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ORIGINAL PAPER

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

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Abstract Supra-physiological levels of vitamin D induce skeletal muscle atrophy, which may be particularly detrimental in already sarcopaenic elderly. Neither the cause nor whether the atrophy is fibre type specific are known. To obtain supraphysiological levels of circulating vitamin D ($1,25(OH)_2D_3$) 27.5-month-old female $Fischer_{344}$ × Brown Norway F1 rats were orally treated for 6 weeks with vehicle or the vitamin D analogue alfacalcidol. Alfacalcidol treatment induced a 22% decrease in body mass and 17% muscle atrophy. Fibre atrophy was restricted to type IIb fibres in the low-oxidative part of the gastrocnemius medialis only (−22%; $P < 0.05$). There was a concomitant 1.6-fold increase in mRNA expression of the ubiquitin ligase MuRF-1 ($P < 0.001$), whereas those of insulin-like growth factor 1 and myostatin were not affected. Circulating IL-6 was unaltered, but leptin and adiponectin were decreased (−39%) and increased (64%), respectively. The treated rats also exhibited a reduced food intake. In conclusion, supraphysiological levels of circulating $1,25(OH)_2D_3$ cause preferential atrophy of type IIb fibres, which is associated with an increased expression of MuRF-1 without evidence of systemic inflammation. The atrophy and loss of body mass in the presence of supra-physiological levels of vitamin D are primarily due to a reduced food intake.

Keywords Vitamin D · Atrophy · IGF-1 · Myostatin · Aging · E3-ligase

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Introduction

Elderly vitamin D-deficient people are at a higher risk of sarcopaenia than those with normal levels of vitamin D [1]. Also, in young people, a positive relationship between vitamin D status and muscle performance has been reported [2, 3]. Given these observations, it is no surprise that 8–12 weeks vitamin D plus calcium supplementation in institutionalized vitamin D-deficient elderly people improved musculoskeletal function by 4–11% [4] and body sway by 9% in ambulatory elderly women [5]. These benefits may contribute to the 22% lower risk of falling after long-term supplementation of vitamin D in vitamin D-deficient people [6].

Vitamin D may exert these beneficial effects via its impact on Ca^{2+} homeostasis, and by proliferation and differentiation of myoblasts [7]. The latter two effects are probably mediated via the interaction of vitamin D with the vitamin D receptor (VDR) as VDR knock-out mice suffer from abnormal muscle development and deregulated expression of myogenic regulatory factors [8]. The impact of vitamin D on muscle is also reflected by the increase in muscle mass and decreased rate of myofibrillar protein degradation after vitamin D administration to deficient rats [9]. Also, in humans, vitamin D administration in vitamin D-deficient people induces increases in muscle size and strength, and an increased proportion and size of type II fibres [10].

CYP27B1 is a hydroxylase that catalyses the hydroxylation of 25D to the active form of vitamin D (1,25D). Although the hydroxylation of 25D occurs mainly in the kidney, 1,25D is also produced extra-renally [11], probably including muscle. The expression of the hydroxylase CYP27B1 in the muscle may serve to dampen the impact of fluctuations in circulating 1,25D levels on the intracellular 1,25D concentration that could otherwise cause dysregulation of gene expression and Ca^{2+} homeostasis.

It has been shown in a human liver cell line that maximal VDR activity occurs at concentrations of 1,25D far above physiological circulating levels [12]. One might thus speculate that supraphysiological 1,25D serum levels would accentuate the hypertrophic effects of vitamin D described above. Yet, we have shown that increased 1,25D serum levels in rats after alfacalcidol supplementation, a vitamin D analogue which is converted into 1,25D, were accompanied by a reduction in body mass, muscle atrophy and muscle weakness [13], rather than hypertrophy and improvements in muscle strength. Elucidating the cause of this muscle atrophy 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 loss of body and muscle mass during alfacalcidol treatment was associated with a reduced food intake [13]. Reductions in food intake may be caused by altered sensations of satiety. The hormone leptin plays an important role in the regulation of food intake and feelings of satiety and is positively related to the amount of body fat [14]. Adiponectin, on the other hand, plays a role in the regulation of metabolism and is inversely related to the amount of body fat [15, 16]. Elevated levels of interleukin 6 (IL-6) may also reduce food intake [17] and contribute to muscle wasting via abolishing the normally anabolic effect of insulin-like growth factor 1 (IGF-1) [18]. The muscle breakdown may be realised by an increased expression of the muscle specific ubiquitin ligases, MAFbx and MuRF-1, that play an important role in protein breakdown, as observed during undernutrition [19–22] and systemic inflammation [23]. It is thus possible that undernutrition and inflammation may induce the expression of the muscle specific E3 ubiquitin ligases and thereby contribute to the loss of body and muscle mass during alfacalcidol supplementation.

The aim of this study was to determine how alfacalcidol supplementation, which was initially thought to counteract sarcopaenia, actually induced muscle wasting. We hypothesised that alfacalcidol supplementation causes (1) the previously observed malnutrition [13] due to increased leptin and decreased 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.

Materials and methods

Animals

Female $Fischer_{344}$ × 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 as it suffers less than other strains from co-morbidities [24]. Rats were housed one to a cage at a 12:12 h light dark cycle with food and standard laboratory chow provided ad libitum. The rats were 7 and 27.5 months old at the end of the experiment. The 27.5-month-old rats were randomly divided in alfacalcidol- or vehicle-treated groups (Table 1). Rats were orally administered vehicle or alfacalcidol (0.1 μg kg^{-1}) (Chugai Pharmaceutical, Japan), 5 days a week during 6 weeks. This dose has been shown to inhibit bone resorption and enhance bone formation in ovariectomized rats treated for 5 weeks [25]. 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 anaesthetised by an initial dose of urethane (0.75 g kg^{-1} i.p). After 10 min, 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 gastrocnemius medialis muscle (Gm) were determined as described previously [13]. Briefly, the sciatic nerve was cut and contractions induced by supramaximal electrical stimulation of the nerve. Subsequently, the muscle was set at optimal length (L_o),

Table 1 Number of animals in each group, with treatment and age of the rats at the end of the experiment

Group	No. of animals	Treatment	Age (months)
CA	9	None	7
A6WO	9	6 weeks alfacalcidol (0.1 μg (in 1 ml) kg^{-1} BM)	27.5
V6WO	9	6 weeks vehicle (1 ml kg^{-1} BM)	27.5

Data from [13]

CA control adult, *A6WO* (alfacalcidol for 6 weeks, old), old rats treated with alfacalcidol for 6 weeks, *V6WO* (vehicle for 6 weeks, old), old rats treated with vehicle for 6 weeks, *BM* body mass

defined as the length at which the active twitch force was maximal. Then, L_o was fine-adjusted with several tetanic contractions (150 Hz, 150 ms). After completion of the contractile measurements (data presented elsewhere [13]), the Gm of the right leg was excised, weighed, stretched to L_o on cork and frozen in liquid nitrogen with vigorous shaking.

Cryosectioning

Muscles were frozen in liquid nitrogen and stored at −80°C until use. Cross-sections (10 μm) of the muscles were cut from 1/3 to 1/2 of its length distally from origin using a cryostat at −20°C. Sections were collected on Vectabond (Vector Laboratories, Burlingame, CA, USA)-coated slides air- dried for at least 10 min at room temperature and stored at −80°C until further use.

Fibre type composition

Sections were stained for myosin ATPase to determine the fibre type composition as described previously [26]. In short, two complementary techniques were used: (1) staining following 10 min preincubation at pH 4.7 at room temperature (acid (ac)-ATPase [27]); (2) staining following consecutive pre-treatments of (a) 5 min fixation with 5% paraformaldehyde at 4°C and pH 7.6 and (b) 15 min preincubation at pH 10.55 at room temperature [28]. The muscle fibres were classified by eye into four types according to their staining properties for mATPase [26]. 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 using the RiboPure kit (Applied Biosystems, Foster City, CA, USA) according to the instructions of the manufacturer. RNA concentrations were determined in duplet by spectroscopy (ND-1000 spectrophotometer; Nanodrop Technologies, Wilmington, DE, USA). RNA purity was verified by the 260:280 ratio (range 1.90–2.10, mean 2.04). The RNA concentration was presented as μg RNA per mg muscle mass.

Reverse transcription (RT)

Five hundred nanograms of total RNA per muscle were reverse transcribed using the high capacity RNA-to-cDNA kit (Applied Biosystems) containing random primers in a 20-μl total reaction volume. Tubes were heated at 25°C for 5 min, followed by 42°C for 30 min. Finally, the tubes were heated to 85°C for 5 min to stop the reaction, and stored at −20°C until used in the qPCR reaction.

qPCR

Quantitative PCR was performed to study the expression of 18S RNA and mRNAs of α-skeletal actin, insulin-like growth factor-I Ea (IGF-I Ea), muscle ring finger-1 (MuRF-1), muscle atrophy F-box (MAFbx), myostatin, c-myc, vitamin D receptor (VDR) and CYP27B1. The sequences for the primers (Invitrogen, The Netherlands) are shown in Table 2. For each target, RT and PCR reactions were carried out under identical conditions by using the same reagent premix for all samples. Five microlitres of each RT reaction were 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 where the yield is expressed as a function of the number of cycles. Amplifications were carried out in duplet in a StepOne real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA) with an initial activation/denaturing step of 23 s at 95°C followed by an annealing step of 30 s at 60°C. 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.

Serum cytokine levels

Blood was collected from the vena cava after the contractile properties of the Gm had been determined. Circulating Leptin and adiponectin (Invitrogen, Camarillo, CA, USA) and IL-6 (Bender MedSystems, Vienna, Austria) levels were determined in serum with ELISAs according to the instruction of the manufacturer. To minimise bias related to the circadian changes in, e.g., leptin concentrations [29, 30], all rats were kept in similar light–dark conditions and blood was collected at the same time of day.

Statistics

SPSS® v.16.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. One-way ANOVA was performed to assess whether there were any significant differences between (1) the alfacalcidol and the age-matched vehicle group, (2) the alfacalcidol and the control adult group, and (3) the old vehicle and control adult group. ANOVA was also used to assess potential differential effects of alfacalcidol on the different fibre types in the low and high oxidative region. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SEM.

Table 2 Sequence of the specific primers used in the quantitative PCR analyses

Target mRNA	PCR primer sequence 5' → 3'	T_m	Efficiency	R^2	Acession number
18S RNA	Forward: CGAACGTCTGCCCTATCAACTT	77.27	102.0	0.998	EU139318.1
	Reverse: ACCCGTGGTCACCATGGTA				
α-skeletal actin	Forward: CGACATCGACATCAGGAAGGA	76.37	94.5	1	NM_019212.2
	Reverse: GGTAGTGCCCCCTGACATGA				
IGF-1 Ea	Forward: AAGCCTACAAAGTCAGCTCG	78.76	91.3	0.997	NM_178866.4
	Reverse: TCAAGTGTACTTCCTTCTGAGTC				
MAFbx	Forward: TGAAGACCGGCTACTGTGGAA	75.46	95.5	0.999	NM_133521.1
	Reverse: CGGATCTGCCGCTCTGA				
MuRF-1	Forward: TGCCCCCTTACAAAGCATCTT	73.38	98.17	0.998	NM_080903.1
	Reverse: CAGCATGGAGATGCAATTGC				
Myostatin	Forward: GGTCCCGGAGAGACTTTGG	77.71	96.2	0.999	NM_019151.1
	Reverse: CGACAGCACCGCGATTC				
c-myc	Forward: CACAACGTCTTGGAACGTCAGA	75.34	91.3	0.998	NM_012603.2
	Reverse: GCGCAGGGCAAAAAAGC				
VDR	Forward: CACCCTTGGGCTCTACTCAC	74.74	112.7	0.98	NM_017058.1
	Reverse: CTGTTGCCTCCATCCCTGAA				
CYP27B1	Forward: CGGGAAAAGGTGTCTGTCCA	75.48	107.2	0.966	NM_053763.1
	Reverse: GTGTCCACTCCAGTAG				

IGF-1 insulin-like growth factor-1 Ea, *MAFbx* muscle atrophy F-box, *MuRF-1* muscle RING finger-1, *VDR* vitamin D receptor

Table 3 Body mass, gastrocnemius muscle (Gm) mass and 1,25D serum levels

Group	Body mass (g)	Gm mass (mg)	[1,25D] (pmol l^{-1})
CA	221 (5)	669 (16)	46.6 (6.88)
A6WO	224 (6)*	515 (12)*	233.8 (60.0)**
V6WO	276 (12)	622 (18)	57.1 (11.5)

Data are represented as mean ± SEM; Data from [13]

* Significantly different from V6WO, ** significantly different from CA and V6WO ($P < 0.05$). (ANOVA, Bonferroni posthoc)

Results

Body and muscle mass

Table 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 ($P < 0.01$). 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 fivefold increased to 234 pmol l^{-1} ($P < 0.01$) (Table 3).

Ageing did not significantly affect the mass of the gastrocnemius (Gm) (Table 3), soleus, plantaris, extensor digitorum longus and tibialis anterior muscles (data not shown). Six weeks of alfacalcidol treatment caused a 17% reduction in Gm mass ($P < 0.001$). 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 to a proportionally similar decrease in the mass of other tissues.

Fibre type distribution and cross-sectional area

The high oxidative part contained approximately 25% type I, 20% type IIa and 55% type IIb/x fibres (Fig. 1a), and the low oxidative part 10% type IIa, 50% type IIx, 40% IIb fibres with almost no type I fibres (Fig. 1c). The fibre type distributions were not significantly affected by age and/or alfacalcidol treatment.

In all groups, the CSA of the different fibre types were similar in the high and low oxidative part of the Gm (Fig. 1b, d). For all fibres pooled, the CSA in high oxidative part of the old vehicle treated Gm was 22% larger than that of the control adult rats ($P = 0.018$) (Fig. 2b). This was primarily due to the increased size of type IIa fibres in

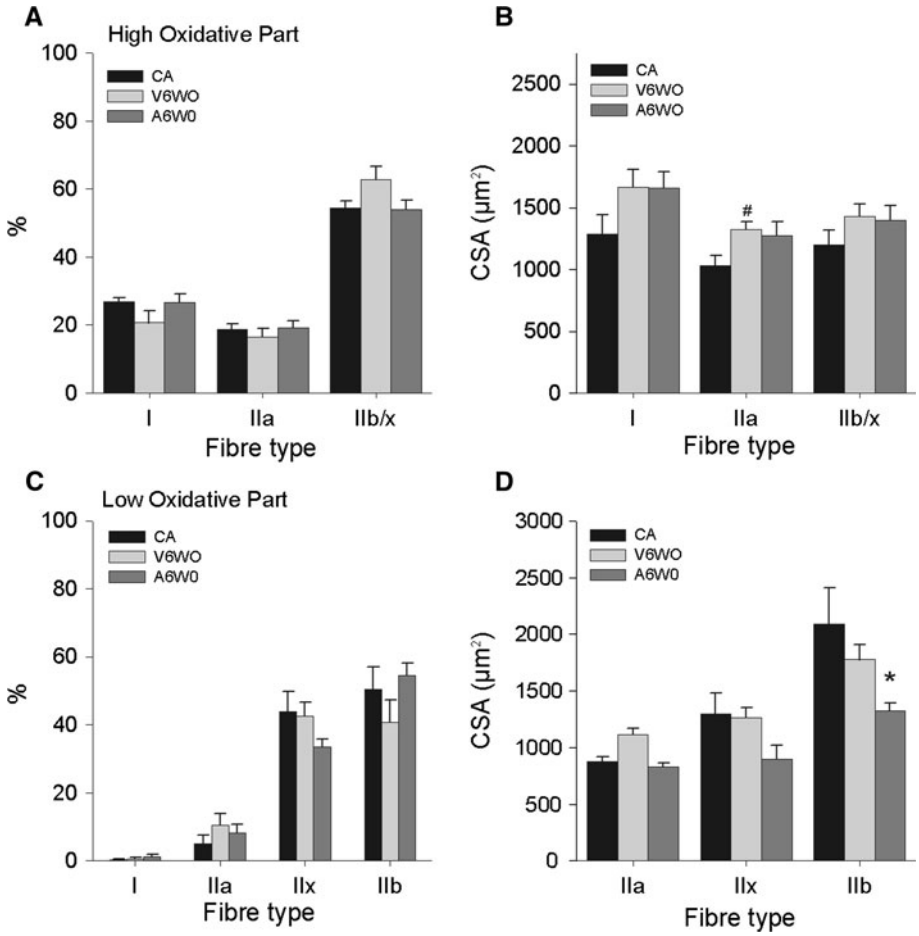

Fig. 1 Fibre type distribution (**a**, **c**) and cross-sectional area (*CSA*) (**b**, **d**) of the fibre types in the high oxidative (**a**, **b**) and low oxidative (**b**, **d**) parts of the gastrocnemius medialis muscle. Fibre type composition (*left*) and CSA of the different fibre types (*right*). There were no significant changes in fibre type composition during ageing or alfacalcidol treatment. The CSA of the type IIa fibres in the high oxidative part of the old vehicle group were larger than that of the adult group. In the low oxidative part, type IIb fibres of the alfacalcidol-treated animals were significantly smaller compared to the age-matched vehicle group. *CA* control adult, *A6WO* (alfacalcidol for 6 weeks, old), old rats treated with alfacalcidol for 6 weeks, *V6WO* (vehicle for 6 weeks, old), old rats treated with vehicle for 6 weeks; #different from CA ($P = 0.018$); *different from V6WO ($P = 0.029$); (repeated-measures ANOVA, Bonferroni posthoc); data are represented as mean ± SEM

this region of the old vehicle group than the control adult group (Fig. 1b; $P = 0.012$). There was no significant difference in overall CSA in the high oxidative part of the Gm between the old vehicle and alfacalcidol groups (Fig. 2a). In the low oxidative region of the Gm, however, the CSA of the old alfacalcidol-treated group was 16% smaller ($P = 0.029$) than that of the old vehicle-treated rats (Fig. 2b), which was primarily due to a decrease of the CSA of type IIb fibres ($P = 0.029$) (Fig. 1d).

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 milligram muscle was similar in the alfacalcidol, vehicle and adult control groups (Fig. 3a). mRNAs were normalized to the 18S ribosomal RNA expression. Figure 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 MuRF-1 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. 3b).

The mRNA level of the autocrine growth factor myostatin, involved in regulating protein synthesis and degradation and satellite cell proliferation, was reduced with ageing ($P = 0.002$). However, myostatin mRNA expression was not significantly affected by alfacalcidol. IGF-1 Ea and c-myc mRNA expression levels as well as that of the vitamin D related markers, VDR and CYP27B1, were similar in all groups (Fig. 3c, d).

Circulating factors

The inflammatory marker IL-6, which is associated with sarcopaenia and muscle wasting [18], was elevated in the old rats ($P < 0.05$), 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 ($P = 0.004$), but was not significantly different from that of the control adult group. The leptin serum concentration was decreased

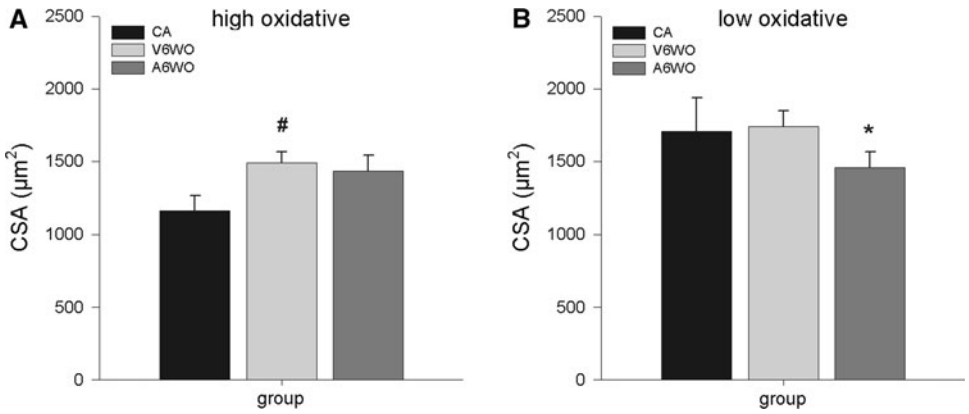

Fig. 2 Combined cross-sectional area of different fibre types of the high oxidative (**a**) and low oxidative (**b**) parts of the Gastrocnemius medialis muscle. In the high oxidative part the CSA of the control adult group was smaller than that of the old vehicle group. In the low oxidative part the CSA of the alfacalcidol-treated animals was smaller compared to the age-matched vehicle group. *CA* control adult, *A6WO* (alfacalcidol for 6 weeks, old), old rats treated with alfacalcidol for 6 weeks, *V6WO* (vehicle for 6 weeks, old), old rats treated with vehicle for 6 weeks; #different from CA ($P = 0.012$); *different from V6WO ($P = 0.030$); (ANOVA, Bonferroni posthoc); data are represented as mean ± SEM

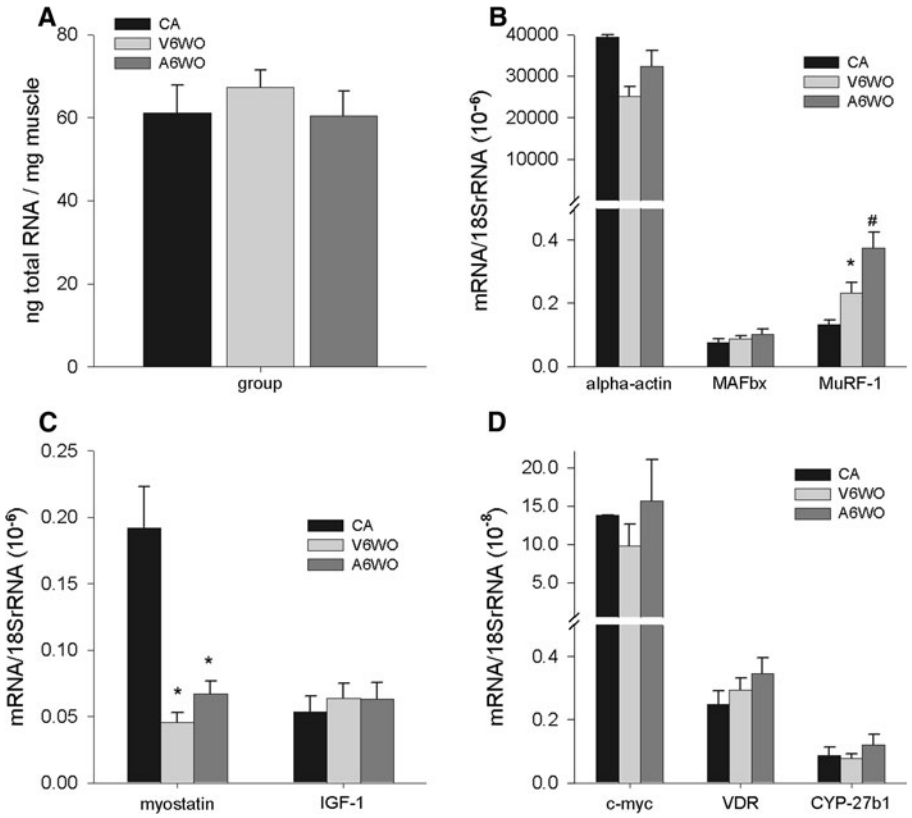

Fig. 3 Quantitative PCR of contractile protein, components of the ubiquitin proteasome pathway, growth factors and vitamin D-associated proteins of the low oxidative, distal part of the Gm. **a** Total RNA per milligram muscle. No differences in total RNA concentration were observed between the groups indicating that it was allowed to normalise the expression of the other factors for 18S RNA. **b** Expression of the structural protein α-actin and the ubiquitin proteasome pathway proteins MAFbx and MuRF-1. The expression of MuRF-1 in the vehicle group was higher compared to the control adult group. MuRF-1 expression was further increased in the alfacalcidol treated group. **c** Expression of the growth factors myostatin and IGF-1 Ea. Myostatin expression was lower in the alfacalcidol and vehicle group than the control adult group. **d** c-myc, vitamin D receptor (*VDR*) and CYP27B1 expression were not different between groups. *CA* control adult, *A6WO* (alfacalcidol for 6 weeks, old), old rats treated with alfacalcidol for 6 weeks, *V6WO* (vehicle for 6 weeks, old), old rats treated with vehicle for 6 weeks; *different from CA at $P < 0.05$; #different from CA and V6WO at $P < 0.001$ (ANOVA, Bonferroni posthoc); data are represented as mean ± SEM

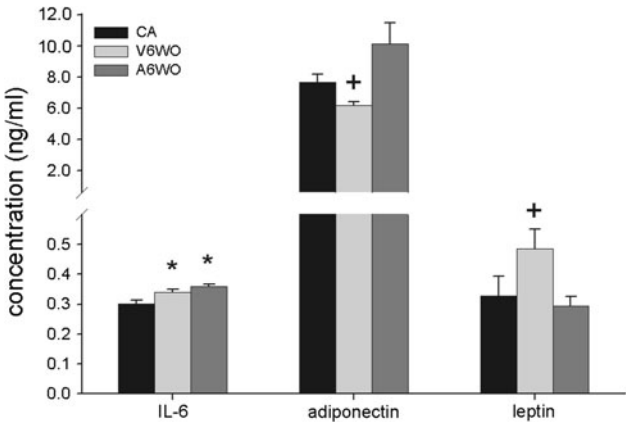

Fig. 4 Serum levels of IL-6, adiponectin and leptin. IL-6 levels were higher in old rats treated with alfacalcidol for 6 weeks (*A6WO*) and vehicle (*V6WO*) than control adult rats (*CA*). Adiponectin levels of the A6WO group were higher than those in the age-matched V6WO group. Leptin levels of the A6WO group were lower than those in the V6WO group. *different from CA at $P \leq 0.05$; $^{+}$different from V6WO at $P < 0.05$ (ANOVA, Bonferroni posthoc); data are represented as mean ± SEM

in the alfacalcidol group compared to the age-matched vehicle group ($P = 0.034$) (Fig. 4).

Discussion

As far as we know this is the first study that addresses some of the potential mechanisms whereby supraphysiological circulating levels of the active form of Vitamin D affect skeletal muscle. The main observation of the present study is that supraphysiological levels of circulating active vitamin D in old rats induce muscle atrophy that is proportional to the decline in body mass. The concomitant increase in the expression of MuRF-1, superimposed on the age-related increase in MuRF-1 expression, is consistent with the hypothesis that MuRF-1 is involved in muscle atrophy. The increased expression of MuRF-1 in rats with supraphysiological levels of circulating vitamin D was not associated with a rise in circulating IL-6. A likely cause of the loss of body mass and increased MuRF-1 expression is the reduced food intake in the animals with supraphysiological levels of circulating vitamin D [13]. Unexpectedly, the decreased food intake was accompanied with reduced, rather than increased, serum leptin levels. 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 systemic inflammation and/or altered satiety set-points.

Effects of ageing

Similar to previous observation in both human [31] and rat muscles [32, 33], we observed that the specific tension (maximum tetanic force per muscle per muscle mass or cross-sectional area) was decreased in the muscles from the old animals [13].

As we observed previously [34], the similar muscle mass but larger fibre cross-sectional area in older Gm suggests that the number of fibres was reduced during ageing. This might be the result of an incomplete denervation–reinnervation process leading to loss of fibres [35] and compensatory hypertrophy of the remaining fibres. Such a process might also explain why muscle mass was not changed even though the (mRNA) expression of the ubiquitin ligase MuRF-1 was elevated (similar as in aging human vastus lateralis muscle [36]). A possible increase in the protein degradation rate consequent to the increased MuRF-1 expression [19, 37] may be attenuated by the reduced expression of myostatin in the old rats, as myostatin enhances protein degradation and reduces protein synthesis [38, 39]. Alternatively, the elevated MuRF-1 occurs primarily in the denervated fibres while the decrease in myostatin occurs primarily in the fibres undergoing compensatory hypertrophy. Other compensatory mechanisms may also have prevented the development of atrophy at this age, but neither c-myc, a transcription factor involved in the regulation of rRNA expression [40], nor the anabolic IGF-1 Ea mRNA [41] were elevated in the old rats in our study.

Another explanation might be that the increased mRNA levels of MuRF-1, the reduced levels of myostatin mRNA and unaltered IGF-I Ea mRNA levels together with the elevated circulating levels of IL-6 in the old animals indicates the beginning of sarcopaenia induced by low grade systemic inflammation [42]. Also, in humans, loss of muscle strength may occur without muscle fibre atrophy, similar to the reduction in specific tension in old rat muscles [43]. Overall, it thus appears that our old rats showed age effects similar to those observed at the onset of sarcopaenia 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 fibres in the low oxidative distal part of the muscle. It is surprising, therefore, that the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles, primarily

consisting of type II fibres, did not show significant atrophy [13]. This might be related to the different functions, where the gastrocnemius and plantaris muscles, which atrophied, are anti-gravity muscles and the EDL and TA are not. The reduced activity and loading due to a loss of body mass would result in a lesser recruitment of the type II fibres in the low oxidative part of Gm and hence type II fibres in this region would be most affected.

The selective type IIb fibre atrophy in our animals with supra-physiological levels of vitamin D was unexpected as vitamin D deficiency in older women was also accompanied by selective type II fibre atrophy [44]. The discrepancy may be related to the reduced food intake in our alfacalcidol-treated animals [13], as in starved rodents particularly the highly glycolytic (presumably IIb/x) fibres have been reported to atrophy [45]. This, and the unaltered fibre type composition during starvation [45], as also observed in our rats, suggest that any effect elevated levels of 1,25D might have on fibre type composition and fibre size is 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 where elevated leptin levels reduce appetite. Yet, serum levels in alfacalcidol-treated rats were decreased, rather than increased, and the decreased food intake can thus not be explained by changes 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 [15], as was the case in our alfacalcidol-treated animals. Since adiponectin enhances glucose uptake and fatty acid oxidation in muscle [46] it might be a response to minimize the use of muscle protein in the face of starvation and ensure that probably elevated levels of circulating fatty acids can be used for energy generation [16].

Chronic low-grade systemic inflammation plays an important role in the weight loss and muscle wasting during ageing and chronic diseases, partly via reducing appetite [42, 47, 48]. IL-6 is an inflammatory cytokine and elevated circulating levels have been associated with loss of muscle strength [18, 49] and atrophy [42, 47]. Treatment with alfacalcidol was, however, not accompanied with an increase in circulating IL-6 levels and alfacalcidol has even been shown to decrease tumor necrosis factor-α [50], another cytokine having a catabolic effect on muscle [42]. It is thus unlikely that the observed atrophy was the result of increased systemic inflammation.

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

We hypothesized that alfacalcidol treatment induces hypertrophy since 1,25D has been shown to activate and induce synthesis of c-myc [51, 52] as well as the MAP kinases ERK1 and ERK 2 in skeletal muscle [53]. However, neither c-myc nor IGF-I Ea, myostatin nor α-skeletal actin expression were changed by alfacalcidol treatment, and we observed atrophy rather than hypertrophy.

It is possible that the intracellular concentration of 1,25D differs from that in the circulation due to the action of CYP27B1, a hydroxylase that catalyses the hydroxylation of 25D to 1,25D. The potential importance of CYP27B1 in muscle wasting has been shown by the muscle atrophy in patients with chronic renal failure, which have sufficient 25D but lack CYP27B1 [54] and thus may suffer from intracellular vitamin D deficiency in the presence of normal circulating 25D levels. These patients did indeed improve muscle size and strength when they were treated with 1,25D [55]. In our study, the CYP27B1 expression in the muscle was not altered despite elevated serum levels of 1,25D (see Fig. 3d) after alfacalcidol treatment and this may have also caused supraphysiological levels of 1,25D within the cells.

Alternatively, the atrophy, rather than the expected hypertrophy after alfacalcidol treatment, may be related to the abundance and/or action of VDR. Yet, despite supraphysiological 1,25D serum levels, the VDR mRNA expression was not altered in the Gm of the alfacalcidol-treated animals (see Fig. 3d). The atrophy of type II fibres was therefore probably not due to a reduced abundance of the VDR.

To our knowledge, there are no studies that have addressed the impact of supraphysiological levels of vitamin D on skeletal muscle. The hypercalcaemia, which we observed previously in these same animals [13] and which accompanies the elevated serum levels of 1,25D, has been associated with muscle weakness and weight loss [56]. Nevertheless, it is likely that the 38% reduction in food intake [13] is the main cause of the muscle wasting and loss of body mass. It remains unclear, however, how elevated levels of 1,25D cause this reduced food intake, but our data indicate that it is not due to increased serum IL-6 and/or altered circulating leptin levels, as discussed above.

During muscle atrophy, including that induced by fasting [19–22], the expression of MuRF-1 and MAFbx have been reported to be increased. MuRF-1 ubiquitinates myofibrillar and cytoskeleton proteins and metabolic enzymes in preparation for their subsequent breakdown in the proteasome [57–60]. In line with this role in muscle protein breakdown during fasting, we observed that

MuRF-1 expression was increased substantially in the alfacalcidol group, without a change in MAFbx mRNA levels (see Fig. 3b). The latter is somewhat surprising as MAFbx is also associated with accelerated muscle protein loss and its mRNA increases 8–40-fold in many types of muscle atrophy [61]. It should be noted, however, that MAFbx and MuRF-1 expression do not always change in a coordinated fashion and their expression is controlled by different signalling pathways [62]. Overall, the data indicate that the alfacalcidol-induced atrophy may at least partly be caused by an increased mRNA expression of the E3 ligases.

Myostatin expression has been shown to be upregulated in type II fibre atrophy [63]. Yet, despite the preferential type II fibre atrophy in our study, no differences in myostatin expression in muscles from the alfacalcidol and vehicle groups were found, suggesting that myostatin had no role in the alfacalcidol-induced atrophy.

In conclusion, the observed decrease in muscle mass in the presence of supraphysiological levels of the active form of vitamin D, induced by alfacalcidol supplementation, is due to preferential atrophy of type IIb. This atrophy was associated with an increased mRNA expression of the ubiquitin ligase MuRF-1 which was superimposed on the age-related increase in MuRF-1 expression. The atrophy and increased MuRF-1 expression were not due to increased systemic inflammation, nor was it accompanied with increased muscular myostatin or reduced IGF-1 Ea expression. The data suggest that reduced food intake is the main cause of the muscle atrophy and loss of body mass when circulating levels of the active form of vitamin D are supraphysiological. Occasionally, vitamin D intoxication is also observed in the clinic [64]. However, the risk of vitamin D intoxication may be exaggerated where the natural/physiological regulation is overridden by supplementation with VitD analogues, such as alfacalcidol, and is less when treated with $VitD_3$ or $VitD_2$. Nevertheless, the clinical implication is that care has to be taken that vitamin D supplementation to vitamin D deficient patients does not result in supraphysiological levels of circulating vitamin D, and it extends the observation that vitamin D supplementation "does not have a significant effect on muscle strength in adults with baseline 25(OH)D $>$ 25 nmol l^{-1}" [65].

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