Vitamin D and skeletal muscle structure and function

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Vitamin D and skeletal muscle structure and function

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Vitamin D and skeletal muscle structure and function

Janwillem Testerink

A thesis submitted in partial fulfilment of the requirements of Manchester Metropolitan University in for the Degree of Doctor of Philosophy

Manchester Metropolitan University
Institute for Biomedical Research into Human Movement and Health
and
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Vitamin D deficiency in humans is associated with a reduction in maximal muscle force and atrophy of type II fibres. Furthermore, it has been shown that vitamin D deficiency changes muscle contractile properties; the half relaxation time is prolonged in rats and chickens. Further support for effects of vitamin D on skeletal muscle is the presence of the vitamin D receptor in skeletal muscle tissue.

The aim of this thesis is to obtain more insight in the effects of vitamin D on the structural and contractile properties of skeletal muscle. Elevated blood serum levels of active vitamin D (1,25D) were obtained in old rats via alfacalcidol supplementation. As a result the rats ate less, which may have contributed to a reduction in maximal force and muscle mass. The supplementation resulted in a leftward shift of the force-frequency relation. The reduction in muscle mass was caused by atrophy of type IIb and IIx fibres, which may be related to the observed increased expression of MuRF1, one of the proteins of the ubiquitin pathway.

Low vitamin D levels were obtained with a vitamin D deficient diet. The deficiency did, in contrast to our hypothesis, not result in a reduction in muscle mass and maximal force.

The finding of a negative influence of addition of 1 nM 1,25D in the medium on maximal force and 10%-relaxation time of *Xenopus* muscle fibres in culture and an unchanged fibre CSA is a further indication that the role of vitamin D on skeletal muscle structure and function is complex. The results suggest that vitamin D itself has not the important direct effects on muscle mass and contractile properties as was thought. Since vitamin D regulates calcium, phosphorus and parathyroid hormone serum levels, these factors might be more important in the regulation of muscle mass and function.

Chapter 1

General Introduction

1.1 Introduction

In the animal kingdom movement is realised by the ability of skeletal muscle to shorten and generate force at the same time. The energy required by the muscles for movement is derived from the breakdown of adenosine-tri-phosphate (ATP). Muscles, however, do not solely play a role in motility, but are also important for respiration, maintenance of body posture and strikingly, in humans and primates, manipulation of objects and the environment. It is therefore not surprising that about 40% of the human body consists of skeletal muscle tissue. The maintenance of the skeletal muscle system comes at a relatively high metabolic cost and it may thus not come as a surprise that the skeletal muscle tissue is highly adaptive; a well-known adagium makes perfect sense in this context: 'Use it or Loose it'. Indeed, in response to decreased use muscle mass decreases (atrophies), thus lowering the metabolic cost to maintain muscle, and only when the muscle is subjected to increased use muscle mass increases (hypertrophies). The atrophy and hypertrophy result from a decrease or increase in the size of existing muscle fibres, respectively. The plasticity of the muscle, however, exceeds changes in muscle mass, and indeed also the phenotype of a muscle, such as its oxidative capacity and fibre type composition, are responsive to altered muscle usage. Muscle phenotype is also changing during ageing, resulting in sarcopenia, the age-related loss of muscle mass. An endocrine factor that is associated with sarcopenia is vitamin D deficiency.

Before discussing muscle plasticity further, a brief overview of the interplay between muscle phenotype and function will be given.

1.2 Muscle structure and function

Muscle has the ability to shorten and generate force at the same time making movement possible. The importance of simultaneous force generation and shortening is evident during simple tasks as moving an object and locomotion. During activities as walking, the muscle has to be able to contract many times and thus have a certain degree of fatigue resistance. There are thus three important functional parameters in skeletal muscle function: the force generating capacity, velocity of shortening and fatigue resistance.

The maximal force generating capacity is mainly determined by the physiological cross-sectional area (CSA) of a muscle, which is determined by the sum of the CSAs of the composing muscle fibres. The maximal shortening velocity of a muscle depends both on the length of the muscle fibre and the proportion of fast and slow fibres within the muscle. The fatigue resistance is little, if at all, determined by the architecture and size of the muscle, but is related to the oxidative capacity of the muscle, which is determined by the mitochondrial density, and fibre type composition. Since the maximal shortening velocity and fatigue resistance of a muscle are at least partly a consequence of the fibre type composition, next the relation between fibre type and fibre function will be discussed.

1.3 Skeletal muscle fibre types

A muscle fibre consists of myofibrils. These myofibrils in turn consist of myofilaments: thin actin, and thick myosin filaments. The myosin filament has myosin heads which are able to bind to tropomyosin, attachment sites on the actin filaments.

The myofibrils are each enveloped in the sarcoplasmic reticulum (SR), which acts as a store for calcium. Activation of muscle contraction is controlled by the nervous system finally resulting in depolarisation of the muscle cell membrane. Depolarization spreads across the cell membrane and into the transverse tubules thereby activating calcium channels in the transverse tubules causing calcium-release channels to open on the adjacent SR. During maximal activation, the cytosolic calcium concentration rises from ~10^{-9} to ~$10^{-4.5}$ M. Calcium then binds to tropomyosin C, tropomyosin C modulates the orientation of the tropomyosin on the actin filament, thereby enabling the myosin head to bind to the actin filament. By bending the myosin head, movement of the actin filaments occurs and thereby shortening of the muscle. ATP is required for this process which binds to the active domain on the myosin head where also the myosin ATPase is located.

It appears that the maximal velocity of shortening is related to the myosin ATPase activity (Barany 1967) and different myosin isoforms have different ATPase activities. In line with this, it has been found that fibres with different isoform composition have different maximal shortening velocities, with the velocity increasing from type I to IIa to IIx to IIb (Larsson and Moss 1993; Bottinelli, Betto et al. 1994; Degens and Larsson 2007; Gilliver, Degens et al. 2009). It appears that the different myosin-ATPases have different pH stability and this characteristic has been used to histochemically classify fibres (Brooke and Kaiser 1970; Guth and Samaha 1970; Lind and Kernell 1991). According to this staining, type I, type IIa, type IIx and IIb fibres can be distinguished. In general one can say that type I fibres are slow fibres with a low glycolytic capacity, and a high oxidative capacity. Type IIa fibres are moderately fast, have a high mitochondrial density and thus a high oxidative capacity and a high glycolytic capacity, while type IIx fibres are fast, have a high glycolytic capacity and a moderate oxidative capacity. The fastest fibres are type IIb fibres and they have a low oxidative capacity and a high glycolytic capacity. It should be noted that this is a simplification and in reality there is a continuum of fibres types (Pette and Staron 1990).

1.4 Skeletal muscle hypertrophy and atrophy

Hypertrophy and atrophy are the result of an increase in protein synthesis and breakdown. The balance between protein synthesis and degradation is regulated by signalling pathways that are influenced by mechanical stress, physical activity, availability of nutrients and growth factors.

In theory skeletal muscle hypertrophy can be realised by both an increase in muscle fibre number (hyperplasia) and muscle fibre size. In adult muscle, however, the contribution of hyperplasia is minimal and thus the increase in muscle mass is mainly the result of an increase in the size of individual muscle fibres (Antonio and Gonyea 1993). The increase in fibre size is accompanied by a net increase in protein synthesis. A factor that plays an important role in enhancing protein synthesis and inhibiting protein breakdown is insulin like growth factor-1 (IGF-1) (Fig 1.1). IGF-1 expression is induced by isometric and dynamic contractions (Kim, Cross et al. 2005; Haddad and Adams 2006), as well as passive stretch (McKoy, Ashley et al. 1999), and acts by stimulating the phospatidylinositol-3 kinase (PI3K)/Akt pathway, resulting in downstream activation of targets that induce protein synthesis and expression of α-skeletal actin. Another important growthfactor is myostatin. Myostatin is involved in regulating protein synthesis and degradation and acts

as a negative regulator of muscle growth influencing the Pax7, myoD and myogenin pathway by inhibiting satellite cell activation and differentiation (Sandri 2008).

Atrophy is largely due to a decrease in cell size and accompanied by a net increase in protein breakdown. An important proteolytic pathway involved in protein breakdown during muscle atrophy is the ubiquitin proteasome pathway. The addition of an ubiquitin to a protein substrate is the basis of the ubiquitin ligase pathway. Three distinct enzymatic components are required, an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin-ligating enzyme (Hershko and Ciechanover 1998). The E3 ubiquiton ligases confer substrate specificity.

Two ubiquitin ligases appeared to be muscle specific and are upregulated during multiple models of muscle atrophy; the muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1) proteins (Fig 1.1) (Sandri 2008).

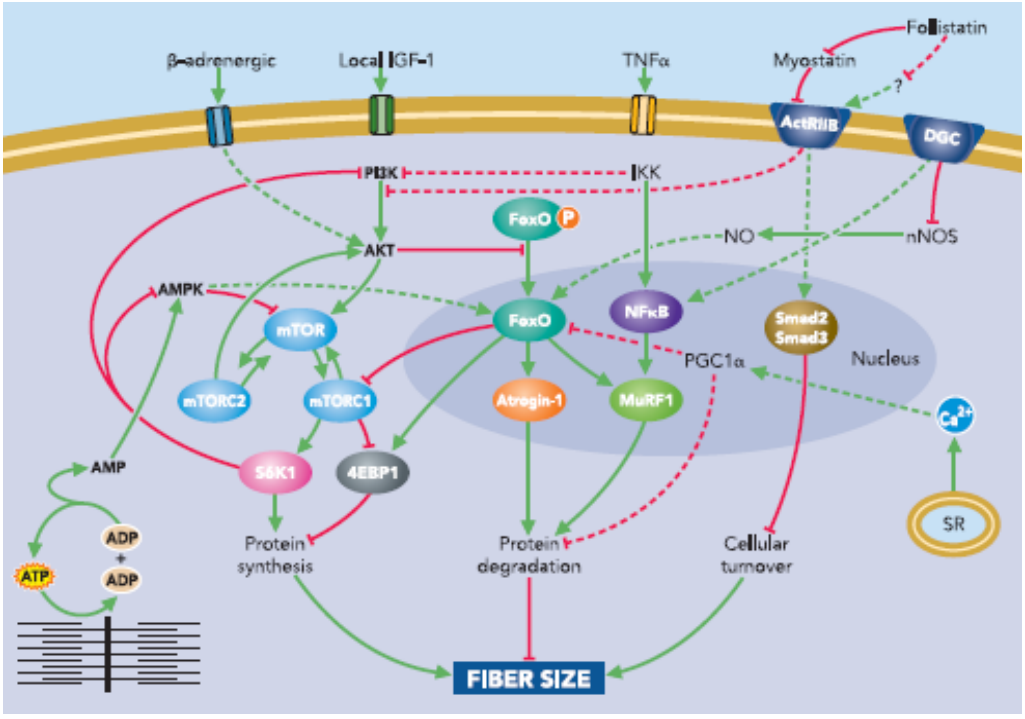

Figure 1.1: Major pathways that control muscle fibre size. (From Sandri 2008; Signaling in muscle atrophy and hypertrophy)

IGF-1: insulin growth factor 1; TNFα: Tumour necrosis factor α; PI3K: phosphatidylinositol 3-kinase; AKT: protein kinase β; AMPK: AMPactivated protein kinase; IKK: IKB kinase; NFKB: nuclear factor kappa-light-chain-enhancer of activated B-cells; MuRF1: muscle RINGfinger 1; FoxO: Forkhead box O; ActRIIB: activin receptor IIB; Smad2; mothers against decapentaplegic homolog 2; Smad3; mothers against decapentaplegic homolog 3; DGC: dystrophin glycoprotein complex; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; SR: sarcoplasmic reticulum; mTOR: mammalian target of rampamycin; mTORC1: mTOR complex 1; mTORC1: mTOR complex 2; S6K1: ribosomal S6 kinase 1; 4EBP1: 4E binding protein 1; ATP: adenosine triphosphate; AMP: adenosine monophosphate; ADP: adenosine diphosphate

1.5 Vitamin D and skeletal muscle

As stated earlier, skeletal muscle is a highly adaptive tissue and responds to many factors, such as altered loading, recruitment and growth factors (Fig 1.1). The focus of this thesis is on the impact of vitamin D on skeletal muscle. Vitamin D is also known as the "sunlight vitamin". It is produced in the skin under the influence of ultraviolet-B radiation which converts 7-dehydrocholesterol to pre-vitamin D3. Pre-vitamin D3 is thereafter rapidly converted to vitamin D3 in the dermis which in turn is converted to 25-hydroxy vitamin D (25D) in the liver. Hydroxylation to 1,25 hydroxy vitamin D (1,25D), the active form of vitamin D, occurs in the kidney (Fig 1.2) (Holick 1998). In chapter 2 and 3 of this thesis alfacalcidol was used to elevate 1,25D serum levels. Alfacalcidol, (1-α-Hydroxycholecalciferol), is a synthetically produced, inactive pro-hormone which is metabolized in the liver and other organs into the active 1,25D form (Kanis 1999; Wu-Wong, Tian et al. 2004), thereby bypassing strict feedback regulation (see Fig 1.2 for details).

Vitamin D plays an important role in phosphate and calcium homeostasis (DeLuca 1988). It exerts its action via influencing the intestine (Van Cromphaut, Dewerchin et al. 2001), kidney (Liu, Yu et al. 1998), parathyroid gland (Fraser 2009) and bone (Panda, Miao et al. 2004) (Fig 1.2). The action of vitamin D is not restricted to those organs and also other tissues, including muscle, are influenced by vitamin D. 1,25D acts through binding to the vitamin D receptor (VDR), but it has been suggested that also 25D acts as an active form (Lou, Laaksi et al. 2004; Tuohimaa 2009).

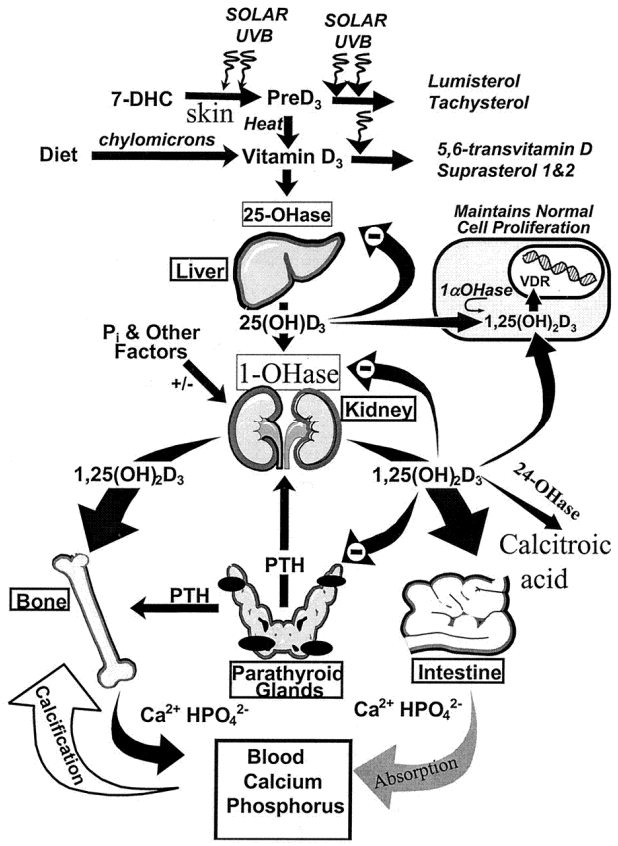

Figure 1.2: Vitamin D has extensive function in different organs and plays an important role in calcium homeostasis (From Holick "How much vitamin D is enough?" 1998).
7DHC: 7-dehydrocholesterol; 25-(OH)D_3: 25-hydroxyvitamin D_3; 1,25$(OH)_2D_3$: 1,25-dihydroxyvitamon D_3; Pi: inorganic phosphate; 25-OHase:25-hydroxyvitamin D3 hydroxylase; 1-OHase: 1,25-dihydroxyvitamin D3 hydroxylase; 24-OHase: 25-hydroxyvitamin D_3-24-hydroxylase; PTH: parathyroid hormone. Alfacalcidol is converted in the liver by 1-OHase to 1,25$(OH)_2D_3$

Two different VDR have been described in muscle tissue, one in the cytoplasm, which acts as a nuclear receptor and the other in the sarcolemma. The cytoplasmic (nuclear) VDR is responsible for genomic effects of vitamin D, while the membrane VDR is thought to be responsible for the non-genomic effects. The fast, non-genomic responses of 1,25D may involve binding to another yet uncharacterised membrane receptor (Nemere, Dormanen et al. 1994; Ceglia 2008) and/or the cytoplasmic VDR which is translocated from the nucleus to the myoblast cell membrane (Capiati, Benassati et al. 2002; Ceglia 2008). It is not known, however, whether the two receptors are different isoforms or are the same and just located at different sites in the cell.

Genomic actions

Genomic actions of 1,25D are long lasting. 1,25D is transported to the nucleus where it heterodimerizes with the retinoid X receptor. This heterodimer binds to the vitamin D response elements (VDREs) in the promoter of 1,25D responsive genes which ultimately result in an altered gene expression (Freedman 1999; Dusso, Brown et al. 2005). Particularly the expression of proteins involved in muscle calcium uptake, phosphate transport across the cell membrane, phospholipid metabolism and muscle cell proliferation and differentiation have been shown to be regulated by 1,25D. In the presence of elevated 1,25D levels this may result in an enhanced activity of calcium pumps in the sarcoplasmic reticulum (SR) and sarcolemma, and thereby affect the rate of sequestration of intracellular calcium (Boland 1986) and hence muscle function (Ebashi and Endo 1968; Ceglia 2008). Also synthesis of calmodulin, a calcium-binding protein that modulates muscle contraction, is enhanced (Brunner and de Boland 1990; Ceglia 2008).

Non-genomic actions

Besides genomic actions, 1,25D also elicits rapid (within seconds to minutes) nongenomic, non-transcriptional responses via activation of several interacting pathways. For instance, 1,25D can activate phospholipase C (PLC) (Morelli, Boland et al. 1996) which generates 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), and together with an acute increase in cyclic AMP (cAMP) levels (Vazquez, Boland et al. 1995) as a result of the activation of adenylyl cyclase this leads to activation of protein kinase A and C (Vazquez and de Boland 1996; Capiati, Vazquez et al. 2000; Ceglia 2008). Resulting in a subsequent release of calcium from intracellular stores and activation of voltage-gated and store-operated calcium channels (Vazquez and de Boland 1993; Vazquez and de Boland 1996; Vazquez, de Boland et al. 1997; Vazquez, de Boland et al. 1998; Capiati, Vazquez et al. 2000; Ceglia 2008).

In addition, the activation of mitogen-activated protein kinase (MAPK) by 1,25D results in initiation of myogenesis, cell proliferation, differentiation, or apoptosis (Wu, Woodring et al. 2000; Ceglia 2008). The outcome depends on which subgroup of the MAPK family is activated. The mammalian MAPK can be divided into 5 families; MAPK extracellular signal-regulated kinase 1/2 (MAPKerk1/2), MAPKp38, MAPK c-JUN N-terminal kinases (MAPKjnk), MAPKerk3/4 and MAPKerk5 (Widmann, Gibson et al. 1999). Activation of ERK's by 1,25D through phosphorylation by different kinases (c-Src, Raf-1, Ras, MAPKK) has been shown to stimulate synthesis of the pro-oncogene c-myc, and consequently growth and differentiation of the muscle cells (Cobb, Robbins et al. 1991; Buitrago, Boland et al. 2001; Morelli, Buitrago et al. 2001; Buitrago, Pardo et al. 2003).

1.6 Vitamin D and skeletal muscle functioning

1,25D has been shown to be involved in the regulation of differentiation of myoblasts acting via both genomic and non-genomic pathways (De Boland and Boland 1994; Whitfield, Hsieh et al. 1995; Vazquez, de Boland et al. 1998; Buitrago, Vazquez et al. 2001; Capiati, Benassati et al. 2002; Ceglia 2008). Vitamin D deficiency in adult humans is associated with a decrease in maximum muscle force (Visser, Deeg et al. 2003) and type II atrophy (Sato, Iwamoto et al. 2005). Furthermore, muscle contractile characteristics are changed during vitamin D deficiency; it was shown that depletion of vitamin D prolonged the relaxation phase of a muscle contraction in rats and chickens (Rodman and Baker 1978; Pleasure, Wyszynski et al. 1979), which might be related to the preferential atrophy

of type II fibres. Normal vitamin D levels therefore seem important in the regulation of the adaptation of muscle phenotype and contractile characteristics. Indeed, reversal of myopathy during vitamin D deficiency by vitamin D supplementation is a strong indicator that vitamin D has an important impact on skeletal muscle tissue (Boland 1986; Sato, Iwamoto et al. 2005). The action of vitamin D on the tissue is mediated through the VDR, which has also been detected in muscle tissue (Zanello, Collins et al. 1997; Bischoff, Borchers et al. 2001). The importance of this receptor and vitamin D in skeletal muscle development is further reflected by the abnormal muscle development and deregulated expression of myoregulatory transcription factors (MRFs) in VDR knock-out mice (Endo, Inoue et al. 2003).

1.7 Ageing and vitamin D

During ageing, vitamin D status is decreasing. About 80-100% of elderly care-home residents in Europe, Australia, and North America are vitamin D deficient, of which a high proportion even has a severe deficiency (Corless, Boucher et al. 1975). Even in independent community dwelling older people mean serum 25D levels are low (Lips 2001). This vitamin D deficiency is due to a decreased dietary intake. In addition, reduced skin thickness and diminished sunlight exposure cause a decline in the cutaneous levels of 7-dehydrocholesterol, resulting in an up to four-fold decrease in vitamin D production in a 70-year-old compared to a 20-year-old (Holick 1985; MacLaughlin and Holick 1985). Also an impaired intestinal absorption and impaired hydroxylation of 25D in liver and kidney and an increased degradation contribute to a lower vitamin D status in the elderly (Lanske and Razzaque 2007). Furthermore, the age-related reduction in vitamin D levels maybe associated with a significant decrease in VDR expression in skeletal muscle, as observed in elderly of women (Bischoff-Ferrari, Borchers et al. 2004). The VDR expression is, however, independent of serum 25D concentration in aged female skeletal muscle (Kinyamu, Gallagher et al. 1997; Bischoff-Ferrari, Borchers et al. 2004). A decreased VDR expression may well reduce the functional response to 1,25D, and aggravate the consequences of reduced vitamin D levels, such as impaired protein synthesis and a decrease in type II fibres, thus contributing to sarcopenia (Bischoff-Ferrari, Borchers et al. 2004).

1.8 Skeletal muscle and ageing

Human ageing is associated with sarcopenia, the age-related loss of muscle mass. The muscle wasting is accompanied by muscle weakness (Blough and Linderman 2000; Degens and Alway 2003) and ultimately results in limited mobility and increased susceptibility to injury (Welle 2002; Rice and Blough 2006). Part of the sarcopenia is attributable to a 30-40% loss of muscle fibres between the second and eighth decade of life (Lexell 1995; Rice and Blough 2006). Especially type II fibres are decreased in size and number (Klitgaard, Zhou et al. 1990).This decrease in the size and number of fast fibres negatively affects the ability to generate muscular power necessary for mobility and daily living activities.

Many explanations have been put forward with respect to the cause and pathogenesis of sarcopenia; reduced physical activity (Degens and Alway 2006), impaired regenerating ability of muscle tissue, increased oxidative stress, loss of motorneurons and reorganisation of neuromuscular junctions, disturbances in the endocrine system, deterioration of the immune system, and development of a chronic inflammatory state (Larsson and Ansved 1995; Linderman and Blough 2002; Degens, Erskine et al. 2009). One endocrine factor that could cause an imbalance in the degeneration-regeneration

processes and thereby sarcopenia is vitamin D deficiency. Indeed, long term vitamin D deficiency results in osteomalacia (Lau and Baylink 1999; Pettifor 2003), a disease characterised by both loss of bone strength and muscle weakness. The muscle weakness increases the risk of falls, and the weaker bones increase the risk of bone fractures during falling. In addition, the decrease in muscle strength will further diminish bone strength, as stronger muscles are associated with stronger bones. In this disorder, the muscle weakness is effectively treated by vitamin D supplementation (Boland 1986).

1.9 Aims and outline thesis

Cross–sectional studies have shown that rodents show the same decrease in muscle strength during ageing (Degens, Veerkamp et al. 1993; Degens, Hoofd et al. 1995; Linderman and Blough 2002; Degens and Alway 2003) and atrophy of type II fibres (Holloszy, Chen et al. 1991; Degens, Veerkamp et al. 1993) as humans. Rodents have therefore often been used as a model to study human muscle ageing. The questions we address in this thesis are:

- 1) assess the effects of vitamin D on whole muscle function and morphology *in vivo* (chapter 2). For this, the synthetically produced vitamin D analogue alfacalcidol was used.
- 2) Study the effects of alfacalcidol on muscle fibre type composition, and explore whether changes in circulating cytokines and changes in factors involved in catabolism and anabolism are associated with the alfacacidol-induced atrophy of aged rat skeletal muscle (chapter 3).
- 3) Determine the effects of vitamin D deficiency on skeletal muscle function and expression of components of the ubiquitin proteasome pathway and growth factors (chapter 4).
- 4) And finally, study the effects of 1,25D on the contractile properties in isolated *Xenopus* skeletal muscle fibres (chapter 5).

Chapter 2

Effects of alfacalcidol on the contractile properties of the Gastrocnemius medialis muscle in adult and old rats

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Abstract

Background

Vitamin D deficiency is associated with muscle weakness. It is unknown, however, how supra-physiological levels of vitamin D affect skeletal muscle.

Methods

To investigate the effects of increased serum vitamin D ($1,25(OH)_2D_3$ or 1,25D) levels on the contractile properties of the medial gastrocnemius muscle, adult and old female $Fischer_{344}$ x Brown Norway F1 rats were orally treated with vehicle or the vitamin D analogue alfacalcidol for 1 or 6 weeks.

Results

Alfacalcidol treatment resulted in elevated 1,25D serum levels. This was accompanied by hypercalcaemia and a reduction in body mass, the latter largely attributable to a reduced food intake. However, kidney function, as reflected by normal creatinine serum levels, as well as heart mass were unaffected. The 17% reduction in maximal isometric force and power was explicable by a similar loss of muscle mass. The force-frequency relation of the 6-week-treated old rats was shifted to the left, but neither the shape of the force-velocity relation nor the fatigability of the muscle were altered.

Conclusion

Supra-physiological doses of vitamin D were accompanied by significant reductions in body and muscle mass, but no improvement in muscle functioning. Weight loss was largely due to a reduced food intake. Although undesirable, this and the maintained kidney function, heart mass and quality of the remaining muscle tissue suggests that quick normalisation of circulating vitamin D would reverse these effects on body and muscle mass. Furthermore, food intake and body mass should be carefully monitored when treating people with vitamin D analogues.

Key words: vitamin D, alfacalcidol, muscle contractile properties, skeletal muscle, fatigue

Introduction

The active form of vitamin D, $1\alpha,25(OH)_2D_3$ (1,25D) elicits cellular responses through both genomic and non-genomic actions (Ceglia 2008). Vitamin D has been shown to be important in phosphate and calcium homeostasis (DeLuca 1988) acting on the intestine (Van Cromphaut, Dewerchin et al. 2001), kidney (Liu, Yu et al. 1998), parathyroid gland (Fraser 2009) and bone (Panda, Miao et al. 2004). It exerts its effects through the vitamin D receptor (VDR) and the discovery of the VDR in muscle tissue (Zanello, Collins et al. 1997; Bischoff, Borchers et al. 2001) 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 (Endo, Inoue et al. 2003). 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 musculoskeletal function in institutionalized elderly by 4-11% within 12 weeks (Bischoff, Stahelin et al. 2003).

It has been found in human and mouse primary hepatocyte cultures that a concentration of 100 $nmol \cdot L^{-1}$ vitamin D elicits maximal activation of the VDR (Reschly, Bainy et al. 2007). The serum vitamin D levels in rats, around 185 $pmol \cdot L^{-1}$ (Anderson, Sawyer et al. 2007), 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 alfacalcidol, 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 (Walters, Sweeney et al. 1991), overload (Degens and Alway 2003) and disuse (Alway, Degens et al. 2003; Degens and Alway 2003) 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. We hypothesized that old rats are vitamin D deficient and that alfacalcidol treatment would reverse the decline in contractile properties in old rats.

Material and Methods

Animals

Female Fischer 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 (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-, 27.5- or 29-month old at the end of the treatment period and divided randomly in 1-week, 6-week, or vehicle treated groups (Table 2.1). Rats were orally administered either vehicle or Alfacalcidol (0.1 $g \cdot kg^{-1}$) (Chugai Pharmaceutical, Japan) daily for 1 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 (Shiraishi, Takeda et al. 2000). Rats were weighed before vehicle and alfacalcidol 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 ethic 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 $g \cdot kg^{-1}$ i.p). After 10 minutes an additional dose of 0.75 $g \cdot kg^{-1}$ urethane was given. If the rats still responded to nociceptive stimuli, supplementary injections of 0.63 $g \cdot kg^{-1}$ 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 (L_0), 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 L_0 on cork and frozen in liquid nitrogen.

group	# animals	treatment	Age (months)
CA	9	none	7
A1WA	6	1 week Alfacalcidol (0.1 g.kg^{-1} BW)	7
A6WO	9	6 weeks Alfacalcidol (0.1 g (in 1 ml) .kg^{-1} BW)	27.5
V6WO	9	6 weeks Vehicle (1 ml. kg^{-1} BW)	27.5
A1WO	9	1 week Alfacalcidol (0.1 g.kg^{-1} BW)	29
V1WO	9	1 week Vehicle (1 ml. kg^{-1} BW)	29

Table 2.1: group arrangement, number of animals in each group, treatment and age of the rats used in this experiment.

CA: control adult; A1WA: (alfacalcidol, 1 week, adult), adult rats treated with alfacalcidol for 1 week; A6WO: (alfacalcidol, 6 weeks, old), old rats treated wih alfacalcidol for 6 weeks; V6WO: (vehicle, 6 weeks, old), old rats treated with vehicle for 6 weeks; A1WO: (alfacalcidol, 1 week, old), old rats treated with alfacalcidol for 1 week
V1WO: (vehicle, 1 week, old) old rats treated with vehicle for 1 week

Protocols
Frequency – force relation
To determine the frequency-force relation 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 a 400-Hz 150-ms trains (de Haan 1998). 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 (De Haan, de Ruiter et al. 1993). 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 was calculated. Half relaxation time 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 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) 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. A bonferroni post hoc test was performed if a significant effect was found. For the force-ferequency relation and force-velocity relation 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 ± SD.

Results

Body and muscle mass

Table 2 shows the body and muscle mass data for the different groups before and after treatment. The old animals were heavier than the adult animals ($P \leq 0.001$). The vehicle treatment did not significantly affect body mass. Treatment with alfacalcidol, on the other hand, caused a 6% reduction in body mass after one week of treatment in adult and old rats ($P < 0.03$), which progressed to a 22% reduction after 6 weeks of treatment in the old rats ($P = 0.001$) (Fig 2.1).

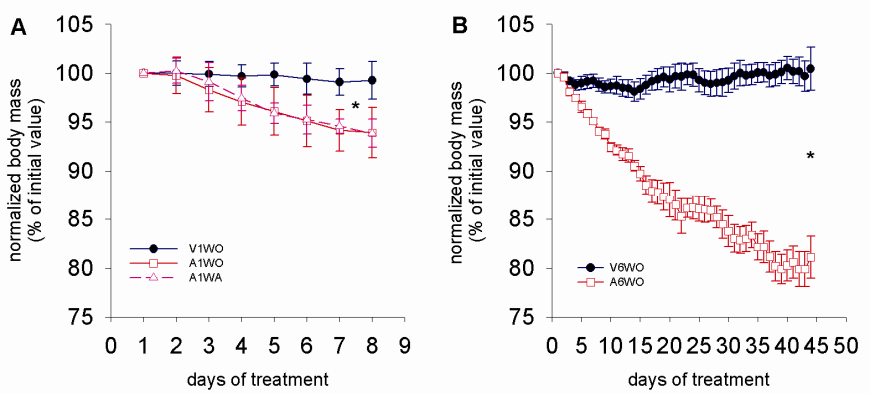

Figure 2.1: Six week Alfacalcidol treatment reduces the body mass old rats. Adult rats were orally treated with vehicle or alfacalcidol for 1 week, and old rats for 1 and 6 weeks while, the adult control group received no treatment. A) Effects of 1 week treatment with alfacalcidol. The adult and old alfacalcidol treated animals had a significant decrease in body mass compared to the old rats receiving vehicle ($P < 0.03$). B) Effects of 6 week alfacalcidol and vehicle treatment on old rats. After 6 weeks alfacalcidol treatment rats showed a substantial loss of body mass compared to initial body mass of the 6 weeks vehicle treated rats ($P = 0.001$, ANOVA, bonferroni post hoc).

Ageing did not significantly affect muscle mass. This applied not only to the gastrocnemius muscle (Table 2), 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 in the old than the adult rats. Treatment with alfacalcidol did not reduce this ratio, indicating that the decrease in body mass is 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. 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

Fmax was lower in the old vehicle treated rats than the young control group ($P < 0.05$) (Table 2). The specific tension was 12% lower in the old (V1WO) than the 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 17% 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 (Fig.2).

	CA	A1WA	V1WO	A1WO	V6WO	A6WO
Body mass before (g)	-	203 (78)	296 (31)	282 (22)	273 (35)	286 (26)
Body mass end (g)	221 (16)	190 (73)~/*	294 (30)*	265 (21)~	276 (36)*	224 (19)~
Gm muscle mass (mg)	669 (47)	638 (44)	627 (46)	603 (50)	622 (54)	515 (37)#
Maximal isometric force Fmax (N)	12.1 (1.3)	11.2 (1.0)	9.8 (1.0)*	10.0 (0.6)	10.7 (0.7)*	8.8 (0.8)**

Table 2.2: Mean (±SD) of body mass, Gastrocnemius muscle (Gm) mass, contractile properties of the Gm (Fmax: maximal tetanic force, Fmax/muscle mass: Maximal tetanic force per unit muscle mass, HRT: half relaxation time (ms), and Pmax: maximal power) and vitamin D blood serum concentrations of the different groups. * significantly different from CA; # significantly different from other groups; ** significantly different from V6WO ($P < 0.05$, ANOVA, bonferroni posthoc).

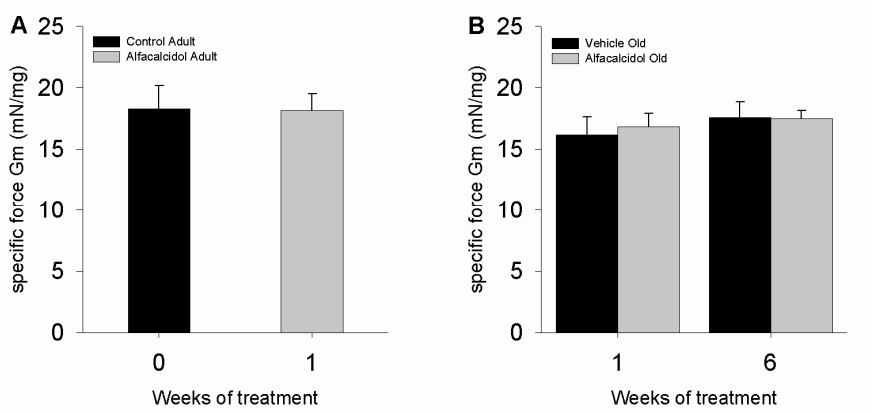

Figure 2.2: Specific force is similar in all groups. A) Adult rats were orally treated with alfacalcidol for 1 week, the control adult group received no treatment B) old rats were treated for 1 and 6 weeks with vehicle or alfacalcidol. No significant differences were found in the specific force between the groups, except a lower specific force was seen for the V1WO group compared to the CA group ($P < 0.05$, ANOVA, bonferroni posthoc).

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 3A). Only alfacalcidol treatment for 6 weeks induced a shift in force frequency curve in to the left compared to their corresponding control group ($P < 0.05$) (Fig. 3B and C).

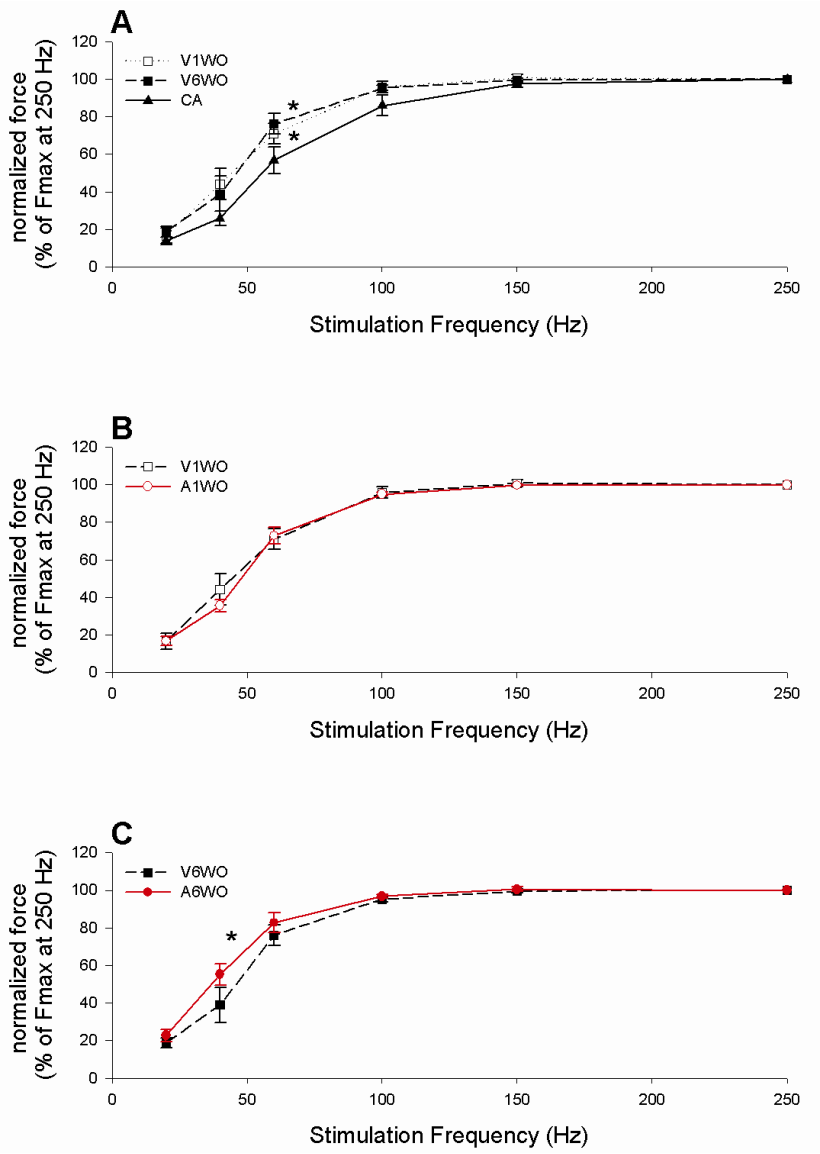

Figure 2.3: Influence of age and alfacalcidol treatment on the force-frequency relation. A) Force frequency relation is shifted to the left in old animals, indicating an age effect. Old rats were treated with vehicle for 1 or 6 weeks, the control adult group received no treatment. * whole curve of V6WO and V1WO different from CA ($P < 0.001$) B) 1 week treatment with alfacalcidol in old animals did not effect the force frequency relation. Old rats were treated for 1 week with vehicle or alfacalcidol. C) 6 weeks alfacalcidol treatment in old rats resulted in a left-shift in the force frequency relation. Old rats were treated with alfacalcidol or vehicle for 6 weeks. Lower frequencies resulted in a higher percentage of the Fmax compared to the old rats treated with vehicle during 6 weeks.

* A6WO curve different from corresponding control group (V6WO) ($P < 0.05$) (Two-way repeated ANOVA)

Force-Velocity Characteristics
No significant differences were found in the force-velocity relations between the groups (data not shown). Figure 2.4 shows a typical example of a force-velocity relation of a control adult animal. 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 max power was similarly reduced after 6 weeks of alfacalcidol treatment ($P < 0.007$).

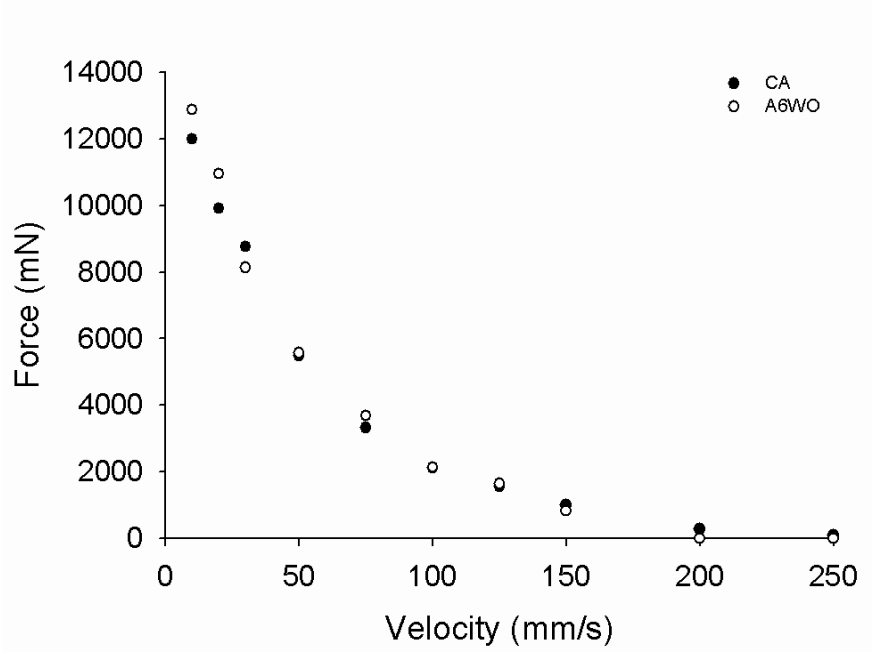

Figure 2.4: A typical example of a force-velocity relation from an adult animal and an old alfacalcidol treated animal.

Fatigue
During 20 repeated isometric contractions a linear decrease to 74% of the initial force was seen in all groups. Concomitantly, the half relaxation (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).

Blood serum levels
Figure 2.5 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. 1,25D serum levels after 6 week treatment in the old rats with alfacalcidol was not different from the 1 week alfacalcidol group. The blood serum levels of 1,25D in the V1WO and V6WO were not significantly different from those of the CA group,

indicating that the vehicle itself had no significant effect on the 1,25D concentration in the blood.
Albumin and creatinine serum levels were similar in all groups (data not shown). Ca^{2+} levels were 1.1-1.3 fold elevated in the alfacalcidol treated groups compared to the corresponding control groups ($P < 0.02$, Fig 2.6).
Pi serum levels of the old animals were lower than that in the adult control animals ($P < 0.001$). Alfacalcidol treatment did not induce significant changes in the circulating levels of Pi in either the adult or old rats, irrespective of duration of treatment.

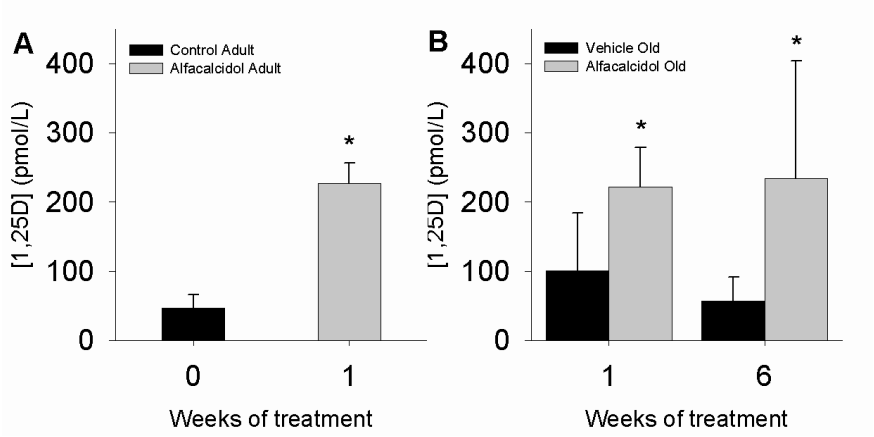

Figure 2.5: 1,25D concentration in blood serum is increased after alfacalcidol treatment. Furthermore, old rats are not vitamin D deficient. A) Adult rats orally treated with alfacalcidol for 1 week, the control adult group received no treatment B) old rats were treated for 1 and 6 weeks with vehicle or alfacalcidol. 1,25D blood serum levels were in the control group 46 pmol/L, the adult animals treated with alfacalcidol for 1 week mean 1,25D level was 226 pmol/L. In the old animals treated with vehicle the 1,25D blood serum was not changed compared to the control group. After 1 and 6 weeks alfacalcidol treatment the 1,25 level was similar to the 1,25D blood serum level of the adult animals treated with alfacalcidol for 1 week.
*different from corresponding control group ($P < 0.05$) (ANOVA, bonferroni posthoc)

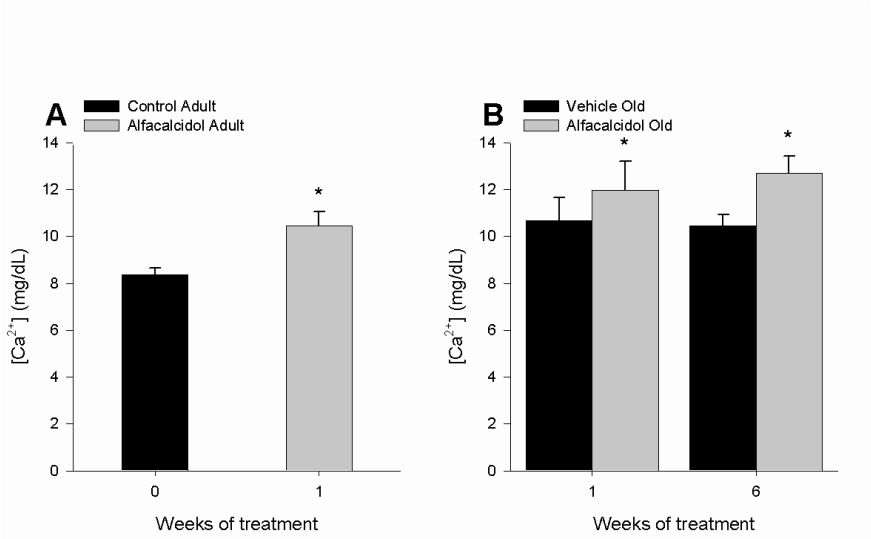

Figure 2.6: Ca^{2+} concentration in blood serum is increased after alfacalcidol treatment. A) Adult rats orally treated with alfacalcidol for 1 week, the control adult group received no treatment B) old rats were treated for 1 and 6 weeks with vehicle or alfacalcidol. *different from corresponding control group ($P < 0.02$) (ANOVA, bonferroni posthoc).

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. 2.7).

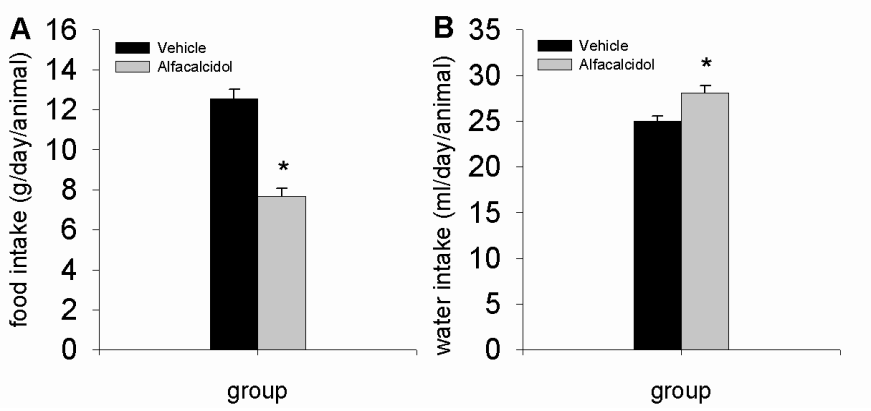

Figure 2.7: Food intake is lower and water intake is higher in alfacalcidol treated rats. A) food intake during vehicle and alfacalcidol treatment in old rats. B) Water intake during vehicle and alfacalcidol treatment in old rats. Food intake was 38% lower and water intake was 12 % higher in alfacalcidol treated animals compared to vehicle treated rats. *different from corresponding control group ($P < 0.05$) (ANOVA, bonferroni posthoc).

Discussion

The aims of this study were 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 relation of the Gm in old rats was found. Thus, while administration of alfacalcidol or vitamin D analogues has been proven beneficial for muscle function in conditions of vitamin D deficiency (Pfeifer, Begerow et al. 2000; Bischoff, Stahelin et al. 2003; Bischoff-Ferrari, Dietrich et al. 2004), 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.

Ageing and blood serum levels

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

During ageing in humans levels of 1,25D in blood serum decrease (Corless, Boucher et al. 1975; Lips 2001), 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 (Holick, Matsuoka et al. 1989), whereas neither the adult nor the old rats were exposed to sunshine and rats were fed the same diet at all ages.

Alfacalcidol and VDR activity

The levels of circulating vitamin D we observed in the control adult and old rats were between 45 and 100 $pmol \cdot L^{-1}$. This concentration has been found to activate approximately 3% of the VDR in mouse and human (Reschly, Bainy et al. 2007). Although the dose response curve for rat is not known it is likely that the activation of the VDR would be in the same range in the rat. By elevating the vitamin D levels with alfacalcidol treatment one would expect, based on the dose response curve of Reschly et al. (Reschly, Bainy et al. 2007), 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 (Endo, Inoue et al. 2003).

Muscle contractile properties

In line with previous observations, we observed a significant slowing of relaxation, a decline in force (Degens and Alway 2003) and power generating capacity, as observed in old mice (Brooks and Faulkner 1991) 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 (Degens, Hoofd et al. 1995; Degens and Alway 2003).

Six weeks of alfacalcidol treatment did cause a left shift in the force frequency relation in old rats. Such a phenomenon would be explicable by an increased relaxation time which we did not observe, however, after alfacalcidol treatment. 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 cross-bridge kinetics, and hence fibre type composition, after alfacalcidol treatment. Another possibility is that the Ca^{2+} sensitivity of the regulatory proteins on the thin filaments is reduced, a situation which has for instance been observed in diaphragm fibres from patients with chronic obstructive pulmonary disease (Ottenheijm, Heunks et al. 2005). Finally, the release of Ca^{2+} by the sarcoplasmic reticulum might be enhanced at low stimulation frequencies and/or the intracellular Ca^{2+} concentration might already be slightly elevated after treatment with alfacalcidol. Indeed, vitamin D has been shown to change the intracellular Ca^{2+} concentration via both non-genomic (Vazquez, Selles et al. 1999) and genomic actions (Boland 1986).

While the treatment with alfacalcidol 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 gastrocnemius muscle mass, but also the mass of the EDL, soleus, plantaris and TA 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: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.

Blood serum levels

In our experiment we saw a progressive decrease in body mass caused by a reduced food intake during the alfacalcidol administration. Clearly, the decreased food intake and body mass indicates that starvation of the rats was a major problem. During starvation, however, also proteins are used in the oxidative metabolism and albumin is an abundant protein and therefore a possible target to metabolize during starvation. Yet, despite the significant decrease in body mass circulating albumin was not reduced (Krantz, Lee et al. 2005). It appears that the body maintains circulating albumin levels at the costs of other protein sources, predominantly muscle tissue (Millward 1979).

Alfacalcidol treatment resulted in elevated serum 1,25D, which facilitates the Ca^{2+} absorption from the intestine and reabsorption in the kidney (Pfeifer, Begerow et al. 2002). It is therefore not surprising that we found elevated Ca^{2+} concentrations in the serum of alfacalcidol-treated rats. The transport protein albumin has a high affinity for calcium and up to 40% is bound and inactive, depending upon albumin concentration and pH (Margarson and Soni 1998). A decrease in albumin concentration can therefore lead to an increase in the free Ca^{2+} concentration in the blood. However, the albumin blood concentration was not decreased in the alfacalcidol groups compared to the vehicle and control groups. Furthermore, Ca was measured, which includes Ca^{2+}. A more plausible

explanation for the hypercalcemia is the increased free 1,25 D serum levels not bound to vitamin D-binding protein (BDP) (Vieth 1990; Pettifor, Bikle et al. 1995).

Further evidence for problems when vitamin D levels are elevated is the increased fluid intake in alfacalcidol-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 alfacalcidol have been associated with a higher risk of hypercalcaemia compared to native vitamin D (Avenell, Gillespie et al. 2009). 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 contributes to the idea that the animals which received alfacalcidol were treated with a too high dose of alfacalcidol leading to a deficit in food intake.

In conclusion, supraphysiological circulating levels of vitamin D are accompanied by significant reductions in body and muscle mass, largely due to a reduced food intake, with no apparent improvement in muscle functioning. Although undesirable, this and the maintained kidney function, heart mass and quality of the remaining muscle tissue suggests that quick normalisation of circulating vitamin D would reverse these effects on body and muscle mass. Furthermore, food intake and body mass should be carefully monitored when treating people with vitamin D analogues.

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Chapter 3

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 $Fischer_{344}$ x Brown Norway F1 rats orally with vehicle or the vitamin D analogue alfacalcidol for 6 weeks to increase serum vitamin D ($1,25(OH)_2D_3$ 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

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 $nmol \cdot L^{-1}$ 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 Ca^{2+} 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 Ca^{2+} 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

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 diabetis. 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 $g \cdot kg^{-1} \cdot day^{-1}$), 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 $g \cdot kg^{-1}$) (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 $g \cdot kg^{-1}$ i.p). After 10 minutes an additional dose of 0.75 $g \cdot kg^{-1}$ urethane was given. If the rats still responded to nociceptive stimuli, supplementary injections of 0.63 $g \cdot kg^{-1}$ 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 (L_0), defined as the length at which the active twitch force was maximal. Then *Lo* 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 L_0 on cork and frozen in liquid nitrogen with vigorous shaking.

group	# animals	treatment	Age (months)
CA	9	none	7
A6WO	9	6 weeks Alfacalcidol (0.1 g (in 1 ml) $.kg^{-1}$ BW)	27.5
V6WO	9	6 weeks Vehicle (1 ml. kg^{-1} BW)	27.5

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.

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.

Target mRNA	PCR primer sequence 5'→ 3'
18S RNA	Forward: CGAACGTCTGCCCTATCAACTT
	Reverse: ACCCGTGGTCACCATGGTA
α-skeletal actin	Forward: CGACATCGACATCAGGAAGGA
	Reverse: GGTAGTGCCCCCTGACATGA
IGF-I (all isoforms)	Forward: CCTACAAAGTCAGCTCGTTCCA
	Reverse: TCCTTCTGAGTCTTGGGCATGT
MAFbx	Forward: TGAAGACCGGCTACTGTGGAA
	Reverse: CGGATCTGCCGCTCTGA
MuRF1	Forward: TGCCCCCTTACAAAGCATCTT
	Reverse: CAGCATGGAGATGCAATTGC
myostatin	Forward: GTTCCCGGAGAGACTTTGG
	Reverse: CGACAGCACCGCGATTC
c-myc	Forward: CACAACGTCTTGGAACGTCAGA
	Reverse: GCGCAGGGCAAAAAAGC
VDR	Forward: CACCCTTGGGCTCTACTCAC
	Reverse: CTGTTGCCTCCATCCCTGAA
CYP27B1	Forward: CGGGAAAAGGTGTCTGTCCA
	Reverse: GTGTCCACTCCAGTAG

Table 3.2: Sequence of the specific primers used in the quantitative PCR analyses. IGF-1: insulin growth actor -1, MAFbx: muscle atrophy F-box, MuRF1: muscle ring finger-1, VDR: vitamin D receptor.

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 alfacalcidol group and the age matched vehicle group, between the alfalcidol 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 ($P \leq 0.001$). 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.

group	Body mass (g)	Gm mass (mg)	[1,25D] (pmol/l)
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)

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).

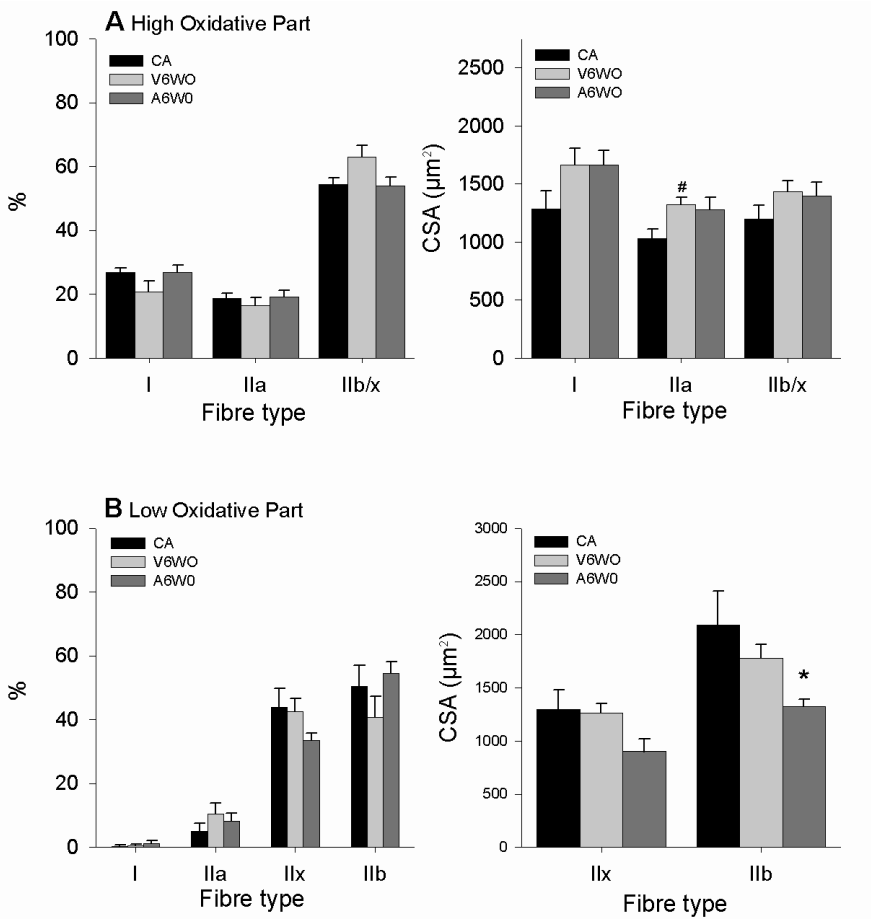

Figure 3.1: Fibre type distribution and cross sectional area (CSA) of the fibre types in the A) high oxidative and B) low oxidative part of the Gm. Fibre type composition (left) and CSA of the different fibre types (right). No differences were observed in fibre type composition. The CSA of the type IIa 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)

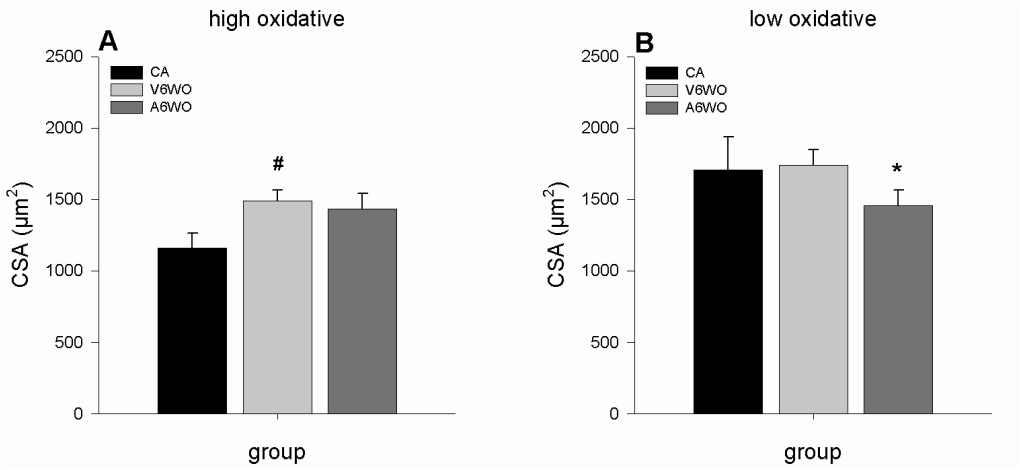

Figure 3.2: combined cross sectional area of different fibre types of the high oxidative (A) and low oxidative (B) part of the Gm. In the high oxidative part the CSA of the control adult group was smaller compared to the old vehicle group. In the low oxidative part the CSA of the alfacalcidol treated 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).

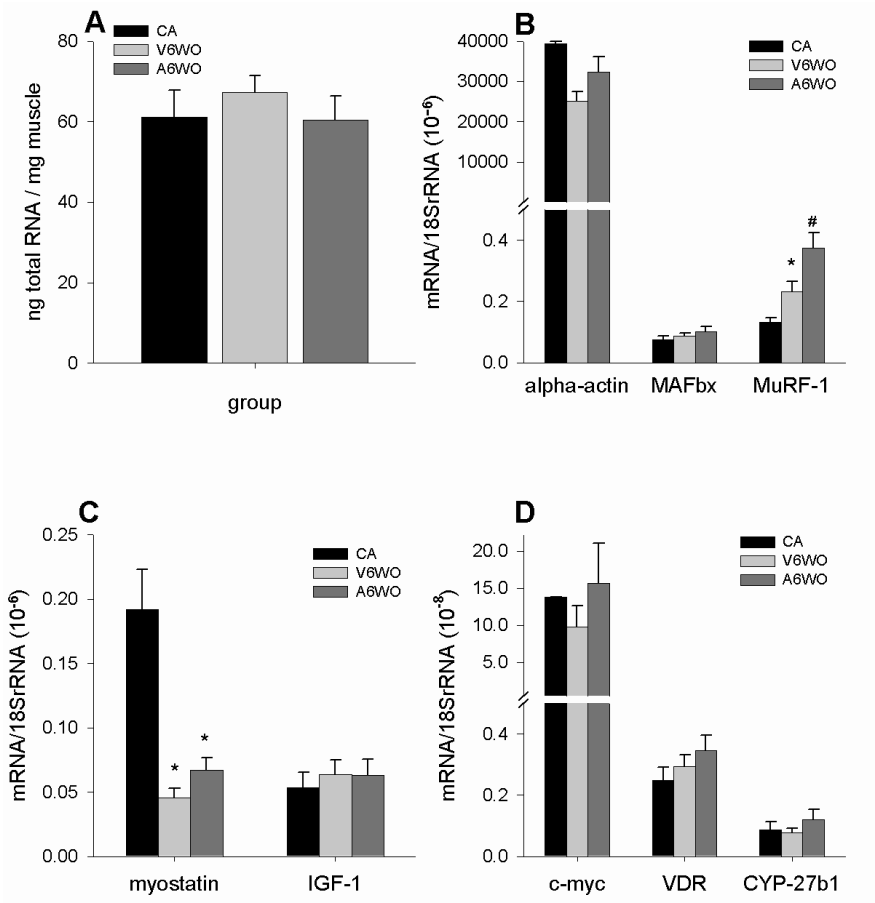

Figure 3.3: Quantitative PCR of structure protein, part of the ubiquiton pathway, growthfactors and vitamin D associated proteins of the low oxidative, distal part of the Gm. A) total RNA per mg muscle. No differences in total RNA concentration were observed between the groups indicating that it was 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).

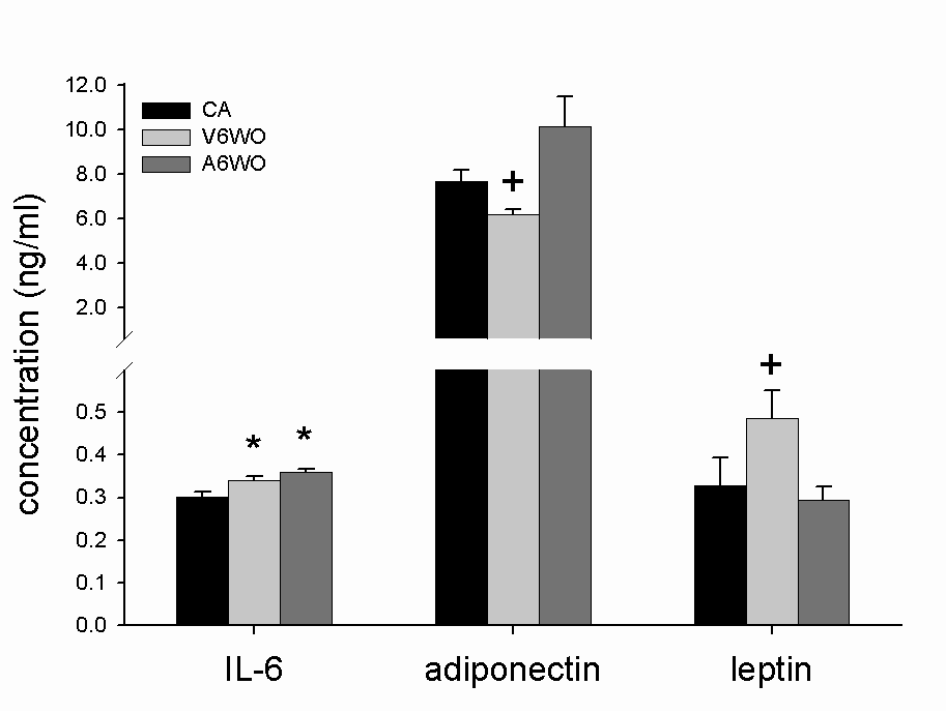

Figure 3.4: Serum levels of IL-6, adiponectin and leptin. IL-6 levels are increased in A6WO and V6WO compared to the control adult group. Adiponectin levels of the alfacalcidol group were increased compared to the age matched vehicle group. Leptin levels of the alfacalcidol group were decreased compared 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 sarcopaenia.

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 sarcopaenia 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,

Ferrucci et al. 2003; Schaap, Pluijm et al. 2006) and atrophy (Goodman 1994; Degens 2009). 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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Chapter 4

Vitamin D deficiency-related muscle atrophy – an indirect effect of low vitamin D levels?

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

Abstract

Background

Vitamin D deficiency has been associated with a decrease in muscle force and fibre type II atrophy. Unknown are the effects of vitamin D deficiency on the skeletal muscle contractile properties.

Methods

To investigate the effects of vitamin D deficiency on the contractile properties and expression factors of the medial gastrocnemius muscle, female 3 week old Wistar rats were provided with special vitamin D deficient food, preventing hypocalcaemia and hyperparathyroidism, and raised in an UV free environment during 10 weeks.

Results

10 weeks of vitamin D deficient food results in undetectable 25-hydroxy-vitamin D blood serum levels. Vitamin D deficiency was not accompanied with a change in muscle contractile properties, only the force frequency relation was shifted to the left. Furthermore no atrophy was observed in different muscles. mRNA expression of α-skeletal actin, MAFbx, MURF1, c-myc , CYP27B1 and VDR was not changed in the Gm.

Conclusion

Vitamin D deficiency *per se* does not result in a decrease in maximal force and muscle mass or atrophy but could be the result of associated hypocalcaemia and hyperparathyroidism.

Key words: vitamin D deficiency, muscle contractile properties, skeletal muscle

Introduction

Vitamin D deficiency is a widespread phenomenon in the elderly; 80-100% of elderly care-home residents in Europe, Australia, and North America have been shown to be deficient, even a high proportion have a severe deficiency (Corless, Boucher et al. 1975). There are several factors contributing to vitamin D deficiency, namely; decreased dietary uptake, diminished sunlight exposure, reduced skin thickness, and thereby a decline in the cutaneous levels of 7-dehydrocholesterol, resulting in an up to four-fold decrease in vitamin D production in a 70 year old compared to a 20 year old (Holick 1985; MacLaughlin and Holick 1985). Also an impaired hydroxylation of 25-hydroxy vitamin D (25D) in the liver and kidney and an increased degradation of vitamin D contributes to a lower vitamin D status in the elderly (Lanske and Razzaque 2007). Furthermore a significant decrease in vitamin D receptor (VDR) expression in skeletal muscle of women is found with ageing (Bischoff-Ferrari, Borchers et al. 2004).

Vitamin D deficiency is associated with a decline in muscle strength (Mowe, Haug et al. 1999) and type II fibre atrophy (Sato, Iwamoto et al. 2005) .Type II fibres are fast-twitch and their recruitment is essential in the strategy to prevent falls (Snijder, van Schoor et al. 2006). Furthermore, vitamin D deficiency is associated with sarcopenia; the degenerative loss of skeletal muscle mass and strength during ageing.

Past vitamin D deficiency studies have been shown that muscle weakness is reversible and that vitamin D supplementation to deficient people resulted in an increase in relative number and size of type II fibres (Sato, Iwamoto et al. 2005). However, it is unknown how contractile properties are affected in vitamin D deficient muscle. In rats it is already shown that vitamin D deficiency did not cause an altered muscle composition and that vitamin D deficiency increased the myofibrillar protein degradation (Wassner, Li et al. 1983). Furthermore, it was shown that depletion of vitamin D prolonged the relaxation phase of a muscle contraction (Rodman and Baker 1978). It is unknown what happens to maximal force, fatigability and transcription factors influencing muscle mass and function during vitamin D deficiency.

During vitamin D deficiency, hypocalcaemia and hyperparathyroidism occur. By providing a special diet, it is possible to maintain normal circulating calcium, phosphorus and parathyroid hormone in vitamin D deficient animals (Kollenkirchen, Fox et al. 1991) and hence study the effects of low vitamin D levels deficiency *per se*. It was our aim to provide a better understanding of the muscular adaptations accompanied with vitamin D deficiency in rats by studying the contractile properties of the medial gastrocnemius muscle (Gm). Furthermore, we aimed to understand muscle adaptations occurring during vitamin D deficiency *per se*, thus without changing calcium and parathyroid serum concentrations, by studying its effect on the expression of α-skeletal actin mRNA and the ubiquitin pathway (MuRF1 and MAFbx) to obtain insight in synthesis and degradation of contractile proteins. In addition, the mRNA expression levels of CYP27B1, the 25D hydroxylase, which transforms the 25D into the active form of vitamin D, 1,25(OH)2D3, (1,25D), and the VDR, were studied to obtain indications for changes in the local intramuscular 1,25D concentration and VDR quantity respectively. We expected that after 10 weeks of vitamin D deficient food uptake and an UV free environment, rats show a change in muscle contractile properties, particularly resulting in an extended half-relaxation time. Furthermore, we expect that the maximal force is reduced which would

be the result of type II atrophy. This atrophy would be the result of an increase in MuRF-1 and MAFbx mRNA expression.

Material and Methods

Animals. Twenty-one-day-old female Wistar rats were obtained from Harlan (Harlan, laboratories, Inc, Netherlands) (n = 16) and raised in an incandescent-lighted environment with a 12:12 light dark cycle. Rats aimed to become vitamin D deficient were maintained on 2.0% calcium, 1.25% phosphorus, 20% lactose semi-synthetic diet deficient in vitamin D. This diet has been reported to cause vitamin D deficiency in rats without concomitant hypocalcaemia, hypophosphatemia and hyperparathyroidism (Kollenkirchen, Fox et al. 1991). Control rats were kept on the same semi-synthetic diet supplemented with 800 IU vitamin D_3. All animals were maintained on their assigned diets for 10 weeks. Rats were weighted 5 times a week and food and water uptake was monitored weekly. At 13 weeks of age the contractile properties of the medial gastrocnemius muscle (Gm) were determined. All experiments were approved by the local ethic 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 $g \cdot kg^{-1}$ i.p.). After 10 minutes an additional dose of 0.75 $g \cdot kg^{-1}$ urethane was given. If the rats still responded to nociceptive stimuli, supplementary injections of 0.63 $g \cdot kg^{-1}$ were applied during the experiment. The medial gastrocnemius muscle (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 the length at which the active twitch force was maximal, with a series of twitch contractions (1 per minute). Then, the optimal length (L_0) was set with a few tetanic contractions (150 Hz, 150 ms). Muscle temperature was maintained at 34-36 ºC with a water-saturated airflow around the muscle, which also kept the muscle moistened (de Haan, Lodder et al. 1989). 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 L_0, pinned on cork and frozen in liquid nitrogen.

Protocols

Frequency – force relation. To determine the frequency-force relation the muscle was stimulated at L_0 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 (de Haan 1998). During the contractions the muscles were allowed to shorten at a constant velocity (10, 20, 30, 50, 75, 100, 125, 150, 200 and 250 $mm \cdot s^{-1}$). Just before the contraction started, the muscle was passively stretched to 0.5-1 mm above L_0. 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 L_0 during shortening (De Haan, de Ruiter et al. 1993). Rest between contractions was 3 minutes.

Fatigue. The fatigue protocol consisted of a serie of 20 isometric contractions (150 Hz; 150 ms; 1 contraction every 500 ms) at L_0.

Data analysis. For all isometric contractions, net peak twitch and tetanic force was calculated. The decrease during the fatigue protocol was expressed relative to the force of the first tetanic contraction and for each 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. After the determination of the contractile properties of the Gm, blood was collected from the vena cava. Serum concentrations for 25D were measured using a DiaSorin radioimmunoassaykit for 25D (DiaSorin, Stillwater, MN).

Total RNA isolation. Total RNA was extracted from the low oxidative, distal part and the high oxidative, proximal part of the Gm (De Ruiter, De Haan et al. 1995) using the RiboPure kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. RNA concentrations were determined in duplicate by spectroscopy (ND-1000 spectrophotometer; Nanodrop Technologies, Wilmington, DE). RNA purity was verified by 260/280 ratio (range 1.97-2.10, mean 2.03). The muscle total RNA concentration was calculated on the basis of total RNA yield (µg) per weight (mg) of the analyzed sample.

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 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. A quantitative PCR analysis method was applied to study the expression of 18S RNA and mRNAs of α-skeletal actin, muscle ring finger-1 (MuRF1), muscle atrophy F-box (MAFbx), c-myc, vitamin D receptor (VDR) and CYP27b1. The sequences for the primers (Invitrogen, The Netherlands) used for the specific RNA and mRNA targets are shown in Table 4.1. For each target, RT and PCR reactions were carried out under identical conditions by using the same reagent premix for all samples. Five microliter of each RT reaction was used for the PCR amplification. cDNA dilutions were set so that both the target mRNA and 18S rRNA 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) 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 rRNA and mRNA data were normalized to total RNA yield per sample as well as to the weight of tissue needed for extracting the amount of RNA used for cDNA synthesis. For each gene, differences in mRNA copy numbers of mRNA expression were shown relative to the Gm 18S rRNA expression level.

Target mRNA	PCR primer sequence 5'→ 3'
18S RNA	Forward: CGAACGTCTGCCCTATCAACTT
	Reverse: ACCCGTGGTCACCATGGTA
α-skeletal actin	Forward: CGACATCGACATCAGGAAGGA
	Reverse: GGTAGTGCCCCCTGACATGA
MAFbx	Forward: TGAAGACCGGCTACTGTGGAA
	Reverse: CGGATCTGCCGCTCTGA
MuRF1	Forward: TGCCCCCTTACAAAGCATCTT
	Reverse: CAGCATGGAGATGCAATTGC
VDR	Forward: CACCCTTGGGCTCTACTCAC
	Reverse: CTGTTGCCTCCATCCCTGAA
CYP27B1	Forward: CGGGAAAAGGTGTCTGTCCA
	Reverse: GTGTCCACTCCAGTAG
c-myc	Forward: CACAACGTCTTGGAACGTCAGA
	Reverse: GCGCAGGGCAAAAAAGC

Table 4.1: Sequence of the specific primers used in the quantitative PCR analyses. IGF-1: insulin growtht actor -1, MAFbx: muscle atrophy F-box, MuRF1: muscle ring finger-1, VDR: vitamin D receptor.

Statistics. SPSS® v16.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. An one-way ANOVA was performed on the data of the treatment group and the corresponding control group to assess whether there were any significant differences. To assess difference in body mass, food and water intake during maintainance of the diets a two-way ANOVA with repeated measures on one factor was performed. Differences were considered significant at $P \leq 0.05$. Data are presented as mean ± SEM.

Results

Blood serum levels. As expected, rats raised to be vitamin D deficient had lower 25D blood levels compared to the control group ($P < 0.001$). 25D blood serum levels of the deficient animals were all below the detection boundary of 12 nmol l^{-1}. The control group had 25D blood serum levels of 43 nmol l^{-1} (data not shown).

Body and muscle mass. Body mass at the start of the experiment was not different between the groups. During the feeding with vitamin D-free food, body mass for the rats raised to be vitamin D deficient was higher during the 10 weeks compared to the control group ($P = 0.018$) from week 7 onwards ($p < 0.05$) (Fig. 4.1A). Food and water intake were monitored weekly and did not differ between the vitamin D deficient group and the control group (Fig 4.1). Gm mass of the vitamin D deficient group was not different from the control group. This was not only the case for the Gm, but also for the other muscles and for the bones (Table 4.2).

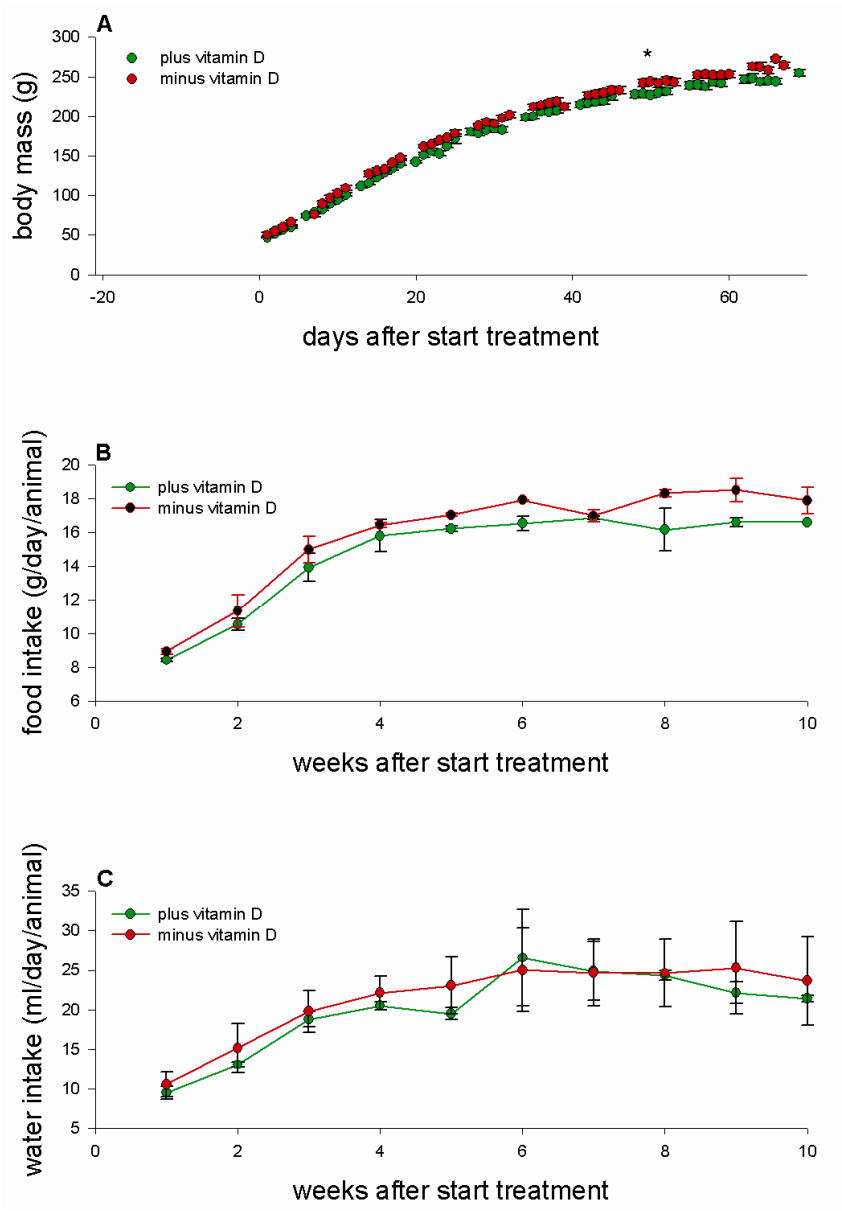

Figure 4.1: Effects of vitamin D deficiency on body mass, food and water uptake for. A) Body mass was monitored 5 times a week from day 21 of birth, at the start of the treatment with vitamin D deficient food or control food, till the day of the experiment. The vitamin D deficient group had a higher body mass during treatment compared to that of the control group *: P = 0.018. B). Food uptake during the 10 weeks of deficient food or control food for the deficient and control group. No differences in food uptake were observed between the deficient and control group. C) Water uptake during the 10 weeks of deficient food or control food for the deficient and control group. No differences in water uptake were observed between the deficient and control group. (repeated ANOVA)

	Control (n = 8)	Deficient (n = 8)

Gm mass left (mg)	663.1 (79)	691.8 (50)
Gm mass right (mg)	770.4 (113)	764.9 (72)
Soleus mass left (mg)	111.6 (11)	135.5 (63)
Soleus mass right (mg)	111.5 (14)	152.0 (64)
Plantarus mass left (mg)	282.3 (19)	257.7 (64)
Plantarus mass right (mg)	292.8 (34)	274.3 (73)
EDL mass left (mg)	130.1 (6)	131.1 (10)
EDL mass right (mg)	133.6 (13)	138.8 (8)
TA mass right (mg)	558.6 (66)	596.6 (51)
Heart (mg)	934.1 (77)	1011.4 (129)
Femur mass right (mg)	926.8 (180)	999.1 (102)
Tibia mass right (mg)	607.0 (144)	579.0 (36)
Gm Maximal isometric force (N)	16.6 (1.5)	15.8 (1.8)

Table 4.2: Effects of vitamin D deficiency on mean (±SEM) of Gastrocnemius medialis (Gm) muscle mass of the left and right leg, Soleus muscle mass of the left and right leg, Plantarus muscle mass of the left and right leg, Extensor digitorum longus (EDL) muscle mass of the left and right leg, Tibialus anterior (TA) muscle mass of the right leg, heart muscle mass, Femur bone mass of the right leg, Tibia bone mass of the right leg and the maximal tetanic force (Fmax) of the right Gm. Note: no significant differences between the groups (ANOVA).

Muscle Functional characteristics

Maximal isometric force. Maximal isometric tetanic force was similar in the vitamin D deficient group and control group ($P > 0.05$; Table 4.2).

Force frequency characteristics. The force-frequency curve of the vitamin D deficient rats was shifted to the left ($P = 0.007$) resulting in higher relative forces at lower stimulation frequencies (Fig 4.2). Figure 4.3 shows typical examples of the observed higher forces during low stimulation frequencies (40 and 60 Hz) for the deficient compared to the control group.

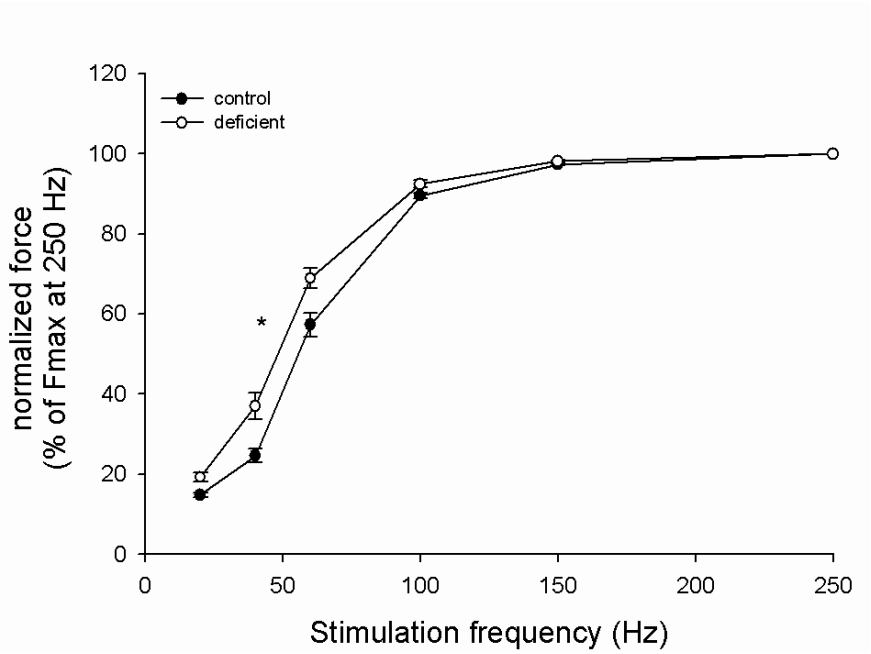

Figure 4.2: Influence of vitamin D deficiency on the force-frequency relation. Vitamin D deficiency resulted in a left-shift in the force frequency relation *: P = 0.007. (repeated ANOVA)

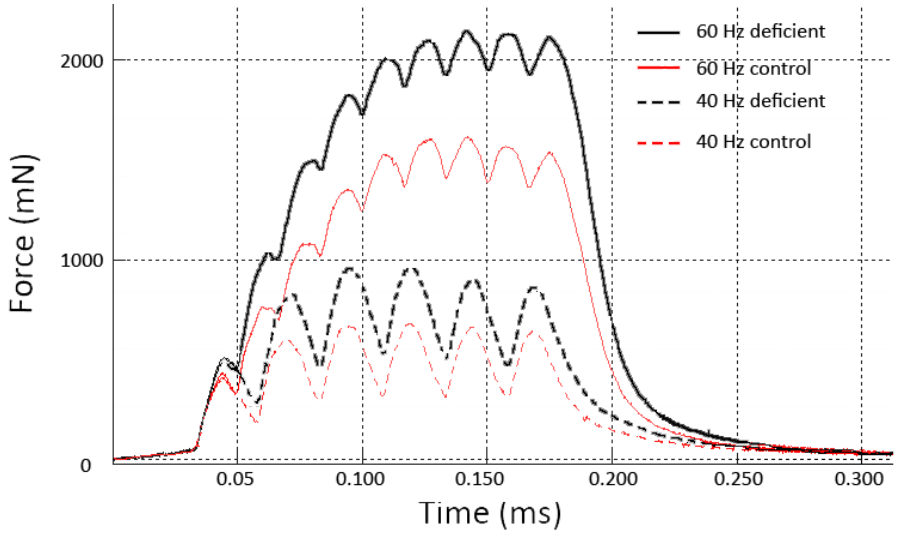

Figure 4.3: Examples of force traces of contractions at 40 and 60 Hz from a deficient and a control muscle. The maximal force for the deficient and control muscle were in the same magnitude (Fmax for the control muscle was 16,7 N and for the deficient muscle 16,6 N).

Force velocity characteristics. No differences were found in the force velocity relation between the vitamin D deficient group and the control group (Fig 4.4). As a consequence, also no differences were found between the groups in the power velocity curve and maximal power output (data not shown).

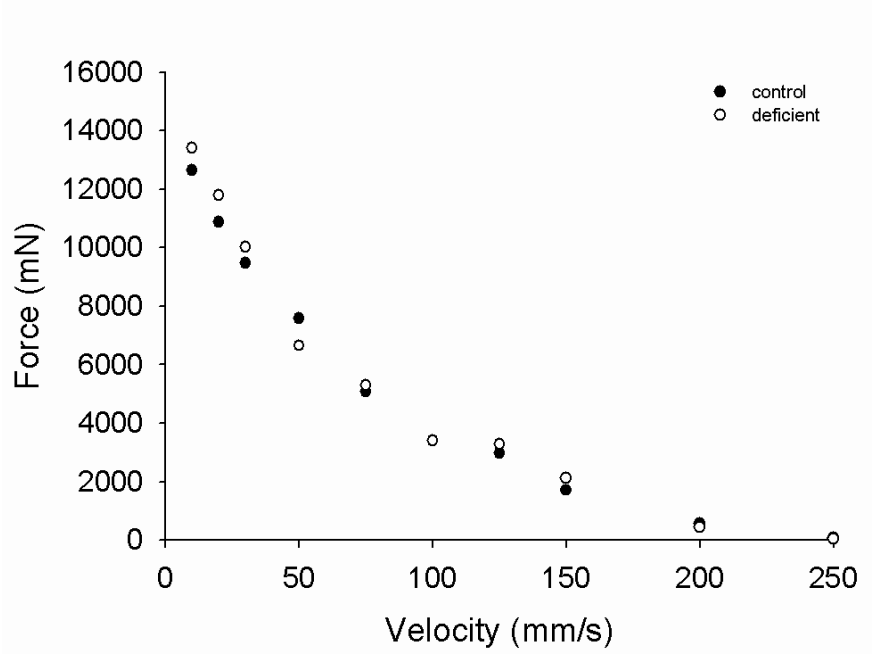

Figure 4.4: A typical example of a force-velocity relation from a control animal and a deficient animal.

Fatigue. During 20 repeated isometric contractions a similar linear decrease to ~75% of the initial force was seen in both groups. Force declined in the control group from 15.9 (0.7) N to 12.0 (0.6) N, and from 16.2 (0.6) N to 12.1 (0.4) N in the vitamin D deficient group ($P > 0.05$) (Fig 4.5). Concomitantly, the half relaxation (HRT) time increased during the isometric repetitions in both groups. There was no significant effect of vitamin D deficiency on initial HRT (31 (2.7) ms for the control group and 35 (5.1) ms for the deficient group, $P > 0.05$), nor on the HRT after 20 contractions (56 (4.0) ms for the control group and 63 (5.1) ms for the deficient group, $P > 0.05$).

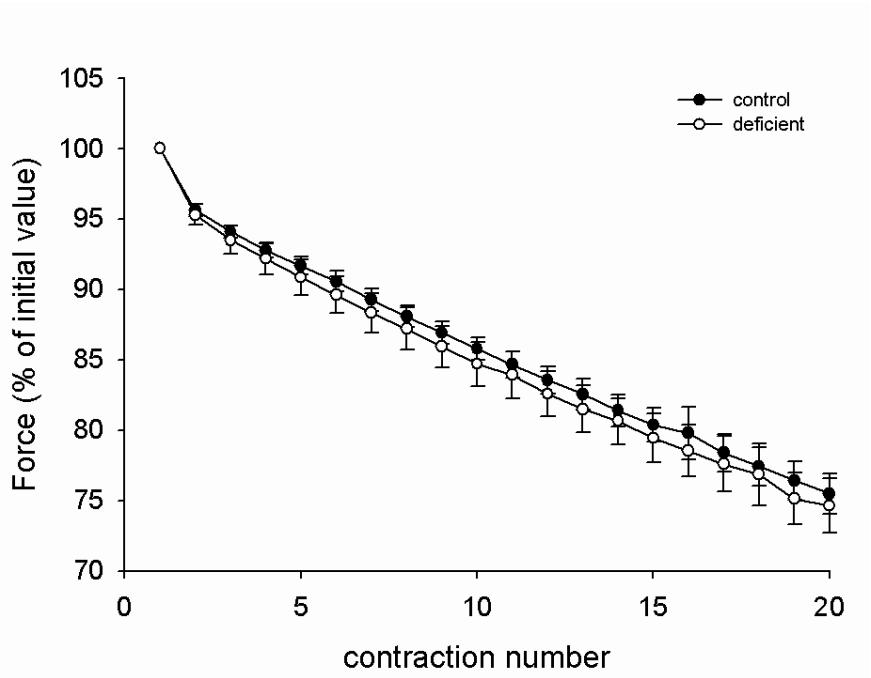

Figure 4.5: Force, as percentage of the initial value, during the fatigue protocol for the control and deficient group. After 20 contractions, force declined to ~75% of the initial value, and was not different between both groups (repeated ANOVA).

qPCR. To clarify how vitamin D deficiency affected the expression and degradation of contractile muscle proteins, different gene transcript levels were assessed in both the high oxidative proximal part and low oxidative distal, part of Gm. Total RNA per mg muscle tissue was not different between the control and vitamin D deficient group in either the proximal or the distal part (Fig 4.6A). This indicates that for comparison of the relative mRNA contents, normalization by the expression of the ribosomal RNA level is justified.

Figure 3B shows the mRNA transcript levels of α-skeletal actin and the E3 ligases MuRF1 and MAFbx of the proteasome system. α-skeletal actin expression in the distal part of the control group was 1.7 fold higher compared to the proximal part of the control group. The expression of the ubiquitin ligases MAFbx and MuRF1 and the vitamin D linked markers, c-myc, VDR and CYP27B1 were determined and did not differ between the proximal and distal part of both groups (Fig 4.6B).

Expression of the structural protein α-skeletal actin and the expression of the ubiquitin ligases MAFbx and MuRF1 was similar between the control group and deficient group. Also the expression of the vitamin D induced c-myc, a transcription factor for ribosomal RNA was not different between the groups as was the mRNA expression level of VDR and CYP27B1 mRNA (Fig 4.6B). These unaltered expression levels of vitamin D related genes indicate that the low serum concentrations of 25D are likely accompanied by low 1,25D levels in the muscle.

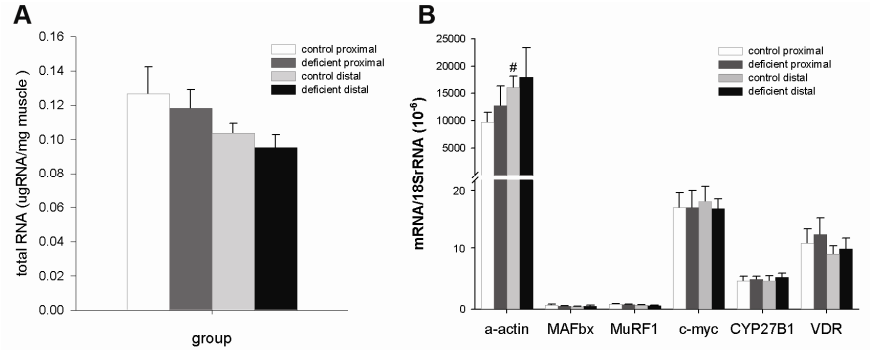

Figure 4.6: Quantitative PCR of structure protein, part of the ubiquitin proteasome system and vitamin D associated proteins of the low oxidative, distal part, and of the high oxidative, proximal part, of the Gm. A) Total RNA per mg muscle. No differences in total RNA concentration were observed between the groups which allowed for normalisation of the mRNA expression levels of the target genes to 18S rRNA. B) Expression levels in both control and vitamin D deficient rats are shown for the contractile protein α-skeletal actin,the E3 ligases MAFbx and MuRF1 of the ubiquitin proteasome system, c-myc, VDR and CYP27b1. No differences in were observed. For all targets, except α-skeletal actin, there was also no difference in expression level between the distal and proximal part. In the control group, α-skeletal actin expression in the distal part was higher compared to that of the distal part (#: P = 0.035) (ANOVA).

Discussion

The aims of this study were to determine the effects of vitamin D deficiency on the Gm contractile properties, the effects of vitamin D deficiency on transcripts of the ubiquitin pathway and on the transcripts of vitamin D related proteins. We observed that vitamin D deficiency resulted in a leftward shift of the force-frequency relation of the Gm. In contrast to our hypothesis, no decreases in maximal force and half-relaxation times were found. Furthermore, no atrophy was observed and also no change in expression levels of genes related to protein synthesis and degradation. This suggests that vitamin D deficiency in rat *per se*, thus without hypocalcaemia and hyperparathyroidism, does not result in muscle atrophy nor in a decrease in the potential to generate force.

Previous studies have shown that a vitamin D free diet after weaning for 6 weeks resulted in low 25D and 1,25D levels (Kollenkirchen, Fox et al. 1991). To be sure that our rats were indeed 25D and 1,25D deficient and to find changes in the skeletal muscle system, we prolonged this period after weaning with another 4 weeks to 10 weeks.

Low serum levels of 25D and 1,25D do not necessarily implicate low vitamin D activities within muscle tissue. Vitamin D activity is determined by its local concentration and the quantity of its receptor (VDR). It was expected that the transcripts of VDR and CYP27B1 were both upregulated in the deficient rats because of the decrease in substrate availability. Regarding the expression of the hydroxylase (CYP27B1), it has been shown that its activity was increased in the kidney of vitamin D deficient rats (Fox, Kollenkirchen et al. 1991). An increase in concentration of the enzyme may have been due to an increase in its mRNA expression. However, in our study we did not find an increase in VDR expression suggesting that the local muscle 1,25D concentration was the same as the control group.

Effects of vitamin D deficiency on Gm contractile characteristics

Vitamin D deficiency induced a leftward shift in the force-frequency relation in rats resulting in higher relative forces during low stimulation frequencies. Such a phenomenon could be explained by an increased relaxation time of Gm, but this was not found. 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 cross-bridge kinetics, and hence fibre type composition. Alternatively, the shift in the force-frequency relation may be caused by a reduction in the Ca^{2+} sensitivity of the regulatory proteins on the thin filaments, a situation which has for instance been observed in diaphragm fibres from patients with chronic obstructive pulmonary disease (Ottenheijm, Heunks et al. 2005). Finally, the release of Ca^{2+} by the SR might be enhanced at low stimulation frequencies and/or the intracellular Ca^{2+} concentration might be slightly elevated. Indeed, 1,25D has been shown to increase the intracellular Ca^{2+} concentration via both non-genomic (Vazquez, Selles et al. 1999) and genomic actions (Boland 1986). Furthermore, other studies suggest changes in intracellular Ca^{2+} transport during vitamin D deficiency; a prolonged half relaxation time was found after twitch and tetanic contraction in rats (Rodman and Baker 1978), the relaxation after tetanic stimulation was slowed in vitamin D deficient chicks (Pleasure, Wyszynski et al. 1979) and it was shown that calcium transport by SR was reduced in chickens in vitro (Pleasure, Wyszynski et al. 1979). Furthermore, the rate of calcium uptake by the SR was lower in vitamin D deficient rabbits (Curry, Basten et

al. 1974). These data all indicate that calcium handling is changed during vitamin D deficiency.

Effects of vitamin D deficiency on body mass and muscle mass

Vitamin D deficiency has been shown to reduce weight gain in growing rats (Rodman and Baker 1978). However; this reduced weight gain was not directly associated with vitamin D deficiency, but rather with the developed hypocalcaemia (Rodman and Baker 1978). In contrast to this, we observed in the present study that vitamin D deficiency did cause a slightly higher body mass as the deficient group was heavier than the control group. We used a special diet which prevents the development of hypocalcaemia, hypophosphatemia and hyperparathyroidism (Kollenkirchen, Fox et al. 1991). This diet has been shown in earlier studies to, besides preventing the occurrence of hypocalcaemia and hyperparathyroidism, lead to a decrease in body mass (Brion and Dupuis 1980; Toromanoff, Ammann et al. 1997). The observed higher body mass in the deficient group was neither the result of a higher food uptake nor of a difference in start weights of the 3 week old animals. Neither start weight nor food uptake during the 10 weeks were different. The type of food was likely also not the cause of the increased body mass because the food of both groups was of the same composition, except for the presence of vitamin D. Moreover, the food had the same caloric value. A decrease in metabolism might have induced an increased body mass, which was however not accompanied by an increase in muscle mass, since after 10 weeks of vitamin D deficient food the mass of the different types of muscles was not different between the groups. Another study showed a decrease in chicken skeletal muscle mass after 3 weeks vitamin D deficiency (Pleasure, Wyszynski et al. 1979). However, these chicks had developed hypocalcaemia (Pleasure, Wyszynski et al. 1979). In line with our observation, vitamin D deficient rats fed with a diet preventing hypocalcaemia, hypophosphatemia and hyperparathyroidism showed no lower muscle mass compared to a control group (Wassner, Li et al. 1983). These results suggest that hypocalcaemia, hypophosphataemia and hyperparathyroidism play an important role in the development of a lower muscle mass. Also a difference in the activity of the animals between the groups could be the cause of the difference in body mass. We did however, not test the activity of the animals.

No effects of vitamin D deficiency on the regulation of synthesis and degradation of muscle contractile proteins

It was reported that vitamin D deficiency is accompanied by a decrease in the CSA of type II fibres (Sato, Iwamoto et al. 2005). To obtain insight in the early effects of vitamin D deficiency in the processes of adaptation of muscle size, we investigated total RNA and the mRNA expression levels of α-skeletal actin, c-myc and the E3 ligases of the ubiquitin proteasome system, MuRF1 and MAFbx. Changes in the expression levels of these genes would provide indications of any atrophic effects of vitamin D deficiency, which may not have been apparent in terms of atrophy and a reduction in the maximum muscle force. Our data indicate that total RNA, c-myc, α-skeletal actin and MuRF1 and MAFbx expression were not changed during vitamin D deficiency.

C-myc is a transcription factor involved in the activation of rRNA expression (Gomez-Roman, Felton-Edkins et al. 2006) and hence the rate of translation. Furthermore, 1,25D has been shown to activate and induce synthesis of c-myc (Buitrago, Vazquez et al.

2001; Morelli, Buitrago et al. 2001). Therefore we hypothesized that c-myc and α-skeletal actin expression were decreased in the vitamin D deficient group. However, neither c-myc nor α-skeletal actin expression were changed by the vitamin D deficiency indicating that α-skeletal actin and c-myc mRNA were not inducing atrophy.

The ubiquitin pathway plays an important role in the induction of muscle atrophy. During atrophy, mRNA expression of MuRF1 and MAFbx has been shown to be increased. 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). As vitamin D deficiency is accompanied by atrophy of type II fibres (Sato, Iwamoto et al. 2005), we hypothesised that the ubiquitin ligases MuRF1 and MAFbx were upregulated in the deficient rats. However, no difference in MAFBx and MuRF1 expression was observed. The unaltered total RNA content, α-skeletal actin, c-myc, MuRF1 and MAFbx mRNA expression is in line with the unexpected observed maintenance of muscle mass and maximal force.

Vitamin D deficiency and its activity in skeletal muscle

Recently, it has been shown that 1,25D serum levels in young animals remained intact even when 25D serum levels were very low and hypocalcaemia and hyperparathyroidism were prevented (Anderson, Sawyer et al. 2007). Based on these data, it was suggested that the mechanisms underlying relatively high 1,25D levels in young animals, despite the low 25D are yet unknown and remain to be determined (Anderson, Sawyer et al. 2007). The results in our study also suggest that 1,25D serum levels of the vitamin D deficient rats may have been high enough to maintain muscle mass and functioning. However, in contrast to Anderson et al. (Anderson, Sawyer et al. 2007), Kollenkirchen et al. (Kollenkirchen, Fox et al. 1991) had shown that 1,25D levels were low in the deficient vitamin D group while maintaining Ca^{2+}, phosphorus, and PTH levels.

The question arises whether vitamin D *per se* is an important determinant of the muscle contractile characteristics and a regulator of adaptation of muscle size. In VDR gene deleted mice without metabolic abnormalities such as hypocalcaemia, had 20% smaller muscle fibres compared to control mice and myogenic transcription factors were increased (Endo, Inoue et al. 2003) suggesting a role for 1,25D on muscle tissue. Whether it was the vitamin D signalling *per se* or other factors that may also have been changed by the receptor deletion remains to be determined. Our experiment suggests that vitamin D likely affects the muscle contractile properties by other factors which are related to the circulating levels of vitamin D. The contrast in effects of isolated reduction in 25D blood serum concentrations and the inhibition of 1,25D function by this VDR gene-deletion also indicate that other unknown factors are likely involved. Extensive protein blood serum analysis between vitamin D deficiency and a control group and measuring local, intramuscular 1,25D concentrations are necessary to obtain more insight to elucidate processes ultimately resulting in vitamin D related muscle atrophy.

Taken together, in contrast to our hypotheses and observations in other vitamin D deficient studies we did not find a decrease in maximal force, muscle mass and also no change in expression levels of factors related to protein synthesis and degradation. Since we used a special diet which prevented hypocalcaemia and hyperparathyroidism we suggest that vitamin D deficiency associated muscle atrophy is not the effect of low serum

levels of vitamin D, but rather induced by its secondary effects. Possibly hypocalcaemia and/or hyperparathyroidism and changed phosphorus serum levels are responsible for the observed muscle atrophy and a decrease in muscle force seen during vitamin D deficiency. Support for this hypothesis is the report that primary hyperparathyroidism is associated with skeletal muscle weakness (Wells 1991; Uden, Chan et al. 1992), and the relation between hypophosphatemia and muscle weakness (Forrester and Moreland 1989). However, no relation was found regarding hypocalcaemia and muscle atrophy or weakness.

In conclusion, 10 weeks of vitamin D deficient food did cause circulating 25D levels to drop below detection limit. A shift to the left was found in the force frequency relation, indicating a change in Ca^{2+} handling. However, no decrease in muscle mass and maximal force was observed. Transcription markers of the ubiquitin pathway, MAFbx and MuRF-1 and the vitamin D specific markers VDR and CYP27b1 were not altered by the deficiency. It is therefore suggested that low vitamin D levels *per se* do not cause muscle atrophy and a decrease in maximal force, but the associated hypocalcaemia and hyperparathyroidism seen during vitamin D deficiency may induce muscle alterations during vitamin D deficiency.

Chapter 5

Effects of the active form of vitamin on contractile properties and size of *ex vivo* cultured mature isolated skeletal muscle fibres

Janwillem Testerink, Hans Degens, Nathalie Bravenboer, Arnold de Haan, Willem van der Laarse and Richard Jaspers

Abstract

Bioactive vitamin D or calcitriol (1,25D) is a steroid hormone that has long been known for its important role in regulating calcium homeostasis and bone mineralization. Since striated muscle cells also express the Vitamin D receptor, 1,25D has been presumed to affect muscle contractile properties. Although 1,25D has been shown to affect signalling in myoblasts and to regulate calcium homeostasis in cardiac myocytes, very little is known about the effects of 1,25d *per se* on mature skeletal muscle fibers. The aim of this study was to investigate the effects of the active form of vitamin D (1,25D) on cultured mature muscle fibres. Single muscle fibres were dissected from m. iliofibularis of *Xenopus laevis* and attached to a force transducer in a culture chamber and kept in serum-free medium at slack length (mean sarcomere length 2.3 μm) for 9 to 16 days. The medium was supplemented with 1 nM 1,25D, 10 nM 1,25D or 0.1% ethanol as a control. Twitch peak force and maximum tetanic force of fibres cultured in the presence of 1,25D were significantly reduced compared to control fibres. The tetanic tetanic 10% relaxation time was significantly decreased compared that of the control group. Muscle fibres cultured with 1,25D did not hypertrophy or increase the number of sarcomeres in series and α-actin mRNA content remained unchanged. We conclude that 1,25D *per se* has no hypertrophic effects on mature *Xenopus* skeletal muscle fibres. Furthermore we suggest that 1,25D reduces the release of calcium from the SR resulting in a reduction in both twitch peak force and maximal tetanic force as well as an increase in the rate of relaxation.

Keywords: skeletal muscle, vitamin D, relaxation, hypertrophy, adaptation

Introduction

Contractile properties of skeletal muscle are largely determined by the type of myosin heavy chains, muscle architecture and fascicle length, and in terms of force generating capacity by the number of muscle fibres and the physiological cross-sectional area of the muscle (see for review (Van Wessel T 2010), (Huijing 1998). Chronic catabolic conditions, such as conditions associated with chronic systemic inflammation and aging are accompanied by severe atrophy (Degens 2010), which limits mobility and increases the morbidity and mortality (Jensen 2008). Adaptation of muscle size is determined by the net effect of protein synthesis and degradation. These processes are regulated by many stimuli, such as mechanical loading, growth factors, cytokines and oxygen tension or pH (see for review (Van Wessel T 2010)). The mechanisms via which growth factors and cytokines affect muscle fibre size have been studied in detail, however their relative contribution is subject to controversy (Huijing and Jaspers 2005). One reason for this is that these factors interact (discussed in (Saini, Al-Shanti et al. 2008; Degens 2010)) and that unknown factors may be involved. Vitamin D, which has been associated with muscle weakness in humans (Mowe, Haug et al. 1999; Visser, Deeg et al. 2003), and may play a role in the regulation of muscle fibre size. Little is known about the mechanisms via which vitamin D affects muscle fibre size and strength. Part of its action may occur via the vitamin D receptor (VDR) expressed in mammalian and amphibian muscle (Zanello, Collins et al. 1997; Bischoff, Borchers et al. 2001); (Reschly, Bainy et al. 2007). It has indeed been shown that vitamin D stimulates the uptake of calcium in the sarcoplasmic reticulum (SR) of *in vivo* in rabbit muscle (Curry, Basten et al. 1974), while depletion of vitamin D prolongs the relaxation phase of rat soleus and chick triceps surae muscles (Rodman and Baker 1978; Pleasure, Wyszynski et al. 1979). Vitamin D may also affect adaptation of muscle fibre size as it has been shown to stimulate proliferation in cultured chick embryo myoblasts and differentiation of myoblasts *in vitro* (Giuliani and Boland 1984; Drittanti, de Boland et al. 1989). *In vivo*, vitamin D receptor (VDR) knock-out mice show a deregulated expression of myogenic regulatory factors (MRFs) and reduced muscle growth (Endo, Inoue et al. 2003). Vitamin D administration to deficient rats (Wassner, Li et al. 1983) and humans (Sato, Iwamoto et al. 2005) has been shown to increase muscle mass and decrease the rate of myofibrillar protein degradation. In humans, there also an increase in the proportion and size of type II fibres has been reported (Sato, Iwamoto et al. 2005). These changes may well contribute to the improvement of muscle contractile function after repletion with vitamin D in vitamin D deficient humans (Rodman and Baker 1978) and suggest that vitamin D may affect muscle function by: 1) increasing the calcium uptake and hence decreasing the rate of relaxation and 2) stimulating muscle fibre hypertrophy (i.e. an increase in the fibre cross sectional area (FCSA)). However, VitD also regulates the plasma concentrations of calcium, phosphorus and parathyroid hormone (see for review (Verhave and Siegert 2010)), indicating that in addition to the presumed direct effects of vitamin D on skeletal muscle, *in vivo* other factors are affected which obscure the effects of vitamin D *per se*. The aim of this study was to investigate the effects of 1,25D *per se* on contractile properties and size of mature muscle fibres. We hypothesized that 1,25D shortens relaxation of contraction and induces hypertrophy that will be accompanied by an increase in tetanic force. To test this, mature isolated *Xenopus laevis* muscle fibres were cultured for two weeks in serum-free medium with and without

1,25D and changes in relaxation times, force generating capacity, FCSA and serial sarcomere numbers were determined.

Material and methods

Animals and preparation of muscle fibres

Treatment of animals was in accordance with the guidelines and regulations concerning animal welfare and experimentation set forth by Dutch law and approved by the Committee on Ethics of Animal Experimentation at the VU University Amsterdam. *Xenopus* laevis (females, 9–10 cm body length) were killed by decapitation after cooling in ice water for 15 minutes and both iliofibularis muscles were excised. The muscles were allowed to recover for 1 h in sterilised (0.22-µm filter), oxygenated Ringer solution (NaCl, 116.5 mM; KCl, 2.0 mM; $CaCl_2$, 1.9 mM; NaH_2PO_4, 2.0 mM; ethylene glycol tetraacetic acid, 0.1 mM; pH 7.2). After recovery, both muscles were transferred to aseptic dissection troughs (Lännergren 1966). Highly oxidative single muscle fibres (type 2/3 according to the classification of Lännergren and Smith (1966) were carefully dissected under aseptic conditions using fine-tipped forceps and scissors under a microscope fitted with dark-field illumination (one or two fibres per animal). Small platinum hooks were tied to the trimmed tendons using 20-µm-diameter sterile polyamide thread. The platinum hooks and tools for dissection were sterilised using 70% ethanol. Unless stated otherwise, chemicals were obtained from Sigma Aldrich (The Netherlands).

Measurement of cross-sectional area and number of sarcomeres in series

Before culture, the number of sarcomeres in series and fibre cross-sectional area (FCSA) were determined as described previously (Jaspers, Feenstra et al. 2001). In the dissection chamber, fibres were stretched to just over slack length. Sarcomere length was determined every millimetre along the length of the fibre using laser diffraction and the mean sarcomere length was calculated. Subsequently, muscle fibre length was adjusted to a length corresponding to a mean sarcomere length of 2.3 µm ($l_{2.3\ \mu m}$, i.e. approximately fibre slack length). At this fibre length, the FCSA was determined by measuring the smallest and largest diameters of the fibre at three positions along the length of the muscle fibre.

The FCSA was calculated assuming an elliptical cross section and muscle fibre FCSA was taken as the mean of these three values. After these measurements, the fibres were mounted in the culture chamber and length was adjusted to $l_{2.3}$ µm.

Culture system and medium

All parts of the culture chamber (Lee-De Groot and Van der Laarse 1996) were sterilized using 70% ethanol. The fibre was mounted between a force transducer (AE801, SensoNor, Horten, Norway) and an adjustable rod.

The chamber contained 0.8 ml of culture medium. The stock solution of the culture medium consisted of 66% Dulbecco's modified Eagle's medium/F12 (GibcoBRL, Life Technologies, Breda, The Netherlands) with 100 $U \cdot mL^{-1}$ penicillin per 100 $\mu g \cdot mL^{-1}$ streptomycin (GibcoBRL), 5 mM sodium phosphate, 1 mM creatine, 0.5 mM L-carnitine and 0.2% bovine serum albumin.

To investigate the effects of 1,25D, the stock medium was supplemented with 0.1% ethanol (n= 5 muscle fibres, control), or 1,25D, dissolved in ethanol. The effects of 1,25D were tested at a high (4 fibres) and low (5 fibres) concentration. The 1,25D concentrations in the culture media were assessed using a $1,25(OH)_2D$ ELISA kit (Immunodiagnostic Systems Ltd., Boldon, England). The final low 1,25D concentration in

the culture chamber was 1.12 nM, SEM 0.25, n = 4, and the high concentration was 11.4 nM , SEM 0.64, n = 4.

The culture chamber was transferred to an incubator kept at 20°C and tubing for gas and culture medium supply were connected. Fresh culture medium was continuously pumped through the chamber at a rate of about 0.5 $mL \cdot h^{-1}$ and was equilibrated with air, containing 2.4% CO_2. Final pH was 7.6 and osmolarity was 235–250 $mOsm \cdot kg^{-1}$. The oxygen tension of the culture medium of the chamber was 120 to 130 mmHg.

Twitch and tetanic forces were measured once every 24 h. The fibre was stimulated (0.4-ms biphasic current pulses, 1.25 times threshold voltage) via platinum plate electrodes flanking the full length of the fibre to produce two twitches, one tetanus (50 Hz, 260 ms duration). Twitch peak force, twitch contraction time and twitch half-relaxation time were determined for the second twitch. For the tetanus, maximal force, the time from the first stimulus pulse to the time at which 50% of the maximal force was attained, (tetanus half rise time) and time from the highest force after the last stimulus pulse to 10% relaxation (tetanus 10%-relaxation time) were determined. The latter has been shown to reflect changes in relaxation speed, predominantly determined by the rate of calcium uptake by sarcoplasmic reticulum (SR) ATPase (Westerblad and Allen 1993). After culture, muscle fibres were transferred back to the dissection chamber filled with Ringer solution and the fibre FCSA as well as the number of sarcomeres in series was determined again. Twitch and tetanic specific forces were calculated at day 2 and at the end of culture (ranging from 9 to 16 days) by dividing fibre force by the FCSA determined before and after culture, respectively.

In situ hybridization for actin mRNA

After culture, fibres were embedded at $l_{2.3\,\mu m}$ in 15% (w/v) gelatine in Ringer solution, pH 7.2, and frozen in liquid nitrogen. Cross-sections, 10 µm thick, were cut using a cryostat at -20°C and mounted on glass slides coated with Vectabond (Vector Laboratories, Burlingame, CA). After drying, the sections (10 µm thick) were stored at -80°C.. The probe for detection of all actin isoforms (cardiac, skeletal and cytoskeletal actin) was amplified from *Xenopus laevis* embryos cDNA using forward primer
5'- CAGCACCAGCTCTCCTGTGCTA-3' and the reverse primer
5'- TCTCAAAGTCCAAAGCCACATA-3' (Eurogentec, Belgium) directed against the N-terminal coding region (721 bp, 11-732 nt, gene ID X03469) (2). The PCR product was cloned into vector pGEMT (Promega, France). Digoxigenin(DIG)-labeled antisense
and sense RNA probes were produced by in vitro transcription using RNA labeling mix (Roche Diagnostics Research, The Netherlands) according to the manufacturer's instructions. After the labelling reaction, the probe was dissolved in diethylpyrocarbonate (DEPC) treated water and its concentration was measured (ND-1000 spectophotometer; Nanodrop Technologies, Wilmington, DE).
The frozen sections were air-dried and fixed in 4% paraformaldehyde solution (in phosphate buffered saline (PBS)) for 20 minutes at 20°C and washed in PBS. The sections were then treated with proteinase K (10µg/ml) for 20 minutes at 20°C, washed 2 times for 3 minutes in PBS and fixed in a 4% formaldehyde solution for 5 minutes, and incubated in tri-ethanolamine solution (1.33% TEA, pH 8.0) for 5 minutes and triethanolamine with acetic anhydride (TEA solution + 100 µl acetic anhydride) for 5 minutes. After TEA incubation, slides were washed twice for 3 minutes incubated for 30 minutes in

prehybridization mix: 50% formamide, 3 x SCC (20x SCC; 3 M NaCl, 0.3 M tri-sodium citrate, pH 4.5), 1% blocking reagent (Roche Diagnostics Research, The Netherlands), 10 mM EDTA, 1 mg/ml torula mRNA, 2.5 mg/ml 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.1 mg/ml heparin, 0.2% Tween-20. Hybridization was performed in the prehybridization solution with 10mM dithiothreitol (DTT) and the DIG-labelled anti-sense and sense RNA probe at a concentration of 10 ng/µl (total volume 12.5 µl). Sections were hybridized overnight at 50°C in a humidified chamber while covered by Hybrislips. After hybridization, sections were washed twice with 2x SSC + 0.02% (w/v) sodium dodecyl sulphate (SDS) for 10 minutes at 50°C, 8 minutes 0.2x SSC + 0.02% (w/v) SDS at 50°C and 8 minutes 0.2x SSC + 0.02% (w/v) SDS + 10 mM DTT at 50°C, slides were washed in MAB buffer (10 mM Maleineacid, 150 mM NaCl, pH 7.5) at room temperature (RT) and incubated with sheep anti-DIG Fab fragments conjugated with alkaline phosphatase (1:4000, Roche) in 10% (w/v) heat inactivated sheep serum, 1 % (w/v) blocking reagent, and 0.1% (w/v) Tween-20 in MAB buffer over night at 4°C.

The sections were washed 5 times 15 minutes at RT in MAB buffer and incubated for 3 minutes in AP buffer (0.1 M NaCl, 0.1 M TRIS HCl, 50 mM $MgCl_2$, 0.1 % (w/v) Tween-20) supplemented with levamisol (Vector Laboratories, UK; final concentration 1 mM). Chromogenesis was performed in the dark using 1 mM levamisol in BM purple (Roche) for 50 hours at 20ºC. Finally, the sections were mounted in glycerine gelatine and stored at 4ºC. Sense probes were used to determine the level of non-specific probe binding.

Microdensitometry

The absorbance values of the final reaction products of the in situ hybridization in the sections was determined using a Leica DMRB microscope (Wetzlar, Germany) fitted with calibrated gray filters using interference filters. Absorbances were determined at 455 nm. Images were recorded with a x20 objective and a Sony XC-77CE camera (Towada, Japan) connected to a LG-3 frame grabber (Scion; Frederick, MD) in an Apple Power Macintosh computer. Recorded images were analysed with the public domain program NIH-Image V1.61 (US National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/). Grey values were converted to absorbance values per pixel using the grey filters and a third-degree polynomial fit in the calibrate option of NIH-image programme. Morphometry was calibrated using a slide micrometer and the set scale option in NIH image, taking the pixel-aspect ratio into account.

Statistical analyses

Data are expressed as the mean ± S.E.M. Two-way ANOVA for repeated measures on one factor was performed to test for effects of 1,25D and culture on the number of sarcomeres in series and FCSA (factor culture had two levels; i.e.pre and post culture) as well as for effects on twitch and tetanic force parameters during culture (factor culture had 8 levels; i.e. day 2 to 9). Correlation coefficients with repeated observations were calculated using the methods described in Bland and Altman (Bland and Altman 1995). A one-way ANOVA was used to compare the pre and post culture values between groups.

Results

Fibre characteristics and culture period

Table 5.1 shows the twitch and tetanic forces at day 2 of culture for the control, 1 nM 1,25D and 10 nM 1,25D groups. The fibres in each group had similar contractile properties corresponding to the type 2/3 characteristics described previously (Lännergren J 1966). The mean culture period was similar for the three groups and varied between 13 and 14 days.

	Control (n = 5)		**1 nM 1,25D (n = 5)**		**10 nM 1,25D (n = 4)**	
	Day 2	**End**	**Day 2**	**End**	**Day 2**	**End**
Twitch peak force (mN)	1.53 (0.25)	2.00* (0.33)	2.10 (0.48)	1.08# (0.36)	1.78 (0.39)	1.92 (0.50)
Twitch half relaxation time (ms)	29.40 (3.19)	45.40* (5.71)	36.20 (7.76)	43.00 (10.32)	26.00 (2.97)	36.75* (2.69)
Twitch contraction time (ms)	29.20 (2.90)	37.60* (4.27)	37.20 (3.43)	39.60 (2.54)	27.70 (1.93)	33.00 (3.34)
Twitch specific force ($kN \cdot m^{-2}$)	156.80 (13.08)	221.12* (11.99)	157.46 (12.74)	88.13# (31.75)	158.47 (5.57)	186.14 (16.39)
Tetanus maximal force (mN)	2.89 (0.51)	2.92 (0.47)	3.74 (0.71)	1.72*# (0.36)	3.34 (0.44)	2.74* (0.40)
Tetanus half rise time (ms)	21.20 (1.83)	18.60 (1.50)	34.80 (11.37)	26.40 (4.82)	22.25 (2.46)	19.50 (2.57)
Tetanus 10% relaxation time (ms)	24.60 (2.84)	35.80 (7.23)	34.60 (3.70)	24.60*# (4.23)	24.00 (1.73)	15.75*# (1.03)
Tetanic specific force ($kN \cdot m^{-2}$)	298.09 (26.47)	311.08* (17.58)	292.10 (17.28)	169.66*# (26.95)	317.79 (17.70)	270.15* (10.98)

Table 5.1: Twitch and tetanic characteristics at the beginning of culture. None of the parameters were different between the groups at day 2. Values are mean ±SEM *: significantly different from the initial value at day 2; #: significantly different from control group at the same time point.

Twitch peak force is reduced by 1,25D

Figure 5.1 shows an example of a force trace for a control and 10 nM 1,25D cultured fibre. This figure shows an example in the increase in twich force for as well the control as 1,25D group, and a decrease in tetanic force after culture. Figure 5.2 en 5.3A show the twitch peak force during culture. For all groups, twitch peak force increased significantly up to 8 days in culture. After this, it remained constant in the control group, but in both the 1 nM twitch peak force decreased to 56% the initial value, whereas in the 10 nM 1,25D group a slight increase of 10 was shown ($P < 0.039$).

Maximum tetanic force is reduced by 1,25D

Maximal tetanic force of the muscle fibres during culture is shown in figure 5.3A. For the control group, tetanic force increased during the first 9 days after which it decreased to

the initial value. During the culture period from day 1 to day 9, tetanic force of the 1 nM 1,25D and 10 nM 1,25D groups was not different from that of the control group. However, after 9 days, for the 1 nM 1,25D and 10 nM group tetanic forces decreased by ~5 % per day down to 68 % and 86% of the initial values at day 2, respectively ($P < 0.031$).

Effects of 1,25D on twitch and tetanic time to peak and relaxation

During the first 9 days of culture and at the end of culture, twitch time to peak and half relaxation time did not differ between the groups (Fig 5.2B and 5.2C). For the control group, the twitch-time-to-peak was significantly increased at the end of the culture period compared to day 2 ($P < 0.008$) (Fig 5.2B). In addition, it was shown for the control and 1,25D groups that the half relaxation time at the end of the culture was increased compared to the initial value ($P < 0.019$), but did not differ between the groups (Fig 5.2C).

Figure 5.3B and 5.3C show the tetanus half rise time and tetanus 10% relaxation time as a function of the culture period. During the first 9 days of culture, both parameters were similar in all groups. The half rise time was similar in all groups. Only for the 1,25D groups, the 10% relaxation time at the end of culture was decreased with respect to the initial value ($P < 0.026$, Fig. 3C). Both 1,25D groups had significantly lower half relaxation times ($P < 0.02$) (data not shown) and 10% relaxation times ($P < 0.02$) compared to the control group.

In each group, twitch force and half relaxation time correlated significantly ($r=0.88$, $p < 0.001$; $r= 0.81$, $P < 0.045$; $r= 0.78$ $P < 0.0001$, for control, 1 nM 1.25D and 10 nM 1.25D, respectively), while tetanic force and the 10% relaxation only correlated significantly in the 1,25D groups ($r=0.92$, $P < 0.0001$; $r= 0.89$, $P < 0.0001$ for 1 nM 1.25D and 10 nM 1.25D, respectively).

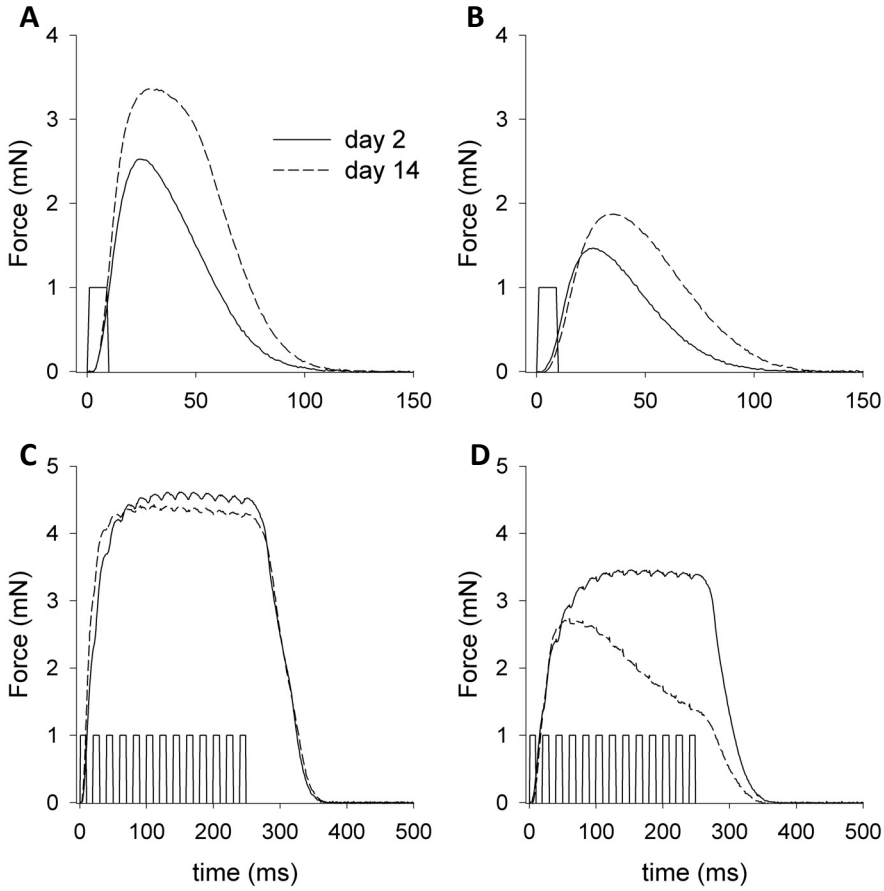

Figure 5.1: Examples of twitch and tetanic forces of muscles fibre during culture in medium with or without 1,25 D. A and B: twitch forces of a fibre cultured in control medium and medium supplemented with 10nM 1,25 VitD, respectively. C and D: Tetanic forces of a fibre cultured in control medium and medium supplemented with 10nM 1,25 VitD, respectively. The differences in the initial maximum tetanic force and twitch peak force at day 2 were mainly explained by the different the cross-sectional area of the muscle fibres.

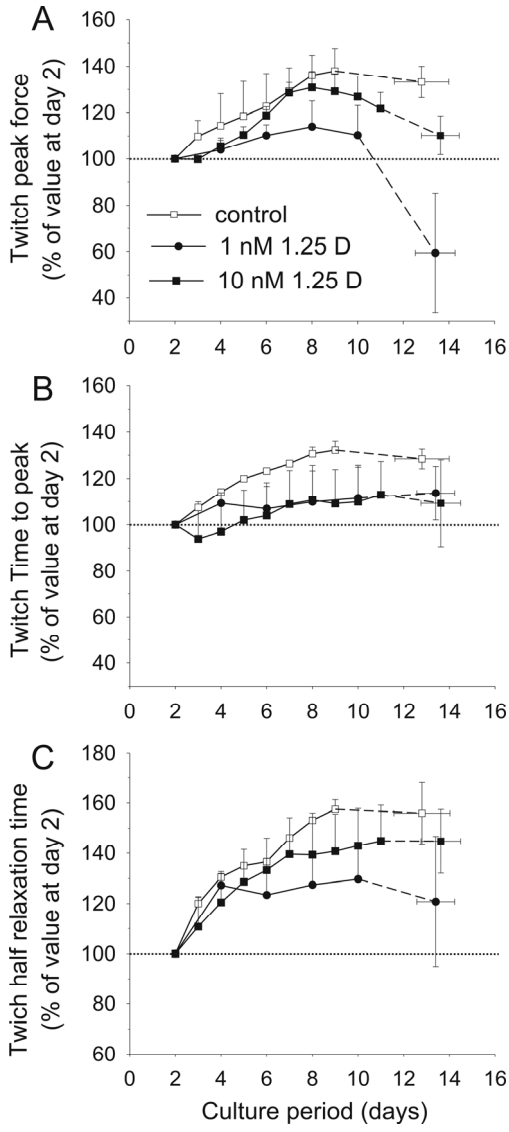

Figure 5.2: Twitch contraction characteristics during culture in serum-free medium of *ex vivo* single, mature *Xenopus laevis* musle fibres supplemented with 1 nM 1,25D, 10 nM 1,25D or 0.1% ethanol as the control. Mean culture period varied from 13 to 14 days. Twitch peak force (A), twitch time to peak (B) and twitch half relaxation time (C) were normalized by the value at day 2 and expressed a function of culture time. For all groups, during the first 9 days of culture, changes in twitch peak force increased. However, at the end of culture, twitch peak force of the 1 nM 1,25D group was significantly lower compared to the control group. Twitch time to peak was not different during the first 9 days between groups and also the values at the end of the culture were not different from the initial values. Twitch half relaxation time did not differ during the first 9 days of culture, while for the 1 nM and 10nM 1,25D groups it was reduced compared to the initial value. The dashed lines represent an interpolation between the mean values at the highest culture time at which all fibres per condition are included and the mean values at the ends of culture. * significantly different from control ($P < 0.05$). Values are mean ±SEM.

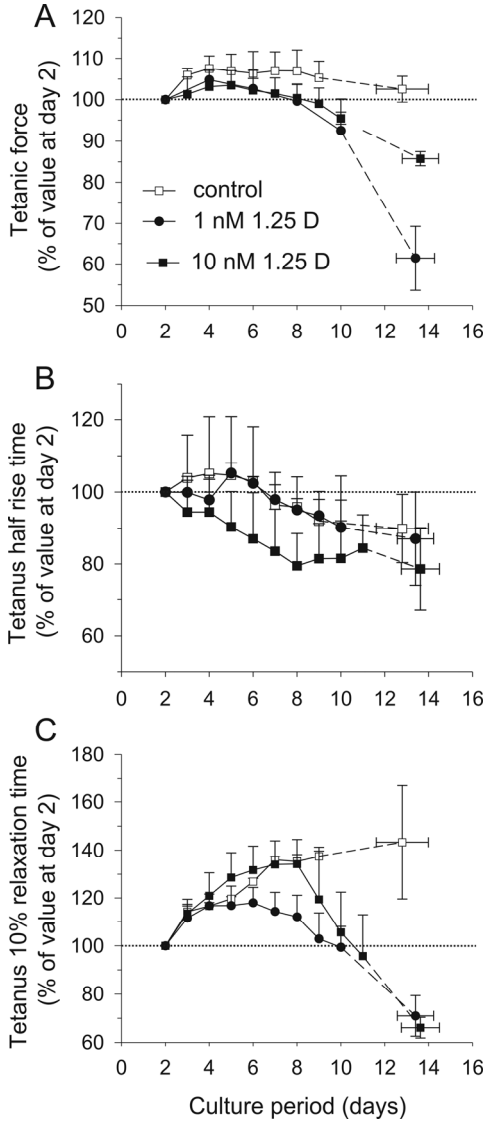

Figure 5.3: Tetanic contraction characteristics during culture in serum –free medium of *ex vivo* single mature *Xenopus laevis* musle fibres supplemented with 1 nM 1,25D, 10 nM 1,25D or 0.1% ethanol as the control. Mean fibres culture period varied from 13 to 14 days. A) Maximal tetanic force. Maximal tetanic force did not differ during the first 9 days of culture between all groups. However, at the end of culture, the maximal tetanic force of the 1 nM 1,25D group was lower compared to the control group. B) Tetanic half rise time. Tetanic half rise time did not differ during the first 9 days of culture between all groups. At the endpoint of culture, the 1 nM 1,25D and 10 nM 1,25D had increased half rise times compared to the control group. C) Tetanic 10%-relaxation time. Tetanic 10% relaxation time did not differ during the first 9 days of culture. At the end of the culture period, the 10%-ralaxation time was decreased in the 1 nM1,25D and 10 nM 1,25D groups compared to the control group. Changes in force are expressed as percentage of the initial value at day 2. The dotted line represents an interpolation between the mean values at the highest culture time at which all fibres per condition are included and the mean values at the ends of culture. * significantly different from the control group $P < 0.05$ Values are mean ±SEM

No effects of 1,25D on fibre CSA and the number of sarcomeres in series

Figure 5.4 shows the change in FCSA during culture. ANOVA revealed a significant reduction in the FCSA during culture ($P < 0.006$), which was similar for all conditions. The number of sarcomeres in seires did not change (Fig 5.4B). These results indicate that the reduction in tetanic force cannot be explained by atrophy.

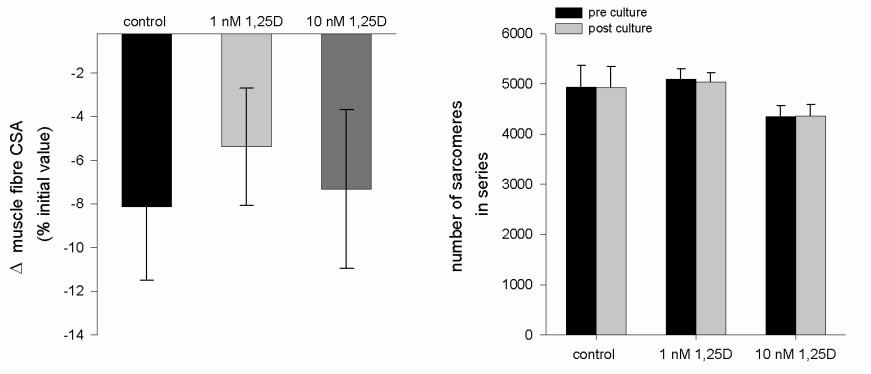

Figure 5.4: Culture in serum-free medium supplemented with 1,25D does not increase the number of sarcomeres in series and the cross sectional area. A) Difference of muscle fibre CSA pre- and post-culture.B) Pre- and post-culture mean number of sarcomeres in series Values are mean ±SEM.

Effects of 1,25D on α-actin mRNA expression

Figure 5.5 shows cross-sections of freshly frozen m. iliofibularis stained for α-actin mRNA. α-actin mRNA appeared to be present in both myonuclei and cytoplasm. Note that for *Xenopus,* myonuclei are located in the cytoplasm and not below the sarcolemma as in mammalian muscle. Figures 5.5 C-E show typical examples of cross-section of the fibres cultured with 1 nM 1,25D, 10 nM 1,25D and control muscle fibres. The mean absorbance values of α -actin for the different conditions were similar (Fig 5.5E) but about four times smaller than the absorbance values measured in freshly frozen muscle fibres (absorbance 0.33 ± SEM 0.01). These results indicate that 1,25D *per se* does not stimulate α-actin expression in *ex vivo* cultured muscles fibres and fits the observation that during culture 1,25D did not induce adaptation of muscle fibre size.

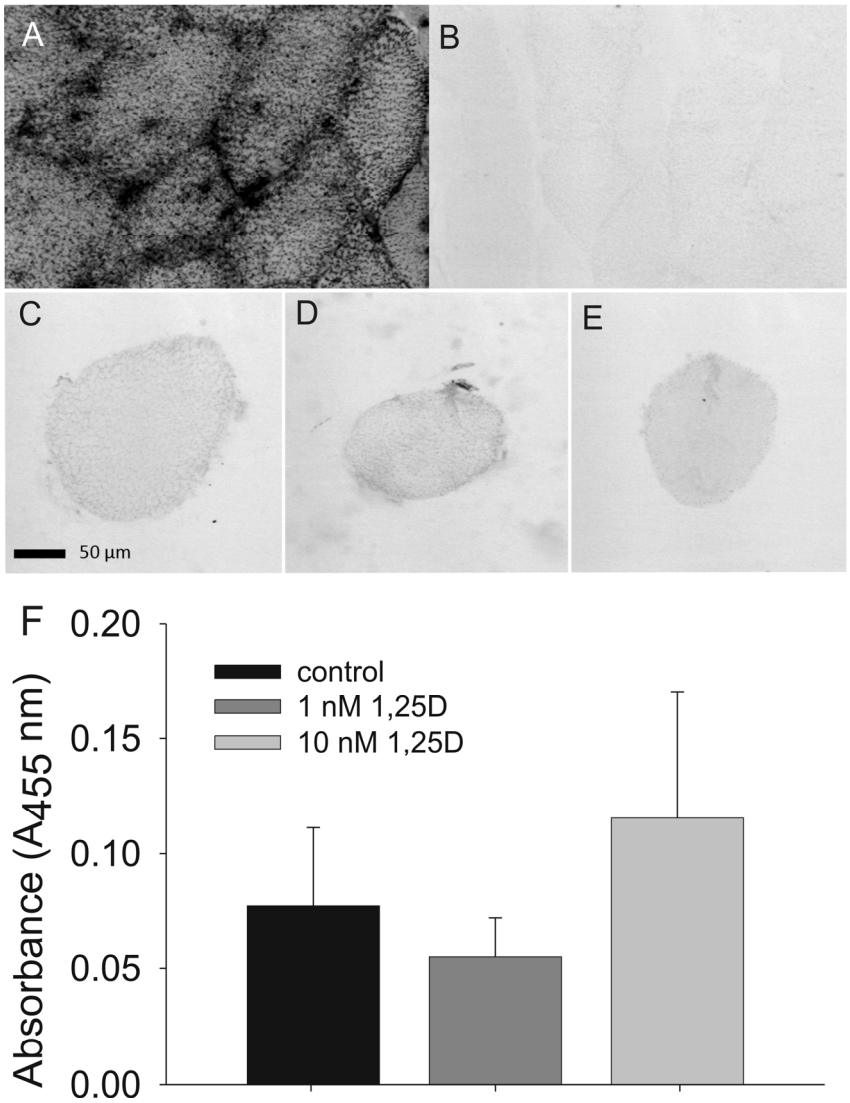

Figure 5.5:1,25D *per se* does not increase the α-actin mRNA expression in *ex vivo* cultured muscle fibres. A) ISH of α-actin on fresly frozen iliofibularis muscle, B) blanco of fresly frozen iliofibularis C, D, E) typical examples of α-actin mRNA stainings on sections of *Xenopus* muscle fibres after culture in serum –free medium containing: C) 1 nM 1,25D D) 10 nM 1,25D E) 0.1% ethanol D) blanco on cross section of the iliofibularis muscle. E) After culture of *ex vivo* single mature *Xenopus Laevis* musle fibres supplemented with 1 nM 1,25D, 10 nM 1,25D or 0.1% ethanol as the control the mRNA content as measured by the absorbance of α-actin mRNA (absorbance at 455 nm) was not different between the groups. Values are mean ±SEM.

Discussion

The aim of this study was to determine the effects of the active form of vitamin D (1,25D) on the size and contractile properties of cultured mature skeletal muscle fibres. We hypothesized that 1,25D stimulates muscle fibre hypertrophy and hence increases the force generating capacity of the muscle and reduces the rate of relaxation of contraction. In contrast to our hypothesis, we have shown that 1,25D caused a reduction, rather than an increase, in twitch and tetanic force, while the fibre CSA remained similar to control. In line with our hypothesis, however, twitch half relaxation and 10% tetanus relaxation times were decreased.

Culture system and 1,25D medium concentrations.

To investigate the effects of 1,25D *per se* on skeletal muscle, without changes in e.g. parathyroid hormone and calcium levels that often accompany changes in circulating vitamin D (Testerink et al., 2010; (Verhave and Siegert 2010)), we exposed single fibres to different concentrations of 1,25D. The advantage of our set up is that fully differentiated mature single fibres can be cultured in a carefully controlled medium. Although others have studied the effects of adding 1,25D to differentiating C2C12 myoblast (Buitrago, Ronda et al. 2006; Ronda, Buitrago et al. 2007), there are no studies on the long term effects of 1,25D on contractile properties of fully differentiated mature skeletal muscle fibres in culture.

We added 1 and 10 nM 1,25D to the culture medium while maintaining calcium, phosphorus and parathyroid hormone levels in the culture medium. It has been found in human and mouse primary hepatocyte cultures that a vitamin D concentration of 100 nM elicits maximal activation of the VDR (Reschly, Bainy et al. 2007), which is far above the 150 pM 1,25D observed in mammalian serum (Anderson, Sawyer et al. 2007). In primary *Xenopus* hepatocytes, the concentration which elicits maximal activation of the VDR is between 0.1 nM and 1 mM nmol·L^{-1} (Reschly, Bainy et al. 2007). As VDR has also been shown to be expressed in *Xenopus* muscle (Li, Bergwitz et al. 1997) it is to be expected that at concentrations of 1 and 10 nM 1,25D would be sufficient to elicit skeletal muscle VDR activation.

Influence of 1,25D on twitch and tetanic contractile characteristics and muscle fibre size

In vitamin D deficient rats the half rise time and the time to peak were increased (Rodman and Baker 1978). Vitamin D restored these parameter values to normal in both vitamin D deficient rat and chicken muscle (Rodman and Baker 1978; Pleasure, Wyszynski et al. 1979), which suggests that vitamin D affects muscle relaxation. This might well be the result of the impact of vitamin D on calcium handling (Boland, Buitrago et al. 2005). In that study the maximal twitch force and maximal tetanic force were not measured and consequently it is not possible to determine whether the restoration of the parameters is solely due to an enhanced rate of calcium uptake, or also due to a reduced calcium release, and/or a change in cross-bridge kinetics. The increased 10%-relaxation time in our control group was similar to that reported for *Xenopus* fibres cultured in medium without albumin and 1,25 D (Jaspers, Feenstra et al. 2001) and could have been the result of the absence of vitamin D in the medium. In line with this suggestion, we observed that supplementation with either 1 nM or 10 nM resulted in a decrease in the twitch half-

relaxation time and tetanic 10%-relaxation time. However, it also reduced twitch peak force and maximal tetanic force.

The latter suggests an impaired excitation contraction coupling, e.g. when the release of calcium from the SR impaired (Konishi and Watanabe 1998). Indeed, an impaired calcium release can also explain the observed shorter relaxation times (Wahr, Johnson et al. 1998). Therefore, the reduced tetanic force together with an increased rate of relaxation may be the result of a combination of a reduced calcium release in response to excitation and/or an enhanced rate of calcium uptake. Given the high correlation between the reduction in twitch and tetanic forces and the half- and 10% -relaxation times, respectively, the contribution of an enhanced calcium uptake may be fairly small. Future experiments should test the effects of 1,25D on the calcium release.

The potential hypertrophic effect of vitamin D was investigated by measuring the FCSA pre and post the culture period. We observed no hypertrophy or atrophy in response to 1,25D, which is in line with the similar α-actin mRNA expression in all groups. The probe we used, was a full length anti-sense α-cardiac actin probe, which has a high homology with the α-skeletal and α-cytoskeleton actin genes. Hypertrophy requires an increased expression of both proteins. The final absorbances were not different between the control and 1,25D groups, suggesting that 1,25D did not enhance the rate transcription and/or translation.

1,25D has been shown to activate the mitogen-activated protein (MAP) kinases ERK1 and ERK 2 in cultured chick skeletal muscle (Morelli, Buitrago et al. 2000). Activation of these pathways by insulin and insulin-like growth factor 1 (IGF-1) of *in vivo* rat plantaris muscle has been shown to result in hypertrophy (c.f. (Haddad and Adams 2004)). The hypertrophic effect of insulin has been shown also for *ex vivo* cultured *Xenopus*. During two weeks culture of *Xenopus* muscle fibres in the presence of insulin, FCSA was increasing by about 2% per day, which was accompanied by at least a doubling of the α-actin mRNA content (Jaspers, van Beek-Harmsen et al. 2008). Here we showed that active 1,25D does not have the hypertrophic potential as insulin and IGF-1, which suggests that *ex vivo* 1,25D *per se* does not activate the insulin/IGF-1 signalling pathways (i.e. phosphatidylinositol 3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR) and MAPK pathways).

In summary we conclude that 1,25D levels in the culture medium resulted in a decrease of twitch and tetanic force, which was not due to muscle fibre atrophy, but probably the result of a diminished calcium release from the SR. Further research is necessary to elucidate whether this decrease is solely due to enhanced SR calcium uptake, an impaired calcium release, or both.

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Chapter 6

Summary and General Discussion

In this thesis we investigated the effects of vitamin D on the morphological and functional characteristics of skeletal muscle. Vitamin D has long been shown to have effects on muscle. It has been shown that vitamin D deficiency is related with muscle weakness and type II fibre atrophy (Mowe, Haug et al. 1999; Sato, Iwamoto et al. 2005). Ageing is associated with muscle wasting, which may at least partly be attributable to vitamin D deficiency that often occurs at old age. It has been reported that aged rats also suffer from muscle weakness (Degens and Alway 2003). Vitamin D supplementation to vitamin D deficient persons has been shown to improve muscle strength and increase the proportion and size of type II fibres (Sato, Iwamoto et al. 2005). These observations provide circumstantial evidence that vitamin D does affect muscle phenotype and function. The aim of this thesis was to obtain insight in the effects of vitamin D on muscle function and molecular mechanisms via which vitamin D affects muscle function, size and phenotype.

We studied the influence of vitamin D on the contractile properties by: (1) increasing the 1,25D serum levels by supplementation with the active vitamin D analog alfacalcidol in adult and aged rats, (2) by making rats vitamin D deficient and finally (3) by studying the influence of 1,25D on *ex vivo* cultured mature *Xenopus laevis* single muscle fibres.

The results of the experiments in this thesis show that in contrast to our hypothesis, *increased* 1,25D serum levels resulted in a *decrease* in muscle mass and maximal force generating capacity. It appeared that old rats had no decreased 1,25D levels and consequently the alfacalcidol supplementation resulted in supraphysiological 1,25D serum levels. While vitamin D deficiency is associated with type II atrophy (Sato, Iwamoto et al. 2005) we found here that also supraphysiological levels of circulating vitamin D induced type II (IIb and IIx) muscle fibre atrophy. The atrophy was accompanied by an increased expression of ubiquitin ligase MuRF1, a component of the ubiquitin-proteasome pathway that plays an important role in protein degradation, which indicates that the rate of protein breakdown was likely enhanced. The loss of force generating capacity was proportional to the loss of muscle mass, indicating that the quality of the remaining muscle tissue in terms of specific tension was maintained. The muscle weakness was at submaximal activation somewhat attenuated as the force-frequency relation was shifted to the left, meaning that at submaximal stimulation frequencies the force generated was relatively increased.

The increased levels of circulating vitamin D were associated with hypercalcaemia and a decrease in food intake making it difficult to assess to what extent the observed muscle wasting and loss of body mass were attributable to the increased vitamin D levels *per se*. Cell culture studies allow one to control composition of the growth medium carefully. Therefore, to test whether these effects were an effect of elevated vitamin D levels and not the effects of other factors influenced by vitamin D, mature isolated *Xenopus laevis* muscle fibres were cultured in medium with and without 1,25D supplementation. These experiments showed that vitamin D does not have a direct effect on the size of the muscle fibres. These experiments showed that 2 weeks of culturing with additional vitamin D did not affect muscle fiber CSA. While *in vivo* specific tension was maintained in rats with elevated circulating vitamin D levels, the specific tension of the single fibres cultured in 1 nM 1,25D was reduced. Furthermore the tetanic 10%-relaxation time and the tetanic half relaxation time appeared to be increased.

In chapter 4 the effects of vitamin D deficiency on rat muscle were studied. In this study the potential confounding effects of vitamin D deficiency on serum levels of calcium, phosphorus and parathyroid hormone were compensated by special food. Unexpectedly, vitamin D deficiency was not associated with muscle wasting and weakness. This suggests that the often observed atrophy and weakness associated with vitamin D deficiency (Mowe, Haug et al. 1999; Sato, Iwamoto et al. 2005), are not caused by low vitamin D serum concentrations *per se* but rather by changes in other factors which accompany a reduced vitamin D status.

Effects of vitamin D on muscle mass and maximal force generating capacity

In vitro, physiological 1,25D serum levels have been reported to activate only a small percentage of the VDR of hepatocyte cells (Reschly, Bainy et al. 2007). An increase in the 1,25D serum concentration is thus expected to increase the VDR activity, and hence transcription of genes under control of the VDR. One of these genes is c-myc and elevated levels of 1,25D have indeed been shown to induce the expression of c-myc (Buitrago, Vazquez et al. 2001). As c-myc in turn is an enhancer of the expression of rRNA (Buitrago, Boland et al. 2001; Morelli, Buitrago et al. 2001) an increased expression of c-myc may thus via this pathway increase the rate of mRNA translation and hence protein synthesis. It was further hypothesized that 1,25D triggers a cascade of events that may finally lead to the activation to 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). It has been shown that the MAP/ERK kinase appeared to be one of the important pathways to induce IGF-1 induced hypertrophy: inhibition of the MAP/ERK kinase prevented IGF-1 induced hypertrophy in rat muscles (Haddad and Adams 2004) which suggests that 1,25D MAP/ERK activation is one of the pathways inducing IGF-1 activation. IGF-1 is an important growth factor of skeletal muscle and enhanced expression results in protein synthesis and the expression of building blocks of skeletal muscle, such as α-skeletal actin. Therefore, we expected an increase in maximal force and muscle mass after elevated 1,25D levels. However, we found the opposite: Supraphysiological serum levels of 1,25D in aged rats caused a reduction of the maximal force and a decrease in muscle mass (chapter 2). Since the reduction in maximal force was of the same magnitude as the decrease in muscle mass, the specific force was unchanged. Furthermore it appeared that the muscle mass to body mass ratio was maintained, indicating that the decrease in muscle mass could be attributed to the observed decrease in food intake, (chapter 2), obscuring a possible direct influence of vitamin D on muscle mass. This idea is further strengthened by the observation (1) that no atrophy was found in rats in which vitamin D deficiency was induced (chapter 4) and (2) that supplementation of 1 or 10 nM 1,25D to the culture medium did not affect the fibre CSA during at least 2 weeks of culture (chapter 5).

From these results we conclude that vitamin D *per se* in the regulation of muscle size is unlikely. However, elevated 1,25D and decreased vitamin D serum levels may have indirect effects on muscle mass, such as those related to altered calcium, phosphorus, parathyroid hormone, and cytokine serum levels which will be discussed below.

Effects of vitamin D on muscle contractile properties and muscle fibre type composition

The force-frequency and force-velocity relation, contraction and relaxation times and fatigability of muscles from rats with elevated circulating 1,25D levels and vitamin D deficient animals were discussed in chapters 2 and 4, respectively. In chapter 3 we studied the fibre type composition after elevated 1,25D levels, and in chapter 5 we studied the influence of different levels of 1,25D in the culture medium on relaxation time of the fibres.

Vitamin D deficiency has been reported to be associated with fibre type II atrophy (Sato, Iwamoto et al. 2005) which is reversible as vitamin D supplementation to normalise vitamin D levels increased the proportion and size of type II fibres (Sato, Iwamoto et al. 2005). Furthermore, vitamin D has been reported to positively effect calcium handling of muscle cells (Curry, Basten et al. 1974; Rodman and Baker 1978; Pleasure, Wyszynski et al. 1979). In vitamin D depleted rabbits, for instance, it has been shown that the calcium uptake by the SR was lower, while after repletion the calcium uptake was increased (Curry, Basten et al. 1974). This altered calcium handling is reflected during vitamin D deficiency of rat and chicken by increased half relaxation times of twitch and tetanic contractions (Rodman and Baker 1978; Pleasure, Wyszynski et al. 1979). We, however, did not find a prolonged half relaxation time during vitamin D deficiency. It should be noted, however, that in the other studies circulating calcium, phosphorus and parathyroid serum levels were not maintained. The absence of a reduction in the half relaxation time when circulating 1,25D levels were elevated, further suggests that vitamin D *per se* does not affect the calcium handling. However, in chapter 5, a decrease in half relaxation time was found as well as a decrease in twitch force which suggests that the calcium release by the SR is diminished, which could be an indication for an altered fibre type composition. Further support for the absence in a change in half relaxation time comes from the unaltered force-velocity relation after periods of both vitamin D deficiency and elevated 1,25D serum levels. In contrast, in chapter 2 and 4 we showed that both elevated 1,25D serum levels and vitamin D deficiency resulted in a leftward shift in the force-frequency relation, which is associated with altered relaxation times. The observed leftward shift in the force-frequency relation could have several causes; 1) a decreased Ca^{2+} sensitivity of the regulatory proteins on the thin filaments 2) a decreased rate of sequestration of Ca^{2+} back into the SR 2) enhanced release of Ca^{2+} from the SR into the cytoplasm 3) increased intracellular Ca^{2+} concentration. Indeed, vitamin D has been shown to influence the intracellular Ca^{2+} concentration by modulating the calcium uptake by the SR (Curry, Basten et al. 1974). So it could be that in an elevation of 1,25D, as studied in chapter 2, was caused by an increased intracellular Ca^{2+} concentration and that during vitamin D deficiency, studied in chapter 3, the activity of the Ca^{2+} pump is decreased. This leftward shift could be an indication for a shift towards a slower fibre type composition. Also the force-velocity relation may give an indication of a change in fibre type composition of the muscle; a decrease in the velocity where power output is maximal may indicate a shift in fibre type composition towards slower fibres. It was found that after elevated 1,25D levels and vitamin D deficiency, the force-velocity relation was not changed (chapter 2 and chapter 4). However, in chapter 3, we showed that the CSA of fibres of the low oxidative part of the Gm was decreased which was the result of a decrease in the CSA of type IIx and type IIb fibres. Because there was a decrease in the CSA of the fast fibres, slower characteristics for the force-velocity relation are expected.

However, it remains unknown why we did not find a change in the force-velocity relation. It is possible that the magnitude of these changes was too small to cause significant changes in the force-frequency and force-velocity relations.

Muscle fatigue resistance was not significantly affected by elevated 1,25D levels or vitamin D deficiency. This suggests that the oxidative capacity and blood supply of the muscle and the fibre type composition are not significantly altered.

Effects of vitamin D on mRNA expression and blood serum levels

The ubiquitin pathway

In chapter 3 and 4 we studied two proteins of the ubiquitin-proteasome pathway; muscle specific MuRF1 and MAFbx. The ubiquitin proteasome pathway is invariably activated during muscle atrophy (Kandarian and Jackman 2006). During supraphysiological levels of circulating vitamin D muscle atrophy occurred. Therefore, it was expected that also in this conditions MuRF1 and MAFbx mRNA were upregulated. In line with this expectation, the expression level of MuRF1 was increased after elevated 1,25D serum levels; this increased expression was on top of an already increased expression of MuRF1 which was found in the aged control group. This increased MuRF1 expression after elevated 1,25D levels can be explained by the decrease in food intake and thereby the state of starvation the animals were suffering. However no differences were observed in the expression of MuRF1 and MAFbx during vitamin D deficiency, which was in line with the absence of muscle atrophy and with the unaltered maximal force. From here we can conclude that vitamin D has no direct effects on the expression on the ubiquitin pathway in skeletal muscle.

Growth factors

Elevated levels of 1,25D appeared to have no effects on IGF-1 and myostatin expression (chapter 3). We did not examine the effects of vitamin D deficiency on these growth factors, but because no atrophy or hypertrophy was observed, no change in expression levels is expected.

Leptin, Adiponectin and IL-6

Elevated 1,25D levels were accompanied by a reduced food intake (chapter 2). However, this reduced food intake was not the result of increased leptin serum levels, a hormone which reduces appetite and plays an important role in food intake and feelings of satiety (Kelesidis, Kelesidis et al.)(chapter 3). Adiponectin, which levels are elevated during weight loss, has been shown to enhance the glucose uptake and fatty acid oxidation in the muscle (Yamauchi, Kamon et al. 2001; Haluzik, Parizkova et al. 2004), serum levels were increased, suggesting that 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). The increased adiponectin levels are thus probably associated with the decreased food intake. Furthermore, IL-6 may also reduce food intake (Kuhlmann and Levin 2008) and increased IL-6 levels have been shown to contribute to muscle wasting (Degens 2007; Degens 2009). IL-6 levels appeared to be upregulated in old rats. However, increased 1,25D levels did not result in a further increase in IL-6 serum levels. 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.

The observed muscle atrophy appeared to be the result of a decreased food intake which was not the result of altered leptin and IL-6 levels, but are probably the result of increased adiponectin serum levels (chapter 3).

Vitamin D associated proteins

It was hypothesized that during elevated 1,25D serum levels the activity, and thus the expression of the hydroxylase CYP27B1 was reduced, and that during vitamin D deficiency the activity, and hence the expression was upregulated to cover the decreased availability of 25D. Elevated levels of 1,25D and vitamin D deficiency, however, appeared to have no effects on expression levels of the vitamin D related proteins VDR and CYP27B1.

C-myc synthesis appeared to be induced by 1,25D (Buitrago, Vazquez et al. 2001; Morelli, Buitrago et al. 2001), and c-myc is a transcription factor involved in the activation of rRNA expression (Gomez-Roman, Felton-Edkins et al. 2006) and therefore the rate of translation. If the rate of translation is increased the α-skeletal actin expression should be increased. Therefore it was hypothesised that elevated 1,25D levels, *in vivo* and *in vitro*, caused an increase, and that vitamin D deficiency resulted in a decrease, in c-myc and α-skeletal actin expression. Yet, the transcription of both genes was unaltered during both elevated 1,25D serum levels and vitamin D deficiency. These results suggest that vitamin D has no effects on the expression of vitamin D related proteins.

Role of hypocalcaemia, hypophosphaetaemi and hyperparathyroidism on muscle contractile properties and muscle mass

Part of the problems with other studies and our study where the effects of elevated levels of vitamin D are investigated is that this is often accompanied with hypercalcaemia, hyperphosphataemia and hypoparathyroidism. These conditions in themselves can have a significant impact on skeletal muscle (Forrester and Moreland 1989; Wells 1991; Uden, Chan et al. 1992). Likewise, vitamin D deficiency is accompanied with hypocalcaemia, hypophosphataemia and hyperparathyroidism (Kollenkirchen, Fox et al. 1991). Indeed, these conditions also occurred in our rats with elevated circulating 1,25D levels and may have obscured the effects of vitamin D *per se*. To minimise this bias in the vitamin D deficiency study of chapter 4, we used a special diet that induced vitamin D deficiency while at the same time preventing changes in the levels of calcium, phosphate and parathyroid hormone (Kollenkirchen, Fox et al. 1991). Also during the *in vitro* culture of skeletal muscle fibres (chapter 5) the calcium and phosphorus levels in the culture medium were kept constant allowing to study the effects of elevated 1,25D levels *per se*, without the occurrence of hypercalcaemia and hypophosphataemia. The results suggest that vitamin D *per se* had no effects on muscle contractile properties and mRNA transcription in either low vitamin D serum levels or elevated 1,25D levels in culture. These results are in line with the results of a study of Wassner et al. in which vitamin D deficiency was studied and where hypocalcaemia, hypophosphataemia and hyperparathyroidism were prevented (Wassner, Li et al. 1983). A very recent study suggested the same, it is not vitamin D, but it is the vitamin D related phosphorus level which decreases during low vitamin D levels (Schubert and DeLuca).

Taken together, the data suggests that it is not vitamin D itself which influences muscle structure and functioning but vitamin D-associated factors such as alterations in circulating calcium, phosphorus and/or parathyroid hormone. This would fit the observation that skeletal muscle contains little VDR (Burmester, Wiese et al. 1988;

Sandgren, Bronnegard et al. 1991) and that 1,25D is not incorporated in skeletal muscle nuclei, which is in contrast with other target tissues of vitamin D (Stumpf 1981).

Future directions to understand the role of vitamin D on skeletal muscle

From the various studies on vitamin D deficiency, the idea came that muscle structure and function are negatively affected by low levels of vitamin D. The results in this thesis, however, suggest that vitamin D itself appears to have no significant direct effects on muscle. The observed effects of vitamin D deficiency in other studies and supraphysiological levels of vitamin D as observed by others and described in chapter 2 most likely are the consequence of other factors, such as changes in circulating levels of calcium, phosphate and parathyroid hormone that often accompany changes in circulating levels of vitamin D. More research is needed to elucidate the role of hypocalcaemia, hypophospataemia and hyperparathyroidism on structural and contractile properties of and mRNA expression in skeletal muscle. With the culture system, used in chapter 5, it is possible to study the role of these factors separately and in combination.

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Vitamine D en skeletspier structuur en functie

Vitamine D, beter bekend als de zonlicht vitamine, heeft een belangrijke invloed op veel verschillende weefsels, ook op spierweefsel. Vitamine D tekort in de mens wordt geassocieerd met een lagere maximale spierkracht en atrofie van type II spiervezels. Verder is het aangetoond dat een vitamine D tekort veranderingen veroorzaakt in de contractiele eigenschappen van spieren. In ratten en kippen is het bijvoorbeeld aangetoond dat de relaxatiesnelheid afneemt tijdens een tekort aan vitamine D. Daarnaast is de vitamine D receptor (VDR) aangetoond in skeletspierweefsel, wat verder suggereert dat vitamine D een direct effect zou kunnen hebben op de genexpressie in skeletspierweefsel en de regulatieve adaptatie processen.

Het doel van dit proefschrift is inzicht te krijgen via welke mechanismen vitamine D de structuur en contractiele eigenschappen van de skeletspier beïnvloedt. Dit werd gedaan door 1) de actieve vorm van vitamine D (1,25D) te verhogen in volwassen en oude ratten, 2) door ratten vitamine deficiënt te maken en 3) de invloed te bestuderen van 1,25D op *ex vivo* gekweekte *Xenopus laevis* spiervezels.

In de studies beschreven in de hoofdstukken 2 en 3 is bij ratten onderzocht wat de effecten zijn van een verhoging van de vitamine D spiegels in het bloed. Gedurende een periode van 6 weken werd bij oude ratten het gehalte van 1,25D verhoogd door alfacalcidol supplementatie. Alfacalcidol is een farmacologische vitamine D analoog die relatief snel kan worden omgezet in de actieve vorm van vitamine D. Zoals verwacht leidde suplementatie van alfacalcidol tot een toename van 300-400% van de 1,25D concentratie in het serum. Naast een verhoging van de 1,25D serum spiegel bleek dat de calcium serum gehaltes ook sterk toenamen. Na een periode van 6 weken alfacalcidol supplementatie bleek dat er een afname was van de spiermassa en kracht wat waarschijnlijk veroorzaakt werd door atrofie van type II (IIb en IIx) spiervezels. Deze atrofie ging gepaard met een verhoogde expressie van de ubiquitin ligase MuRF1, een eiwit dat deel uitmaakt van de ubiquitin-proteosome pathway dat een belangrijke rol speelt in eiwit degradatie, wat een aanwijzing is dat de eiwit afbraak is verhoogd. De spierkracht afname was in dezelfde orde van grote als de afname in spiermassa, wat betekent dat de specifieke kracht van de spier niet was aangedaan. Verder bleek dat de frequentie-kracht relatie naar links was verschoven, hetgeen betekent dat na alfacalcidol supplementatie en de gepaard gaande verhoging van de 1,25D serum concentratie bij lagere stimulatie frequenties de spier (Gastrocnemius medialis) een hogere kracht leverde.

De toegenomen serum concentraties van 1,25D waren geassocieerd met hypercalcemie alsmede een afname in de voedselinname waardoor het niet mogelijk is om de gevonden adaptaties toe te schrijven aan verhoogde serum concentratie van 1,25D *per se*. Mogelijk is een deel van de spieradaptaties en krachteigenschappen bepaald door andere veranderingen, bijvoorbeeld hypercalcemie.

Om het effect van een verhoogde 1,25D gehalte *per se* te onderzoeken vereist een aanpak waarbij alleen 1,25D concentraties rond spiervezels verhoogd wordt en de overige factoren onveranderd blijven. In hoofdstuk 5 staan experimenten beschreven, waarbij in een kweeksysteem voor volgroeide spiervezels het effect van 1,25D *per se* op de maximale spiervezel kracht en de vezelgrootte is onderzocht. Geïsoleerde, volgroeide spiervezel van de *Xenopus laevis* werden gekweekt in een medium met en zonder 1,25D supplementatie. Deze experimenten lieten zien dat 1,25D *per se* niet leidt tot hypertropfie

en een toename in het aantal sarcomeren in serie. Hoewel *in vivo* bij ratten de specifieke kracht gelijk bleef gedurende verhoogde 1,25D serum concentraties, bleek de specifieke maximale tetanische kracht van de *Xenopus* spiervezels tijdens de kweek af te nemen. Verder bleek dat de 10% relaxatie tijd en de half relaxatietijd afnamen, hetgeen vermoedelijk het gevolg is van een verminderde excitatie-contractie koppeling als gevolg van de hoge 1,25D in het kweekmedium.

Hoofdstuk 4 beschrijft de effecten van een laag vitamine D gehalte bij ratten. Door ratten voor lange tijd (10 weken) een vitamine D deficiënt dieet te geven en in kunstlicht te huisvesten bleek het mogelijk om de vitamine D concentratie te verlagen tot minder dan de detectiegrens van 12 nM. In tegenstelling tot onze hypothese resulteerde vitamine D gebrek niet in een afname van de spiermassa en maximale spierkracht. Afgezien van de kracht-frequentie relatie, die naar links was verschoven, waren er geen verschillen in de contractiele eigenschappen van vitamine D deficiënte dieren. Ook bleken de mRNA expressie levels van α-skeletal actin, MAFbx, MURF1, c-myc , CYP27B1 en de VDR onveranderd. Deze onveranderde expressie niveaus laten zien dat *in vivo* vitamine D deficiëntie niet leidt tot vroege verandering in signaal transductieroutes die leiden tot hypertropfie en veranderde contractiele eigenschappen.

De resultaten in dit proefschrift suggereren dat vitamine D *per se* geen hypertroof effect heeft op skeletspieren en dat het de relaxatiesnelheid niet verhoogt. Omdat vitamine D de calcium, fosfor, en parathyroid hormoon serum concentraties reguleert, zijn waarschijnlijk een of meerdere van deze factoren belangrijker in de regulatie van spiermassa en spierfunctie dan vitamine D zelf. Vitamine D lijkt voornamelijk een indirect effect op de regulatie van spiermassa en functie te hebben. Om inzicht te krijgen in de onderliggende mechanismen via welke vitamine D betrokken is bij de regulatie van spiermassa en kracht verreist een nadere studie van de effecten van deze factoren en hun interacties.

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