Chapter 1

General Introduction
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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 phosphatidylinositol-3 kinase (PI3K)/Akt pathway, resulting in downstream activation of targets that induce protein synthesis and expression of \(\alpha\)-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 rapamycin; 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)$_2$D$_3$: 1,25-dihydroxyvitamin D$_3$; Pi: inorganic phosphate; 25-OHase: 25-hydroxyvitamin D$_3$ hydroxylase; 1-OHase: 1,25-dihydroxyvitamin D$_3$ 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)$_2$D$_3$

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 adenyl 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 may be 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 motoneurons 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).