protein content of the muscle extract was ascertained using the bichinchonic acid method. Clastolactacystin considerably reduced (>90%) the activity of all three proteasomal enzymes.

Measurement of lysosomal and calpain activities in muscle

According to a study by Furono *et al*., 1990 [25] cathepsin B/L activity was measured fluorometrically in the GM and SOL muscles using the substrate Z-Phe-Arg-4-AMC. At 380 nm for excitation and 460 nm for emission, fluorescence was measured against a free AMC standard. For these data, the unit of measurement is micromoles/hr/mg protein. The GM and SOL muscles' calpain activity, according to Sultan *et al*, was assessed (Sultan *et al*., 2000) [26]. In the absence of $CaCl_2$, we performed control studies using 10 mM EDTA and 10 mM EGTA. Calpain activity was discovered to be caused by Ca_2^+, which is dependent on calpain cleaving the calpain-specific substrate Suc-LY-AMC. The activity of the protein was measured in nanomoles/min/mg.

Muscle histology

The isopentane in which the GM muscle samples were frozen was chilled using liquid nitrogen. The photos were frozen in a cryostat (Leica-CM 3050S) and maintained at -20 °C until they were processed in sections (10 m). The types I and II of fibre were stained with ATPase using the method described by Round *et al*., 1980 [27]. The pictures were taken with an Olympus 1X-51 inverted microscope and a Jenoptik CCD digital camera (prog RESR C3). With a 40x magnification, we utilised ProgRes capture pro version 2.8.8 to count the quantity and area of fibres (Jenoptik AG).

Results and Discussions

In this work, we investigated the role of several proteolytic pathways in vitamin D deficiency-induced muscle protein breakdown, which results in muscle wasting. Due to an imbalance in protein breakdown and synthesis, muscle atrophy occurs. The UPP, the lysosomal route, and the calpain pathway are the three proteolytic mechanisms by which muscle proteins are degraded. We don't know how vitamin D deficiency affects muscle protein lysis through the three routes described above. In the study, researchers used a vitamin D deficient rat model and found that despite eating the same amount of food as their vitamin D-supplied counterparts, the deficient rats weighed less than their counterparts. Deficiency in the vitamin D metabolites 25(OH)D3 and 1,25(OH)2D3 and hypocalcemia are the results of a malfunctioning vitamin D metabolism. After 18 weeks on the vitamin D deficient diet, the animals developed significant 1,25(OH)2D3 deficit (10pg/ml) and hypocalcemia, confirming the animals' vitamin D shortage and correlating with previous studies. Vitamin D deficiency causes hypocalcemia, which is in agreement with past research and our own findings. A vitamin D deficiency has been shown to affect serum phosphorous levels in a variety of ways. Alkaline phosphatase levels in the serum are consistent with our previous research and published reports (Ismail and Namala, 2000) [18], which indicate that the enzyme is leaking from the bone. Calcium has long been recognised as an important component of muscular function. The VDR has been studied in muscle tissue from a variety of animals. Reports on VDR muscle localisation are often conflicting, with some supporting the idea while others rejecting it (Bischoff *et al*., 2001; Simpson *et al*., 1985 Boland *et al*., 1985) [3, 15-2, 4]. Consequently, we looked at how calcium supplementation alone, without vitamin D, affected muscle function to better understand calcium's role in muscle. When the concentration of calcium in the lumen is high, previous investigations have revealed that calcium absorption is predominantly facilitated by passive diffusion.

Adults with vitamin D insufficiency have been shown to have considerable muscular weakening and morphological abnormalities (Girgis *et al*., 2013) [21]. The loss of muscle mass and lean body mass was accompanied by a reduction in body weight due to vitamin D insufficiency. As a consequence of increased protein breakdown or reduced protein production, muscle loss occurs. The effects of vitamin D insufficiency on muscle wasting were studied. Tyrosine release from muscle was shown to be elevated in D-deficient muscle, but protein production was reduced, as measured by total protein degradation. Vitamin D supplementation restored the decrease in TPD. A rescue diet strong in calcium, it turns out, can only partly reverse this alteration. Myofibrillar protein breakdown produces 3-MH,

an amino acid that is solely found in actin and myosin and is neither destroyed or reutilized for protein synthesis following its release during proteolysis (Young *et al*., 1978) [20]. Consequently, *in vivo* measurements of muscle protein breakdown based on urine 3-MH excretion have become popular. We found higher excretion of 3-MH in the urine of D-deficient rats compared to control rats, which was totally reversed by vitamin D supplementation and somewhat rectified by high calcium consumption, which is in accordance with muscle protein breakdown. It was also revealed that myofibrillar protein level in the deficient muscle was reduced; treatment with vitamin D and a high calcium diet corrected this effect. Vitamin D insufficiency has been linked to an increase in muscle protein breakdown, according to the results of this study.

Vitamin D insufficiency has been shown to cause type II (fast twitch) fibre atrophy in clinical studies (Ward *et al*., 2009) [19]. Our investigation also found a reduction in type II fibre area in the defective muscle, although type I fibre area was not affected. Only supplementation with vitamin D could correct this alteration in muscle structure indicating that vitamin D may be vital in preserving the muscle's structural design. Muscle-specific transcription factors such as MyoD, Myogenin, and Myf5 govern myogenesis and have been shown to promote muscle development. Compared to control muscle, the expression of these myogenic factors was reduced in deficient muscle, which suggests a reduction in muscle mass (Weintraub, 1993) [7]. Intriguingly, the expression of myogenic genes was reversed by both vitamin D supplementation and a high calcium consumption. The expression of the myostatin gene, a muscle growth inhibitor, was also examined. Vitamin D deficiency seems to have no effect on myostatin gene expression, indicating that the TGF- pathway may not play a function in regulating muscle mass.

Since the UPP is responsible for the vast majority of cellular protein lysis, we started by looking at the proteasome's 20S catalytic core enzyme activity in two different muscle types. Ch-L, T-L, and Cp-L enzyme activity in the 20S proteasome were all elevated in vitamin D deficient rats' mixed fibre gastrocnemius muscles. Only the Ch-L activity was elevated in the soleus muscle, which has a high percentage of type I fibres as its main constituent. Even though it was previously believed that the Ch-L activity was the rate-limiting one, new investigations have shown that all three enzyme activities are necessary and contribute to protein breakdown (Kisslev *et al*., 2006) [6]. Vitamin D supplementation and rescue with a high-calcium diet were both able to restore enzyme activity to that of controls, which is interestingly enough. When it comes to the ubiquitination of proteins, the E2-14kDa ubiquitin conjugating enzyme is the most common enzyme. In vitamin D deficiency-induced muscle atrophy, increased protein production of the E2 enzyme and high molecular weight ubiquitin conjugates support the notion that the UPP is more active than usual. The mRNA levels of two proteasome subunits, PSC2 and PSC8, were also examined and found to be higher in the deficient group compared to the vitamin D-administered group. Molecular markers for muscle atrophy have recently been identified as Atrogin-1 and MuRF1 E3 ligases. In the group with the deficiency, there was an increase in the expression of both atrophy gene markers. Vitamin D supplementation and a high-calcium diet may both correct the abnormalities in the proteasome that occur when vitamin D levels are low. During vitamin D deficiency-induced muscle atrophy, we found that the UPP was elevated.

The significance of the lysosomal and calpain pathways in vitamin D-induced muscle protein breakdown was also examined. Deficient muscle had no effect on the enzyme activity of any of these pathways. The expression of genes coding for enzymes in the lysosomal and calpain pathways was likewise unaffected by our findings. There was no evidence of a change in any of these pathways in vitamin D-deficient muscles, which suggests that the UPP is the primary mechanism for degradation of muscle protein in this condition.

Conclusion

From this study it is clear that vitamin D deficiency leads to muscle atrophy through the ubiqutin proteasome pathway, as previously reported (Kandarian and jackman, 2006) [1]. It does not appear that the lysosomal or calpain proteolytic mechanisms are at play. Supplementation with a control diet or rescue with a high calcium diet may either completely or partially cure the effects, depending on the severity of the vitamin D insufficiency. An increase in muscle protein carbonyls suggests that protein oxidation has been triggered by a vitamin D deficit. In addition, vitamin D therapy reversed an increase in protein oxidation brought on by oxidative stress in the C2C12 murine muscle cell line. SOD and CAT activity decreased in the deficient muscle, indicating a different anti-oxidant state, whereas GPx and GR activity, which are related to glutathione, were both increased. Supplementing with vitamin D caused all enzyme activity to revert to normal. In living cells, SOD serves as the first line of defence against free radicals by catalysing the conversion of superoxide into oxygen and hydrogen peroxide.

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