Effects of alfacalcidol on circulating cytokines and growth factors in rat skeletal muscle

Janwillem Testerink, Richard T. Jaspers, Jörn Rittweger, Arnold de Haan, Hans Degens
Abstract

Background
Supra-physiological levels of vitamin D induce skeletal muscle atrophy. However, the cause of the atrophy and which muscle fibres are affected are unknown. The atrophy may be particularly detrimental in the already sarcopenic elderly.

Methods
Therefore, we treated 27.5-month-old old female Fischer344 x Brown Norway F1 rats orally with vehicle or the vitamin D analogue alfacalcidol for 6 weeks to increase serum vitamin D (1,25(OH)2D3 or 1,25D) levels. In histological sections of the medial gastrocnemius muscle we assessed the atrophy of each fibre type and whether circulating IL-6, adiponectin and leptin were altered.

Results
The alfacalcidol-treated animals had a reduced food intake. The treatment resulted in a 22% decrease in body mass and 17% muscle atrophy. Especially the low oxidative part of the Gm, which consist mainly of type IIb and IIx fibres, was atrophied. This atrophy was accompanied with a 1.6 fold increase in mRNA of the ubiquitin ligase MuRF1, whereas insulin-like growth factor 1 and myostatin mRNA levels were not affected. The circulating IL-6 was unaltered, leptin levels decreased and adiponectin elevated.

Conclusion
Supraphysiological circulating levels of 1,25D cause a preferential atrophy of type IIb and IIx fibres. This atrophy is associated with an increased expression of MuRF1, without evidence of systemic inflammation. The atrophy and loss of body mass are primarily due to a reduced food intake.

Key words: vitamin D, alfacalcidol, skeletal muscle, atrophy
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Introduction

In vitamin D deficient community-dwelling older people the vitamin D status is related to measures of physical performance (Visser, Deeg et al. 2003). Also in young people a relation between vitamin D status and muscle performance has been reported, though not consistently (Garnero, Munoz et al. 2007; Allali, El Aichaoui et al. 2009; Foo, Zhang et al. 2009; Ward, Das et al. 2009). It has been shown that older adults with lower 25-hydroxyvitamin-D3 (25D) status are at increased risk of a decline in physical performance over 3 years compared to those with higher levels (Visser, Deeg et al. 2003). Given these observations it is no surprise that vitamin D supplementation, especially in combination with calcium, has been shown to improve body sway by 9% in ambulatory elderly women within 8 weeks (Pfeifer, Begerow et al. 2000). Musculoskeletal function in institutionalized elderly people with 25D serum levels lower than 50 nmol·L⁻¹ was improved by 4-11 % after vitamin D supplementation in combination with calcium within 12 weeks (Bischoff, Stahelin et al. 2003) while long term supplementation of 25D lowered the risk of falling with 22% (Bischoff-Ferrari, Dietrich et al. 2004).

The discovery of the vitamin D receptor (VDR) in muscle tissue (Zanello, Collins et al. 1997) provided a direct link of how vitamin D could affect muscle tissue. Meanwhile it has been shown that vitamin D affects Ca²⁺ homeostasis, and cell proliferation and differentiation in myoblasts (reviewed in (Boland, de Boland et al. 1995)). The latter two effects are probably mediated via its effect on myogenic regulatory factors (MRFs) as indicated by the abnormal muscle development and deregulated expression of MRFs in VDR knock-out mice (Endo, Inoue et al. 2003). Vitamin D administration in deficient rats leads to an increase in muscle mass and decrease in the rate of myofibrillar protein degradation (Wassner, Li et al. 1983). The same has been shown in humans, where vitamin D administration in D-deficient people leads not only to an increase in muscle size and strength, but also to an increased proportion and size of type II fibres (Sato, Iwamoto et al. 2005).

The active form of vitamin D, 1,25 hydroxy vitamin D (1,25D), may be higher concentrated within the muscle than in the circulating blood due to the activity of CYP27B1. CYP27B1 is a hydroxylase that catalyses the hydroxylation of 25D to 1,25D. This process occurs mainly in the kidney, but extra-renally produced 1,25D has been reported in many cells and tissues (Peterlik and Cross 2005), probably also including muscle. It is thus possible that the expression of the vitamin D receptor (VDR) and CYP27B1 may be altered when circulating levels of 1,25D are altered to attenuate fluctuations in intracellular 1,25D that could cause dysregulated gene expression and Ca²⁺ homeostasis.

In a human liver cell line it has been shown that maximal VDR activity occurs at concentrations of 1,25D in the medium that are far above physiological circulating levels (Reschly, Bainy et al., 2007). This suggests that elevation of circulating levels of 1,25D could enhance VDR. However, we have shown that increasing serum levels of 1,25D in rats, by supplementation of alfacalcidol, a vitamin D analog which is converted into 1,25D, was accompanied by a reduction in body mass, muscle atrophy and muscle weakness (Testerink et al., 2010). The mechanisms underlying this muscle atrophy remains to be established and would enhance our understanding of the role of vitamin D in the regulation of skeletal muscle size and its contractile characteristics.

Previously we have observed that the reduced food intake during alfacalcidol treatment contributed to the loss of body and muscle mass (Testerink et al., 2010). Such a
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reduced food intake could be caused by altered sensations of satiety. The hormone leptin plays an important role in the regulation of food intake and feelings of satiety while reduced plasma levels of adiponectin, an adipokine that is elevated during weight loss may play a role in pathogenesis of obesity and diabetes. However, a physiological role for adiponectin has not been fully established (Haluzik, Parizkova et al. 2004). Elevated levels of interleukin 6 (IL-6) may also reduce food intake (Kuhlmann and Levin 2008) and contribute to muscle wasting. In fact, the presence of elevated IL-6 and e.g. tumor necrosis factor α may even cause the normally anabolic impact of insulin-like growth factor 1 (IGF-1) to become detrimental (Barbieri, Ferrucci et al. 2003). Muscle specific ubiquitin ligases, MAFbx and MuRF-1, play an important role in protein breakdown and have been shown to be upregulated during undernutrition (Bodine, Latres et al. 2001; Dehoux, van Beneden et al. 2003; Wray, Mammen et al. 2003; Nikawa, Ishidoh et al. 2004), systemic inflammation (Saini, Al-Shanti et al. 2008) and type II atrophy. Thus changes in the expression of these factors could contribute to the loss of body mass during alfacalcidol supplementation.

The aim of this study was to determine why alfacalcidol supplementation (0.1 g·kg⁻¹·day⁻¹), which was initially thought to be a countermeasure against age-related muscle atrophy, actually induced muscle wasting. We hypothesised that alfacalcidol supplementation causes 1) malnutrition due to decreased leptin and increased adiponectin levels 2) systemic inflammation contributing to the loss of body and muscle mass 3) muscle atrophy via a decrease in the expression of anabolic and an increase in the expression of catabolic factors and 4) a reduced expression of the VDR and CYP27B1 in the muscle which would attenuate the increase in the level of 1,25D within the muscle fibres.
Material and Methods

Animals
Female Fischer x Brown Norway F1 rats were obtained from Harlan (USA) (n = 16). This strain of rats is recommended by the National Institute of Ageing as the strain of choice for the study of ageing processes as it suffers less than other strains from co-morbidities (Lipman, Chrisp et al. 1996). Rats were housed one to a cage at a 12:12 light dark cycle with food and standard laboratory chow provided ad libitum. The rats were 7- and 27.5-month old at the end of the experiment. The 27.5-month-old rats were randomly divided in alfacalcidol or vehicle treated groups (Table 3.1). Rats were orally administered vehicle or alfacalcidol (0.1 g·kg⁻¹) (Chugai Pharmaceutical, Japan) for 5 days during 6 weeks. This dose has been shown to inhibit bone resorption and enhance bone formation in ovariectomized rats treated for 5 weeks (Shiraishi, Takeda et al. 2000). Rats were weighed before vehicle and alfacalcidol administration to determine the dose. The 7-month-old rats did not receive any treatment. Food and water consumption were monitored in the 6-week-treatment groups. All experiments were approved by the local ethics committee of the VU University Amsterdam and conform to the Dutch Research Council’s guide for care and use of laboratory animals.

Rats were anaesthetized by an initial dose of urethane (0.75 g·kg⁻¹ i.p). After 10 minutes an additional dose of 0.75 g·kg⁻¹ urethane was given. If the rats still responded to nociceptive stimuli, supplementary injections of 0.63 g·kg⁻¹ were applied during the experiment. Contractile properties of the Gm were determined as described previously (Testerink et al., 2010). Briefly, the sciatic nerve was cut and contractions induced by supramaximal electrical stimulation of the nerve. Subsequently, the muscle was set at optimal length (L₀), defined as the length at which the active twitch force was maximal. Then L₀ was fine-adjusted with several tetanic contractions (150 Hz, 150 ms). After completion of the contractile measurements, the Gm of the right leg was excised, weighted, stretched to L₀ on cork and frozen in liquid nitrogen with vigorous shaking.

<table>
<thead>
<tr>
<th>group</th>
<th># animals</th>
<th>treatment</th>
<th>Age (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>9</td>
<td>none</td>
<td>7</td>
</tr>
<tr>
<td>A6WO</td>
<td>9</td>
<td>6 weeks Alfacalcidol (0.1 g (in 1 ml).kg⁻¹ BW)</td>
<td>27.5</td>
</tr>
<tr>
<td>V6WO</td>
<td>9</td>
<td>6 weeks Vehicle (1 ml. kg⁻¹ BW)</td>
<td>27.5</td>
</tr>
</tbody>
</table>

Table 3.1: Number of animals in each group, with treatment and age of the rats at the end of the experiment. CA: control adult; A6WO: (alfacalcidol, 6 weeks, old), old rats treated with alfacalcidol for 6 weeks; V6WO: (vehicle, 6 weeks, old), old rats treated with vehicle for 6 weeks.

Cryosectioning
Cryosectioning of the proximal part of the Gm was done within 1 week after freezing in liquid nitrogen. Cross-sections (10 µm) were cut in a cryostat at -20 °C and collected on Vectabond (Vector Laboratories; Burlingame, CA) coated slides, air dried for at least 10 minutes at room temperature and stored at – 80 °C until further use.
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**Fibre type composition**
Sections were stained for myosin ATPase to determine the fibre type composition as described in detail by Lind & Kernell (1991) (Lind and Kernell 1991). In short, two complementary techniques were used: (i) staining following 10 min preincubation at pH 4.7 and at room temperature (acid (ac)-ATPase; cf. Brooke & Kaiser, 1970) (Brooke and Kaiser 1970); (ii) staining following consecutive pretreatments of (a) 5 min fixation with 5% paraformaldehyde at +4 °C and pH 7.6, and (b) 15 min preincubation at pH 10.55 and at room temperature (fixed alkaline (alk)-ATPase; cf. Guth & Samaha, 1970 (Guth and Samaha 1970)). The absorbance of the final reaction product was measured and expressed as arbitrary absorbance units. The muscle fibres were classified into four types according to their staining properties for mATPase (see Results) as described by Lind & Kernell (1991) (Lind and Kernell 1991). In the same fibres we determined the cross sectional area (CSA).

**Total RNA isolation.**
Total RNA was extracted from the low oxidative, distal part of the Gm (De Ruiter, De Haan et al. 1995) using the RiboPure kit (Applied Biosystems, Foster City, USA) according to the instructions of the manufacturer. RNA concentrations were determined in duplo by spectroscopy (ND-1000 spectrophotometer; Nanodrop Technologies, Wilmington, DE). RNA purity was verified by the 260:280 ratio (range 1.90-2.10, mean 2.04). The muscle RNA concentration was presented as µg RNA per mg muscle mass.

**Reverse Transcription (RT).**
Five hundred ng of total RNA per muscle were reverse transcribed using the high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA) containing random primers in a 20-μl total reaction volume. Tubes were heated at 25 ºC for 5 min, followed by 42 ºC for 30 min. Finally the tubes were heated to 85 ºC for 5 min to stop the reaction and stored at -80 ºC until used in the qPCR reaction.

**qPCR.**
Quantitative PCR was applied to study the expression of 18S RNA and mRNAs of α-skeletal actin, insulin-like growth factor-I (IGF-I; all isoforms), muscle ring finger-1 (MuRF1), muscle atrophy F-box (MAFbx), myostatin, c-myc, vitamin D receptor (VDR) and CYP27B1. The sequences for the primers (Invitrogen, The Netherlands) used for the specific targets are shown in Table 3.2. For each target, RT and PCR reactions were carried out under identical conditions by using the same reagent premix for all samples. Five µl of each RT reaction was used for the PCR amplification. cDNA dilutions were set so that both the target mRNA and 18S product yields were in the linear range of the semi-log plot when the yield is expressed a function of the number of cycles. Amplifications were carried out in a StepOne real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA) with an initial activation/denaturing step of 23 s at 95 ºC followed by an annealing step of 30 s at 60 ºC. The range of cycle threshold values was 15-30. Specificity was confirmed by melting curve analysis after amplification. 18S RNA and mRNA data were normalized to total RNA per sample as well as to the mass of tissue used to extract the RNA used for cDNA synthesis. Differences in mRNA for each primer are shown relative to 18S RNA.
### Table 3.2: Sequence of the specific primers used in the quantitative PCR analyses. IGF-1: insulin growth factor-1, MAFbx: muscle atrophy F-box, MuRF1: muscle ring finger-1, VDR: vitamin D receptor.

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>PCR primer sequence 5’ → 3’</th>
</tr>
</thead>
</table>
| 18S RNA                  | Forward: CGAACGTCTGCCCTATCAACTT  
                          | Reverse: ACCCGTGTCACCACCATGGTA  |
| α-skeletal actin         | Forward: CGACATCGACATCAGGAAGGA  
                          | Reverse: GGTAGTGCCCCCCTGACATGA  |
| IGF-I (all isoforms)     | Forward: CCTACAAAAGTCAGCTCGTTCCA  
                          | Reverse: TCCTTCTGAGTCTTTGGGCGATGT  |
| MAFbx                    | Forward: TGAAGACCAGGCTACTGTTGGA  
                          | Reverse: CGGATCTGCGCCGTCCTGA  |
| MuRF1                    | Forward: TGCCCCCTTACAAACATCTTT  
                          | Reverse: CAGCATGGAGATGCAATTGC  |
| myostatin                | Forward: GTTCCCCGGAGAGACTTTGG  
                          | Reverse: CGACAGCGCGCGATTC  |
| c-myc                    | Forward: CACAAACGTCTTGGAACGTCAGA  
                          | Reverse: GCGCAGGGGAAAAAGGC  |
| VDR                      | Forward: CACCCTTGAGGCCCTACTCAG  
                          | Reverse: CTGTTGCTCCATCCCTGAA  |
| CYP27B1                  | Forward: CGGGAAAAGGTGTCTGTCCA  
                          | Reverse: GTGTTGACCTCCAGTGA  |

Serum levels.
Leptin and adiponectin (Invitrogen, Camarillo, CA, USA) and IL-6 (Bender MedSystems, Vienna, Austria) levels in serum were determined with ELISA’S according to the instruction of the manufacturer.

Statistics.
SPSS® v16.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. One-way ANOVA was performed to assess whether there were any significant differences between the alfalcaldiol group and the age matched vehicle group, between the alfalcaldiol group and the control adult group and between the vehicle group and the control adult group. A repeated measures ANOVA was performed with muscle region (2 levels) and fibre type (3 levels) as within factors and with age and treatment as between subject factors to assess the effects on fibres of different types in the low and high oxidative region. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SEM.
Results

Body and muscle mass

Table 3.3 shows the mean body and muscle mass as well as the 1,25D serum concentrations. The old animals were 20% heavier than the adult animals (P ≤ 0.001). Vehicle treatment did not significantly affect body mass. Six weeks treatment with alfacalcidol, however, caused a 22% loss of body mass in the old rats (P = 0.001).

The serum concentration of 1,25D in the V6WO was comparable to those of the CA group, indicating that age and vehicle had no effect on the 1,25D serum concentration. After 6 weeks of treatment with alfacalcidol, 1,25D serum levels were increased 5 times to 234 pmol/L (P < 0.01) (Table 3.3).

Ageing did not significantly affect the mass of the gastrocnemius (Gm) (Table 3.3), soleus, plantaris, extensor digitorum longus and tibialis anterior muscles (data not shown). Gm mass was reduced by about 17% after 6 weeks of alfacalcidol treatment compared to the controls (P < 0.001). Plantaris muscle mass was reduced in the same order of magnitude (P < 0.05), but the mass of the other muscles was not significantly affected by alfacalcidol. The Gm mass : body mass ratio was lower in the old than in the adult rats (P < 0.001). Treatment with alfacalcidol did not change this ratio, indicating that the decrease in body mass was not only due to a decrease in muscle mass, but also due to a proportionally similar decrease in other tissue.

<table>
<thead>
<tr>
<th>group</th>
<th>Body mass (g)</th>
<th>Gm mass (mg)</th>
<th>[1,25D] (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>221 (5)</td>
<td>669 (16)</td>
<td>46.6 (6.88)</td>
</tr>
<tr>
<td>A6WO</td>
<td>224 (6)#</td>
<td>515 (12)#</td>
<td>233.8 (60.0)**</td>
</tr>
<tr>
<td>V6WO</td>
<td>276 (12)</td>
<td>622 (18)</td>
<td>57.1 (11.5)</td>
</tr>
</tbody>
</table>

Table 3.3: Mean (±SEM) of body mass, Gastrocnemius muscle (Gm) mass and 1,25D serum levels. # significantly different from V6WO; ** significantly different from CA and V6WO (P < 0.05). (ANOVA, bonferroni posthoc).

Fibre type distribution and cross sectional area

In the high oxidative part, there were approximately 25% type I, 20% type IIa 60% type IIb/x fibres (Fig 3.1A), while in the low oxidative part there were 5% type IIa, 40% type IIb and 40% IIx fibres and almost no type I fibres (Fig 3.1B). The fibre type distributions were not significantly affected by age and/or alfacalcidol treatment. The CSA of the different fibre types were similar in the high and low oxidative part of the Gm (Fig 3.1). For all fibres pooled, the CSA in high oxidative part of the control adult Gm was 22% smaller than that of the old vehicle treated rats (P = 0.018) (Fig 3.2A). This was due to the larger size of type IIa fibres, but not fibres of other types, in this region of the old vehicle group than the control adult group (P = 0.012). No difference in CSA was found between the old vehicle and alfacalcidol group (Fig 3.2A). In the low oxidative region of the Gm, however, the CSA of the old alfacalcidol treated group was 16 % smaller (P = 0.029) compared to that of the old vehicle treated rats (Fig. 3.2B) which was due to a decrease of the CSA of type IIx (n.s.) and type IIb fibres (P = 0.029) (Fig 3.1B).
Figure 3.1: Fibre type distribution and cross sectional area (CSA) of the fibre types in the A) high oxidative and B) low oxidative part of the Gm. Fibre type composition (left) and CSA of the different fibre types (right). No differences were observed in fibre type composition. The CSA of the type IIa fibres in the high oxidative part of the control adult group were smaller compared to the old vehicle group. However, in the high oxidative part, type IIb fibres of the alfacalcidol treated animals were significantly smaller compared to the age matched vehicle group.

# different from CA (P = 0.018)
* different from V6WO (P = 0.029)
(Repeated ANOVA, Bonferroni posthoc)
Figure 3.2: combined cross sectional area of different fibre types of the high oxidative (A) and low oxidative (B) part of the Gm. In the high oxidative part the CSA of the control adult group was smaller compared to the old vehicle group. In the low oxidative part the CSA of the alfacalcidol treated animals was smaller compared to the age matched vehicle group indicating that the observed decrease in muscle mass was the result of type atrophy of the low oxidative part.

# different from CA (P = 0.012)
* different from V6WO (P = 0.030)
(ANOVA, bonferroni posthoc)

**Effects of age and alfacalcidol on mRNA expression**

To clarify how alfacalcidol induces atrophy of the fibres in the low oxidative part of the Gm, mRNA levels of genes involved in the regulation of protein synthesis and degradation were determined. As the atrophy was apparent in the distal, low oxidative, part of the Gm, qPCR analyses were performed on this part only. Total RNA per mg muscle was similar in the alfacalcidol, vehicle and adult control group (Fig 3.3A). mRNAs were normalized to the 18S ribosomal RNA expression. Figure 3.3B shows that the expression levels of the structural protein α skeletal actin and the ubiquitin ligase MAFbx were similar in all groups. In contrast, the ubiquitin ligase MuRF1 was upregulated in the old vehicle group compared to the control adult group (P < 0.05) and was further elevated after alfacalcidol treatment (P < 0.001) (Fig 3.3B).

The mRNA level of the autocrine growth factor myostatin, involved in regulating protein synthesis and degradation, was reduced with ageing (P = 0.002). However, myostatin mRNA expression was not significantly affected by alfacalcidol. IGF-1 and c-myc mRNA expression levels as well as that of the vitamin D linked markers, VDR and CYP27B1, were similar in all groups (Fig 3.3C and 3.3D).
Figure 3.3: Quantitative PCR of structure protein, part of the ubiquiton pathway, growthfactors and vitamin D associated proteins of the low oxidative, distal part of the Gm. A) total RNA per mg muscle. No differences in total RNA concentration were observed between the groups indicating that it was allowed to normalise the expression of the other factors for 18S RNA. B) Expression of the structural protein alpha actin and the ubiquitin pathway proteins MAFbx and MuRF1. MuRF1 expression was increased in the alfacalcidol treated group compared to the age matched vehicle group and control adult group. Furthermore the expression of MuRF1 in the vehicle group was higher compared to the control adult group, suggesting an age effect. C) expression of the growth factors myostatin and IGF-1. Myostatin expression was decreased in the alfacalcidol and vehicle group compared to the control adult group, however, no differences were observed between the alfacalcidol group and age matched vehicle group. D) c-myc expression was not different between the group as was the VDR and CYP27B1 expression. *: P <0.05 #: P <0.001 (ANOVA, bonferroni posthoc).
**Circulating factors**

The inflammatory marker IL-6, which is associated with sarcopaenia and muscle wasting, was elevated in the old rats ($P \leq 0.05$), but was not significantly elevated further after treatment with alfacalcidol. The adiponectin serum concentration was 64% higher in the alfacalcidol group compared to that of the vehicle group ($P = 0.004$). The serum adiponectin concentration in the alfacalcidol group did not differ from that of the control adult group. The leptin serum concentration, which decreases during fasting (review (Kelesidis, Kelesidis et al.)), was decreased in the alfacalcidol group compared to the age matched vehicle group ($P = 0.034$). (Fig 3.4).

**Figure 3.4**: Serum levels of IL-6, adiponectin and leptin. IL-6 levels are increased in A6WO and V6WO compared to the control adult group. Adiponectin levels of the alfacalcidol group were increased compared to the age matched vehicle group. Leptin levels of the alfacalcidol group were decreased compared to the age matched vehicle group. (ANOVA, bonferroni posthoc).
Discussion
Administration of alfacalcidol to rats with normal vitamin D levels resulted in elevated levels of 1,25D. This was accompanied by a reduction in maximal force and power generating capacity, atrophy and weight loss in both adult and old rats (Testerink et al., 2010). Since ageing is accompanied by muscle wasting and weakness we were interested how factors that may already be affected by the ageing process may be further affected by elevated levels of alfacacidol, thereby aggravating sarcopenia.

The main observation of the present study is that the loss of muscle mass after treatment with alfacalcidol was fibre type and muscle region specific; only type IIB/X fibres in the low oxidative, distal part of the muscle were atrophied. The atrophy was accompanied by an increase in the expression of MuRF1 on top of the age-related increase in MuRF1 expression. Note that this increase in MuRF1 expression is not a direct proof of the cause of atrophy, but is consistent with the hypothesis that MuRF1 is upregulated during atrophy. While ageing is accompanied by low-grade systemic inflammation, as reflected by elevated circulating IL-6 levels, alfacalcidol did not cause a further rise in IL-6. In addition, food intake in rats treated with alfacalcidol was reduced (Testerink et al., 2010). Here we show that the decreased food intake was not related by increased serum leptin levels, although adiponectin was elevated. Despite elevated circulating levels of 1,25D, the VDR and CYP27b1 mRNA expression levels remained unaltered during ageing and alfacalcidol treatment. This suggests that the main cause of atrophy induced by elevated levels of circulating vitamin D is a reduced food intake and not an increased systemic inflammation and/or altered satiety set-points.

Effects of ageing
It has been shown that the specific tension of rat (Degens, Hoofd et al. 1995; Degens and Alway 2003) and human (Review (Degens, Erskine et al. 2009)) muscles decreases with age. In our previous work on the very same animals we have shown that this was also the case in our animals (Testerink et al., 2010). Here we found that MuRF-1 mRNA was elevated and myostatin mRNA reduced in the old rats. Elevated MuRF1 mRNA expression during aging has been shown before in both rat (Clavel, Coldefy et al. 2006) and human muscle (Raue, Slivka et al. 2007). Also an increase in myostatin has been shown in skeletal muscle (Dennis, Przybyla et al. 2008), but this is not unequivocal as also unchanged or even increased myostatin mRNA levels have been reported in muscles from both elderly and old rats (Clavel, Coldefy et al. 2006; Leger, Derave et al. 2008). Both MuRF1 and myostatin play a role in muscle atrophy (Watt, Jaspers et al.; Bodine, Latres et al. 2001; McFarlane, Hennebry et al. 2008) and since the Gm did not show any atrophy, compensatory mechanisms might have been activated. One such factor might be c-myc, a transcription factor involved in the regulation of rRNA expression (Gomez-Roman, Felton-Edkins et al. 2006), and is elevated in C2C12 myotubes cultured with 1,25D (Buitrago, Vazquez et al. 2001). Here we did not see a change in c-myc mRNA expression with age, which fits with our earlier observation that the old rats were not vitamin D deficient (Testerink et al., 2010). We also did not find an increase in the anabolic IGF-1 mRNA during ageing and several studies even reported an age-related decrease in IGF-I mRNA levels in both rats (Clavel, Coldefy et al. 2006) and human (Leger, Derave et al. 2008). It does seem unlikely that compensatory mechanisms attenuated the atrophy that is expected to follow the rise in MuRF1 and myostatin expression.
Another explanation might be that the increased mRNA levels of MuRF1, myostatin and unaltered IGF-I mRNA together with the elevated circulating levels of IL-6 in the old animals indicates the beginning of sarcopenia induced by low grade systemic inflammation (Degens 2007; Degens 2009). A similar situation has been observed in humans where loss of muscle strength was associated with increased apoptosis, but yet no significant muscle fibre atrophy (Whitman, Wacker et al. 2005). Overall, our old rats showed age-effects similar to those observed in humans and suggests that the rat can serve as a model to study the mechanisms and type of atrophy caused by elevated levels of vitamin D superimposed on the age-related muscle dysfunction and onset of atrophy.

Effects of alfacalcidol and muscle fibre atrophy
The alfacalcidol-induced decrease in Gm muscle mass was due to fibre type-specific atrophy and limited to type Iib and Iix fibres in the low oxidative distal part of the muscle. This was somewhat unexpected as in contrast to supraphysiological levels of 1,25D in our study, vitamin D deficiency in older women was accompanied by type II fibre atrophy (Verhaar, Samson et al. 2000).

The discrepancy may be related to the reduced food intake in our alfacalcidol-treated animals (Testerink et al. 2010), as in starved rodents particularly the highly glycolytic (presumably Iib/x) fibres have been reported to atrophy (Goldspink and Ward 1979). This, and the unaltered fibre type composition during starvation (Goldspink and Ward 1979), also observed in our rats, suggests that any effect of elevated levels of 1,25D may be overruled by the effects of a reduced food intake.

Alfacalcidol treatment-related decreased food intake and muscle fibre atrophy
Leptin plays a key role in regulating energy intake and energy expenditure and inhibits appetite. Indeed, the levels of leptin decrease rapidly decrease during fasting (for a review see (Kelesidis, Kelesidis et al.)). Furthermore it has been shown that leptin is secreted in a pulsatile manner in humans (Sinha, Sturis et al. 1996) and a circadian rhythm for the concentration of plasma leptin levels has been shown for rats (Saladin, De Vos et al. 1995) and humans (Sinha, Ohannesian et al. 1996). The leptin concentration is highest between midnight and early morning and lowest between noon and early afternoon. Because rats were kept in similar light-dark conditions and blood was collected at the same timepoint a difference in leptin levels due to this rhythm can be excluded. One might therefore expect that the reduced food intake in our animals is due to increased leptin levels, giving the animal a reduced appetite. Yet, serum levels were decreased, rather than increased, and the decreased food intake can thus not be explained by a change in leptin levels.

Another factor that may play a role in the regulation of body mass is adiponectin, an adipokine that is elevated during weight loss (Yang, Lee et al. 2001), as was the case in our alfacalcidol-treated animals. Since adiponectin enhances glucose uptake and fatty acid oxidation in muscle (Yamauchi, Kamon et al. 2001) it might be a response to minimize the use of muscle protein in the face of starvation and ensure that the probably elevated levels of circulating fatty acids can be used for energy generation (Behre 2007).

Another condition which may contribute to loss of body mass and muscle wasting is chronic low-grade systemic inflammation, which might also reduce appetite (Goodman 1994; Wust and Degens 2007; Degens 2009). IL-6 is an inflammatory cytokine and elevated circulating levels have been associated with loss of muscle strength (Barbieri,
Because IL-6 levels were not different between the alfacalcidol and vehicle group, muscle wasting during treatment with alfacalcidol was not due to elevated circulating levels of IL-6. Since alfacalcidol did not increase circulating IL-6 levels and alfacalcidol has been shown to decrease tumor necrosis factor α (Scharla, Schacht et al. 2005), it is unlikely that the observed atrophy was the result of inflammation.

**Effects of alfacalcidol and reduced food intake on signalling pathways for protein synthesis and degradation**

We hypothesized that alfacalcidol treatment induces hypertrophy because of the presumed stimulative effects of 1,25D on hypertrophic signalling in C2C12 myotubes. 1,25D has been shown to activate and induce synthesis of c-myc (Buitrago, Boland et al. 2001; Morelli, Buitrago et al. 2001). C-myc is involved in stimulating the expression of rRNA and hence the rate of translation. In addition, it was hypothesized that 1,25D trigger a cascade of events which may lead to activation of IGF genes (Whitelaw and Hesketh 1992). Furthermore it has been shown that 1,25D activates the MAP kinases ERK1 and ERK 2 in skeletal muscle (Morelli, Buitrago et al. 2000). The inhibition of MAP/ERK kinase has been shown to prevent IGF-1 induced hypertrophy in rat muscles (Haddad and Adams 2004) which suggests that 1,25D MAP/ERK activation induces IGF-1 activation. However, neither c-myc nor IGF-I, myostatin and α-skeletal actin expression were changed by alfacalcidol treatment, despite elevated circulating 1,25D and we observed atrophy rather than hypertrophy.

A possible explanation is that the intracellular concentration differs from that in the circulation. Indeed, the intracellular concentration of 1,25D is controlled by CYP27B1, a hydroxylase that catalyses the hydroxylation of 25D to 1,25D and the potential importance of CYP27B1 in muscle wasting has been shown by the atrophy in patients with chronic renal failure, which have sufficient 25D but lack CYP27B1 (McElroy, Silver et al. 1970). These patients do not exhibit improvement in muscle size and strength when they were treated with 1,25D (Gordon, Sakkas et al. 2007). Despite elevated serum levels of 1,25D, CYP27B1 expression in the muscle was not altered after alfacalcidol treatment and thus does not seem to play a role in the muscle atrophy we observed in our study. Part of the discrepancy between the observation on CYP27B1 in our study and those patients is that the patients also had neuropathy and had sub-physiological levels of circulating vitamin D (McElroy, Silver et al. 1970), whereas our alfacalcidol treated rats had physiological levels of vitamin D to start with. However, the data indicate that caution should be considered during supplementation with active vitamin D.

Alternatively, a decrease in 1,25D serum levels may lead to a decrease in stimulation of the VDR resulting in a decrease in transcription of VDR mRNA (Costa, Blau et al. 1986). A reduced VDR expression in turn may impair protein synthesis in muscle leading to a preferential atrophy of type II fibres (Boland 1986; Costa, Blau et al. 1986). Despite supraphysiological 1,25D serum levels the VDR mRNA expression was not altered in the Gm of the alfacalcidol-treated animals, and the decrease in the CSA of type II fibres was therefore probably not due to an altered abundance of the VDR and down-stream signalling.

The lack of a hypertrophic response with elevated levels of 1,25D raised the question whether vitamin D has any atrophic effects. To our knowledge such effects have not been
reported. From the discussion so far it appears that 38% reduction in food intake (Testerink et al. 2010) is the main cause of the muscle wasting and 22% loss of body mass and not systemic inflammation and/or altered circulating leptin levels. It remains unclear how elevated levels of 1,25D cause this reduced food intake. Nevertheless, the data suggest that it is not these elevated 1,25D levels, but starvation that is the main cause of the observed atrophy.

During muscle atrophy, including that induced by fasting (Bodine, Latres et al. 2001; Dehoux, van Beneden et al. 2003; Wray, Mammen et al. 2003; Nikawa, Ishidoh et al. 2004), two muscle specific ubiquitin ligases, muscle RING-finger protein-1 (MuRF1) and atrogen-1/muscle atrophy F-box (MAFbx) are upregulated. MuRF1 ubiquitinates (Kedar, McDonough et al. 2004) myofibrillar and cytoskeleton proteins and energy metabolic enzymes (Witt, Granzier et al. 2005) in preparation for their subsequent breakdown in the proteasome (Saini, Al-Shanti et al. 2006). In line with this role in muscle protein breakdown, we observed that MuRF1 expression was increased in the alfacalcidol group, without a change in MAFbx mRNA levels. The latter is somewhat surprising as MAFbx is essential for accelerated muscle protein loss and its mRNA increases 8-40 fold in all types of muscle atrophy inducing conditions studied (Gomes, Lecker et al. 2001). It is unclear why only MuRF1 and not MAFbx was upregulated, but overall the data indicate that the alfacalcidol-induced atrophy is at least partly caused by an increased proteosome activity.

Myostatin expression has been shown to be upregulated in type II fibre atrophy (Wojcik, Nogalska et al. 2008). Yet, despite the preferential type II fibre atrophy in our study, no difference in myostatin expression in muscles from the alfacalcidol and vehicle group were found, suggesting that myostatin had no role in the alfacalcidol-induced atrophy.

In conclusion, the observed decrease in muscle mass during alfacalcidol supplementation which leads to elevated serum levels of 1,25D is the result of a preferential atrophy of type IIb and IIx fibres. This atrophy is probably the result of an upregulation of the ubiquitin ligase MuRF1 mRNA. This increased expression is on top of the age-related increase in MuRF1 expression. This increased expression of MuRF1 is not due to altered muscular myostatin and IGF-1 expression and is not associated with increased systemic inflammation. It appears that reduced food intake is the main factor.

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