INTRODUCTION

The active form of vitamin D, 1α,25(OH)2D3 (1,25D) elicits cellular responses through both genomic and non-genomic actions (1). Vitamin D has been shown to be important in phosphate and calcium homeostasis (2) acting on the intestine (3), kidney (4), parathyroid gland (5) and bone (6). It exerts its effects through the vitamin D receptor (VDR) and the discovery of the VDR in muscle tissue (7, 8) suggests that vitamin D will also act on muscle tissue. Indeed, the deregulated expression of myoregulatory transcription factors and abnormal muscle development in VDR knock-out mice demonstrates the importance of vitamin D in skeletal muscle development (9). This suggests that vitamin D deficiency could have important consequences for muscle metabolism and functioning. Indeed, vitamin D supplementation during vitamin D deficiency has been shown to improve muscular/skeletal function in institutionalized elderly by 4-11% within 12 weeks (10). It has been found in human and mouse primary hepatocyte cultures that a concentration of 100 nmol·L⁻¹ vitamin D elicits maximal activation of the VDR (11). The serum vitamin D levels in rats, around 185 pmol·L⁻¹ (12), are far below this level. Although it is known that vitamin D supplementation during vitamin D deficiency enhances muscle function, it remains to be established what the impact on muscle function is when circulating vitamin D levels are elevated in non-deficient adult and old rats. To do so, in this study alfalcacidol, which is a synthetic calcitriol analogue, is administered to adult and old rats. We used old rats, as many responses to e.g. electrical stimulation (13), overload (14) and disuse (14, 15) are reduced. This might be due to reduced circulating levels of vitamin D. The aims of this study were to determine the effects of increased serum 1,25D on muscle contractile properties in old and adult rats. A decrease in vitamin D concentration observed during ageing in humans, is the result of a decreased sunlight exposure, dietary intake, decreased uptake of vitamin D in the food and a decrease in hydroxylation capacity (16). Because of the last two factors it was hypothesized that the vitamin D concentration in the rat is lower in aged rats. Further, we hypothesized that alfalcacidol treatment would reverse the decline in contractile properties in old rats.

MATERIAL AND METHODS

Animals

Female Fischer344 x Brown Norway F1 rats were obtained from Harlan (USA) (n=52). 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 (17). Rats were housed four to a cage at a 12:12 light dark cycle with food and standard laboratory chow provided ad libitum. The rats were adult (7 months old), and old (27.5- or 29-months old) at the end of the treatment period and divided randomly in 1-week, 6-week, or vehicle treated groups (Table 1). Rats were orally administered either vehicle or alfalcacidol (0.1 µg kg⁻¹) (Chugai Pharmaceutical Co Ltd, Japan) daily for one week, to study short term effects, or for 5 days during 6 weeks to study long term effects. This dose has been shown to inhibit bone resorption and enhance bone formation in ovariectomized rats treated for 5 weeks (18). Rats were weighed before vehicle and alfalcacidol administration to determine the dose. Food and water consumption were monitored in the 6-week treatment groups. At the end of the treatment period the contractile properties of the medial gastrocnemius muscle (Gm) were determined. 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. The Gm of the right leg was dissected while keeping the proximal origin and the blood supply intact. The femur was fixed and the distal tendon, with a small part of the calcaneus, connected to a force transducer. Length changes of the Gm were controlled by a servomotor connected to the lever arm to which the force transducer was mounted. The sciatic nerve was cut and contractions induced by supramaximal electrical stimulation (1 mA, pulse width 200 µs), defined as the current above which the twitch force did not increase further. Subsequently, the muscle was set at optimal length (Lₒ), defined as the length at which the active twitch force was maximal, with a series of twitch contractions (1 per minute). Then Lₒ was fine-adjusted with several tetanic contractions (150 Hz, 150 ms). Muscle temperature was maintained at 34-36°C with a water-saturated airflow around the muscle, which also kept the muscle moistened. Stimulation and length changes were computer controlled. Force and length signals were digitized using an AD-converter at a sampling rate of 10 kHz. At the end of the measurements the Gm was excised, weighed, stretched to Lₒ on cork, and frozen in liquid nitrogen.

### Protocols

#### Frequency - force relation

To determine the frequency-force relationship the muscle was stimulated at the following frequencies in random order: 20, 40, 60, 100, 150 and 250 Hz. The stimulation duration was 150 ms. The time between each contraction was 3 minutes to prevent the development of fatigue and minimize potentiation.

#### Force - velocity relation

To determine the force-velocity relation, the muscles were maximally stimulated with 400-Hz 150-ms trains (19). During the contractions the muscles were allowed to shorten at a constant velocity (10, 20, 30, 50, 75, 100 and 125 mm s⁻¹). Just before the contraction started, the muscle was passively stretched to a length 0.5-1 mm above Lₒ. Each contraction started with a short isometric phase during which the force increased to the level that could be sustained during the subsequent shortening at the specific imposed velocity. This ensured that the force was constant when the muscle passed Lₒ during shortening (20). Rest between contractions was 3 minutes.

#### Fatigue

The fatigue protocol consisted of a series of 20 isometric contractions (150 Hz; 150 ms; 1 contraction every 500 ms).

#### Data analysis

For all isometric contractions, net peak force was calculated. Subsequently, the maximal tetanic force was normalized to muscle mass. The decrease during the fatigue protocol was expressed relative to the force of the first tetanic contraction and for every tetanus during the fatigue protocol, the half relaxation time (HRT) was calculated. HRT was calculated as the time for force to decrease from the maximum to 50% of the maximum at the end of the stimulation.

#### Blood serum values

Blood was collected from the vena cava inferior after the contractile properties of the Gm had been determined. Serum values for 1,25D were measured using a 1,25(OH)₂D ELISA kit (Immunodiagnostic Systems Ltd., Boldon, England). Albumin, calcium (Ca), creatinine, and inorganic phosphate (Pi) serum concentrations were determined with a Hitachi Biochemical Automatic Analyzer 7070 (Hitachi Co., Ltd., Tokyo, Japan).

#### Statistics

To determine whether there were any statistical differences a three way ANOVA with as factors age (three levels: 7, 27.5 and 29 months) and duration of alfacalcidol treatment (control, 1 and 6 weeks) was performed on the treatment group and the corresponding control group (SPSS 17.0). A bonferroni post hoc test was performed if a significant effect was found. For the force-frequency relation, force-velocity relation and body mass as function of treatment duration a two-way ANOVA with repeated measures on one factor was performed to test for differences in the whole curve. Differences were considered significant at p<0.05. Data are presented as mean ±S.D.
RESULTS

Food and water consumption

Food and water intake were monitored daily. The food intake was 38% lower (p<0.001) in the alfacalcidol than the vehicle groups. The water intake, on the other hand, was increased by 12.4% (p=0.002) (Fig. 1).

Blood serum levels

Fig. 2 shows that the 1,25D blood serum level was 3-4-fold higher in both adult (p=0.004) and old rats (p=0.002) treated for 1 week with alfacalcidol compared with vehicle treated rats resulting in supra-physiological 1,25D levels. 1,25D serum levels after 1 and 6 week treatment with alfacalcidol in the old rats did not differ. 1,25D in the old V1WO and V6WO were not significantly different from those of the adult CA group, indicating that administration of the vehicle itself had no significant effect on the 1,25D concentration in the blood. Hence, there was no age-related reduction in 1,25D levels.

Albumin and creatinine serum levels were similar in all groups (Table 2). Ca levels were 1.1-1.3 fold elevated in the alfacalcidol treated groups compared to the corresponding control groups (p<0.02, Table 2).

Pi serum levels of the old animals were lower than those in the adult control animals (p<0.001). Alfacalcidol treatment induced a significant increase in the circulating levels of Pi in the old rats, irrespective of duration of treatment (p<0.001) (Table 2).

Body and muscle mass

Table 3 shows the body and muscle mass data for the different groups before and after treatment. The old animals were heavier than the adult animals (p<0.001). The vehicle treatment did not significantly affect body mass. Treatment with

---

**Table 2.** Mean (±S.D.) of blood serum values at the end of the experimental period. * significantly different from corresponding control group (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (mg/dL)</td>
<td>2.1 (0.1)</td>
<td>2.2 (0.2)</td>
<td>2.3 (0.1)</td>
<td>2.2 (0.2)</td>
<td>2.4 (0.1)</td>
<td>2.1 (0.2)</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.4 (0.4)</td>
<td>1.2 (0.2)</td>
<td>1.7 (0.2)</td>
<td>1.8 (0.2)</td>
<td>1.8 (0.2)</td>
<td>1.7 (0.3)</td>
</tr>
<tr>
<td>Pi (mg/dL)</td>
<td>8.5 (0.9)</td>
<td>9.1 (0.5)</td>
<td>5.0 (1.2)</td>
<td>8.0 (1.0)*</td>
<td>5.3 (0.7)</td>
<td>7.5 (1.1)*</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>8.4 (0.3)</td>
<td>10.5 (0.6)*</td>
<td>10.7 (1.0)</td>
<td>12.0 (1.2)*</td>
<td>10.5 (0.5)</td>
<td>12.7 (0.7)*</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** Food intake is lower and water intake is higher in alfacalcidol treated rats. A) food intake during vehicle and alfacalcidol treatment in old rats. B) Water intake during vehicle and alfacalcidol treatment in old rats. Food intake was 38% lower and water intake was 12% higher in alfacalcidol treated animals compared to vehicle treated rats. Data is represented as mean ± S.D.*different from corresponding control group (p<0.05).

**Fig. 2.** 1,25D concentration in blood serum is increased after alfacalcidol treatment. A) Adult rats orally treated with alfacalcidol for one week, the control adult group received no treatment. B) old rats were treated for 1 and 6 weeks with vehicle or alfacalcidol. 1,25D blood serum levels of the alfacalcidol treated animals were 3-4 fold higher compared to the corresponding control group. Data is represented as mean ± S.D.*different from corresponding control group (p<0.05).
alfacalcidol, on the other hand, caused a 6% reduction in body mass after 1 week of treatment in adult and old rats (p<0.001), which progressed to a 22% reduction after 6 weeks of treatment in the old rats (p<0.001) (Fig. 3). Ageing did not significantly affect muscle mass. This applied not only to the Gm (Table 3), but also to the soleus, plantaris, extensor digitorum longus and tibialis anterior muscles (data not shown). While 1 week of alfacalcidol treatment did not cause a significant reduction in Gm mass, it was reduced by about 17% after 6 weeks of alfacalcidol treatment (p<0.001). The same was found for the plantaris muscle (p<0.05), but in the other muscles it did not reach significance. The Gm mass normalized to body mass was lower (p<0.05) in the old than in the adult rats. Treatment with alfacalcidol did not reduce this ratio, indicating that the decrease in body mass was not only due to a decrease in muscle mass, but also due to a decrease in other tissue, e.g. fat tissue, to a similar extent (data not shown). The heart mass increased with age, but when expressed per unit body mass it decreased (p<0.05; Table 3). The heart mass was not significantly affected by alfacalcidol, while as a consequence of the loss of body mass the heart:body mass ratio was increased after alfacalcidol treatment (p<0.002).

**Muscle functional characteristics**

**Maximal isometric force**

Maximal isometric force (Fmax) was lower in the old vehicle treated rats than the control adult group (p<0.05) (Table 3). Fmax was 19% lower in the old (V1WO) than the control adult group (p<0.05). Treatment with alfacalcidol for 1 week did not affect Fmax, but after 6 weeks of treatment Fmax was reduced by 18% in the old rats. These age- and treatment related reductions in Fmax were explicable by the decrease in muscle mass, as the specific force (Fmax normalized by muscle mass) was similar in all groups (Table 3).

**Force-frequency characteristics**

The force-frequency curve of the old rats treated with vehicle for 1 and 6 weeks was shifted to the left compared to the control adult group, indicating an age effect (p<0.001) (Fig. 4A). Alfacalcidol treatment for 6 weeks induced a shift in the force-frequency curve to the left compared to their corresponding control group (p<0.05) (Fig. 4C).

![Fig. 3. Six weeks alfacalcidol treatment reduced the body mass of old rats. Adult rats were orally treated with vehicle or alfacalcidol for 1 week, and old rats for 1 and 6 weeks while the adult control group received no treatment. A) Effects of 1 week treatment with alfacalcidol. The adult and old alfacalcidol treated animals (A1WA and A1WO respectively) had a significant decrease in body mass compared to the old rats receiving vehicle (V1WO) (p<0.03). B) Effects of 6 week alfacalcidol and vehicle treatment on old rats. After 6 weeks alfacalcidol (A6WO) treatment rats showed a substantial loss of body mass compared to initial body mass of the 6 weeks vehicle (V6WO) treated rats (p<0.001). Data is represented as mean ±S.D.](image)

**Table 3.** Mean (±S.D.) of body mass, gastrocnemius muscle (Gm) mass and maximal tetanic force (Fmax) of the Gm at the end of the experimental period. * significantly different from CA; ~ significantly different from value before treatment; # significantly different from other groups; ** significantly different from V6WO (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass before (g)</td>
<td>-</td>
<td>203 (78)</td>
<td>296 (31)</td>
<td>282 (22)</td>
<td>273 (35)</td>
<td>286 (26)</td>
</tr>
<tr>
<td>Body mass end (g)</td>
<td>221 (16)</td>
<td>190 (73)<del>/</del></td>
<td>294 (30)*</td>
<td>265 (21)~</td>
<td>276 (36)*</td>
<td>224 (19)~</td>
</tr>
<tr>
<td>Gm muscle mass (mg)</td>
<td>669 (47)</td>
<td>638 (44)</td>
<td>627 (46)</td>
<td>603 (50)</td>
<td>622 (54)</td>
<td>515 (37)#</td>
</tr>
<tr>
<td>Maximal isometric force</td>
<td>12.1 (1.3)</td>
<td>11.2 (1.0)</td>
<td>9.8 (1.0)*</td>
<td>10.0 (0.6)</td>
<td>10.7 (0.7)*</td>
<td>8.8 (0.8)**</td>
</tr>
<tr>
<td>Fmax (N)</td>
<td>18.3 (1.9)</td>
<td>18.1 (1.4)</td>
<td>16.2 (1.5)</td>
<td>16.8 (1.1)</td>
<td>17.6 (1.3)</td>
<td>17.5 (0.7)</td>
</tr>
<tr>
<td>Specific force (mN/mg)</td>
<td>684 (46)</td>
<td>640 (64)</td>
<td>925 (61)</td>
<td>902 (92)</td>
<td>794 (41)</td>
<td>791 (40)</td>
</tr>
</tbody>
</table>
Force-velocity characteristics

No significant differences were found in the force-velocity relationships between the groups (data not shown). The Gm of the oldest animals (V1WO) had lower maximal power outputs than the adult control group (p<0.001) (data not shown). As a consequence of the reduction in Fmax after 6 weeks of treatment with alfacalcidol the maximal power was similarly reduced after 6 weeks of alfacalcidol treatment (p<0.007).

Fatigue

During 20 repeated isometric contractions a linear decrease to 74% of the initial force was seen in all groups. Concomitantly, half relaxation time (HRT) time increased during the isometric repetitions in all groups. The HRT of the non-fatigued non-treated adult muscles was shorter than that of both old control groups (p<0.02). There was no significant effect of alfacalcidol in the HRT (data not shown).

DISCUSSION

The aim of this study was to determine the effects of an increase in serum 1,25D on the Gm muscle contractile properties in old and adult rats. We observed that ageing resulted in a reduction in force and power generating capacity of the muscle. Administration of the vitamin D analogue alfacalcidol did increase the 1,25D serum levels but in contrast to our hypothesis the old rats showed no vitamin D deficiency. Furthermore, the increased 1,25D serum levels did not improve the contractile characteristics of the Gm. Rather, a decrease in maximal force and power generating capacity and a left-shift of the force frequency relationship of the Gm.
in old rats was found. Thus, while administration of alfalfacalcidol and other vitamin D analogues has been proven beneficial for muscle function in conditions of vitamin D deficiency (10, 21, 22), our data indicate that elevated vitamin D levels may negatively affect muscle function. This suggest that the effect of vitamin D on muscle and the whole body may have a U-shape where below and above normal physiological levels circulating vitamin D or their analogues have a negative, rather than a beneficial effect.

Effects of ageing: serum levels and muscle contractile properties

Albumin, calcium and creatinine levels were not affected by ageing in our study, but the inorganic phosphate serum concentrations in serum were significantly lower in the old than the adult animals.

During ageing in human levels of 1,25D in blood serum decrease (23, 24), but we did not find lower 1,25D levels in our old rats. It is possible that part of the discrepancy between the decrease in vitamin D during ageing in men and the unaltered levels we found in rats is explicable by an age-related reduction in sun-exposure and dietary intake in humans (25), whereas neither the adult nor the old rats were exposed to sunshine and rats were fed the same diet at all ages. In contrast to humans, the vitamin D levels in rats may only depend on nutrition, the quality of which was maintained in our experimental setting. In line with previous observations, we observed a significant slowing of relaxation, and decline in force (14) and power generating capacity, as observed in old mice (26) with ageing. These changes were largely due to a reduction in specific tension as muscle mass was maintained, similar to what has been observed previously (14, 27).

Six weeks of alfalfacalcidol treatment did cause a left shift in the force frequency relation in old rats. If, as a result of atrophy sarcoplasmic reticulum proteins were less reduced than contractile proteins, this might contribute to the higher forces observed at low stimulation frequencies, because of a possible relatively higher Ca2+ release. If atrophy would lead to more reduction in sarcoplasmic reticulum proteins, this might increase half relaxation times, which was not found in this study. Further support for the absence of changes in the rate in relaxation comes from the unaltered force-velocity relation, suggesting that there is no major change in crossbridge kinetics, and hence fibre type composition, after alfalfacalcidol treatment. Another possibility is that the Ca2+ sensitivity of the regulatory proteins on the thin filaments was increased, a situation which has for instance been observed in diaphragm fibres from patients with chronic obstructive pulmonary disease (28). Finally, the release of Ca2+ by the sarcoplasmic reticulum might be enhanced at low stimulation frequencies and/or the intracellular Ca2+ concentration might already be slightly elevated after treatment with alfalfacalcidol.

Indeed, vitamin D has been shown to change the intracellular Ca2+ concentration via both non-genomic (29) and genomic actions (30). However, because we have not used pair-feeding in this study, we cannot exclude an influence of reduced food intake with resultant loss of body mass.

Effects of alfalfacalcidol treatment: short-term and long-term effects on blood serum levels, animal characteristics (physiology) and muscle properties

The levels of circulating vitamin D we observed in the control adult and old rats were between 45 and 100 pmoL·L−1. This concentration has been found to activate only about 3% of the VDR in mouse and human hepatocytes (11). Although the dose response curve for rat is not known it can be assumed that the activation of the VDR would be in the same range in the rat. By elevating the vitamin D levels with alfalfacalcidol treatment one would expect, based on the dose response curve of Reschly et al. (11), that the activation of the VDR would increase to approximately 12-20% at the levels of vitamin D we observed. Such an increase may have a significant impact on skeletal muscle structure and function as VDR knock-out mice, for instance, show abnormal muscle development (9).

While the treatment with alfalfacalcidol did not significantly affect the fatigue resistance of the muscle, it did cause a significant reduction in the force generating capacity of both adult and old muscles. This muscle weakness was solely due to a concomitant loss of muscle mass, while the force generating capacity, or force per unit muscle mass, of the remaining muscle tissue was unaffected. Not only Gm mass, but also the mass of the extensor digitorum longus, soleus, plantaris and tibialis anterior was reduced, suggesting that muscle wasting occurred irrespective of the fibre type composition of the muscle. There was also a significant loss of body mass. The fact that the muscle mass: body mass ratios remained constant indicates that there was a proportional loss of fat and lean body mass. The heart mass, however, remained unaffected. The loss of body mass was largely due to a reduced food intake during the alfalfacalcidol administration. The lower food intake is probably responsible for the decrease in muscle and body mass and maximal force, however, the qualitative result, the left shift in the force-frequency relation, may be due to increased vitamin D levels.

Alfalfacalcidol treatment resulted in elevated serum levels 1,25D, which may have facilitates the Ca2+ absorption from the intestine and reabsorption in the kidney (31). It is therefore not surprising that we found elevated Ca2+ concentrations in the serum of alfalfacalcidol-treated rats. The transport protein albumin has a high affinity for Ca2+ up to 40% is bound and inactive, depending upon albumin concentration and pH (32). A decrease in albumin concentration can therefore lead to an increase in the free Ca2+ concentration in the blood. However, the albumin blood concentration was not decreased in the alfalfacalcidol groups compared to the vehicle and control groups. A more plausible explanation for the hypercalcemia is the increased free 1,25D serum not bound to vitamin D-binding protein (33, 34).

Further evidence for problems when vitamin D levels are elevated is the increased fluid intake in alfalfacalcidol-treated animals. Nevertheless, the blood analysis showed that the creatinine concentration was not significantly changed indicating that the kidney function was normal. Interestingly, also the heart was spared from the toxic effect, in contrast to the significant loss of skeletal muscle tissue.

Supplementation with active forms of vitamin D such as calcitriol and alfalfacalcidol have been associated with a higher risk of hypercalcaemia compared to native vitamin D (35). Symptoms of vitamin D poisoning, which work via hypercalcaemia, include among other things diarrhoea, lethargy, weakness, polyuria, polydipsia and anorexia. In this study we observed polydipsia, hypercalcaemia and anorexia, which support the idea that the animals which received alfalfacalcidol were treated with a too high dose of alfalfacalcidol leading to a deficit in food intake. The mechanisms underlying the adverse effects of alfalfacalcidol treatment warrant further investigation. Future research should test whether the adverse effects of supraphysiological levels of vitamin D can be reverted by immediate cessation of alfalfacalcidol administration, by VDR antagonist supplementation or by nutritional administration of Ca2+ to normalize its blood serum concentration.
Consequence of treatment with alfalcaciloid for elderly people health

Supraphysiological circulating levels of vitamin D are accompanied by significant reductions in body and muscle mass, largely due to a reduced food intake. This indicates that food intake and body mass should be carefully monitored when treating people with vitamin D analogues. Further, vitamin D supplementation did not lead to apparent improvement in muscle functioning. The quantitative results of this study such as the decrease in muscle and body mass and maximal force production are probably related to lower food intake, whereas the qualitative results, the left shift in the force-frequency relation, may be related to increased vitamin D levels.

Acknowledgements: The present study was financially supported by a grant from Chugai Pharmaceuticals Co. LTD, Tokyo, Japan. We further want to thank Junko Hashimoto and Tinelies Busé for their support during the set-up and execution of the experiments.

Conflict of interests: None declared.

REFERENCES

35. Avenell A, Gillespie WJ, Gillespie LD, O’Connell D. Vitamin D and vitamin D analogues for preventing fractures
associated with involutional and post-menopausal osteoporosis. Cochrane Database of Systematic Reviews (online). 2009; CD000227.

Received: March 24, 2010
Accepted: February 23, 2011

Author’s address: Dr. A. de Haan, VU University Amsterdam, Research Institute MOVE, Faculty of Human Movement Sciences, 9 Van der Boechorststraat Str., 1081 BT Amsterdam, The Netherlands; E-mail: A.de.Haan@vu.nl