CHAPTER 1

General Introduction
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Vitamin D was identified in the early 20th century after a long search for a cure for rickets, a disease in growing children characterized by skeletal deformities and muscle weakness [1]. An important step forward was made in 1968, when 25-hydroxyvitamin D (25(OH)D) was isolated, the major circulating form of vitamin D [2]. A few years later, in 1971, the most active form of vitamin D was identified: 1,25-dihydroxyvitamin D (1,25(OH)₂D) [3;4]. The metabolite 1,25(OH)₂D was shown to have an essential role in the regulation of calcium homeostasis [3;4]. The 1α-hydroxylase enzyme in the kidney was demonstrated to be responsible for the synthesis of 1,25(OH)₂D and appeared to be under strict hormonal control [5].

Nowadays, it is known that, in addition to the kidney, 1α-hydroxylase is expressed in numerous extra-renal tissues including bone [6] and muscle [7]. The locally synthesized 1,25(OH)₂D in bone appears to be important for cell proliferation and differentiation in an autocrine and paracrine way [6;8]. These positive effects of locally synthesized 1,25(OH)₂D on bone cell function urge for the need to investigate which factors can stimulate the local synthesis of 1,25(OH)₂D, especially for the prevention and treatment of metabolic bone diseases such as osteoporosis. However, the regulation of 1α-hydroxylase in bone cells is poorly understood. In muscle, the synthesis of 1,25(OH)₂D has not completely been elucidated, but local vitamin D metabolism might be an important contributing mechanism for an adequate muscle function. The aim of this thesis was to gain more insight into the activity of locally synthesized 1,25(OH)₂D and the regulation of 1α-hydroxylation of 25(OH)D to 1,25(OH)₂D in bone and muscle. In this chapter, the background of local vitamin D metabolism in bone and muscle is described. First, vitamin D synthesis and metabolism, mechanism of action, biological actions and the consequences of vitamin D deficiency are introduced. Then, the local hydroxylation of 25(OH)D to 1,25(OH)₂D is described and the outline of the thesis is provided.

VITAMIN D SYNTHESIS AND METABOLISM

The daily vitamin D requirement for most humans is obtained from sunlight exposure (Fig. 1) [9]. During exposure to solar ultraviolet B radiation, 7-dehydrocholesterol in the skin is photolyzed to previtamin D₃ [10]. Previrtamin D₃ is thermodynamically unstable and isomerizes to vitamin D₃ [11;12]. After binding to the vitamin D-binding protein, vitamin D₃ is translocated into the circulation. Vitamin D can also be absorbed from dietary sources (either D₂ or D₃) after which vitamin D is transported into the circulation in chylomicrons [13]. Vitamin D from skin and dietary sources are taken up by the liver and metabolized to 25(OH)D through the action of 25-hydroxylase [14]. The metabolite 25(OH)D is transported to the kidney and hydroxylated at the 1α-position to the most active metabolite 1,25(OH)₂D [14]. The enzyme 1α-hydroxylase, encoded by the gene CYP27B1, is responsible for the hydroxylation to 1,25(OH)₂D [14]. The most pronounced effect of 1,25(OH)₂D is to maintain calcium homeostasis by increas-
ing the intestinal calcium absorption [15]. Because serum calcium needs to be kept within narrow limits, the activity of 1α-hydroxylase is regulated by parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and 1,25(OH)₂D itself [16-19]. PTH is released from the parathyroid glands in response to a low serum calcium concentration and induces the 1α-hydroxylase expression in the kidney by a cyclic AMP-dependent mechanism [20]. FGF23 is secreted by osteocytes in response to phosphate loading or a high serum 1,25(OH)₂D concentration and suppresses 1α-hydroxylase expression in the kidney [21-23]. The metabolite 1,25(OH)₂D reduces the expression of 1α-hydroxylase through its receptor [19;24]. An excess of serum 1,25(OH)₂D is prevented by the action of renal 24-hydroxylase which is also regulated by PTH [25], FGF23 [23] and 1,25(OH)₂D [26] but in opposing directions. This enzyme hydroxylates 1,25(OH)₂D and also 25(OH)D at the C24-position leading to respectively 1,24R,25-trihydroxyvitamin D (1,24R,25(OH)₃D) and 24R,25-dihydroxyvitamin D (24R,25(OH)₂D) [27;28].

Figure 1. Vitamin D synthesis and metabolism
MECHANISM OF ACTION OF 1,25(OH)\(_2\)D

The genomic activity of 1,25(OH)\(_2\)D is mediated by the vitamin D receptor (VDR) which is expressed in almost all cell types [29]. The VDR belongs to the nuclear receptor gene superfamily of ligand-activated transcription factors [30;31]. The VDR contains an A/B domain, a zinc-finger containing DNA binding domain (DBD), a flexible hinge region and a C-terminal ligand binding domain (LBD) [12;29]. After binding to 1,25(OH)\(_2\)D, the VDR is phosphorylated and associates with the retinoid X receptor (RXR) through dimerization domains in the LBD [32;33]. The 1,25(OH)\(_2\)D-VDR-RXR complex binds to vitamin D response elements (VDREs) in the DNA sequence of vitamin D-regulated genes and recruits complexes of either coactivators or corepressors leading to positive or negative regulation of gene transcription [29;32;33].

In addition to the genomic actions which take a few hours to days to be fully manifest, 1,25(OH)\(_2\)D is able to induce rapid non-genomic responses which take seconds to minutes [34]. The non-genomic activity of 1,25(OH)\(_2\)D may be mediated by the VDR associated with caveolae of the plasma membrane [35]. After binding of 1,25(OH)\(_2\)D to the caveolae-associated VDR, signal transduction pathways are activated leading to rapid responses such as opening of voltage-gated calcium and chloride channels in osteoblasts [35;36].

BIOLOGICAL ACTIONS OF 1,25(OH)\(_2\)D

The metabolite 1,25(OH)\(_2\)D plays a key role in the maintenance of calcium homeostasis [37]. To maintain adequate serum calcium concentrations, 1,25(OH)\(_2\)D acts on the classical target tissues intestine, kidney, parathyroid gland and bone by binding to the VDR [37]. In addition to the classical target tissues, the presence of the VDR has also been found in many non-classical target tissues including skeletal muscle tissue [38]. The biological actions of 1,25(OH)\(_2\)D on intestine, kidney, parathyroid gland, bone and muscle are discussed below. For a better understanding of the actions of 1,25(OH)\(_2\)D on bone and skeletal muscle, an overview of bone and skeletal muscle biology is included.

Intestine

One of the major functions of 1,25(OH)\(_2\)D is stimulation of calcium absorption from the intestine [37]. Calcium absorption occurs along two routes, a transcellular and a paracellular pathway [39;40]. The transcellular pathway is largely present in the duodenum [39]. This multistep process starts with the entry of calcium across the brush border membrane of the enterocyte via calcium channel TRPV6 [41]. The second step is the intracellular diffusion by the calcium-binding protein, calbindin-D\(_{9k}\) [41]. Thereafter, calcium is extruded from the enterocyte mediated by the plasma membrane calcium ATPase (PMCA) [40;42]. The transcellular pathway is regulated by 1,25(OH)\(_2\)D which
stimulates the transcription of the genes encoding for TRPV6 [43;44], calbindin-D_{9K} [43] and PMCA [44;45]. The paracellular pathway of calcium absorption occurs throughout the length of the intestine [39]. It has been suggested that 1,25(OH)_{2}D facilitates the paracellular pathway of calcium absorption as well [46;47]. 1,25(OH)_{2}D enhances the transcription of claudin-2 and claudin-12 [48], which are transmembrane components of tight junctions [46]. 1,25(OH)_{2}D also suppresses cell adhesion proteins such as cadherin-17 and cadherin-14 [49]. This indicates that 1,25(OH)_{2}D stimulates the paracellular pathway by increasing junction ion permeability [46;48].

**Kidney**

In the kidney, 1,25(OH)_{2}D stimulates the reabsorption of calcium [50]. 1,25(OH)_{2}D enhances the actions of PTH on the active transcellular epithelial calcium transport by upregulating the expression of the PTH receptor in the distal tubule of the kidney [51;52]. In the distal tubule, 1,25(OH)_{2}D is also able to induce the expression of epithelial calcium channel TRPV5 [53], calcium-binding protein calbindin-D_{28K} [50], and the Na^{+}/Ca^{2+} exchanger (NCX1) [54;55].

**Parathyroid glands**

The parathyroid glands play a central role in the regulation of calcium homeostasis [12]. A decrease of serum calcium is sensed by the calcium sensing receptor (CaSR) in the parathyroid glands resulting in the secretion of PTH [52;54]. Subsequently, PTH stimulates the intestinal calcium absorption indirectly by enhancing the synthesis of 1,25(OH)_{2}D in the proximal tubules of the kidney [20;50;52]. PTH also enhances the reabsorption of calcium in the thick ascending limbs and the distal tubules [52;54]. In bone, PTH promotes the release of calcium by stimulating osteoclastic bone resorption [52]. Restoration of serum calcium to normal levels is sensed by the CaSR in the parathyroid glands leading to an inhibition of PTH synthesis and secretion [52]. Moreover, 1,25(OH)_{2}D directly acts on the parathyroid glands through its receptor which suppresses the synthesis and secretion of PTH by inhibiting PTH gene transcription [52]. 1,25(OH)_{2}D also increases the expression of the VDR which leads to further suppression of PTH gene transcription and 1,25(OH)_{2}D increases the expression of the CaSR resulting in an increased sensitivity to changes in serum calcium [52]. In addition, 1,25(OH)_{2}D indirectly decreases PTH expression by induction of FGF23 expression in bone [47;52].

**Bone**

Bone is a specialized connective tissue which serves a variety of functions in the human body: it provides structural support, allows movement, protects vital internal organs, serves as a storage for minerals and harbors hematopoiesis [56;57]. The human skeleton is composed of cortical (or compact) bone and trabecular (or cancellous) bone [57]. Cortical bone, which comprises the outer part of all skeletal structures, has a dense and organized structure. It is composed of lamellae concentrically
arranged around blood vessels and nerves to form Haversian systems [58]. Cortical bone has an outer surface, called periosteum, and an inner surface, the endosteum [57]. Trabecular bone is a less dense type of bone found internal to cortical bone [58;59]. It is composed of plates and rods surrounded by bone marrow [57].

Bone consists of extracellular matrix (90% of bone volume) and bone cells (10% of bone volume) [60]. The major components of the extracellular matrix are mineral matrix, organic matrix, lipids and water [60]. Hydroxyapatite is the main component of the mineral phase which strengthens the organic matrix, resists compressive forces and serves as an ion reservoir [60-62]. The organic matrix predominantly consists of collagen type 1 (90%), but also consists of lipids and non-collagenous proteins including proteoglycans, glycosylated proteins (e.g. alkaline phosphatase, osteonectin, SIBLINGs such as matrix extracellular phosphoglycoprotein, osteopontin and dentin matrix protein I) and Gla-containing proteins (e.g. osteocalcin) [62;63]. The relative amounts of these components in bone is dependent on age, site, gender and health status [63]. The three major bone cells in bone are osteoclasts, osteoblasts and osteocytes [56]. Osteoclasts are large, multinucleated cells derived from mononuclear cells of the hematopoietic stem cell lineage under the influence of factors such as macrophage colony-stimulating factor (M-CSF) and RANKL [60;64]. The function of osteoclasts is bone resorption [56]. Osteoclasts attach to the mineralized matrix via integrin receptors to form a microcompartment between the ruffled border and the bone surface [57;65]. In this microcompartment, the mineral component solubilizes by acidification followed by protein degradation of the organic matrix [65]. Osteoblasts are derived from mesenchymal stem cells and located along the bone surface [64]. Osteoblasts secrete collagen type I as well as non-collagenous proteins to form the organic matrix where after mineralization of the matrix takes place [64]. At the end of the bone formation phase, osteoblasts undergo apoptosis or become bone-lining cells or osteocytes [74]. Bone-lining cells are flat-shaped osteoblasts located on the quiescent bone surfaces [74]. Osteocytes, the most abundant cells in bone, are terminally differentiated osteoblasts located in lacunae within mineralized matrix [57;66;67]. With their dendritic processes through the canaliculi, osteocytes form a lacuno-canalicular network [66;68]. This network is ideal for sensing of mechanical signals and for the release of biochemical signals to neighboring cells [66;68]. By the production of signaling molecules such as nitric oxide, osteocytes can affect osteoblast and osteoclast activity leading to an adequate bone remodeling [67;69].

Bone is a highly dynamic tissue undergoing constant remodeling throughout life. Remodeling is the process by which old bone is removed and replaced by new bone in order to prevent accumulation of microdamage, to adapt to changing mechanical demands and to subserve the metabolic function of the skeleton [57;70;71]. The remodeling cycle consists of four phases: activation, resorption, reversal and formation [57]. The bone remodeling process is initiated in response to different stimuli, including microdamage, mechanical stimuli, hormones, growth factors or cytokines [56;72]. Os-
teoblast-lineage cells may produce local factors that recruit and activate circulating osteoclast precursors [73]. The osteoclast precursors fuse to form multinucleated osteoclasts which bind to the mineralized matrix [73;74]. Resorption by osteoclasts results in the formation of Howship lacunae on the trabecular bone surface and cylindrical Haversian canals in cortical bone [57;74]. After completion of the bone resorption process, mononuclear cells prepare the surface for new osteoblasts and release signals to initiate bone formation [71;74]. During the formation phase, osteoblasts deposit layers of osteoid in the resorption cavity, followed by mineralization of the matrix [74]. At the end of a bone remodeling cycle a new osteon is produced [57].

Extracellular matrix mineralization involves a rapid primary phase leading to 50% to 70% of the maximum level of mineralization followed by a slow secondary phase characterized by a gradual maturation of the mineral component until full mineralization is reached [75;76]. The mineralization process starts with the formation of hydroxyapatite crystals within matrix vesicles budding from the plasmamembrane of osteoblasts, whereafter the membrane ruptures and hydroxyapatite is released into the extracellular matrix [77;78]. The formation of hydroxyapatite crystals and its deposition is dependent on the balance between promotors and inhibitors [57;77-80]. Alkaline phosphatase, an important mineralization promotor, hydrolyses pyrophosphate leading to an increase of the local phosphate concentration [57]. Another example of a mineralization promotor is bone sialoprotein [57;79]. Pyrophosphate, generated by pyrophosphatase phosphodiesterase 1 (NPP1) and transported by the transmembrane protein ANKH, inhibits the formation of hydroxyapatite [77;78]. Other identified inhibitors include osteonectin, osteopontin, osteocalcin and matrix Gla protein [78;79].

The effects of 1,25(OH)$_2$D on bone are primarily accomplished by increasing the intestinal absorption of calcium, which in turn stimulates mineral deposition in bone matrix indirectly [37]. In addition to these indirect effects, direct effects of 1,25(OH)$_2$D on bone cells are exerted by binding to the VDR. In human osteoblasts in vitro, 1,25(OH)$_2$D inhibits proliferation [81] and enhances osteoblast differentiation by regulating the transcription of genes associated with osteoblast maturation [6;82;83]. Treatment of human osteoblasts with 1,25(OH)$_2$D results in an increased alkaline phosphatase activity [6;81;84;85], and an increased synthesis and secretion of collagenous proteins and non-collagenous proteins such as osteocalcin and osteopontin [6;81]. Moreover, 1,25(OH)$_2$D enhances in vitro mineralization of the extracellular matrix made by osteoblasts [6;84;85]. In mouse osteoblasts, 1,25(OH)$_2$D stimulates the NO production in the absence of mechanical stimuli, while in the presence of mechanical stimuli 1,25(OH)$_2$D inhibits the production of NO [86]. Whether 1,25(OH)$_2$D affects the response to mechanical loading in human osteoblasts has not been investigated yet. In addition to the effects on osteoblasts, 1,25(OH)$_2$D is able to stimulate osteoclast differentiation by increasing the expression of RANKL in osteoblasts [87]. In osteocytes, 1,25(OH)$_2$D stimulates the transcription and synthesis of fibroblast growth factor 23 (FGF23) [88].
Muscle

Skeletal muscle is composed of muscle fibers which are surrounded by connective tissue [89]. An individual muscle fiber is a multinucleated cell and contains myofibrils in which actin and myosin myofilaments are arranged in a highly organized manner [90]. These filaments are arranged in compartments, the sarcomeres, which are the contractile units of the cell [91]. Muscle contraction results from an interaction of the actin and myosin filaments [92]. In response to an action potential in the plasma membrane, Ca\(^{2+}\) is released from the sarcoplasmatic reticulum into the cytosol through ion channels [91;93]. Ca\(^{2+}\) binds to troponin and, subsequently, troponin modulates the orientation of tropomyosin, thereby allowing myosin heads to bind to the actin filament [90]. The myosin head pulls the actin filament towards the center of the sarcomere resulting in shortening of the muscle [93;94]. Each attachment and release of the bond between actin and myosin requires ATP to convert chemical energy into movement [90;94]. The speed of muscle shortening is related to the myosin ATPase activity which is influenced by different myosin heavy chain (MHC) isoforms [95]. The major adult MHC isoforms expressed in skeletal muscle include type I, type IIA, type IIX and type IIB [91;95]. During embryonic and early postnatal development, skeletal muscle also express embryonic MHC isoforms [91]. The muscle fiber types are generally classified as being slow oxidative (type I), fast oxidative (type IIA), fast oxidative glycolytic (type IIX) and fast glycolytic (type IIB) [96].

To maintain muscle mass, a delicate balance between the rates of protein synthesis and proteolysis exists to maintain muscle mass [97;98]. A balance in favor of protein synthesis results in an increase of the muscle fiber size (hypertrophy), while a balance in favor of protein degradation leads to a decrease in muscle fiber size (atrophy) [97]. The rate of protein turnover is regulated by signaling pathways which are activated by growth factors, hormones, cytokines, nutrients and mechanical loading [97]. The major anabolic pathway involved in protein synthesis is the Akt/mTOR pathway and the major catabolic pathway involved in protein degradation is the ubiquitin-proteasome system [97;98].

Adult skeletal muscle is a post-mitotic tissue, but has the ability to regenerate in response to muscle damage [99]. Regeneration is possible by the presence of myogenic stem cells, called satellite cells, which are located between the sarcolemma and the basal lamina [100]. Satellite cells are activated after exposure to signals from a damaged area, and start to proliferate as myoblasts, differentiate and fuse with damaged muscle fibers or form new multinucleated muscle fibers to restore the muscle structure and function [101]. The process of muscle regeneration is controlled by the expression of myogenic regulatory factors, i.e. transcription factors, such as MyoD and myogenin [102].

In skeletal muscle, effects of vitamin D have been suggested since severe vitamin D deficient patients show, in addition to bone pain, clinical signs as a waddling gait and proximal muscle weakness which can lead to immobility [103]. Moreover, the symptoms of myopathy disappear in response to vitamin D treatment [104]. Detec-
tion of the VDR in skeletal muscle cells has led to further support of the view that vitamin D has direct effects on skeletal muscle cell function [38;105]. Studies in chick myoblasts show that 1,25(OH)\(_2\)D regulates calcium uptake by sarcoplasmic reticulum and sarcolemma via calcium pumps [106-109]. Calcium influx is stimulated by 1,25(OH)\(_2\)D via calcium channels leading to alterations in the intracellular calcium concentration which is necessary for muscle relaxation and contraction [106-109]. In addition to calcium transport, 1,25(OH)\(_2\)D stimulates phosphate transport across the cell membrane of chick myoblasts [110;111]. Furthermore, 1,25(OH)\(_2\)D has been demonstrated to be involved in myogenesis, a process that is essential for muscle regeneration, growth and hypertrophy [102]. In vitro studies show that 1,25(OH)\(_2\)D reduces myoblast proliferation most likely by altering expression levels of cell cycle genes [108;112]. However, whether 1,25(OH)\(_2\)D affects myoblast differentiation and hypertrophy of differentiated myotubes is not well known (Fig. 2). In addition, which regulatory mechanisms, including myogenic regulatory factors and signaling pathways, are involved in possible effects of 1,25(OH)\(_2\)D on myoblast proliferation, differentiation and myotube hypertrophy is also not well known.

**VITAMIN D STATUS**

**Assessment of vitamin D status**

Assessment of serum 25(OH)D provides the best estimate of an individual’s vitamin D status [113]. Serum 25(OH)D reflects synthesis in the skin and dietary intake, has a long half-life of 2 to 3 weeks and is not strictly regulated by hormones [114;115]. Although 1,25(OH)\(_2\)D is the most active metabolite of vitamin D, serum 1,25(OH)\(_2\)D is not appropriate for the assessment of an individual’s vitamin D status due to the very short half-life of about 4 hours [114]. Moreover, serum 1,25(OH)\(_2\)D concentrations are strictly regulated by PTH, FGF23 and 1,25(OH)\(_2\)D itself and thus kept within a narrow reference range [114]. Only in case of severe vitamin D deficiency, serum 1,25(OH)\(_2\)D concentrations drop to lower levels [114].

**Vitamin D deficiency**

Vitamin D deficiency is a highly prevalent condition worldwide [116]. In Europe, the prevalence of vitamin D deficiency in adults ranges between 2 to 30% [117], but in the elderly percentages up to 90% have been reported [118]. Risk factors for vitamin D deficiency are a lack of exposure to sunshine, a low dietary vitamin D intake, obesity, malabsorption and a pigmented skin [37]. In the elderly, a decline of the cutaneous production of vitamin D\(_3\) and an impaired 1α-hydroxylase activity in the aging kidney are also involved in the development of vitamin D deficiency [119]. Vitamin D deficiency can have serious consequences for skeletal health [118]. Low 25(OH)D serum concentrations result in lower 1,25(OH)\(_2\)D serum concentrations...
and a reduced intestinal calcium absorption [120]. In response to lower serum calcium concentrations, PTH is secreted by the parathyroid glands to restore calcium homeostasis by stimulating the renal synthesis of 1,25(OH)₂D, by increasing the renal tubular reabsorption of calcium and by increasing bone resorption [118]. Due to the higher PTH serum concentrations, serum 1,25(OH)₂D concentrations can be kept within normal ranges [118]. The secondary hyperparathyroidism causes an increase of bone turnover and bone loss [118]. Longstanding severe vitamin D deficiency (serum 25(OH)D < 25 nmol/l) leads to the development of rickets in children which is characterized by widening of the epiphyseal plates, bowing of lower extremities and muscle weakness [1;121]. In adults, osteomalacia may develop during longstanding severe vitamin D deficiency [121]. Osteomalacia is characterized by osteoid accumulation and a reduced mineralization [118]. In addition to osteomalacia, severe vitamin D deficient patients show signs including bone pain, waddling gait and muscle weakness [118]. Muscle weakness has not only ascribed to low serum 1,25(OH)₂D levels [122], but also to other factors such as hypocalcemia, hyperparathyroidism and hypophosphatemia [123-125].

**Optimal serum 25(OH)D concentration**

Optimal vitamin D status is essential for an adequate skeletal and extraskeletal health. However, controversy exists on the optimal serum 25(OH)D concentrations in humans and related guidelines for supplementation [126-129]. For instance, the Institute of Medicine recommends serum 25(OH)D levels above 50 nmol/l [130], while The Endocrine Society defines 25(OH)D levels above 75 nmol/l as the optimal level [131]. The absence of international consensus is caused by uncertainty about the different thresholds that may exist for different health outcomes, for instance bone mineral density, physical performance and parathyroid hormone suppression [127;132;133]. One study showed that the threshold for serum 25(OH)D in relation to BMD of total hip and femoral trochanter appeared to be around the level of 50 nmol/l, while the threshold for serum 25(OH)D in relation to physical performance was around 60 nmol/l [132]. For PTH, a continuous decline was found with increasing 25(OH)D serum concentrations, without reaching a plateau [132]. Different thresholds in different subgroups, i.e. men and women, different age and BMI groups, have been shown as well [133].

A recent analysis of randomized clinical trials shows that serum levels above 50 nmol/l are sufficient to normalize calcium and bone homeostasis [134]. Regarding extraskeletal health, a threshold could not be established due to insufficient evidence for a causal relationship between vitamin D and extraskeletal outcomes [134].
LOCAL VITAMIN D METABOLISM IN BONE AND MUSCLE

Physiological role
Originally, it was believed that the kidney was the sole tissue capable of converting 25(OH)D to 1,25(OH)_2D. In the early 1980s, the first evidence of extra-renal 1α-hydroxylase activity was found in lymph node homogenates and pulmonary macrophages from patients with the granulomatous disease sarcoidosis [135-137]. Around that time, in vitro studies also showed 1,25(OH)_2D synthesis from 25(OH)D in several non-renal cells including human melanoma cells [138], human alveolar macrophages [139], human keratinocytes [140], chicken calvarial cells [141] and human bone cells [142]. Although the conversion of 25(OH)D to 1,25(OH)_2D in bone cells was demonstrated, the physiological role remained unclear.

In 1997, the 1α-hydroxylase enzyme in bone cells was sequenced and it was found to be identical to that in the kidney [143-146]. However, renal 1α-hydroxylase was shown to be regulated by PTH, FGF23 and 1,25(OH)_2D itself [16-19], while the regulation of 1α-hydroxylase in bone appeared to be different from that in the kidney [6]. In human osteoblast cultures, PTH and increasing concentrations of extracellular calcium appeared not to affect 1α-hydroxylase expression as was observed in the kidney [6]. The difference in regulation of 1α-hydroxylase between bone and kidney supported the view that 1,25(OH)_2D has not only calcium-regulating activities systemically, but also other local activities [6;87]. Indeed, local activity of 1,25(OH)_2D metabolized from 25(OH)D was shown in a human osteoblast cell line in which the cells respond to 25(OH)D incubation by an increased alkaline phosphatase activity, an increased osteocalcin expression and enhanced mineralization [6]. Additionally, primary human osteoblasts showed a decreased proliferation, an increased expression of osteocalcin, osteopontin and RANKL and an enhanced mineralization in response to 25(OH)D incubation [8]. Thus, under physiological circumstances, locally synthesized 1,25(OH)_2D may not enter the circulation but binds to the vitamin D receptor in the same cell or in neighboring cells thereby affecting bone cell function. Moreover, osteoblasts in culture treated with 25(OH)D or 1,25(OH)_2D increase their expression of 24-hydroxylase which is responsible for the reduction of concentrations of 1,25(OH)_2D or 25(OH)D [6;8;142]. Thus, osteoblasts are able to metabolize 25(OH)D to 1,25(OH)_2D leading to actions on cell growth and differentiation in an autocrine and/or paracrine way, and to reduce 25(OH)D and 1,25(OH)_2D concentrations by 24-hydroxylation.

Correlations of serum 25(OH)D with endpoints such as bone turnover parameters and bone mineral density may be explained by the hydroxylation of 25(OH)D in bone [127;132]. Correlations of serum 25(OH)D do not only exist between serum 25(OH)D and endpoints such as bone turnover parameters and bone mineral density, but also between serum 25(OH)D and physical performance [127;132]. The expression of 1α-hydroxylase in skeletal muscle could account for this observation. In this way, the local conversion of 25(OH)D to 1,25(OH)_2D may be involved in the regulation of muscle fiber size and contractile function in vivo. Supportive are studies which show that 25(OH)D is able to affect skeletal muscle function regarding calcium uptake
[107] and phosphate uptake [110]. However, whether skeletal muscle cells are able to convert 25(OH)D to 1,25(OH)₂D by 1α-hydroxylase has not been investigated yet (Fig. 2). In addition, whether skeletal muscle cells are able to reduce intracellular 25(OH)D and 1,25(OH)₂D concentrations by 24-hydroxylase is unknown.

**Figure 2.** Local vitamin D metabolism in a skeletal muscle cell

**Regulation**

The positive effects of locally synthesized 1,25(OH)₂D on osteoblast function urge for the need to investigate by which factors bone 1α-hydroxylase is regulated and how the local synthesis of 1,25(OH)₂D can be stimulated, especially for the prevention and treatment of metabolic bone diseases such as osteoporosis. After all, an increased local availability of 1,25(OH)₂D may stimulate osteoblast activities which may be beneficial in osteoporotic bone. However, until now the regulation of 1α-hydroxylase expression in bone is poorly understood (Fig. 3). As mentioned above, the regulators of renal 1α-hydroxylase, calcium and PTH, appear not to affect the expression of CYP27B1 in human osteoblasts, however whether the other renal regulators FGF23, phosphate and calcitonin are able to affect CYP27B1 expression has not been fully elucidated yet.
When $1\alpha$-hydroxylase activity in bone is not regulated by renal regulators such as calcium and PTH, substrate availability may become very important. Increasing the substrate, i.e. 25(OH)D, would then lead to an increased local availability of $1,25(OH)_2D$ and a greater response of bone cells [147]. Higher doses of vitamin D and higher serum 25(OH)D levels would be advantageous for the local synthesis of $1,25(OH)_2D$ in bone cells [6;127]. Since the hydroxylation of 25(OH)D in bone cells can not be measured in the circulation, in vitro models are a valuable tool to investigate whether bone cells are able to synthesize more $1,25(OH)_2D$ after 25(OH)D addition. In case of vitamin D deficiency, low serum 25(OH)D concentrations may be a limiting factor for the intracellular synthesis of $1,25(OH)_2D$ [147]. When 25(OH)D hydroxylation in bone is an important local source for $1,25(OH)_2D$, then a low serum 25(OH)D concentration may lead to a decreased or altered bone cell activity. However, whether the expression of $1\alpha$-hydroxylase in bone is affected in case of low serum 25(OH)D concentrations is unknown.

When $1\alpha$-hydroxylase activity in bone is important for local activities regarding cell growth and differentiation, it is likely that local regulatory mechanisms exist. An important local factor that affects the activity of bone cells is mechanical loading [67]. In response to mechanical stimuli, osteocytes produce signaling molecules to
modulate the activity of osteoblasts and osteoclasts in an autocrine or paracrine way [148]. This may lead to adaptation of bone mass and structure to mechanical stimuli [67;149]. Mechanical loading stimulates a number of responses in bone cells which eventually may result in osteoblast differentiation [150]. Osteoblast differentiation is also stimulated by locally synthesized 1,25(OH)$_2$D by human osteoblasts [6;8]. However, whether mechanical loading stimulates osteoblast differentiation through an increased 1α-hydroxylase expression and activity by human osteoblasts has not been investigated yet.

When skeletal muscle cells are able to convert 25(OH)D to 1,25(OH)$_2$D, locally synthesized 1,25(OH)$_2$D may affect myoblast differentiation and muscle fiber size. In case of a stimulating effect of locally synthesized 1,25(OH)$_2$D on muscle fiber size, higher local 1,25(OH)$_2$D concentrations may result in a greater response of skeletal muscle cells. Higher local concentrations of 1,25(OH)$_2$D may be accomplished by increasing the activity of 1α-hydroxylase or increasing the substrate. However, whether 1α-hydroxylase expression is affected by vitamin D metabolites needs to be resolved, as well as whether skeletal muscle cells have the ability to increase the synthesis of 1,25(OH)$_2$D after treatment with higher concentrations of 25(OH)D.

In conclusion, autocrine and paracrine activities of locally synthesized 1,25(OH)$_2$D appear to be important for cell growth and differentiation. However, the regulation of 1α-hydroxylase in bone and muscle is poorly understood. The primary aim of this thesis is to explore the response of osteoblast and skeletal muscle cell function to 25(OH)D, to investigate the ability of osteoblasts and skeletal muscle cells to metabolize 25(OH)D and to determine by which factors the expression of 1α-hydroxylase is affected. Since the local availability of 1,25(OH)$_2$D and 25(OH)D is also determined by the activity of 24-hydroxylase, we also determined by which factors the expression of 24-hydroxylase is affected.
OUTLINE

The aim of this thesis was to gain more insight into the activity of locally synthesized \(1,25(\text{OH})_2\)D and the regulation of local vitamin D metabolism in bone and muscle. In this thesis the following research objectives were addressed using in vitro and in vivo models:

In vitro:
- To determine the effects of 25(OH)D, besides \(1,25(\text{OH})_2\)D, on osteoblast function as well as skeletal muscle cell function.
- To investigate the ability of osteoblasts and skeletal muscle cells to synthesize \(1,25(\text{OH})_2\)D from 25(OH)D and 24R,25(OH)\(_2\)D from 25(OH)D.
- To investigate whether CYP27B1 and CYP24 mRNA expression in osteoblasts and skeletal muscle cells are affected by vitamin D metabolites.
- To investigate whether CYP27B1 and CYP24 mRNA expression in osteoblasts are affected by renal regulators and mechanical loading.

In vivo:
- To investigate the effects of vitamin D deficiency on CYP27B1 and CYP24 mRNA expression in bone tissue.

Both in vitro and in vivo models were used to gain more insight into the activity of locally synthesized \(1,25(\text{OH})_2\)D and to investigate by which factors local vitamin D metabolism in bone and muscle is affected. With respect to osteoblasts, a primary human osteoblast culture model was used to determine the proliferative and differentiative activity of osteoblasts to 25(OH)D, compared to \(1,25(\text{OH})_2\)D, and to investigate the ability of osteoblasts to synthesize \(1,25(\text{OH})_2\)D and 24R,25(OH)\(_2\)D from 25(OH)D (chapter 2). The next step was to investigate by which factors local vitamin D metabolism in osteoblasts is affected. We investigated whether vitamin D metabolites affect the mRNA expression of CYP27B1 and CYP24 in osteoblasts and whether osteoblasts are able to synthesize more \(1,25(\text{OH})_2\)D after treatment with increasing doses of 25(OH)D (chapter 2). In chapter 3, we investigated whether CYP27B1 mRNA expression in primary human osteoblasts is affected by the renal regulators PTH, FGF23, calcium, phosphate, calcitonin and matrix extracellular glycoprotein (MEPE). In chapter 4, we examined whether mechanical loading increases the mRNA expression of CYP27B1 and the synthesis of \(1,25(\text{OH})_2\)D in primary human osteoblasts. With respect to skeletal muscle cells, a murine C2C12 myoblast culture model was used to determine whether both \(1,25(\text{OH})_2\)D\(_3\) and 25(OH)D\(_3\) affect myoblast proliferation, differentiation and myotube size, and which regulatory mechanisms are involved. We also investigated whether C2C12 myotubes were able to
synthesize 1,25(OH)$_2$D from 25(OH)D and 24R,25(OH)$_2$D from 25(OH)D (Chapter 5). Two animal models were used to examine whether CYP27B1 and CYP24 mRNA expression in bone tissue are modulated by vitamin D deficiency. In Chapter 6, we investigated in adult rats whether vitamin D deficiency affects bone structure and remodeling as well as mRNA levels of genes involved in local vitamin D metabolism in bone. In Chapter 7, we determined in older adult mice the effects of long-term vitamin D deficiency on bone structure, remodeling and mineralization as well as on mRNA levels of genes involved in local vitamin D metabolism in bone. Chapter 8 provides a general discussion of this thesis.
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