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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\textsuperscript{2+} sensitivity of the regulatory proteins on the thin filaments 2) a decreased rate of sequestration of Ca\textsuperscript{2+} back into the SR 2) enhanced release of Ca\textsuperscript{2+} from the SR into the cytoplasm 3) increased intracellular Ca\textsuperscript{2+} concentration. Indeed, vitamin D has been shown to influence the intracellular Ca\textsuperscript{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\textsuperscript{2+} concentration and that during vitamin D deficiency, studied in chapter 3, the activity of the Ca\textsuperscript{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 alfalcacldol and vehicle group, muscle wasting during treatment with alfalcacldol 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, hypophosphataemia 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 hyperparathyroidism. 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, hypophosphataemia 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.