THE EFFECT OF DIETARY COMPONENTS ON OSTEOCYTE MECHANOSENSITIVITY:

IMPLICATIONS FOR BONE HEALTH

The studies described in this thesis were carried out at the section Oral Cell Biology of the Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Amsterdam, The Netherlands. The studies were performed as part of the project "The combined effect of physical activity and nutrition on bone metabolism: Nutribone".

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THE EFFECT OF DIETARY COMPONENTS ON OSTEOCYTE MECHANOSENSITIVITY:

IMPLICATIONS FOR BONE HEALTH

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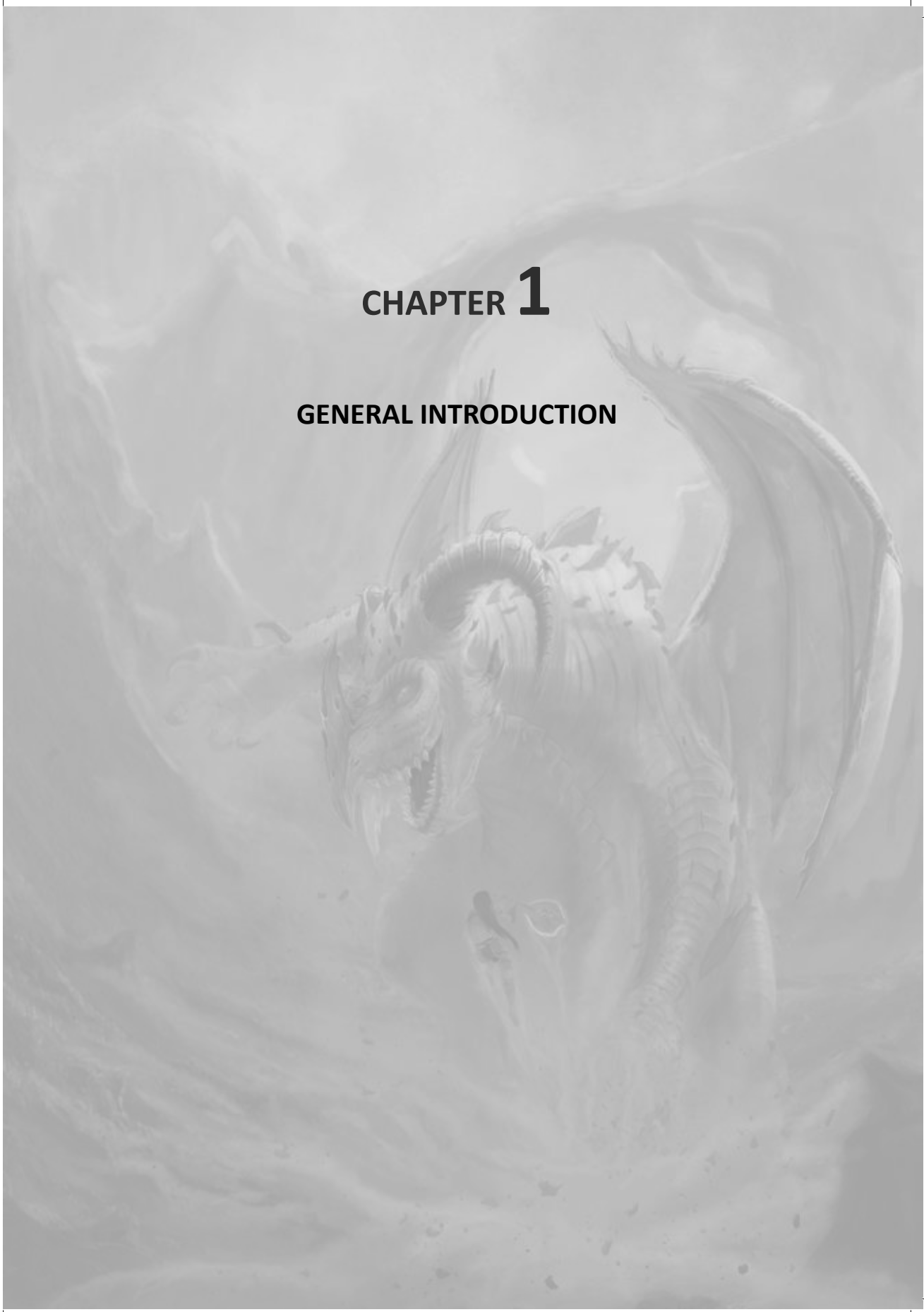
CONTENTS



CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Physical activity and diet are among the most commonly advocated lifestyle measures to improve skeletal health[1]. With respect to physical activity, Julius Wolff and Wilhelm Roux at the end of the nineteenth century already observed that bone mass and structure change under influence of the mechanical load that bone carries. While Julius Wolff focused on the mathematical rules predicting bone shape, Wilhelm Roux proposed a concept of functional adaptation as a self-organized process based in the tissues[2].

While increased mechanical loading (e.g. due to exercise) results in a higher bone mass, unloading (e.g. due to bed rest) results in a decrease in bone mass. It is currently believed that mechanotransduction, the process by which a mechanical signal is transduced into a chemical signal by cells in bone that sense mechanical loading, proceeds as follows. When bones are loaded, the resulting matrix strains force the interstitial fluid that surrounds the osteocytes to flow over the osteocyte processes, which are positioned in the canaliculae[3,4]. Osteocytes sense and translate this fluid flow into a chemical signal that alters the bone remodeling activities of osteoblasts and osteoclasts[5,6]. Among the most widely studied signaling molecules produced by osteocytes in response to mechanical stimulation are nitric oxide (NO) and prostaglandin E_2 (PGE_2).

PGE_2 is abundantly expressed and active in bone and is generated in cells by PGE-synthase from prostaglandin H_2[7]. The rate-limiting enzyme in prostaglanding H_2 synthase is cyclooxygenase (COX). Although several PGE-synthases have been identified, prostaglandin E synthase-1 is known as the key enzyme in the formation of PGE_2. PGE_2 regulates bone resorption by affecting osteoclast activity as well as bone formation by inducing osteoblast proliferation[8] and differentiation[9]. *In vitro*, PGE_2 production primarily leads to bone resorption. In contrast, PGE_2 stimulates both bone formation and resorption *in vivo*[10].

NO is a short-lived, highly reactive free radical involved in many biological processes, including the regulation of bone metabolism[11]. NO inhibits osteoclast activity[12], and mediates the anabolic response of bone to mechanical loading in vivo[13]. The loading-induced NO production in osteocytes results from the activity of endothelial cell nitric oxide synthase (ecNOS)[14]. In cell culture experiments, osteocytes produce high levels of NO in response to mechanical loading in the form of fluid shear stress[14-16].

Bone morphogenic proteins (BMPs) are produced in response to mechanical loading, and BMPs might have a relevant role in bone mechanotransduction. BMP gene expression is upregulated after mechanical loading in chondrocytes[17], rat achilles tendon[18], and vascular endothelial cells[19]. Moreover the BMP antagonist noggin inhibits mechanical loading-induced chondrocyte proliferation[17]. Production of molecules involved in bone remodeling such as BMPs, NO, and PGE_2 by bone cells is affected by mechanical stimuli, but may be also

affected by nutrition. This could, at least partially, explain how nutrition contributes to a lower risk for bone-related diseases[20].

A striking example of how nutrition affects bone health is provided by the lower incidence of osteoporosis in Mediterranean countries[21,22]. An environmental factor determining this lower risk is the Mediterranean diet, which includes a high intake of olives and olive oil. Soybean isoflavones, an important dietary component of the Asian diet, are currently thought of as a potential alternative therapy for osteoporosis as well. There is convincing evidence for the potential role of the soybean isoflavone genistein in the prevention of osteoporosis and reduction of fraction risk from human studies[23] and animal studies[24,25]. Together the findings described above indicate that a balanced diet and an active life style can be beneficial to improve bone health. Whether nutrition and mechanical loading are able to interact in their effect on bone turnover and thereby synergistically affect bone mass maintenance and accrual is currently unknown, and therefore the major question addressed in this thesis.

This thesis focuses on the effect of nutrients on the response of mechanosensitive bone cells to mechanical stimuli. The overall aim was to identify nutritional factors that alter the bone cell response to mechanical loading. We also assessed whether the combination of certain nutrients and mechanical stimuli can counteract the effects of inflammatory cytokines on mechanosensitive bone cells, since bone loss after the menopause and generalized bone loss associated with systemic inflammatory diseases may be partially attributed to an increase in cytokines in the circulation (for a review see Geusens and Lems, 2011)[26].

In this thesis the following scientific questions were addressed:

1. What are the pathways leading to the release of signaling molecules, specifically bone morphogenetic proteins, by bone cells in response to mechanical loading?
2. How do dietary components that are used as agents in osteoporosis treatment, such as 1,25-dihydroxyvitamin D_3 and fluoride, affect the response of osteocytes to mechanical stimulation?
3. Could nutrients such as oleuropein potentially counteract inflammation-induced bone loss by restoring the altered response of bone cells to mechanical stimuli in an environment of inflammation?

In chapter 2, the effect of mechanical loading on the production of bone morphogenetic protein 2 and 7 by human bone cells was investigated. In chapter 3 we tested the effect of fluoride on the response of bone cells to mechanical loading. Another metabolic bone agent present in the diet is $1,25(OH)_2D_3$. In chapter 4 we assessed whether $1,25(OH)_2D_3$ affects NO production via the vitamin D receptor (VDR), in the presence and absence of a mechanical stimulus. In chapter 5 we investigated whether oleuropein alters the effect of the cytokine interleukin-1b on the osteocyte response to mechanical stimulation. Nutrients

also have an effect on gut hormones, which in turn can affect bone mass. In chapter 6 we explored the effect of the gut hormone glucose dependent inhibitory peptide (GIP) on the response of bone cells to mechanical stimulation. Finally, in chapter 7, we discuss how our results provide new insights in the molecular pathways that are activated in osteocytes subjected to mechanical loading, and how nutrients affect this process. These insights could contribute to the development of products, i.e. a personalized exercise and diet programme, to maintain existing bone mass and aid the gain of new bone mass in people that are likely to develop osteoporosis.

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CHAPTER 2

NUTRIENTS AND OSTEOCYTES, A MATCH MADE IN BONE

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ABSTRACT

Considering our ageing population and the expected concomitant increase incidence of bone loss-associated fractures, it is of the utmost importance to develop novel therapies that enhance bone strength in a safe and efficient way. Dietary intake of dairy products positively affects bone mineral density in early life, and reduces loss of bone mass with ageing. In addition, regular weight-bearing physical activity has a strong positive effect on bone. This review focuses on the possible synergistic effects of nutrients and mechanical stimuli for bone health. Bones contain a vast number of interconnected living cells embedded within the calcified extracellular matrix. These cells, the osteocytes, perceive the mechanical stimuli evoked during weight-bearing exercise. In response to mechanical stimuli, osteocytes produce potent signaling molecules that affect the formation and activity of bone forming osteoblasts and bone resorbing osteoclasts. As a consequence, any factor that alters the response of osteocytes to mechanical stimuli, potentially affects bone mass and structure. We identify a number of nutrients, which are expected to enhance the beneficial effect of physical activity on bone mineral density by enhancing osteocyte mechanosensitivity. Such nutrients may have a synergistic effect on bone health when combined with a regime of physical activity, providing an efficient and safe way to combat fractures in the elderly.

INTRODUCTION

Skeletal diseases such as osteoporosis increase the risk of bone fracture[1]. Since osteoporosis is primarily a disease of the elderly, the concern is growing that with the increased aging of the human population, the incidence of osteoporosis-related fractures will substantially rise over the coming decades[2]. In 2000 the costs of osteoporotic fractures in Europe alone have been estimated at €31.7 billion, and these costs are expected to increase up to €76.7 billion in 2050[3]. More importantly osteoporosis-related fractures often lead to a diminished quality of life, disability, discomfort, and even death. Thus prevention of these fractures is required[4].

Daily physical activity and a healthy diet are among the most commonly advocated lifestyle measures to improve (skeletal) health[5]. The European Food Safety Authority (EFSA) states the importance of a balanced nutrition for healthy bones. Vitamin D and calcium are well known for decades for their contribution to bone health[6]. Recently other nutrients like magnesium, vitamin K, and carotenoids, have been shown to positively affect bone health as well[7]. It has been proven that bone health benefits from a balanced diet consisting of fruit, vegetables, adequate dairy and other protein-rich food intake, and a limited intake of low nutrient-density food[7].

Regarding the contribution of daily mechanical loading to bone health, Roux (1850-1924) proposed a concept in which daily mechanical loading of bone leads to the adaptation of bone structure to optimize the load-bearing capacity of bone[8]. How much physical activity exactly contributes to bone mass is difficult to predict, but a lack of physical activity, e.g. as is seen in bed-ridden patients, quite obviously results in a rapid and substantial loss of bone mass[9]. Moreover compelling evidence exists showing that high impact physical activity in adults has an anabolic effect on bone mineral content (BMC) and bone mineral density (BMD)[10]. The BMC and BMD and have been shown to increase up to 20% in the loaded bone regions in athletes[11-15].

Bone mass at the age of 70 largely depends on peak bone mass reached before the age of 30[21]. High impact physical activity in childhood, especially when initiated before puberty, results in increased bone width and increased bone mineral content in girls and adolescent females[22]. Therefore a physical activity regime that has started before puberty may be used for the prevention of osteoporosis and fractures at a later age[23]. During growth, not only high impact activity but also low impact activity contributes to skeletal health[16-18]. High magnitude mechanical loads as well as loads applied at high frequency are known to increase bone mass, but low impact activities have less effect on bone even when applied for a long duration[19,20]. It is much debated whether the efficiency of physical stimuli for increasing bone strength diminishes with age, especially after the menopause. Exercise may

benefit bone health in adults since bone resorption and bone turnover markers are reduced by one month of moderate intensity exercise, i.e. 3 times per week and 30 minutes per day, in both pre- and postmenopausal woman[24]. Important bone health biomarkers are also favorably affected by a high intake of dairy, calcium, and protein during diet- and exercise-induced weight loss in premenopausal overweight and obese women[25], indicating that both exercise and diet contribute to the maintenance of bone health.

It seems reasonable to expect that a combination of dietary components and physical activity will efficiently lead to stronger and thicker bones. However, it is still largely unknown which nutrients actually enhance the gain in bone mass achieved with moderate daily physical activity. Therefore we aimed to identify nutrients with the potential to enhance the effect of physical activity on bone mass. We focus on osteocytes, the bone cells responsible for the adaptive response of bone to mechanical signals.

THE ROLE OF OSTEOCYTES IN BONE HEALTH

The idea that bone is a dead tissue could not be further from the truth. Bone is made up of a combination of organic and anorganic matrix, and contains four types of living cells, i.e. osteoblasts, osteoclasts, osteocytes, and bone lining cells. Osteoblasts are bone forming cells that produce collagens and proteins that play a role in the initiation of bone matrix calcification[26]. Osteoclasts degrade bone by matrix demineralization and degradation of collagen and non-collagenous proteins. The osteoclasts and osteoblasts act in concert to continuously repair micro-damage caused by fatigue loading, through targeted remodeling[27]. This process is believed to be orchestrated by the osteocytes[28]. The process of bone remodeling (Figure 1) facilitates the adaptation of bones to their mechanical environment[29]. During bone remodeling excess bone is removed in regions exposed to low loads and new bone is deposited in regions exposed to high loads. Osteocytes are the mechanosensor cells in bone. During bone formation, a subpopulation of osteoblasts undergoes terminal differentiation and become embedded within the bone as osteocytes[30,31]. The osteocytes constitute 95% of all cells in bone and form a network of numerous cell extensions ("fingers") extending throughout the mineralized bone, which enables cell-cell contact[32]. The cell "fingers" lie within the canaliculi in the calcified bone matrix and are surrounded by a thin layer of interstitial fluid[33].

It has been subject to much debate how a mechanical signal in intact bone is transduced into a signal that activates the osteocytes[34]. The deformations in healthy bones as a result of normal daily activity are quite small[35]. Deformation in animal and human long bones never exceed 2000-3000 microstrain (a 0.2-0.3% change in length when compared to the original length of the bone)[19], and on a daily basis deformations of this order of magnitude are

rare. It is uncertain whether bone cells can directly perceive those small daily deformations, even though it is certain that such stimuli maintain bone mass. Thus a biological system must exist that somehow amplifies the mechanical signals. According to the canalicular flow hypothesis, mechanical stimuli deform the bone tissue, causing the interstitial fluid to flow through the lacuno-canalicular network over the cell "fingers"[32]. This can be compared with squeezing a stiff, water-soaked sponge. The osteocytes sense this fluid flow and start producing signaling molecules such as nitric oxide (NO) to alter the activity of osteoblasts and/or osteoclasts. NO is produced by the enzyme nitric oxide synthase (NOS), and NO production is a well known and essential early response of osteocytes to mechanical loading in vivo[36]. Thus NO can be measured to get an impression of the magnitude of the biological response of osteocytes to mechanical loading. The mechanical signal can be mimicked in vitro by means of a laminar fluid flow exerted on a monolayer of cultured osteocytes or osteocyte-like cells. Osteocytes are often subjected to a pulsatile fluid flow (PFF) in vitro[37]. Within minutes after PFF application, the osteocytes start to release NO and prostaglandin E_2 (PGE_2)[38,39]. PGE_2 production is mediated by the enzyme cyclooxygenase (COX)[40]. There are several isoforms of COX, of which COX-2 is the isoform that is induced by mechanical loading *in vitro*[41] and *in vivo*[42].

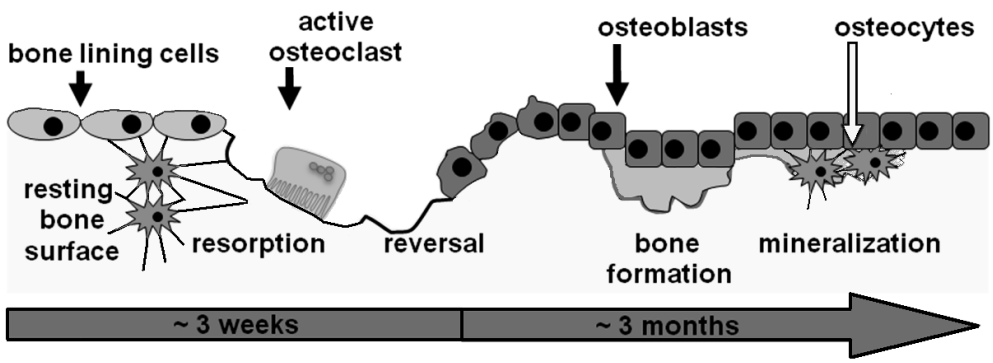

Figure 1. The adaptation of bone to the ever changing mechanical demands takes place during the complicated bone remodeling process. Bone remodeling involves four main processes, i.e. activation, resorption, reversal, and formation. The remodeling cycle is initiated by osteocyte apoptosis and activation of bone lining cells. Osteoclast precursor cells are recruited to the activated surface and fuse to form mature, bone resorbing osteoclasts, where they attach and start to dissolve bone matrix, creating a resorption pit. When bone resorption eventually subsides, osteoclasts disappear and mononuclear cells arrive that differentiate into osteoblasts. The remodeling cycle ends with the synthesis and deposition of bone matrix by osteoblasts. The newly formed bone will then be covered by bone lining cells, until the next cycle.

THE ROLE OF NUTRIENTS IN THE DETERMINATION OF OSTEOCYTE SHAPE

Lately the view of the cytoskeleton as a structure responsive to external physical and chemical stimuli has become more prominent[43]. The cytoskeleton is involved in mechanosensing and is a key determinant of the material properties of the cell. The effects of microstrain (με) stresses on a cell largely depend on the material properties of that cell. In bone, low magnitude (<10 με) and high frequency (10–100 Hz) loading stimulates bone mass and strength *in vivo*[44]. For bone cells it has been shown that the production of signaling molecules in response to fluid shear stress and vibration stress *in vitro* is correlated with the applied stress rate[45]. The faster the stress was applied, the stronger the observed response of the cells. Moreover, osteocytes with round suspended morphology require lower force stimulation in order to show an increase in NO production, while they are an order-of-magnitude more elastic compared to flat adherent cells[43]. Apparently, round osteocytes seem to require less mechanical forces in order to respond than stiffer cells. These findings indicate that differences in mechanosensitivity in osteocytes are related to the shape and material properties of the cell[43]. The shape of cytoskeletal structures depends on the dynamics of actin fibers and microtubules[46,47]. Actin fibers are most abundant in eukaryotic cells and form a framework that supports and shapes the plasma membrane. Microtubules exhibit structural and functional polarity and are important components of primary cilia[48], a mechano-sensitive structure that is likely also present on osteocytes[49].

The question arises whether it is possible that nutrients affect the cytoskeleton of bone cells, thereby affecting their mechanosensitivity. Fluoride is known to disrupt the actin cytoskeleton of protozoa[50] and actin fibers in ameloblasts[51], leading to a disrupted actin-cytokeleton and thus more rounded cells. Therefore fluoride might enhance osteocyte mechanosensitivity. The polyphenol oleuropein disrupts microtubules in tumor cells, also resulting in rounded cells and altered cytoskeletal organization[52]. Genistein, an isoflavone, mostly present in soybeans, depolymerizes the microtubules in human A549 epithelium cancer cells, and inhibits microtubule polymerization *in vitro*[53]. These findings suggest a role for fluoride, oleuropein, and genistein in the mechanoresponse of osteocytes.

THE ROLE OF (PHYTO-) ESTROGEN IN BONE CELL MECHANOSENSITIVITY

Estrogen is known to have profound anabolic effects on bone. Decreased estrogen levels following menopause have a strong negative effect on bone mass[54]. On the other hand, administration of exogenous estrogen increases bone mineral density in humans[55,56], and high estrogen concentrations induce new bone formation in mice[57,58]. These effects of estrogen on bone can be explained by its opposite effects on osteoclasts and osteoblasts.

Estrogen can suppress bone resorption and stimulate bone formation after binding to its receptor present on both osteoclasts and osteoblasts[54,59]. In addition, estrogen likely alters the response of osteocytes to mechanical loading via modulation of the estrogen receptor α[60], thereby affecting bone mass. It has been suggested that osteocytes become less sensitive to mechanical stimuli in woman after menopause, due to the estrogen loss. This would explain the rapid loss in bone mass associated with menopause[61]. Phytoestrogens are plant-derived compounds with estrogen-like activity, and therefore it is possible that these agents affect osteocyte mechanosensitivity. Daidzein and genistein are two examples of phytoestrogens derived from soy-beans. Genistein stimulates bone-nodule formation and the release of osteocalcin via the estrogen receptor-dependent pathway in rat osteoblasts *in vitro* [62], suggesting that genistein potentially affects osteocyte mechanosensitivity. Like genistein, daidzein stimulates bone-nodule formation and osteocalcin release in rat osteoblasts *in vitro*, but this effect is likely not mediated by the estrogen receptor[62].

NUTRIENTS AFFECTING THE DIFFERENTIATION OF OSTEOBLASTS INTO OSTEOCYTES

Osteocytes are derived from osteoblasts. Osteocytes are highly mechanosensitive, but osteoblasts and osteoblastic cell lines also show mechanosensitivity[63]. Osteoblasts differentiate into osteocytes, and as a result become more sensitive to mechanical loading[63]. Nutritional components that are able to stimulate osteoblast differentiation might therefore enhance the mechanoresponse, which ultimately will lead to adaptive changes in bone mass.

The component fluoride is well known for its effect on osteoblast differentiation. It increases osteoblast proliferation and differentiation in a rat osteosarcoma cell line[64]. Osteoblast mitochondrial activity is also stimulated by fluoride, which also leads to osteoblast differentation[65]. Lactoferrin, a pleiotropic factor, stimulates both osteoblast proliferation and differentiation[66]. Lactoferrin potently increases differentiation of osteoblasts into osteocytes[67]. Since both genistein and daidzein seem to stimulate osteoblast differentiation[68], it is possible that they affect the mechanoresponse of osteoblasts as well. Other components that are known to enhance osteoblast differentiation are strontium, isoflavones, and whey protein[69-71].

NUTRIENTS AFFECTING THE PRODUCTION OF SIGNALING MOLECULES

NO and prostaglandins are early parameters for bone formation and essential for the anabolic response of bone to mechanical loading *in vivo*[72]. NO is a short-lived molecule involved in the regulation of bone metabolism[73], and is formed by the conversion of L-arginine in L-citrulline by NOS[74]. PFF-induced NO production by bone cells results from the activity of endothelial NOS[75]. NO is known to inhibit osteoclast activity[76]. Both animal studies and pilot studies in humans support the use of NO donors to prevent bone loss[77]. Since NO is formed during the conversion of L-arginine by NOS, it is feasible that the amino acid arginine plays a role in the process of adaptive bone formation. Several studies have shown that arginine administration significantly increased alkaline phosphatase, NO, and insulin like growth factor-I production, as well as type I collagen synthesis by human osteoblasts, and in osteoblasts derived from calvaria of newborn rats[78].

Prostaglandins are generated by the release of arachidonic acid from phospholipids in the cell membrane, followed by conversion of arachidonic acid into prostaglandin G2 and subsequently prostaglandin H_2 by COX[79]. Prostaglandin H_2 is further isomerised to the biological active prostanoids, such as PGE_2[79]. COX-2, but not COX-1 is likely essential for the anabolic effect of mechanical loading in bone[40]. Any nutrient that affects COX-2 activity thereby potentially affects the anabolic response of bone to mechanical loading. PGE_2 (10^{-8} M) affects bone formation by inducing osteoblast proliferation and differentiation[80-82]. Higher PGE_2 concentrations (10^{-7} M) stimulate bone resorption *in vivo* and osteoclastogenesis *in vitro*[83]. Moreover PGE_2 and COX-2 at high concentrations play an important role in inflammatory processes, often associated with trabecular bone loss, and leading to osteoporosis. The role of arachidonic acid and phospholipids in this important regulatory pathway suggest that fatty acids in the diet may influence the process of mechanical adaptation of bone. Especially the dietary ratio of omega 6 to omega 3 fatty acids is important, i.e. lowering this ratio seems to diminish PPAR-γ activation and thereby promotes osteoblastogenesis at the expense of bone marrow adiposity. Only few studies have been conducted addressing the prevention or treatment of osteoporosis by dietary poly-unsaturated fatty acids[84,85]. Epidemiological evidence indicates that diets containing omega 3 fatty acids are beneficial to skeletal health. A recent review concludes that any potential benefit of omega 3 fatty acids on skeletal health may be enhanced by concurrent administration of calcium[85]. However whether fatty acids, either or not in combination with calcium, modulate osteocyte mechanosensitivity is unknown.

Osteoporosis-related fractures are lowest in Southern Europe, which is likely related to dietary influences[86]. Olive oil and its main compound oleuropein are abundantly present in the Mediterranean diet. Oleuropein at 10-100 μg/ml reduces the levels of COX-2 seen in

inflammation[87]. Lower concentrations of oleuropein (10^{-4}-10^{-6} M) increase NO production via induction of inducible NOS by macrophages in a mouse infection model, thus increasing functional activity of these cells[88]. Another polyphenolic compound, chlorogenic acid (CGA), decreases the inflammation-induced production of PGE_2 as well as the expression of COX-2 in RAW 264.7 macrophages[89], implementing a role for polyphenols in inflammation-induced bone resorption. Strontium is known for its effect on the proliferation of pre-osteoblasts, and the stimulation of bone formation[90]. In both MC3T3-E1 osteoblasts and PC-3 cells, strontium indirectly induces the production of PGE_2 via IL-6[91]. Together these findings suggest a role for strontium in bone turnover. The components discussed above are known to affect PGE_2, COX-2, and NO production, and therefore they are likely to increase the response of bone cells to mechanical stimulation.

Bone remodeling strongly depends on the communication between osteocytes and osteoblasts and osteoclasts. Osteoclasts are formed by the fusion of mononuclear osteoclast precursor cells[92] and express tartrate-resistant acid phosphatase, an osteoclast marker. RANKL is a membrane-bound cytokine and binds to its receptor RANK on osteoclast precursors[93]. Binding of osteoprotegerin (OPG) to RANKL results in suppression of osteoclast formation *in vivo* and *in vitro*[94]. The ratio OPG/RANKL is therefore considered crucial in osteoclast formation[95]. RANKL is also expressed by osteocytes in bone microdamage[96], and apoptotic osteocytes directly and indirectly initiate bone resorption by recruitment of osteoclast precursor cells to the local damage site[97]. Vitamin K is present as phylloquinone (vitamin K1) and menaquinones (vitamin K2), e.g. MK-7, MK-8, and MK-9[98]. Vitamin K2 inhibits osteoclast formation by decreasing RANKL[99]. It also inhibits PGE_2-stimulated osteoclast formation[100]. Incubation of human monocytes with RANKL and M-CSF leads to inhibition of osteoclast formation. MK-7 suppresses proliferation, but enhances OPG, RANKL, and RANK gene and protein expression in MC3T3-E1 osteoblasts[101]. Recent data show that osteocytes express much more RANKL and have a greater capacity to support osteoclastogenesis *in vitro* than osteoblasts or bone marrow stromal cells[102]. The severe osteopetrotic phenotype observed in adult mice specifically lacking osteocyte-derived RANKL indicates that osteocytes are the major source of RANKL during bone remodeling *in vivo* thereby determining bone mass[102].

Expression of the PTH receptor is not limited to a specific tissue, and both osteoblasts and osteocytes but not osteoclasts have been shown to express PTH receptors[103]. PTH is secreted by the parathyroid glands during bone resorption to maintain serum calcium levels. PTH binds to and activates the PTH receptor on osteoblasts in bone[104]. It activates protein kinase A and protein kinase C together with intracellular signaling pathways leading to osteoclast recruitment[105]. This recruitment of osteoclasts to the remodeling site is a signal for the osteoblasts to deposit bone matrix, and is generated by osteocytes via PTH. Oral administration of glucose rapidly decreases serum levels of PTH[106].

The role of 1,25-dihydroxyvitamin D_3 in bone mineral homeostasis is to promote intestinal absorption of calcium and phosphate[107]. 1,25-Dihydroxyvitamin D_3 masters calcium and phosphate homeostasis through cross-regulation of PTH, but also via osteoclastogenesis and osteoblastogenesis, calcium and phosphate acquisition, and regulation of anabolic and catabolic gene expression, in order to achieve a proper mineral and skeletal metabolism[108]. Vitamin D deficiency results in inadequate intestinal calcium absorption and increased PTH levels, which possibly leads to accelerated bone loss[109]. Daily supplementation of postmenopausal women with 400 IU vitamin D per day reduces PTH levels and increases bone mineral density at the femoral neck[110,111]. It has been shown that PTH reduces the mechanical stress-induced NO production[112]. Since glucose and vitamin D decrease serum levels of PTH[106, 111], these nutrients might positively affect the mechanical loading-induced NO production.

THE COMBINED EFFECT OF NUTRITION AND MECHANICAL LOADING ON BONE IN VIVO

Cortical bone contains osteons, which are composed of a central Haversian canal surrounded by bone matrix. The Haversian canal encompasses blood vessels and nerves throughout the bone and communicates with osteocytes in lacunae through canaliculi[113]. After ingestion, nutrients enter the blood stream and are delivered to the bone, where the reach the bone cells. A balanced diet and other nutrients in combination with an active life style can thus be beneficial to improve bone health by affecting osteocytes[114].

The major drawbacks of studies on bone *in vivo* are the associated high costs and the long-term duration of the experiments. Therefore only few studies have been conducted on the effect of a combination of physical activity and a balanced diet on bone health under normal daily circumstances. Bone mass status in athletes is definitely determined by a combination of systemic hormones, nutrition, and exercise. The total body mineral density/ bone mineral content ratio of the calcified matrix is relatively high in female dancers, which is caused by high levels of physical activity and a balanced diet[115]. Physical activity and a high-fat diet results in increased trabecular bone mineral density and improved three-dimensional (3D) micro-structure in obese mice[116]. Low calcium intake diminishes the increased bone mass resulting from exercise in prepubertal girls[117]. Limited evidence exists regarding a relationship between vitamin D intake and fracture prevention by physical activity[118]. One of the few studies conducted on the effect of diet and physical activity in healthy subjects (age 14-18 yrs) shows that the main dietary variables related to bone mass are energy intake, calcium, vitamin D, and servings of dairy products, in combination with vigorous (jumping) physical activity[119]. The *in vivo* experiments described above suggest

that nutrients and physical exercise have at least an additive effect on bone health. Further studies should reveal whether nutrients and physical exercise synergistically enhance bone health in humans *in vivo*. The consumption of vitamin-enriched dairy products might be of great benefit when the natural intake is not sufficient, e.g. in older people (age >50 yrs) or professional athletes.

CONCLUDING REMARKS

The increasing number of patients suffering from osteoporosis is accompanied by high costs and decreased quality of life, and therefore new therapeutic approaches are urgently needed. Most approaches focus on the prevention of osteoporosis. Given the crucial importance of nutrients and physical activity for bone health in general, we suggest a new approach existing of a 'change in lifestyle', i.e. a regime of daily exercise and a balanced diet, possibly fortified with bone formation-stimulating nutrients. A better understanding of the pathways involved in the process of mechano-transduction and the regulating factors in daily nutrition is necessary to develop a life style advise. In this review several mechanisms have been identified by which nutrients could modulate the beneficial effect of mechanical stimuli on bone health. The establishment of the effects of nutrients on mechanical loading-induced bone formation *in vivo* needs further investigation. This review also identifies a number of nutrients, i.e. fluoride, oleuropein, (phyto)estrogens, lactoferrin, strontium, vitamin K, vitamin D, and glucose, with great potential for enhancing bone health when applied in combination with mechanical stimuli. It is to be expected that this list can be expanded in the future as research progresses, holding great promise for finding a balanced therapeutic approach for the prevention of age-related bone loss.

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CHAPTER 3

MECHANICAL LOADING STIMULATES BMP7, BUT NOT BMP2, PRODUCTION BY OSTEOCYTES

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ABSTRACT

Bone mechanical adaptation is a cellular process that allows bones to adapt their mass and structure to mechanical loading. This process is governed by the osteocytes, which in response to mechanical loading produce signaling molecules that affect osteoblasts and osteoclasts. Bone morphogenic proteins (BMPs) are excellent candidates as signaling molecules, but it is unknown whether mechanically stimulated osteocytes affect bone adaptation through BMP production. Therefore, the aim of this study was to assess whether osteocytes produce BMPs in response to mechanical loading. In addition, since BMP7 has a vitamin D receptor (VDR) response element in the promoter region, we also investigated whether VDR is involved in the BMP7 response to mechanical loading. Human or $VDR^{-/-}$ mouse primary bone cells were submitted *in vitro* to 1 h pulsating fluid flow (PFF) and postincubated without PFF (PI) for 1–24 h, and gene and protein expression of BMP2 and BMP7 were quantified. In human bone cells, PFF did not change BMP2 gene expression, but it upregulated BMP7 gene expression by 4.4- to 5.6-fold at 1–3 h PI and stimulated BMP7 protein expression by 2.4-fold at 6 h PI. PFF did not stimulate BMP7 gene expression in $VDR^{-/-}$ mouse bone cells. These results show for the first time that mechanical loading upregulates BMP7, likely via the VDR, but not BMP2, gene and protein expression in osteocytes *in vitro*. Since BMP7 plays a major role in bone development and remodeling, these data might contribute to a better understanding of the mechanism leading to the mechanical adaptation of bone.

Keywords Bone adaptation; Bone morphogenic protein 2; Bone morphogenic protein 7; Osteocyte; Mechanical loading.

INTRODUCTION

Mechanical adaptation of bone is a cellular process that allows bones to adapt their mass and structure to their mechanical environment[1,2]. It is currently believed that this process of adaptation is governed by the osteocytes[3-6]. Osteocytes are terminally differentiated osteoblasts that become embedded deep within the mineralized bone matrix during bone formation. They are regularly distributed throughout the bone matrix and connected to each other by cytoplasmatic protrusions that run through the canaliculi[7]. This way the osteocytes form a unique dendritic network that enables contact not only with the bone surface but also with other cells[7]. When bones are loaded, the resulting deformation causes a flow of interstitial fluid through the lacunocanalicular network[8,9]. This flow of fluid results in mechanical stimulation of the osteocytes[6,8,9]. The mechanically stimulated osteocytes then produce signaling molecules that are potent regulators of the other types of bone cells, i.e., osteoblasts and osteoclasts, during bone remodeling[10,11]. A multitude of interacting signaling pathways are involved in translating the load applied on bone into the production of signaling molecules by osteocytes[12].

Bone morphogenic proteins (BMPs) are excellent candidates as signaling molecules that could be produced in response to mechanical loading. BMPs belong to a family of genetically conserved secreted signaling molecules of the transforming growth factor-β superfamily of polypeptides (for review, see Chen et al.[13])[14]. BMPs signal through heteromeric type I and type II serine-threonine receptors and activate the intracellular signalling molecules Smad1, Smad5, and Smad8 through serine phosphorylation[13]. Activated Smad proteins form heterodimers with the co-smad, Smad 4, and translocate to the nucleus to mediate the transcription of BMP-dependent target genes[13].

BMPs might have a relevant role in bone mechanotransduction. BMP2/4 is upregulated by mechanical loading through the induction of Indian hedgehog in chondrocytes, and the BMP antagonist noggin inhibits mechanical loading-induced chondrocyte proliferation[15]. This indicates that BMPs are part of the transduction pathway that is activated in response to mechanical loading in chondrocytes[15]. Mechanical loading of the rat achilles tendon upregulates BMP7 gene expression[16]. It upregulates BMP2 gene expression in endothelial cells of the vascular system[17]. This suggests that BMPs might be part of the signaling pathways that are activated upon mechanical stimulation. Remarkably, the data available on the role of BMPs in bone mechanotransduction are extremely limited.

Systematic administration of BMP7 enhances endosteal bone formation in response to mechanical loading in a rat tibial bending model, suggesting a possible role of BMP7 in the response of bone to mechanical loading *in vivo*[18]. A loss-of-function mutation at the BMP7 locus resulted in BMP7 knockout mice with specific skeletal defects. The most prevalent

effect was the failure of one or both of the seventh-pair ribs to fuse with the sternum. These results suggest that although BMP7 is not limited to skeletal development, BMP7 is involved in bone formation and patterning[19,20]. Furthermore, primary osteoblasts derived from C57BL/6J mice show upregulation of BMP-related genes, i.e., BMP4, BMP receptor 1, and BMP receptor 2, after mechanical loading by fluid shear stress[12]. Moreover, treatment with noggin ceases the fluid flow-induced osteoblast proliferation[12], indicating that the BMP signaling pathway likely contributes to the translation of fluid shear stress into the anabolic response of bone. However, thus far it is unknown whether mechanical adaptation of bone occurs through BMPs produced by mechanically stimulated osteocytes. To assess whether BMPs play a role in the process of mechanotransduction in bone, we studied the effect of mechanical loading on BMP production by osteocytes, which are considered the prime bone mechanosensor cells[1,5,6]. We focused on BMP2 and BMP7 since these BMPs are currently available as clinically approved recombinant human proteins for bone healing[21,22]. In addition, since BMP7 has a vitamin D receptor (VDR) response element (VDRE)[23], we also investigated whether VDR is involved in the BMP7 response to mechanical loading.

MATERIALS AND METHODS

Immunohistochemical Analysis

Four 12-week-old female Wistar rats (235 ± 12 g; Harlan, Zeist, the Netherlands) were killed for immunohistochemical analysis. The animal experiment was performed in accordance with the governmental guidelines for care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the VU University Medical Center (Amsterdam, the Netherlands). Tibiae from rats were retrieved and fixed with 4% (w/v) formaldehyde (buffered in phosphate-buffered saline [PBS], pH 7.4) at 4°C for 24 hours. After fixation, the tibiae were decalcified in 10% EDTA containing 0.5% formaldehyde in PBS at 4°C for 4.5 weeks. Tibiae were washed in PBS, dehydrated through an ascending series of ethanol and xylene at room temperature, and embedded in paraffin. After deparaffination in xylene and rehydration in a descending series of ethanol, tissue sections were rinsed in PBS. Endogenous peroxidase was blocked by incubation in 3% H2O2 in water for 3 min at room temperature. Potential endogenous binding sites for avidin and biotin were blocked using a biotin avidin blocking kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. Nonspecific binding sites were blocked by incubating tissue sections with normal goat serum (30%) for 30 min. Then, sections were incubated overnight at 4°C with primary antibodies to BMP2[24] (1:50) and BMP7[25] (1:100), kindly donated by Dr. J. Maliakal. Recombinant hBMP2 (COP-16, cloned in Escherichia coli) was used to raise the rabbit anti-BMP2 (rabbit 88) and recombinant hBMP7 (mature form, cloned in Chinese hamster

ovary cells) to raise chicken anti-BMP7 (chicken 881). After rinsing in PBS, tissue sections were incubated with biotinylated goat anti-rabbit IgG or biotinylated goat anti-chicken IgG (ABC peroxidase technique, Vectastain, Elite kit; Vector Labs) for 1 h at room temperature, rinsed, and incubated with ABC–peroxidase complex for 1 h at room temperature. After rinsing, the bound peroxidase complex was developed for 6 min at room temperature with DAB kit (Vector Labs) and sections were counterstained with hematoxylin. Kidney sections were used as a positive control. As a negative control, primary antibodies were replaced by normal rabbit or chicken IgG (Vector Labs).

Primary Human Bone Cells and Primary Mouse Bone Cells

For primary human bone cell isolation, trabecular bone samples (surgical waste) from seven female and two male donors (mean age 43, SD 23.7, range 18–84 years) were obtained from the proximal femoral shaft during hip replacement surgery for coxarthrosis. The protocol was approved by the Ethical Review Board of the VU University Medical Center, and all subjects gave informed consent.

Mouse long bone cells were obtained from the limbs of adult VDR knockout ($VDR^{-/-}$) mice[26]. To isolate $VDR^{-/-}$ mouse bone cells, $VDR^{-/-}$ mice were generated as described previously[26] (kindly provided by Dr. G. Carmeliet, Katholieke Universiteit, Leuven, Belgium). All mice were bred at the local animal housing facility (Proefdierencentrum, Leuven, Belgium) and lived in conventional conditions: 12-h light/dark cycle, standard diet (1% calcium, 0.76% phosphate), and water ad libitum. All experimental procedures were conducted after obtaining formal approval from the ethical committee of the Katholieke Universiteit Leuven.

Human trabecular bone biopsies were placed in sterile PBS, chopped into small fragments, and washed extensively with PBS. Long bones from $VDR^{-/-}$ mice were aseptically harvested, epiphyses were cut off, and bone marrow was flushed out using a syringe and needle. The diaphyses were chopped into small fragments and washed extensively with PBS. Human and mouse bone fragments were treated in exactly the same way, as described previously[27]. To obtain outgrowth of the bone cells, bone fragments were cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 100 U/ml penicillin (Sigma, St. Louis, MO), 50 µg/ml streptomycin sulfate (GIBCO), 50 µg/ml gentamicin (GIBCO), 1.25 µg/ml fungizone (GIBCO), 100 µg/ml ascorbate (Merck, Darmstadt, Germany), and 10% FBS. After reaching subconfluence, outgrowth bone cells were replated at 5 × 103 cells/75 cm2 flask until enough cells were obtained. All cells used were from passage 2 or less. For pulsating fluid flow (PFF) experiments outgrowth bone cells from humans or $VDR^{-/-}$ mice were harvested and seeded at a density of 5 × 105 cells per polylysine-coated (50 µg/ml; poly-l-lysine hydrobromide, mol wt 15 to 30 × 104; Sigma) glass slide (5 cm2) and incubated overnight to promote cell attachment.

Pulsating Fluid Flow

Pulsating fluid flow was generated by pumping 13 ml of culture medium, using a roller pump, through a parallel-plate flow chamber as described previously[5,8,28]. Briefly, fluid shear stress of 0.7 ± 0.3 Pa at 5 Hz was induced for 1 h on the monolayer of human and mouse primary bone cells by circulating 13 ml of DMEM containing 10% FBS plus antibiotics. Static control cultures (Co) were kept in a Petri dish under similar conditions as the experimental cultures. After 1 h of PFF or control treatment, human primary bone cells were postincubated without PFF (PI) for 1–24 h in 2.5 ml fresh medium and lysed for total RNA isolation or medium was collected for extracellular BMP2 and BMP7 protein quantification. $VDR^{-/-}$ mouse primary bone cells were lysed directly after 1 hour of PFF for total RNA isolation.

Nitric Oxide

Nitric oxide (NO) was measured as nitrite (NO^{2-}) accumulation in the medium using Griess reagent containing 1% sulfanylamide, 0.1% naphthylethelene-diamine-dihydrochloride, and 2.5 M H3PO4. Serial dilutions of NaNO2 in nonconditioned medium were used as a standard curve. Absorbance was measured at 540 nm with a microplate reader (Bio-Rad Laboratories, Richmond, CA).

Total DNA Content

Human primary bone cells were lysed with 0.5 ml of ice-cold Milli-Q water, harvested on ice, sonicated for 10 min, and centrifuged for 10 min at 2,000 rpm at room temperature. The supernatants were analyzed for total DNA content, which was quantified using the Cyquant Cell Proliferation Assay (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

Analysis of Gene Expression

Gene expression of rat and human BMP2 and BMP7 was analyzed using Taq-Man® Gene Expression Assays (Applied Biosystems, Foster City, CA) in an ABI Prism 7700 DNA sequence detector. Gene-expression values were normalized for human or mouse GAPDH (Inventoried TaqMan, Gene Expression Assays).

BMP2 and BMP7 Protein Quantification

Immediately after PFF treatment or static control culture, human primary bone cells were postincubated without PFF for 6 or 24 h in 1.5 ml fresh culture medium. At 6 and 24 h, human BMP2 was quantified in the culture medium using the Quantikine® BMP2 Assay (R&D Systems, Minneapolis, MN), and human BMP7 was quantified using the Quantikine BMP7 Assay (R&D Systems) according to the manufacturer's protocol.

Statistical Analysis

For statistical analysis of total DNA content and NO data, differences between control and PFF groups were tested with Student's two-tailed t test for paired groups. NO data were log-transformed in order to obtain normality. For statistical analysis of PFF treatment or mechanical loading-over-static control ratios (PFF/Co), human BMP2 and BMP7 gene expression values are expressed as mean ± SEM. Differences of PFF/Co ratios were tested with a one-tailed t-test for single group mean and compared to 1 (PFF/control or loading/control = 1, no difference). Values of the total amount of human BMP2 and BMP7 protein are provided as mean ± SEM. Differences between control and PFF groups were tested with t test for paired comparison of means. Differences were considered significant if $P < 0.05$.

RESULTS

To determine whether osteocytes express BMP2 *in vivo*, immunohistochemical staining was performed in rat tibia (Fig. 1). BMP2 staining was intense in chondrocytes of the resting zone and hypertrophic chondrocytes of the growth plate (Fig. 1A). BMP2-positive cells were found in bone marrow (Fig. 1B) and trabecular and cortical bone (Fig. 1B). In cortical bone, osteoblasts stained positive for BMP2 (Fig. 1B, C), while osteocytes stained either clearly positive or completely negative (Fig. 1B, C). No staining was found in negative controls, in which nonimmune IgG was replaced by the primary antibody (Fig. 1D).

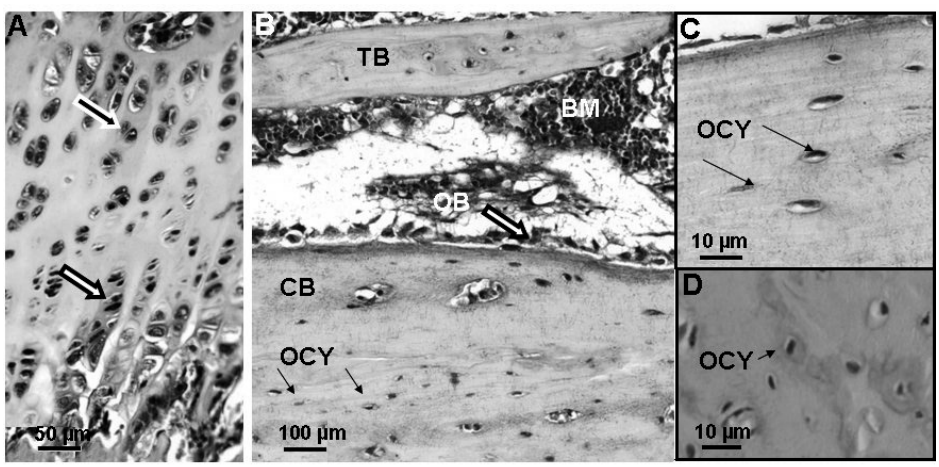

Figure 1. BMP2 expression in rat tibia. (A) BMP2-positive staining (brown) was intense in chondrocytes of the resting zone (white arrow) and in hypertrophic chondrocytes of the growth plate (black arrow). (B) BMP2-positive cells were found in bone marrow (BM), trabecular bone (TB), and cortical bone (CB). In cortical bone, osteoblasts (OB, white arrow) stained positive for BMP2, while osteocytes (OCY, black arrows) stained either clearly positive or completely negative. (C) High magnification of cortical bone showing osteocytes that stained positive or negative for BMP2. (D) Negative control.

To assess whether osteocytes express BMP7 in vivo, immunohistochemical staining against BMP7 was performed in rat tibia (Fig. 2). BMP7 staining was intense in chondrocytes of the resting zone, proliferative zone, and hypertrophic chondrocytes of the growth plate (Fig. 2A). BMP7-positive cells were found in bone marrow (Fig. 2B) and cortical bone (Fig. 2B). In cortical bone osteoblasts stained positive for BMP7 (Fig. 2C). In osteocytes of cortical bone, a gradient of staining intensity in BMP7 was observed from the endosteal to the periosteal side, with osteocytes showing the highest staining intensity on the endosteal side (Fig. 2B, C). No staining was found in negative controls, in which nonimmune IgG replaced the primary antibody (Fig. 2D).

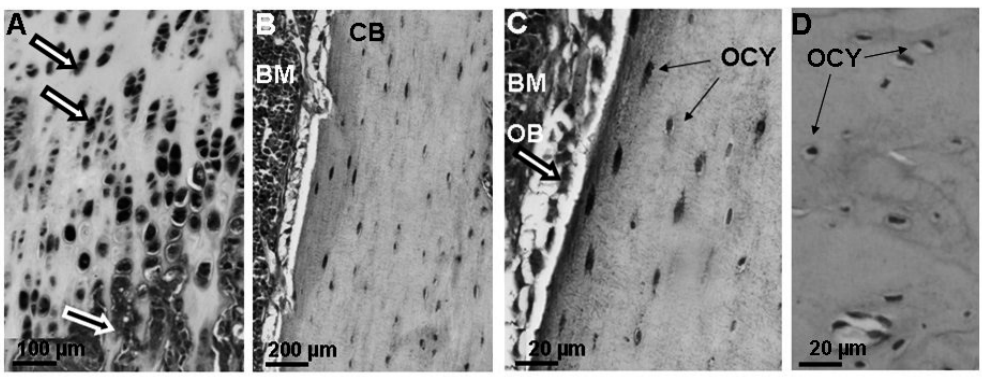

Figure 2. BMP7 expression in rat tibia. (A) BMP7-positive staining (brown) was intense in chondrocytes of the resting zone (white arrow) and in hypertrophic chondrocytes of the growth plate (black arrow). (B) BMP7-positive cells were found in bone marrow (BM) and cortical bone (CB). (C) High magnification of cortical bone. (D) Negative control. In cortical bone, a gradient staining intensity of BMP7-positive cells was observed, with osteoblasts (OB, white arrow) and osteocytes (OCY, black arrows) at the endosteal surface mostly staining positive for BMP7.

To determine whether osteocytes respond to mechanical loading with modulation of BMP2 and BMP7 gene expression, human primary bone cells were submitted to 1 h of PFF (Fig. 3). No cells were removed by the fluid flow treatment, as assessed by quantification of the DNA content (control 33.4 ± 3.8 ng/ml, PFF 32.5 ± 3.6 ng/ml; P = 0.5). NO release was measured after 5 min of PFF as a parameter for bone cell responsiveness. Five minutes of PFF treatment rapidly increased NO production by 3.2-fold (P = 0.036, Fig. 3A). One hour PFF increased BMP2 gene expression by 1.3-fold, but this value did not reach significance (P = 0.2, Fig. 3B). Although BMP2 gene expression levels decreased after 1 h of PFF treatment followed by 1–3 h of postincubation without PFF (PI), the values did not reach significance (P = 0.1 and P = 0.2, Fig. 3B). In contrast, 1 h PFF followed by 1–3 h of PI upregulated BMP7 gene expression by 4.4- to 5.6-fold (Fig. 3C), indicating that mechanical loading upregulates BMP7 gene expression in human primary bone cells.

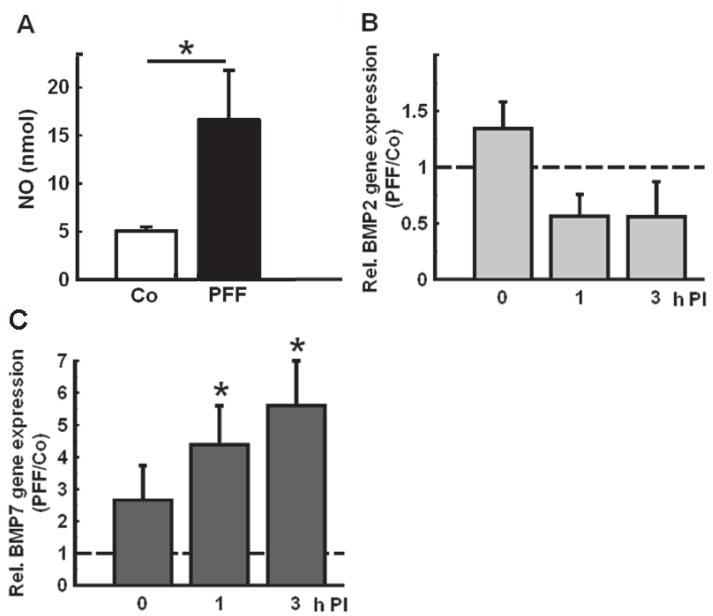

Figure 3. PFF increases NO production and upregulates gene expression of BMP7, but not BMP2, in human primary bone cells. (A) Five minutes of mechanical loading by PFF enhanced NO production. (B) PFF did not affect BMP2 gene expression. (C) One hour of PFF followed by 1 and 3 hours of PI upregulated BMP7 gene expression. Values were normalized for human GAPDH and expressed as mean ± SEM of absolute values and PFF-over-control ratios of five independent donors. PFF pulsating fluid flow, Co static control, PI postincubation without PFF. *Significant effect of PFF, $P < 0.05$.

To verify whether PFF-induced changes in gene expression result in increased protein expression, human primary bone cells were submitted to 1 h PFF and postincubated for 6–24 h without PFF (Fig. 4). Under static control conditions, human bone cells produce 144.6 ± 1.6 pM BMP2 protein (Fig. 4A), while basal levels of BMP7 protein are 12.2 ± 1.1 pM (Fig. 4B). This indicates that human primary bone cells produce 11.8-fold more BMP2 than BMP7 ($P = 0.001$) under static control conditions. One hour PFF followed by 6–24 h of PI did not change BMP2 protein levels when compared to static controls (Fig. 4A). However, 1 h PFF followed by 6 h PI significantly upregulated BMP7 protein levels by 2.4-fold (Fig. 4B).

To investigate how osteocytes increase BMP7 production in response to mechanical loading, $VDR^{-/-}$ mouse primary bone cells were submitted to mechanical loading by PFF in vitro (Fig. 5). NO release was measured after 5 min of PFF as a parameter for bone cell responsiveness. Five minutes of PFF treatment increased NO production by 1.8-fold ($P = 0.023$, Fig. 5A). Our preliminary data on BMP7 gene expression show that PFF did not change BMP7 gene expression in these cells (Fig. 5B), suggesting that the VDR mediates mechanical loading-induced upregulation of BMP7 in osteocytes.

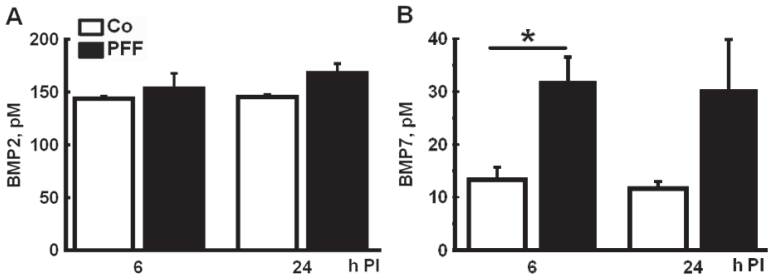

Figure 4. PFF upregulates protein expression of BMP7, but not BMP2, in human primary bone cells. (A) PFF did not affect protein expression of BMP2. (B) One hour of PFF followed by 6 and 3 hours of PI upregulated BMP7 protein expression. Values were expressed as mean ± SEM of PFF-over-static control ratios of five independent donors. PFF, pulsating fluid flow; Co, static control; PI, postincubation without PFF. *Significant effect of PFF, $P < 0.05$.

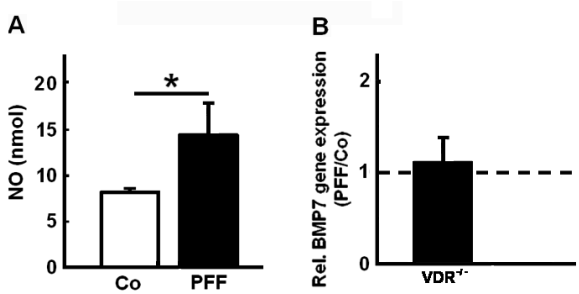

Figure 5. PFF increases NO production but does not alter gene expression of BMP7 in $VDR^{-/-}$ mouse primary bone cells. (A) 5 min of mechanical loading by PFF enhanced NO production. (B) PFF did not affect gene expression of BMP7 in $VDR^{-/-}$ mouse primary bone cells. Values were normalized for mouse GAPDH and expressed as mean ≤ SEM of absolute values and PFF-over-control ratios of three independent donors. PFF pulsating fluid flow, Co static control. *Significant effect of PFF, $P < 0.05$.

DISCUSSION

Bone is a living tissue that is able to adapt its mass and structure to mechanical demands throughout life[2]. Osteocytes are proposed as the key cells that convert the physical stimuli resulting from daily loading into a biological signal by the secretion of signaling molecules in a process known as mechanotransduction[1,5,8]. These mechanostimulated molecules activate a wide spectrum of signaling pathways fundamental for bone resorption by osteoclasts and/or bone formation by osteoblasts[12]. BMPs represent a class of key proteins for the regulation of cell–cell communication, cell differentiation, and morphogenesis of several organs[13,29]. In bone, BMPs are multifunctional regulators of proliferation and differentiation during development[30,31] and are required for osteoblast gene expression and differentiation[30–33]. BMPs represent one of the most studied growth factors due to their

relevance in bone regeneration. However, it is unclear what role BMPs, specifically BMP2 and BMP7, play in the process of bone mechanotransduction. Therefore, we studied the effect of mechanical loading on BMP production by osteocytes, which are considered the prime bone mechanosensor cells[1, 3–5].

We used human primary bone cells obtained as outgrowth from collagenase-stripped bone pieces to study whether osteocytes in vitro respond to mechanical loading with production of BMP2 and BMP7. Although the exact nature of the outgrowth cells is not completely known, human primary bone cells may be considered bone cells since they express alkaline phosphatase, cfba-I[32,33], and osteocalcin[32-34]. In addition, these cells respond to mechanical loading with increased expression of COX-2[23], indicating that these bone cell cultures have an osteocyte-like phenotype since mechanical loading induces increased gene expression of COX-2 in chicken osteocytes but not osteoblasts[35]. Furthermore, these cells express detectable levels of MEPE, DMP1, and SOST (unpublished observations), which are considered osteocyte markers[36].

We found that PFF, representing a physiological loading regime[28], significantly increased BMP7, but not BMP2, gene and protein expression in human osteocytes, indicating that upregulation of BMP7 represents a physiological response to mechanical loading of bone. Our findings are supported by the observation of others that load-induced bone formation in vivo is enhanced by BMP7[18], indicating that BMP7 might be an essential molecule in the process of mechanically driven bone formation.

Contradictory data have been reported on the effects of mechanical loading on BMP production in bone. Compressive forces and shear stress upregulate expression of BMPs 2, 4, 6, and 7 in osteoblasts in vitro[12,37]. Upregulation of BMPs 2, 4, and 6 was observed during distraction osteogenesis; but BMP7 expression did not change during the distraction[38]. Mechanical tension during distraction osteogenesis *in vivo* increases BMP2 and BMP4, but not BMP7, gene expression[39]. These observations differ from our results, showing that mechanical loading stimulated BMP7, but not BMP2, expression in cultured osteocytes. In addition, we assessed expression of BMP4 and BMP6 (data not shown). BMP4 gene expression seemed to be reduced after PFF treatment. BMP6 gene-expression data were not consistent; therefore, we cannot exclude a role for BMP6 in the anabolic response of osteocytes to mechanical loading. It is generally accepted that osteocytes are the primary sensors of mechanical loading in bone, rather than osteoblasts[1,5-8]. Osteocytes and osteoblasts respond differently in terms of loading-induced production of signaling molecules[2,8,11]. Therefore, differences in mechanical loading regimes and characteristics between osteocytes and osteoblasts might explain the differences in loading-induced BMP expression between experiments.

To investigate how osteocytes increase BMP7 production in response to mechanical loading, we searched for signaling pathways that are activated upon loading and could mediate this response. Since both BMP7 protein expression and gene expression were affected by loading, we focused on signaling pathways that activate the BMP7 promoter sequence. BMP2 gene expression remained unchanged, but BMP7 gene expression and protein expression were affected by mechanical loading, although BMPs are highly conserved proteins. Thus, we focused on possible differences between the promoter region of BMP2 and BMP7. The BMP7, but not the BMP2, promoter does contain VDREs[23]. Therefore, we hypothesized that the VDR is involved in the increase in BMP7 gene expression in response to mechanical loading. To test our hypothesis, $VDR^{-/-}$ mouse primary bone cells[26] were submitted to mechanical loading by PFF *in vitro*. Our data show that PFF does not change BMP7 gene expression in these cells (Fig. 5), suggesting that the VDR mediates mechanical loading-induced upregulation of BMP7 in osteocytes. Although $VDR^{-/-}$ mouse primary bone cells were derived from mice rather than humans, they were obtained in exactly the same manner. In addition, mouse and human primary bone cells show the same response to mechanical loading as illustrated by the increased NO production in response to mechanical loading.

It is well established that bone cells and chondrocytes produce BMPs[13]. Similar to observations by others, we also show moderate to intense expression of BMP2 and BMP7 in bone marrow cells, bone lining cells, osteoblasts, and osteocytes[40,41]. We observed a gradient of BMP7 expression, varying from intense staining at the endosteal side to no staining at the periosteal side of cortical bone. In addition, basal levels of BMP2 expression were significantly higher than basal BMP7 expression in cultured human osteocytes, and BMP7, but not BMP2, was produced in response to mechanical loading. Since BMP2 is essential for recruitment of bone marrow cells and initial cell lineage commitment, while BMP7 has a relevant role in the maturation process of preosteoblasts, these finding might have implications for bone remodeling. However, the role of BMPs in bone remodeling is not fully understood. BMP2 is produced by bone marrow stromal cells and necessary for osteoblast differentiation[40,41]. Cheng and colleagues[42] reported an osteogenic hierarchy where BMPs 2, 6, and 9 are the most potent agents to induce osteoblast lineage-specific differentiation of mesenchymal progenitor cells, while most BMPs can promote the terminal differentiation of committed osteoblast precursors and osteoblasts. BMP7 increases bone formation primarily by promoting preosteoblast growth and differentiation without affecting bone matrix production by individual osteoblasts *in vitro*[43]. Systemic administration of BMP7 stimulates the differentiation of bone lining cells, fibroblasts, and other committed cells to osteoblasts but does not increase putative stem cell proliferation[18]. Thus, it is possible that the mature cells of the osteoblastic lineage, i.e., the osteocytes, respond to mechanical

loading with increased BMP7 production to enhance differentiation of already committed osteogenic cells, while high basal BMP2 expression guarantees the differentiation of bone marrow cells into preosteoblasts. In other words, BMP2 seems to be essential for general bone formation, while BMP7 is required for local bone formation in response to mechanical loading. Future functional studies are needed to assess the precise functional role of loading-induced BMP7 production in bone (re)modeling.

In bone biology, the mechanisms by which bones adapt to mechanical demands are still a challenging area of research. We show for the first time that physiological loading regimes induce increased BMP7 gene and protein expression in osteocytes, the bone mechanosensor cells. Basal BMP2 protein expression levels were significantly higher than BMP7 levels in osteocytes, while mechanical loading did not affect BMP2 gene or protein expression. Thus, it is likely that BMP7 plays a role in mechanotransduction in osteocytes. Our data might contribute to a better understanding of the mechanisms that lead to bone mechanical adaptation. Unraveling the signaling pathways that lead to an optimal response to physiological loading regimes might provide new insights for therapeutic approaches in bone biology.

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CHAPTER 4

VDR DEPENDENT AND INDEPENDENT EFFECTS OF 1,25-DIHYDROXYVITAMIN D_3 ON NITRIC OXIDE PRODUCTION BY OSTEOBLASTS

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ABSTRACT

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) strongly mediates bone mass. Mechanical stimulation also affects bone mass, partly via enhancing nitric oxide (NO) production by osteoblasts. We aimed to determine whether $1,25(OH)_2D_3$ affects NO production by osteoblasts in the presence or absence of mechanical stimulation. We hypothesised that $1,25(OH)_2D_3$ stimulates NO production via nuclear actions of the vitamin D receptor (VDR), which requires hours of incubation with $1,25(OH)_2D_3$ to occur. MC3T3-E1 osteoblasts and long-bone osteoblasts of adult wildtype and $VDR^{-/-}$ mice were pre-incubated for 24h with or without $1,25(OH)_2D_3$ (10^{-13}-10^{-9} M), followed by 30 min pulsating fluid flow (PFF; 0.7±0.3 Pa, 5 Hz) or static culture with or without $1,25(OH)_2D_3$. NO production and NO synthase (NOS) expression were quantified. 10^{-11} M $1,25(OH)_2D_3$ for 24h, but not 30 min, stimulated NO production by MC3T3-E1 osteoblasts (8-fold). $1,25(OH)_2D_3$ for 24 h increased inducible-NOS gene-expression (2-fold), suggesting that $1,25(OH)_2D_3$ stimulated NO production via activation of NOS gene transcription. PFF rapidly increased NO production by MC3T3-E1 osteoblasts, wildtype osteoblasts, and $VDR^{-/-}$ osteoblasts. This PFF effect was abolished after incubation with $1,25(OH)_2D_3$ for 24 h, or during PFF only. Our results suggest that $1,25(OH)_2D_3$ stimulates inducible-NOS expression and NO production by osteoblasts in the absence of mechanical stimulation, likely via genomic VDR action. In contrast, $1,25(OH)_2D_3$ may affect mechanical loading-induced NO production independent of genomic VDR action, since $1,25(OH)_2D_3$ diminished PFF-induced NO production in $VDR^{-/-}$ bone cells. In conclusion, $1,25(OH)_2D_3$ and mechanical loading interact at the level of mechanotransduction, whereby $1,25(OH)_2D_3$ seems to act independently of VDR genomic mechanism.

Key words Osteoblasts; mechanical loading; 1,25-dihydroxyvitamin D_3; vitamin D receptor; $VDR^{-/-}$ mice; nitric oxide synthase.

INTRODUCTION

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a steroid hormone, which serves as a signalling molecule in almost every human tissue, including bone[1]. The action of $1,25(OH)_2D_3$ on bone is ambiguous. On the one hand, $1,25(OH)_2D_3$ increases bone mineralization, stiffness, and strength directly via stimulation of calcium and phosphate supply, mainly by absorption from the gut[2]. On the other hand, $1,25(OH)_2D_3$ has clear direct effects on osteoblasts and osteoclasts[3]. $1,25(OH)_2D_3$ not only stimulates osteocalcin and alkaline phosphatase production by osteoblasts[3], but also affects osteopontin expression in osteoblasts[4], and bone resorption in vitro. Considering the biological significance of $1,25(OH)_2D_3$ in the regulation of bone mass, it is surprising how little is known about the mechanism by which it stimulates bone mass, and how the actions of $1,25(OH)_2D_3$ on bone interact with other bone mass-regulating factors, such as mechanical loading.

The genomic responses to $1,25(OH)_2D_3$ are mediated by the formation of a ligand-receptor complex of $1,25(OH)_2D_3$ with the vitamin D receptor (VDR), a member of the super-family of steroid hormone nuclear receptors[5]. The specific binding of $1,25(OH)_2D_3$ to nuclear components of small intestinal mucosa was demonstrated for the first time in vitamin D-deficient chicks[6]. In addition to the VDR-mediated actions, $1,25(OH)_2D_3$ seems to mediate cellular responses via a non-VDR mediated mechanism. These non-VDR mediated cellular responses to $1,25(OH)_2D_3$ appear to be facilitated by a variety of receptor types located within or near the plasma membrane or caveolae[7]. The existence of a specific $1,25(OH)_2D_3$ membrane coupled receptor is subject of debate[8], although a possible receptor for $1,25(OH)_2D_3$ was isolated from chick intestinal basolateral membranes and called $1,25(OH)_2D_3$-membrane-associated rapid response to steroid ($1,25D_3$-MARRS)[9]. The membrane receptor associated rapid responses to $1,25(OH)_2D_3$, which are mediated by $1,25D_3$-MARRS or other receptors, include activation of phospholipase A_2, increased membrane fluidity, and activation of phospholipase C, leading to activation of protein kinase C and release of intracellular calcium[10,11], and vary in response time from seconds (opening of ion channels) to 10-60 min. In contrast, the VDR-mediated genomic actions of $1,25(OH)_2D_3$ take generally much longer, i.e. hours, to occur. Recently it was demonstrated that $25(OH)D_3$ is equipotent to $1,25(OH)_2D_3$ in opening calcium channels[12]. These new findings suggest the ability of $25(OH)D_3$ as well as $1,25(OH)_2D_3$ to generate biological responses *in vivo*[12].

The VDR-mediated genomic actions of $1,25(OH)_2D_3$ occur by coupling of the VDR to VDR response elements (VDRE) in the promoter regions of $1,25(OH)_2D_3$ target genes[1]. Genes containing a VDRE often encode for proteins involved in the regulation of osteoclast formation and/or activity, or osteoblast differentiation, such as RANKL or nitric oxide synthase (NOS)[13]. Three NOS isoforms are known, endothelial NOS (ecNOS), neuronal NOS (nNOS),

and inducible NOS (iNOS)[14,15]. NOS is responsible for the synthesis of nitric oxide (NO) from L-arginine[16]. NO is a short-lived free radical with an important physiological role in the skeletal system[15]. NO stimulates osteoblast activity, e.g. 17β-estradiol dose-dependently stimulates proliferation and differentiation of cultured primary human and rat osteoblasts, which is abolished by inhibition of NOS activity[17]. $1,25(OH)_2D_3$ is a steroid hormone like 17β-estradiol and androgens, which are known to stimulate NO production in osteoblasts[18,19]. Therefore $1,25(OH)_2D_3$ might also stimulate NO production. Indeed $1,25(OH)_2D_3$ has been shown to modulate iNOS expression in rat brain cells[20,21]. Therefore some of the effects of $1,25(OH)_2D_3$ on bone might be mediated by enhanced NO production by osteoblasts and/or osteoclasts, or by bone cells in close proximity to osteoblasts and osteoclasts, such as osteocytes.

Both $1,25(OH)_2D_3$ and mechanical loading of bone positively affect bone mass. Importantly, the NO production by mechanosensitive cells is essential for the anabolic effect of mechanical loading on bone. The increase in bone mass as a result of mechanical loading-induced NO production likely occurs as follows. When load is applied to bone, the deformation of the bone matrix will result in a flow of the thin layer of interstitial fluid that surrounds the network of osteocytes lying in the calcified bone matrix[22,23]. As a result, mechanosensitive bone cells produce signalling molecules, amongst others NO, that signal to the osteoblasts and/or osteoclasts to change their bone remodelling activities[24]. Interestingly, $1,25(OH)_2D_3$ and mechanical loading may interact at the cellular level to increase bone mass[25].

It is unknown whether $1,25(OH)_2D_3$ affects NO production by osteoblasts, and if so, whether this effect occurs via genomic actions of the VDR. Since mechanical stimulation is known to enhance NO production by osteoblasts, and $1,25(OH)_2D_3$ and mechanical loading may show interaction at the cellular level in the regulation of bone mass, the aim of the present study was to determine whether $1,25(OH)_2D_3$ affects the production of NO by osteoblasts that are either or not mechanically stimulated. We hypothesised that $1,25(OH)_2D_3$ stimulates NO production by osteoblasts via nuclear actions of the vitamin D receptor (VDR), rather than via rapid membrane receptor-mediated mechanisms.

MATERIALS AND METHODS

Osteoblast culture

MC3T3-E1 cells were cultured in α-minimal essential medium (α-MEM; Gibco, Paisly, UK) supplemented with 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 µg/ml streptomycin (Sigma-Aldrich), 1.4 mM L-glutamine (Sigma), 9 mM β-glycerophosphate (Sigma-Aldrich), 1.9 mM ascorbate (vitamin C; Merck, Darmstadt, Germany), and 10% fetal bovine serum (FBS; Gibco). Cells were cultured with or without $1,25(OH)_2D_3$ and used for pulsating fluid flow (PFF) experiments as described below.

Osteoblasts from wildtype and VDR$^{-/-}$ mice

Mice were bred at the local animal housing facility ("Proefdierencentrum", Leuven, Belgium), and living under conventional conditions, i.e. standard diet (1% calcium, 0.76% phosphate), and water *ad libitum*. All experimental procedures were conducted after approval was obtained from the ethical committee of the Katholieke Universiteit Leuven. Mouse long bone cells were obtained from the limbs of adult VDR$^{-/-}$ [26] and wildtype (wt) littermates. Long bones were aseptically harvested, chopped into small fragments, and incubated with collagenase II (Sigma) at 2 mg/ml in Dulbecco's modified Eagle's medium (DMEM; Gibco) at 37°C in a shaking water bath. Bone fragments were washed with medium containing 10% FBS and transferred to 25 cm^2 flasks (Nunc, Roskilde, Denmark). Bone fragments were cultured in DMEM supplemented with 100 U/ml penicillin (Sigma), 50 µg/ml streptomycin sulfate (Gibco), 50 µg/ml gentamicin (Gibco), 1.25 µg/ml fungizone (Gibco), 100 µg/ml ascorbate, and 10% FBS. Cells were used at passage 1 for 1,25(OH)$_2$D$_3$ treatment and PFF-experiments.

1,25(OH)$_2$D$_3$ treatment

Cells were seeded onto polylysine-coated (50 µg/ml; poly-L-lysine hydrobromide, mol wt 15-30 x 10^4; Sigma) glass slides (size 2.5x6.5 cm) at 2x10^5 cells/glass slide, and cultured overnight in petridishes with 13 ml α-MEM plus supplements as described under "osteoblast culture". Next the culture medium was replaced by α-MEM (MC3T3-E1) or DMEM (osteoblasts from wt or VDR$^{-/-}$ mice) containing 0.2% bovine serum albumin (BSA), antibiotics, ascorbate, and 0, 10^{-9}, 10^{-11}, or 10^{-13} M 1,25(OH)$_2$D$_3$ for 24 h. 1,25(OH)$_2$D$_3$ was dissolved in 100% ethanol, and media for all treatment and control groups contained 0.001% ethanol. Cells were then either lysed for total RNA isolation or used for PFF treatment.

Pulsating fluid flow (PFF)

PFF was applied as described earlier[18]. Briefly, PFF was generated by pumping 13 ml of culture medium containing 0, 10^{-9}, 10^{-11}, or 10^{-13} M 1,25(OH)$_2$D$_3$ through a parallel-plate flow chamber containing osteoblasts. Osteoblasts were subjected for 30 min to a 5 Hz pulse with a mean shear stress of 0.7 Pa, a pulse amplitude of 0.3 Pa, and a peak shear stress rate of 8.4 Pa/s. Stationary control cultures were kept in a petridish under similar conditions as experimental cultures. After 5, 10, 15, and 30 min of PFF or static control culture, medium was collected and assayed for NO concentrations.

Nitric oxide (NO)

NO was measured as nitrite (NO_2^-) accumulation in the medium using Griess reagent containing 1% sulfanylamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in non-conditioned medium were used as a standard curve. Absorbance was measured at 540 nm with a microplate reader (BioRad Laboratories Inc.).

Total RNA, total protein, and cDNA synthesis

RNA and protein were isolated using TRIzol reagent (Gibco) according to the manufacturer's instructions. The RNA content was determined by measuring the absorbance in water at 260 nm using an Ultrospec III spectrophotometer (Amersham). cDNA synthesis was performed using 1 µg total RNA in a 20 µl reaction mix consisting of 5 units Transcriptor Reverse Transcriptase (Roche Diagnostics), 0.08 A_{260} units random primers (Roche Diagnostics), 1 mM of each dNTP (Invitrogen), and 1x concentrated Transcriptor RT reaction buffer (Roche Diagnostics).

Analysis of gene expression

Gene expression of mouse iNOS, nNOS, and ecNOS was analyzed using Taqman® Gene expression assays (Inventoried Taqman® Gene expression assays, Applied Biosystems) in an ABI Prism 7700 sequence detector (Applied Biosystems). Gene expression values were normalized for mouse GAPDH (Inventoried Taqman® Gene expression assays, Applied Biosystems).

Statistical analysis

Data on the effect of 24 h $1,25(OH)_2D_3$ was obtained from 6 experiments, and data on the effect of 30 min $1,25(OH)_2D_3$ during PFF or static culture from 5 experiments. Data using primary mouse bone cells was obtained from 6 $VDR^{-/-}$ and 4 wild type mice. NO data are expressed as absolute values, and as PFF-treated-over-static control ratios. Data were analysed using the Wilcoxon signed-rank test for paired observations. Differences were considered significant if $p<0.05$. All data are expressed as mean ± SEM.

RESULTS

We first tested whether $1,25(OH)_2D_3$ affects NO production by MC3T3-E1 osteoblasts cultured under static conditions. Twenty-four hour incubation with $1,25(OH)_2D_3$ stimulated NO production by MC3T3-E1 osteoblasts (Fig. 1A), with a maximal 8-fold stimulation by 10^{-11} M $1,25(OH)_2D_3$ at 30 min.

Thereafter we investigated whether $1,25(OH)_2D_3$ stimulated NOS gene expression. MC3T3-E1 osteoblasts were cultured with or without $1,25(OH)_2D_3$ for 24 h, and NOS gene expression was determined. $1,25(OH)_2D_3$ at 10^{-9} M increased iNOS gene expression by 2 fold ($p=0.049$, $n=3$), and 10^{-11} M $1,25(OH)_2D_3$ showed a similar trend ($p=0.06$, $n=3$, Fig. 1B). $1,25(OH)_2D_3$ at all concentrations tested did not alter nNOS or ecNOS gene expression (data not shown).

We then investigated whether $1,25(OH)_2D_3$ affects mechanical loading-induced NO production. MC3T3-E1 osteoblasts were cultured for 24 h in the presence or absence of $1,25(OH)_2D_3$ and subjected to 30 min of PFF. PFF caused a rapid increase in NO production immediately after the start of PFF treatment in the absence of $1,25(OH)_2D_3$ (Fig. 1C). Twenty-four h incubation with $1,25(OH)_2D_3$ at all concentrations tested (10^{-9}, 10^{-11}, 10^{-13} M) significantly inhibited the PFF-induced NO production by 3-9 fold at 10 min (Fig. 1C).

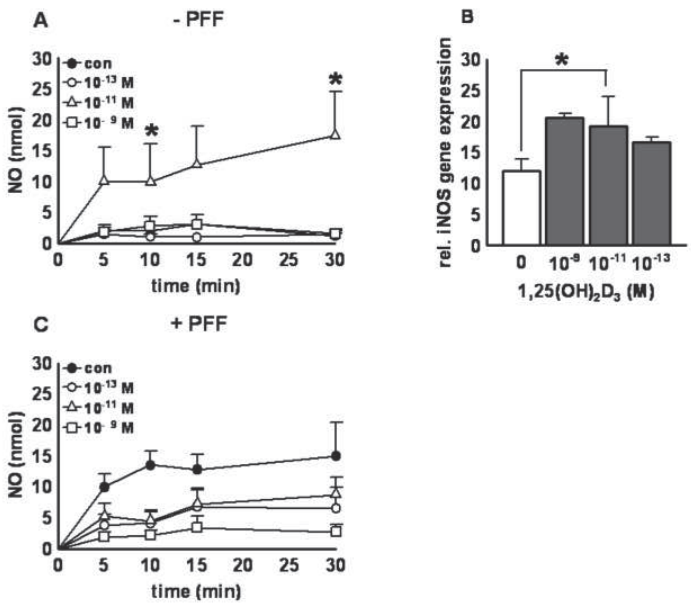

Figure 1. Twenty-four hour incubation with $1,25(OH)_2D_3$ (10^{-11}M) stimulated NO production and iNOS gene expression, and inhibited mechanical loading-stimulated NO production by MC3T3-E1 osteoblasts. (A) $1,25(OH)_2D_3$ induced NO production measured during the last 30 min of a 24 h incubation period with $1,25(OH)_2D_3$. (B) $1,25(OH)_2D_3$ induced iNOS gene expression. (C) $1,25(OH)_2D_3$ inhibited mechanical loading-stimulated NO production. Values are mean ± SEM of 6 independent experiments. * Significant effect of $1,25(OH)_2D_3$ on NO-production, $p<0.05$.

We verified whether genomic actions of the VDR were responsible for the inhibiting effect of $1,25(OH)_2D_3$ on PFF-induced NO production. To this end, we incubated wt and $VDR^{-/-}$ osteoblasts for 24 h with 10^{-13} M $1,25(OH)_2D_3$, which is the lowest concentration affecting PFF-induced NO production in MC3T3-E1 osteoblasts, and subjected the cells to PFF. $1,25(OH)_2D_3$ did not stimulate NO production by wt or $VDR^{-/-}$ osteoblasts under static culture conditions, which is similar to our findings in MC3T3-E1 osteoblasts. PFF stimulated NO production in wt osteoblasts ($p=0.006$) (Fig. 2A) and $VDR^{-/-}$ osteoblasts ($p= 0.006$) (Fig. 2B). Remarkably $1,25(OH)_2D_3$ inhibited mechanical loading-induced NO production in both wt ($p=0.02$) and $VDR^{-/-}$ osteoblasts ($p=0.04$) (Fig. 2B).

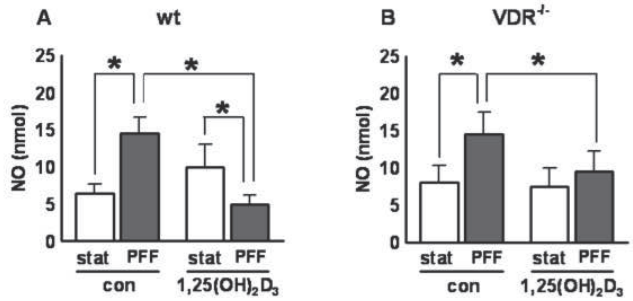

Figure 2. Effect of 24 h incubation with $1,25(OH)_2D_3$ (10^{-13}M) on mechanical loading-induced NO-production by wildtype and $VDR^{-/-}$ osteoblasts. (A) PFF stimulated NO producyion by wt mouse long bone cells by 2-fold (p=0.006). $1,25(OH)_2D_3$ inhibited the mechanical loading-induced NO production (p=0.02). (B) PFF stimulated NO production by $VDR^{-/-}$ mouse long bone cells by 2-fold (p=0.02). $1,25(OH)_2D_3$ decreased mechanical loading-induced NO production by $VDR^{-/-}$ mouse long bone cells (p=0.04). Values are mean ± SEM of 4 independent experiments. wt, wildtype; $VDR^{-/-}$, VDR knockout.

These results suggest that the effect of $1,25(OH)_2D_3$ on PFF-induced NO production is not mediated via genomic actions of the VDR, but possibly via membrane-associated actions. Since membrane receptor-mediated responses to $1,25(OH)_2D_3$ occur within seconds to minutes, we investigated whether incubation with $1,25(OH)_2D_3$ for only 30 min is sufficient to stimulate NO production in MC3T3-E1 osteoblasts, and whether this short incubation affects PFF-induced NO production. Thirty min of incubation with $1,25(OH)_2D_3$ did not induce NO production by MC3T3-E1 osteoblasts at any of the concentrations tested (Fig. 3A). $1,25(OH)_2D_3$ at 10^{-11} M significantly inhibited the PFF-induced NO production by 2-fold at 10 min (p=0.004) and 30 min (p=0.006) (Fig. 3B). A similar trend was observed at later time points, where $1,25(OH)_2D_3$, at all concentrations tested (10^{-9}, 10^{-11}, 10^{-13} M) seemed to decrease PFF-induced NO production (Fig. 3B).

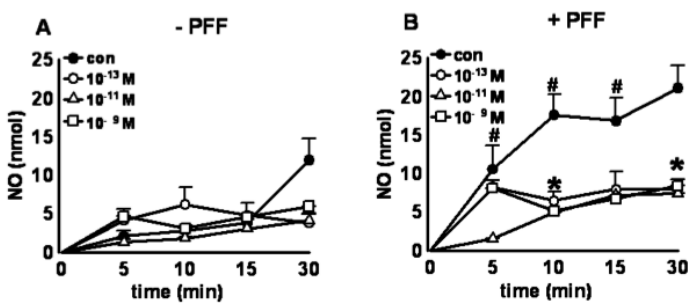

Figure 3. Effect of 30 min of 1,25(OH)2D3 (10^{-13}M) treatment on NO production and mechanical loading-induced NO production by MC3T3-E1 osteoblasts. (A) Thirty min of 1,25(OH)2D3 (10^{-13}M) did not induce NO production in osteoblasts. (B) PFF stimulated NO production by MC3T3-E1 osteoblasts. Thirty min of 1,25(OH)2D3 (10^{-13}M) treatment inhibited the mechanical loading-induced NO production by 4-fold. Values are mean ± SEM of absolute values of 6 independent experiments. * Significant effect of 1,25(OH)2D3 on NO production, p<0.05.

DISCUSSION

Both $1,25(OH)_2D_3$ and mechanical loading are essential regulators of bone mass. Disturbances of $1,25(OH)_2D_3$ metabolism as well as disturbed mechanical adaptation have been linked to the emergence of osteoporosis[27,28]. It is known that the positive effect of mechanical loading on bone mass is mediated, at least in part, by NO. Whether NO plays a similar role in mediating the effects of $1,25(OH)_2D_3$ on bone mass is unknown, but several arguments derived from literature are in favour of this hypothesis[20,21,29]. Therefore we investigated whether $1,25(OH)_2D_3$ affects NO production by osteoblasts, as well as the effect of $1,25(OH)_2D_3$ on the NO response to mechanical loading, and whether this effect is dependent on genomic actions of the steroid receptor VDR. We found a stimulating effect of 10^{-11} M $1,25(OH)_2D_3$ on NO production by osteoblasts under static culture without mechanical stimulation, but the highest concentration tested (10^{-9} M) did not affect NO production. Often a biphasic response to $1,25(OH)_2D_3$ is seen, with an optimum at mid-range concentrations, i.e. higher concentrations of $1,25(OH)_2D_3$ are not necessarily leading to a stronger effect. We reasoned that the NOS isoform responsible for $1,25(OH)_2D_3$-induced NO production may be iNOS, since $1,25(OH)_2D_3$ modulates iNOS expression in microglial cells and the brain[20,21,29]. We found that 24 h incubation with $1,25(OH)_2D_3$ indeed increased iNOS gene expression in osteoblasts, but did not change nNOS or ecNOS gene expression. This suggests that the effect of 10^{-11} M $1,25(OH)_2D_3$ on NO production was mediated via enhanced iNOS gene expression, although we cannot exclude that $1,25(OH)_2D_3$ enhanced ecNOS or nNOS activity rather than transcription. iNOS gene transcription, but not NO production, was increased by 10^{-9} M $1,25(OH)_2D_3$. We speculate that the concentration of 10^{-9} M $1,25(OH)_2D_3$, which is higher than the free available concentration of $1,25(OH)_2D_3$ in the human body (50-180 pmol/L)[30], may be supra-physiologic and either inhibits iNOS activity by limiting substrate availability or inhibits iNOS protein synthesis, as $1,25(OH)_2D_3$ affects protein translation by targeting the mTOR pathway[31].

$1,25(OH)_2D_3$ has direct actions on bone cells via the VDR[32]. However, it seems that not all actions of $1,25(OH)_2D_3$ on bone are mediated via the VDR. For example, studies in VDR knockout mice show that $1,25(OH)_2D_3$ affects skeletal mineralization independent of the VDR[33]. These studies do not clarify whether a membrane-associated VDR is responsible for the VDR-independent actions of $1,25(OH)_2D_3$. However earlier studies did show evidence for a rapid, i.e. within seconds to minutes, activation of signalling pathways by $1,25(OH)_2D_3$, possibly indicating activity of a membrane-type receptor [12]. However, it was demonstrated that $1,25(OH)_2D_3$ elicited electrical activity via a functional VDR[32]. The kinase and second messenger pathways activated by rapid $1,25(OH)_2D_3$-mediated signalling include phospholipase A and C, protein kinase C and increase of intracellular calcium[10,11]. Similar

signalling cascades are activated by mechanical stimuli[34,35], supporting the notion that $1,25(OH)_2D_3$ and mechanical loading may interact. In addition, an important early response of osteoblasts to mechanical loading is a rise in intracellular calcium concentration, which is necessary for activation of calcium/calmodulin-dependent enzymes such as ecNOS and nNOS. When serum levels of calcium are low, $1,25(OH)_2D_3$ stimulates calcium absorption from the gut, thereby increasing serum calcium[36]. This increase in serum calcium may also facilitate an increase in intracellular calcium concentrations in osteoblasts, which could be beneficial to the activation of NOS in response to mechanical loading. Interestingly, the NO response of MLO-Y4 osteocytes to mechanical loading is inhibited in the presence of EGTA, which lowers extracellular calcium concentrations[18]. This also supports the notion that $1,25(OH)_2D_3$ and mechanical loading may interact at the cellular level in the regulation of bone mass.

It has long been a matter of debate whether the non-genomic actions of $1,25(OH)_2D_3$ are mediated by another receptor than the classical VDR[13]. We found that $1,25(OH)_2D_3$ inhibited the NO-response to mechanical loading in $VDR^{-/-}$ osteoblasts. These $VDR^{-/-}$ osteoblasts might still express a truncated form of the VDR that is unable to exert its genomic actions[37], but can still bind $1,25(OH)_2D_3$ and exert a rapid membrane response. It is also possible that there is a separate membrane-associated receptor e.g. MARRS, mediating the inhibition of mechanical loading-induced NO production by $1,25(OH)_2D_3$. Moreover, the VDR was also found to be localized near the plasma membrane in caveolae[38], leading to the proposal that the VDR directs also non-genomic signalling. Modelling studies of the VDR ligand-binding domain showed that the VDR could possibly accept and favour non-bonding interactions with vitamin D sterols in a distinct ligand-binding pocket from the genomic pocket that was previously defined by x-ray crystallography[39,40]. NO production by osteoblasts was differentially affected by $1,25(OH)_2D_3$ dependent on the time of incubation. Only 24 h of incubation with $1,25(OH)_2D_3$ increased NO production by osteoblasts cultured under static conditions, suggesting that the response was facilitated via the genomic VDR. In contrast, $1,25(OH)_2D_3$ administered for only 30 min during PFF reduced mechanical loading-stimulated NO production by osteoblasts. This response is rapid, and therefore it is unlikely that the genomic pathway mediated the effects of $1,25(OH)_2D_3$ on PFF-induced NO production in osteoblasts.

In summary, our results suggest that $1,25(OH)_2D_3$ stimulates iNOS expression and NO production by osteoblasts, likely via genomic actions of the VDR (Fig. 4). Moreover $1,25(OH)_2D_3$ and mechanical loading interact at the level of mechanotransduction, whereby $1,25(OH)_2D_3$ modulation of mechanical loading-induced NO production occurs via a mechanism independent of genomic actions of the VDR.

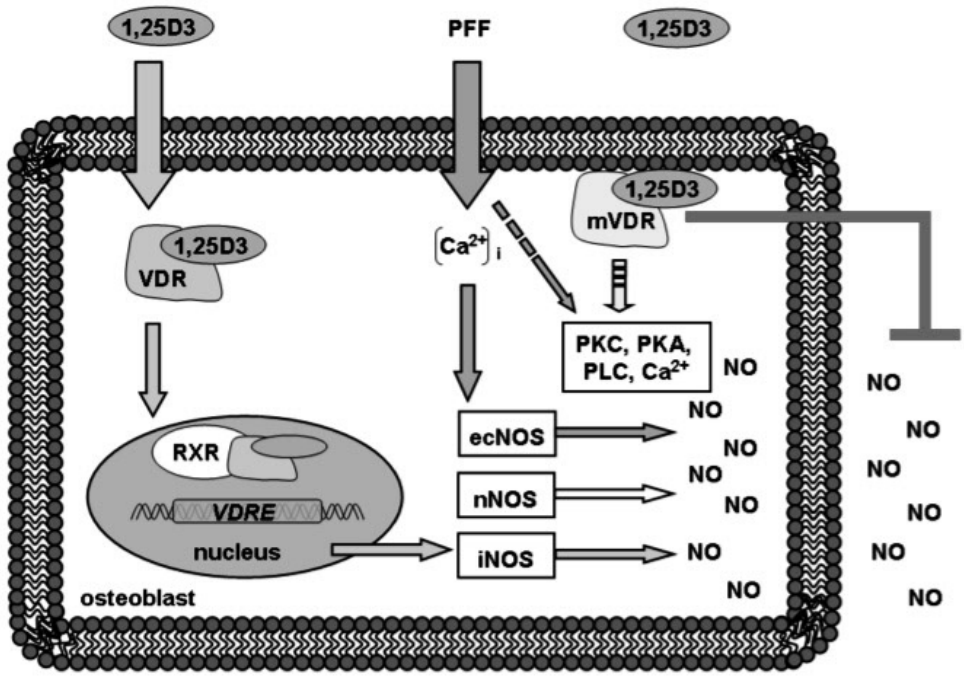

Figure 4. 1,25(OH)2D3 (1,25D3) stimulates iNOS expression and NO production by osteoblasts, likely via genomic actions of the VDR. Mechanical loading (PFF) stimulates NO production via an increase in intracellular calcium and ecNOS activation. 1,25(OH)2D3 and mechanical loading interact at the level of mechanotransduction, whereby 1,25(OH)2D3 inhibits mechanical loading-induced NO production, likely independent of genomic actions of the VDR. Other second messenger pathways activated by this non-genomic 1,25(OH)2D3-mediated signalling include PLA, PLC, PKC and intracellular calcium (Ca^{2+}_i), which are also activated by mechanical loading.

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CHAPTER 5

FLUORIDE INHIBITS THE RESPONSE OF BONE CELLS TO MECHANICAL LOADING

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ABSTRACT

The response of bone cells to mechanical loading is mediated by the cytoskeleton. Since the bone anabolic agent fluoride disrupts the cytoskeleton, we investigated whether fluoride affects the response of bone cells to mechanical loading, and whether this is cytoskeleton-mediated. The mechano-response of osteoblasts was assessed *in vitro* by measuring pulsating fluid flow-induced nitric oxide (NO) production. Osteocyte shape was determined in hamster mandibles *in vivo* as parameter of osteocyte mechanosensitivity. Pulsating fluid flow (0.7±0.3 Pa, 5 Hz) stimulated NO production by 8-fold within 5 min. NaF (10-50 μM) inhibited pulsating fluid flow-stimulated NO production after 10 min, and decreased F-actin content by ~3-fold. Fluid flow-induced NO response was also inhibited after F-actin disruption by cytochalasin B. NaF-treatment resulted in more elongated, smaller osteocytes in interdental bone *in vivo*. Our results suggest that fluoride inhibits the mechano-response of bone cells, which might occur via cytoskeletal changes. Since decreased mechanosensitivity reduces bone mass, the reported anabolic effect of fluoride on bone mass *in vivo* is likely mediated by other factors than changed bone cell mechanosensitivity.

Key words: Fluoride; mechanical loading; nitric oxide; cytoskeleton; osteocytes.

INTRODUCTION

Bone is a living tissue capable of adapting its mass and structure to the demands of mechanical usage[1]. Mechanical adaptation is orchestrated by the osteocytes[2]. When bones are loaded, the resulting deformation of the bone matrix will drive the thin layer of interstitial fluid surrounding the network of osteocytes to flow from regions under high pressure to regions under low pressure[3,4]. This fluid flow is sensed by the osteocytes, which subsequently produce signaling molecules, e.g. nitric oxide (NO),[5-7] which affect osteoblasts and osteoclasts to change their bone remodeling activities leading to changes in bone mass[8-10]. The response of bone cells to mechanical loading is mediated by the cytoskeleton[11]. Moreover cell shape affects the NO response to mechanical loading, i.e. osteocytes with a round morphology require lower force stimulation in order to increase NO production than osteocytes with a flat morphology[12]. Thus any factor changing bone cell cytoskeleton or shape may affect bone mass.

Fluoride has been used for decades to enhance bone mass in osteoporosis[13]. Fluoride treatment enhances bone mass by stimulation of bone matrix deposition. Serum concentrations of fluoride in osteoporotic patients treated with fluoride are only 5-15 μM[14]. Fluoride at these concentrations stimulates osteoblast proliferation, differentiation, and matrix production[15-18]. Higher fluoride concentrations increase fracture risk and peripheral bone loss in humans, and therefore fluoride has not become routine therapy for osteoporosis[19]. Fluoride is present in varying amounts in air, water, and food, and is an essential trace element in the skeletal system and teeth[20]. Fluoride disrupts the cytoskeleton of protozoa[21], and F-actin in ameloblasts[22]. Since fluoride affects bone mass and cytoskeleton, we hypothesized that fluoride inhibits the response of mechano-sensitive bone cells to mechanical loading.

The aim of our study was to determine whether fluoride affects the mechano-response of bone cells *in vitro*, and whether this effect is modulated via the actin cytoskeleton. Mechano-sensitive MC3T3-E1 osteoblastic cells were treated with or without sodium fluoride (NaF) for 24 h. Subsequently cells were subjected to pulsating fluid flow for 30 min. We used NO production as a parameter for bone cell activation. We also quantified cellular F-actin after fluoride treatment. In addition we determined the effect of fluoride on osteocyte shape in developing hamster mandibles *in vivo*. Osteocyte shape was determined as parameter for osteocyte mechanosensitivity.

MATERIALS AND METHODS

MC3T3-E1 osteoblast culture

MC3T3-E1 osteoblast-like cells were cultured in α-MEM medium (Gibco, Paisly, UK) supplemented with 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 µg/ml streptomycin (Sigma-Aldrich), 1.4 mM L-glutamine (Sigma), 9 mM β-glycerophosphate (Sigma-Aldrich), 1.9 mM ascorbic acid (vitamin C; Merck, Darmstadt, Germany), and 10% fetal bovine serum (FBS; Gibco) at 37ºC, in a humidified atmosphere of 5% CO_2 in air. Medium was exchanged every 3-4 days. Upon confluence, cells were harvested using 0.25% trypsin and 0.1% EDTA in phosphate buffered saline (PBS), seeded at $2x10^5$ cells per 75 cm^2 culture flask (Greiner Bio-One, Kremsmuenster, Austria), and cultured until the cell layer reached confluency again. Then cells were used for pulsating fluid flow experiments as described below.

Disruption of the actin cytoskeleton.

Prior to fluid flow stimulation, plated cells were incubated for 1 h in 13 ml of the appropriate medium with the total serum content reduced to 2%. Cells were then divided at random into two groups: (i) untreated control medium; (ii) actin-disrupted: medium supplemented with 1 µM cytochalasin B (Sigma–Aldrich), a toxin that prevents actin polymerization[11]. We observed that this concentration of cytoskeletal-disrupting agent had a significant non-toxic effect on cell morphology within 1 h.

Pulsatile fluid flow (PFF)

Two days before PFF treatment, cells were harvested from the culture flasks and seeded onto polylysine-coated (50 µg/ml; poly-L-lysine hydrobromide, mol wt 15-30 x 10^4; Sigma) glass slides (size 2.5x6.5 cm) at $2x10^5$ cells/glass slide, and cultured overnight in petridishes with 13 ml culture medium containing 10% fetal bovine serum (FBS). Then the culture medium was replaced by α-MEM medium containing 0.2% bovine serum albumin (BSA), antibiotics, ascorbate (Merck), and either or not 10, 25, or 50 µM NaF. Cells were incubated for 24 h, as well as during 30 min of PFF-treatment or static control conditions.

PFF was applied as described earlier.[5] Briefly, PFF was generated by pumping 13 ml of culture medium through a parallel-plate flow chamber containing MC3T3-E1 osteoblast-like cells.[23] Cells were subjected to a 5 Hz pulse with a mean shear stress of 0.7 Pa, pulse amplitude of 0.3 Pa and a peak shear stress rate of 8.4 Pa/s.[12] Stationary control cultures were kept in a petridish under similar conditions, i.e. at 37ºC in a humidified atmosphere of 5% CO_2 in air. After 5, 10, 15, and 30 min of PFF or static control culture, medium was collected and assayed for NO concentrations.

Nitric oxide

NO was measured as nitrite (NO_2^-) accumulation in conditioned medium using Griess reagent containing 1% sulfanylamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in non-conditioned medium were used as a standard curve. Absorbance was measured at 540 nm with a microplate reader (BioRad Laboratories Inc.).

DNA content

DNA was isolated directly after PFF from bone cell cultures using TRIzol reagent (Gibco) according to the manufacturer's instructions. DNA in the cell lysate was determined by CyQUANT Cell Proliferation assay (Molecular Probes Inc., Eugene, OR, USA).

Staining of filamentous actin (F-actin)

MC3T3-E1 osteoblasts were seeded in 24 well plates at $8.0x10^3$ cells/cm^2 (CELLSTAR®, Greiner Bio-one, Alphen a/d Rijn, The Netherlands), incubated overnight, followed by an additional 24 h in α-MEM with 0.2% BSA and with or without 10, 25, or 50 µM NaF. Osteoblasts were washed with PBS, fixed with 4.0% formaldehyde, washed again with PBS, and incubated with 1:250 dilution of donkey anti-rabbit IgG alexa 488-phalloidin (Invitrogen) for 1 h in PBS. F-actin was extracted using methanol and quantified as described[24].

Tissue harvesting

A mandibular model of 4-days-old hamsters was used. In short, a single injection of 20 mg NaF/kg body weight was used to test whether NaF affects osteocyte size (5 hamsters). This concentration affects enamel development[25]. Controls (4 hamsters) received NaCl. NaF and NaCl were administered as one single intraperitoneal injection between 10.30-11.30 AM. Seventy-six hours later, hamsters were sacrificed, mandibles excised, fixed, demineralized, dehydrated in ascending ethyl-alcohol series, and embedded in Historesin (Leica Microsystems, Mannheim, Germany)[25]. Sections of 5 µm thickness were cut with a microtome in a sagittal plane, mounted on glass slides, and stained with haematoxylin according to Mayer, or toluidine blue. Ethical permission was obtained from the VU University Amsterdam.

Histology

Histologic and histomophometric analysis was performed using a Leica light microscope with electronic stage table (Leica Microsystems, Wetzlar, Germany). Images were captured using a Leica digital camera directly connected to a computer. All measurements were done using the Leica QWin Pro V 3.5.0 quantitative imaging computer program (Leica Microsystems, Heerbrugg, Switzerland). Within each section, three areas of interest were distinguished:

1) mesial of the first molar, 2) proximal of the first and second molar, and 3) distal of the second molar. In every section these areas of interest were located at 100x magnification, and photographed at 400x magnification. Osteocyte morphology was determined by measuring length and width. Osteocyte shape is expressed as length-over-width ratio in >500 osteocytes per hamster, and the mean shape calculated per animal.

Statistical analysis

PFF data was obtained from 4 experiments. NO production data are expressed as PFF-over-static control. All data is expressed as mean±SEM. Data was analysed using Wilcoxon signed-rank test for paired observations. The unpaired *t*-test was used for analyzing data from individual hamsters. The paired t-test was used to analyze measurements between subjects in both control and experimental animals. Differences were considered significant if $p<0.05$.

RESULTS

Application of fluid shear stress for 30 min to MC3T3-E1 osteoblasts in the presence or absence of NaF did not result in visible changes in bone cell shape or alignment of cells in the flow direction (data not shown). NaF treatment did not affect the total amount of DNA (control, $1.6x10^{-7}$ g; NaF, $1.6x10^{-7}$ g). No cells were removed by PFF as assessed by microscopic inspection of the cell cultures.

We investigated whether fluoride affects the mechanoresponse of MC3T3-E1 osteoblasts. Cells were cultured for 24 h in the presence or absence of NaF, and then treated with or without PFF for 30 min. PFF caused a rapid and significant increase in NO production at 5 min (Fig. 1A). Twenty-four h incubation with 10 μM NaF significantly inhibited PFF-induced NO production by 8-fold (Fig. 1A). After 10 min PFF, NaF (10, 25, 50 μM) significantly inhibited the PFF-stimulated NO response (Fig. 1B). We also investigated the effect of PFF and cytoskeletal disruption by cytochalasin B on the NO response of MC3T3-E1 osteoblasts, and found that the PFF-induced increase in NO release at 10 min was inhibited by disrupting F-actin (Fig 1C).

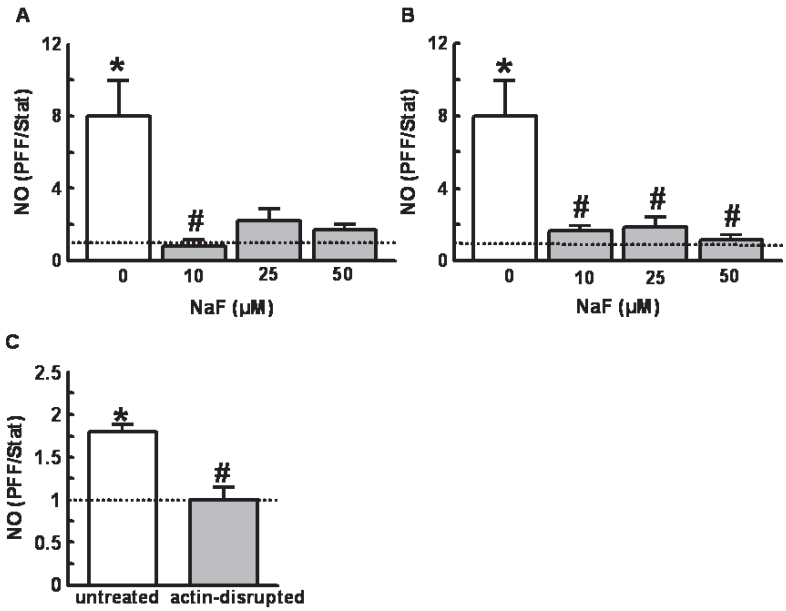

Figure 1. Effect of 24 h NaF treatment (10, 25 or 50 μM) or actin cytoskeleton disruption on PFF-stimulated NO production by MC3T3-E1 osteoblasts. NaF inhibited the PFF-stimulated NO production by MC3T3-E1 osteoblasts, (A) after 5 min PFF, and (B) after 10 min PFF. (C) F-actin cytoskeleton disruption inhibited PFF-stimulated NO production at 10 min.[11] Values are mean ± SEM of PFF-treated-over-static control ratios (T/C of 5 independent experiments). Dashed line, T/C=1 (no effect). Stat, stationary control culture; PFF, pulsating fluid flow. *Significantly different from 1; #Significantly different from untreated.

We then questioned whether the decrease in PFF-stimulated NO production by NaF for 24 h in osteoblasts is mediated via the actin-cytoskeleton. In untreated cells, actin fibers were aligned throughout the whole cell (Fig. 2A). After 24 h NaF (10, 25, 50 μM) treatment, actin fibers were at the edge of the cells and less abundant (Fig 2B). NaF (10, 25, 50 μM) also reduced the amount of F-actin per well by 3-fold (Fig. 2C).

We finally tested whether fluoride affects the mechanosensitivity of bone cells *in vivo,* and therefore osteocyte shape was determined in developing hamster mandibles. Three areas of interest were defined, i.e. distal, mesial, and proximal area (Fig. 3A), and the osteocyte length and width were measured (Fig. 3B).

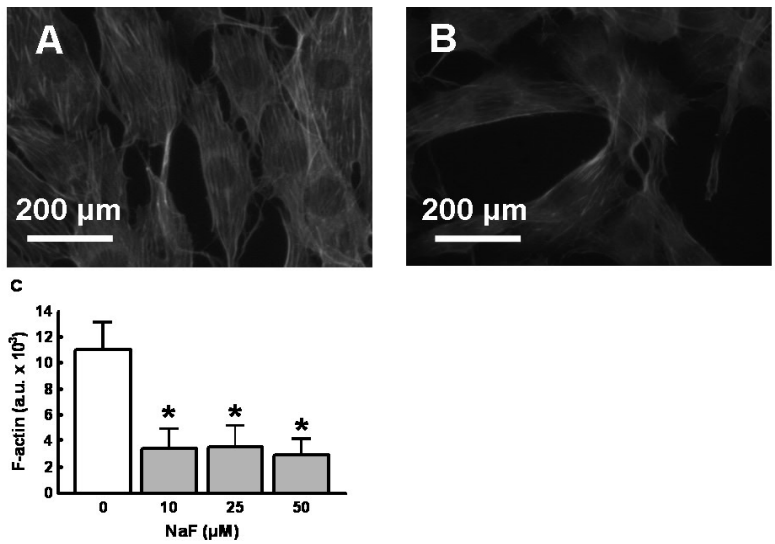

Figure 2. Immunofluorescence analysis and quantification of actin cytoskeleton in MC3T3-E1 osteoblasts. F-actin (green) was visualized with alexa fluor 488 phalloidin. Representative images of (A) control MC3T3-E1 osteoblasts, and (B) F-actin cytoskeleton in MC3T3-E1 osteoblasts after 24 h NaF treatment. (C) Quantification of F-actin in MC3T3-E1 osteoblasts. Values are mean ± SEM of 4 cultures from 2 independent experiments. *Significant effect of NaF on F-actin, $p<0.05$.

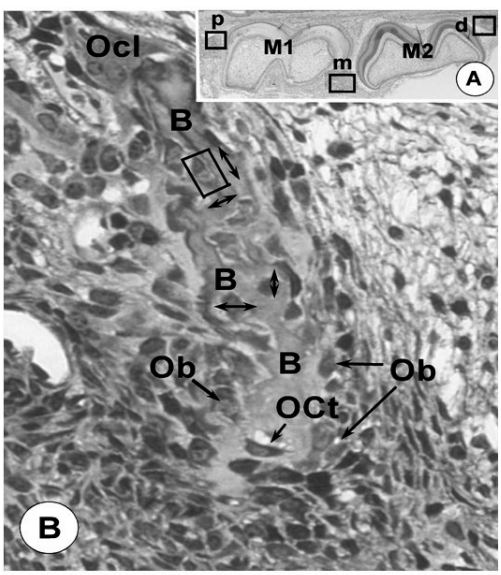

Figure 3. Histological section of a hamster first and second molar showing the areas of interest for determining osteocyte morphology. (A) Undecalcified, haematoxylin-eosin stained section of a first (M1) and second (M2) hamster molar at post-natal day 4 from an animal injected with 20 mg NaF/kg body weight, and killed 76 h later, showing the three areas of interest, i.e. proximal area (p), mesial area (m), and distal area (d) (magnification, 50x). (B) Higher magnification of (A) stained with toluidine blue (magnification, 400x). The square shows a single osteocyte, and lines with double arrow heads indicate length and width of the osteocyte. Ocl, osteoclast; Ob, osteoblast; Ocy, osteocyte; B, bone.

In the distal, mesial, and proximal area, NaF did not affect osteocyte length (Fig. 4A). In the proximal area, but not in the other areas, osteocyte width was 9% smaller when treated with NaF compared to control (marginally significant, p= 0.07) (Fig. 4B). NaF increased the osteocyte length/width ratio by 3% in the mesial area (marginally significant, p = 0.08), but not in the other areas (Fig. 4C).

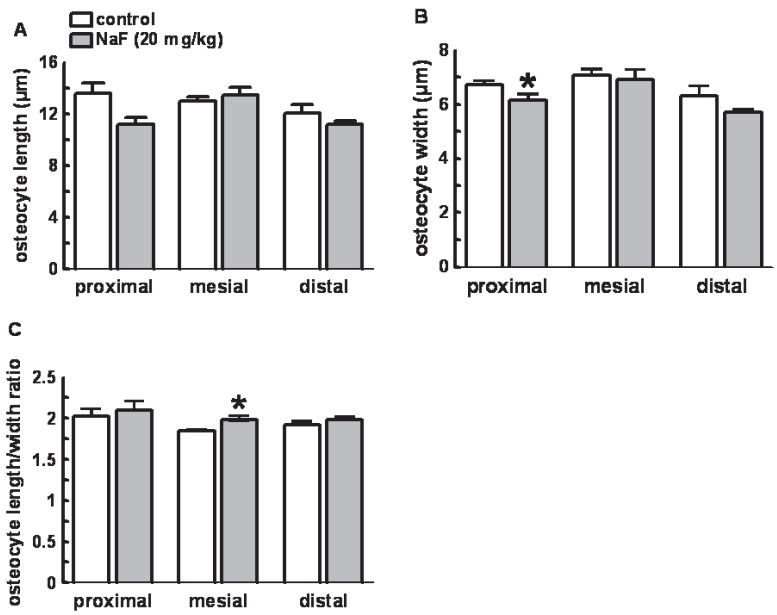

Figure 4. Effect of a single high dose of NaF (20 mg/kg bodyweight) on osteocyte length and width in hamster mandibles. (A) Osteocyte length, (B) Osteocyte width, and (C) Osteocyte length/width ratio. Values are mean ± SEM of 5 NaF-treated hamsters and 4 controls. *Marginally significant effect of fluoride, p=0.07 (Fig. 4B), p=0.08 (Fig. 4C).

DISCUSSION

Mechanosensitive bone cells play a key role in bone remodeling. The cytoskeleton mediates the response of bone cells to mechanical loading, and cytoskeletal changes can therefore affect bone remodeling[11]. Since the bone anabolic agent fluoride affects the cytoskeleton, we hypothesized that 1) fluoride reduces the mechano-response of osteoblasts to mechanical loading by pulsating fluid flow *in vitro*, and that this is modulated via the actin-cytoskeleton, and 2) that fluoride affects osteocyte mechanosensitivity, the bone mechanosensors par excellance, in hamster mandibles *in vivo*. In this report, we present compelling evidence that treatment with fluoride at physiological concentrations *in vitro* inhibits PFF-stimulated NO production by MC3T3-E1 osteoblasts. The same micromolar concentrations of fluoride

affected the arrangement and amount of F-actin in these cells. Inhibition of the NO response to PFF was also observed after cytoskeletal disruption by cytochalasin B. The NO production (PFF/Stat) in the control (0 micro M) is about 8 in Fig. 1A and Fig 1B, while in Fig. 1C, NO production (PFF/Stat) in "untreated" is about 1.8. We adopted Figure 1C from McGarry et al.[11] In these experiments, a low percentage of serum (2%) was present in the culture medium during the mechanical loading (PFF) experiments. In our experiments (Figure 1A and 1B) 0.3% BSA was added to the medium rather than serum. Serum in culture medium will not alter the activation of biological pathways by PFF in osteoblasts, but serum does alter the magnitude of the response of osteoblasts to pulsating fluid flow (unpublished observations within our laboratory). In addition we found that fluoride marginally changes osteocyte shape in the mesial region in hamster mandibles *in vivo.* Fluoride treatment resulted in more elongated and smaller osteocytes. Why this occurs only in the mesial region is not clear. Our results suggest that fluoride inhibits the mechano-response of bone cells by cytoskeletal changes.

We applied mechanical loading by PFF of 0.7±0.3 Pa at 5 Hz. This stimulus is based on the currently available knowledge on physiological bone loading stimuli. The frequency spectra after loading of the hip bone in living humans have been calculated to range between 1 and 3 Hz for walking cycles and reaching 8 to 9 Hz for running cycles[26]. The fluid shear stress amplitude around osteocytes, resulting from daily mechanical loads, have been determined theoretically to range from 0.8 to 3 Pa due to physiological strains in humans[23]. Although the accuracy of these calculations are currently under debate, it is noteworthy that bone cells *in vitro* seem to be highly sensitive to shear stress in the order of this magnitude.

We showed that fluoride affects the arrangement and amount of F-actin in MC3T3-E1 osteoblasts. These findings agree with a study showing that fluoride affects the activity of several g-proteins involved in the microtubular system and other cytoskeletal compounds in protozoa[21]. Since the response of bone cells to mechanical loading is mediated by the cytoskeleton[11], our findings suggest that fluoride affects the mechano-response of osteoblasts via modulation of cellular F-actin.

Another explanation for the fluoride-induced decrease in NO response to mechanical loading by PFF in MC3T3-E1 osteoblasts could be increased activity of superoxide dismutase (SOD). Fluoride induces oxidative damage in mouse osteoblasts[27]. Oxidative damage in cells by reactive oxygen species is controlled by increasing SOD activity. SOD scavenges free radicals such as NO, and turns these into oxygen and hydrogen peroxide[28]. It is possible that in the presence of NaF osteoblasts experienced oxidative stress and did respond with increased SOD activity, thereby eliminating the NO produced as a result of PFF treatment.

The effect of fluoride on the arrangement and amount of F-actin in osteoblasts *in vitro* is in agreement with our *in vivo* observations. Fluoride did not affect osteocyte length in the

mesial, distal, and proximal regions of the hamster mandible, but it decreased osteocyte width in the proximal region and increased osteocyte length-over-width ratio in the mesial region, indicating that a single high dose of NaF in hamster pups resulted in formation of more elongated and smaller osteocytes in the growing mandible. Bone around developing molar teeth has a high turnover, and osteoblasts briefly exposed to fluoride turn into osteocytes within three days. Our results suggest that fluoride inhibits the mechanoresponse via cytoskeletal changes resulting in changes in osteocyte morphology, since differences in osteocyte morphology indicate differences in mechanosensitivity[12].

The decreased NO response to mechanical loading by PFF, in combination with the affected cytoskeleton and the smaller osteocytes *in vivo*, as a result of fluoride treatment suggest that bone cell mechanosensitivity is affected by fluoride. We conclude that fluoride causes rearrangement of the actin cytoskeleton, thereby changing osteocyte shape and mechanical loading-induced NO production. The known anabolic effect of fluoride on bone mass is therefore likely mediated by other factors than changed bone cell mechanosensitivity.

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CHAPTER 6

OLEUROPEIN MODULATES THE COMBINED EFFECT OF INTERLEUKIN-b AND MECHANICAL STIMULATION IN OSTEOCYTES

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ABSTRACT

Osteocytes orchestrate the adaptation of bone mass and structure to mechanical stimulation. The inflammatory cytokine interleukin-1b (IL-1b) reduces the response of osteocytes to mechanical stimuli. Since phenolic dietary compounds counteract the effects of inflammatory cytokines, we investigated whether the phenolic compound oleuropein affects the response of IL-1b–treated osteocytes to mechanical stimulation. MLO-Y4 osteocytes were treated with or without 10^{-8} M oleuropein and/or 1 ng/ml IL-1β. Human osteoblasts obtained from long bones of osteoporotic patients and controls, and MLO-Y4 osteocytes were subjected for 1 h to pulsating fluid flow (PFF: 0.7±0.3 Pa, 5 Hz). The response to PFF was assessed by quantification of nitric oxide (NO) production and measuring cyclooxygenase-2 (COX-2) gene expression. In addition, effects of oleuropein and IL-1β on the cytoskeleton were assessed by quantification of filamentous actin. PFF stimulated NO production within 5 min in MLO-Y4 osteocytes, and in human control osteoblasts. The NO response to PFF was lower in human osteoblasts from osteoporotic patients than in control bone cells. In MLO-Y4 osteocytes, IL-1β reduced the PFF-stimulated NO production by 1-1.5-fold. This inhibition could not be reversed by oleuropein treatment. Furthermore IL-1b decreased F-actin content and COX-2 gene expression in response to PFF. Oleuropein prevented the effect of IL-1b on PFF-induced COX-2 gene expression but not on F-actin content. Our results show that the effect of IL-1b on the mechano-response of osteocytes might be mediated by changes in F-actin, and that oleuropein prevents the IL-1b–modulated COX-2 gene expression in response to PFF. Therefore we conclude that oleuropein may reduce the inflammation-induced bone loss *in vivo* by reducing the inhibitory effect of IL-1b on PFF-stimulated COX-2 gene expression.

Key words: osteocytes, mechanical loading, oleuropein, NO, COX-2, IL-1β, cytokines.

INTRODUCTION

Bone is a living tissue, which adapts its mass and structure to the demands of mechanical loading.[1,2]. This adaptation to the mechanical demands is a cellular process, needing a biological system to function. In this active process of mechanical adaptation, the sensing of the mechanical signals occurs by the osteocytes[3-5], the professional mechanosensory cells within the bone. Osteocytes are distributed in the bone matrix and connected to each other via long slender cell processes that run through the canaliculi[6], forming a unique network that facilitates contact with cells on the bone surface[6]. Slight deformation of bone occurs as a result of mechanical loading, causing a flow of interstitial fluid through this lacuno-canalicular network[7], which result in a shear stress on the osteocyte cell membrane[5,7]. As a response to this shear stress, osteocytes produce signaling molecules, such as nitric oxide (NO), which is essential for adaptive bone formation[8], and prostaglandin E_2, which are early components of the bone cells' response to mechanical strain[9]. It is likely that the cytoskeleton determines the manner by which osteocytes respond to a fluid shear stress[10]. The cytoskeleton determines both cell stiffness and cell shape. Moreover the NO response of osteocytes to mechanical loading is affected by the cell shape, i.e. osteocytes with a round morphology require lower force stimulation in order to increase NO production than osteocytes with a flat morphology[11].

Another important parameter in the process of bone formation after mechanical loading is cyclooxygenase (COX). A 15 min treatment of osteocytes with fluid shear stress already leads to a three-fold increase of COX-2 mRNA expression[12]. Moreover, COX-2 expression is a crucial step in the induction of bone formation by mechanical stimuli *in vivo*[9].

Bone mass peaks between the age of 20 to 30 years, after which it declines. A prompt decline in estrogen levels after menopause in women plays an important role in the etiology of osteoporosis, which occurs if bone resorption exceeds bone formation[13,14]. The decrease in estrogen levels after menopause is accompanied by a chronic inflammatory status[15], and the production of cytokines[16]. The cytokines IL-1β, TNF-a, and IL-6 are known to affect bone turnover by increasing bone resorption[17], and IL-1β and TNF-a are also known to reduce bone formation. Although the pathogenesis of post-menopausal osteoporosis is not fully understood, there is increasing evidence that inflammation contributes to osteoporosis by causing increased osteoclast activity[13,16,18].

Phenolic compounds have been shown to decrease the production of inflammatory cytokines in macrophage cell lines[17]. Oleuropein is the most representative catecholic component of olives and their derivates in olive oil[19]. In Mediterranean countries the average intake of oleuropein is approximately 25 mg/day[20]. In these countries, a lower incidence of cardiovascular disease has been observed[19]. Since the development of cardiovascular

disease, e.g. atherosclerosis, includes a strong inflammatory component[21], these findings suggest that phenolic compounds reduce the negative effects of inflammatory cytokines on cells. Oleuropein also inhibits cytokine-mediated bone loss in a postmenopausal bone model of ovariectomized rats[22]. Oleuropein stimulates the production of NO by macrophages via a direct effect on the inducible form of nitric oxide synthase[24]. In addition, oleuropein induces cell rounding in different cell lines *in vitro* and in mice tumor cells *in vivo*. The effect of oleuropein on the shape of tumor cells was explained by an immediate effect on the actin cytoskeleton[25]. Therefore oleuropein could potentially affect the response of bone cells to mechanical stimulation via an effect on the cytoskeleton.

Both oleuropein and mechanical loading are able to increase bone mass via the production of signaling molecules involved in bone formation, but it is unknown whether the combination of mechanical loading and oleuropein affects COX-2 gene expression by osteocytes. We investigated whether oleuropein affects the inhibitory effect of IL-1b on the mechanoresponse of osteocytes *in vitro* by measuring COX-2 gene expression and NO production in osteocytes subjected to mechanical loading. Since oleuropein likely affects f-actin we aimed to determine whether oleuropein modulates the combined effect of IL-1b and mechanical stimulation on the mechanoresponse of osteocytes, and if this potential modulation was mediated by the f-actin cytoskeleton. MLO-Y4 osteocytes were treated with or without oleuropein and/or IL-1β, and human osteoblastic cells obtained from long bones were subjected to 1 h of pulsating fluid flow (PFF). The bone cell response to mechanical loading was assessed by quantifying NO production, and measuring COX-2 gene expression. Finally we tested whether oleuropein did affect the f-actin cytoskeleton of MLO-Y4 osteocytes.

MATERIALS AND METHODS

Human osteoblast isolation and culture

For the isolation of human osteoblasts, trabecular bone samples (surgical waste) were obtained from 6 female osteoporotic patients. Trabecular bone samples were obtained from the proximal femur shaft after hip replacement surgery for cox-arthrosis or after surgery for subcapitalhip fracture. The protocol was approved by the Ethical Review Board of the VU University Medical Center, Amsterdam, The Netherlands, and all subjects gave informed consent.

Human trabecular bone biopsies were placed in sterile phosphate buffered saline (PBS), chopped into small fragments, and washed extensively with PBS. To obtain bone cells, bone fragments were cultured in Dulbecco's minimal essential medium (DMEM; Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin (Sigma, St Louis, MO), 50 µg/ml

streptomycin sulfate (Gibco), 50 µg/ml gentamicin (Gibco), 1.25 µg/ml fungi-zone (Gibco), 100 µg/ml ascorbate (Merck, Darmstadt, Germany), and 10% fetal bovine serum (FBS). After reaching subconfluency, human osteoblasts were trypsinized with 0.25% trypsin (Difco, Detroit, MI), and replated at 5 x 10^3 cells / 75 cm^2 flask until subconfluency was reached again. All cells used were from passage 3 or lower. For PFF experiments, human osteoblasts were harvested and seeded at 5 x 10^5 cells/cm^2 of polylysine-coated (50 µg/ml; poly-L-lysine hydrobromide, mol wt 15-30 x 10^4; Sigma) glass slides (15 cm^2), and incubated overnight to promote cell attachment.

Osteocyte culture

MLO-Y4 osteocytes, a cell line derived from mouse long bones[25], were cultured in α-minimum essential medium (αMEM) supplemented with 5% FBS (Gibco), 5% calf serum (Gibco), 50 µg/ml gentamicin (Gibco), 1.25 µg/ml Fungizone (Gibco), 60 µg/ml penicillin (Sigma), and 50 µg/ml streptomycin (Sigma) at 37°C and 5% CO2 in air in a humidified incubator. Cells were harvested between passages 30 and 35 using 0.25% trypsin (Difco) and 0.1% EDTA (Sigma) in PBS, and seeded at 2 x 10^4 cells/cm^2 on poly-L-lysine hydrobromide–coated glass slides (total area 15 cm^2). Cells were incubated overnight in medium with serum to allow cell adherence to the glass surface, followed by 24 h pre-incubation before PFF treatment with 10^{-7}, 10^{-8}, or 10^{-9} M oleuropein (Frutarom Netherlands BV, Veenendaal, The Netherlands) and/or 1 ng/ml IL-1β (Sigma). The concentrations of IL-1β used for this study were based on the concentrations found in plasma of patients with rheumatoid arthritis[26].

Pulsating fluid flow

PFF was applied for 1 h as described earlier[4]. Briefly, PFF was generated by pumping 13 ml of aMEM medium without or with 10^{-7}, 10^{-8} or 10^{-9} M oleuropein and/or 1 ng/ml IL-1β through a parallel-plate flow chamber containing the MLO-Y4 osteocytes or by pumping DMEM medium through a parallel-plate flow chamber containing human osteoblasts. Cells were subjected for 1 h to a 5 Hz pulse with a mean shear stress of 0.7 Pa, and a pulse amplitude of 0.3 Pa. Stationary control cultures were kept in a petridish under similar conditions as the experimental cultures, i.e. at 37˚C in a humidified atmosphere of 5% CO_2 in air. After 5 min of PFF or static control culture, medium was collected and assayed for NO concentrations.

Nitric oxide

NO was measured as nitrite (NO_2^-) accumulation in medium using Griess reagent containing 1% sulfanylamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in non-conditioned medium were used as standard curve. Absorbance was measured at 540 nm with a microplate reader (BioRad Laboratories Inc.).

Total RNA, and cDNA synthesis

RNA was isolated from MLO-Y4 cell cultures after 1 h of PFF or static culture, using TRIzol reagent (Gibco) according to the manufacturer's instructions. The RNA content was determined by measuring the absorbance in water at 260 nm using an Ultrospec III spectrophotometer (Amersham). cDNA synthesis was performed using 1 μg total RNA in a 20 μl reaction mix consisting of 5 units Transcriptor Reverse Transcriptase (Roche Diagnostics), 0.08 A_{260} units random primers (Roche Diagnostics), 1 mM of each dNTP (Invitrogen), and 1x concentrated Transcriptor RT reaction buffer (Roche Diagnostics).

Analysis of gene expression

MLO-Y4 gene expression of COX-2 was analyzed using Taqman® Gene expression assays (Inventoried Taqman® Gene expression assays, Applied Biosystems) in an ABI Prism 7700 sequence detector (Applied Biosystems). Gene expression values were normalized for mouse GAPDH (Inventoried Taqman® Gene expression assays, Applied Biosystems).

Staining of filamentous actin (F-actin)

MLO-Y4 osteocytes were seeded in 24 well plates at 8 x 10^3 cells/cm^2 (CellStar®, Greiner Bio-one, Alphen a/d Rijn, The Netherlands), incubated overnight, followed by an additional 24 h incubation with or without 10^{-8} M oleuropein and/or 1 ng/ml IL-1β. Osteocytes were washed with PBS, fixated with 4% formaldehyde, washed again with PBS, and incubated with 1:250 dilution of donkey anti-rabbit IgG alexa 488-phalloidin (Invitrogen) for 1 h in PBS. F-actin was extracted using methanol and quantified as described by Pritchard and Guilak (2006)[27].

Statistical analysis

PFF data was obtained from 3 or 4 separate experiments. NO production was expressed as PFF-over-static control values. All data is expressed as mean ± SEM. NO production data was analyzed using Wilcoxon signed-rank test for paired observations. Mann-Whitney U tests or Student's unpaired 2-tailed t-tests were used to test the effect of oleuropein and IL-1b. A $p<0.05$ was considered significant.

RESULTS

Human primary bone cells obtained from patients with osteoporosis and from non-osteoporotic patients were submitted to 1 h of pulsating fluid flow (PFF; Figure 1A). No cells were removed by the fluid flow treatment, as assessed by quantification of the DNA content (data not shown). NO release was measured after 5 min of PFF treatment as a parameter

for bone cell responsiveness to mechanical loading. Five minutes of PFF-treatment tended to increase the NO production by 3-fold (p=0.08; Figure 1A) in cells isolated from non-osteoporotic patients. In cells from osteoporotic patients, 5 min PFF-treatment increased NO production by 2-fold (p=0.01; Figure 1A). The absolute NO production in response to mechanical loading was higher in cells from non-osteoporotic patients than in cells from osteoporotic patients (p=0.04; Figure 1A). To show that primary human bone cells and MLO-Y4 osteocytes respond in a similar manner to PFF-treatment, we subjected MLO-Y4 osteocytes to PFF and measured the NO response (Figure 1B). We found that PFF increased NO production by 10-fold (p=0.01; Figure 1B). Addition of 1 nM IL-1ß abolished the upregulation of NO production in response to PFF (Figure 1B). Twenty-four h incubation with oleuropein did not counteract this inhibitory effect of IL-1β on NO production after mechanical loading by MLO-Y4 osteocytes (Figure 1C). Oleuropein alone did not affect the PFF-stimulated NO production (data not shown).

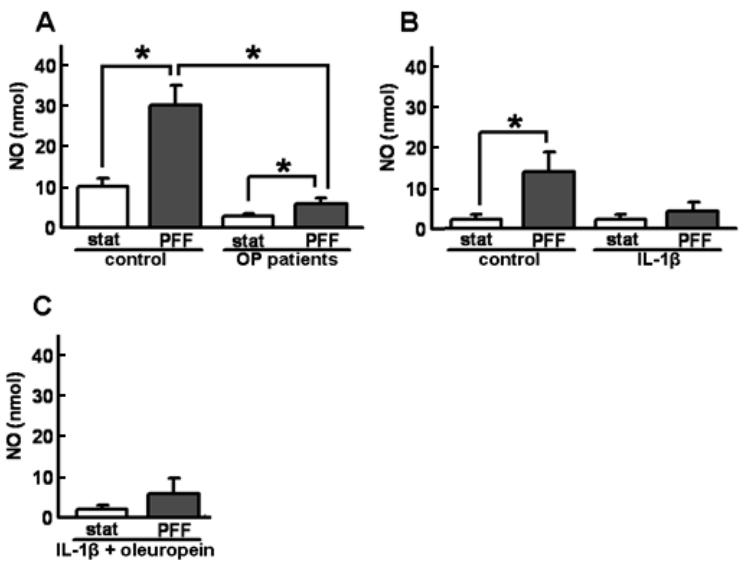

Figure 1. Effect of PFF on NO production by human primary bone cells and MLO-Y4 osteocytes. PFF-stimulated NO production after 5 min in (A) human primary bone cells from healthy subjects and osteoporotic patients, (B) MLO-Y4 osteocytes, and (C) MLO-Y4 osteocytes treated with IL-1β and oleuropein. Values are mean ± SEM of absolute NO values obtained from 5 independent experiments. Stat, stationary control culture; PFF, pulsating fluid flow; control, non-osteoporotic subjects; OP patients, osteoporotic patients. *Significant effect of PFF or oleuropein, $p<0.05$.

To establish whether IL-1β and oleuropein alter PFF-induced gene expression in MLO-Y4 osteocytes, basal COX-2 gene expression as well as COX-2 gene expression after PFF treatment were determined. PFF increased COX-2 gene expression by 2-fold. Twenty four h incubation with IL-1β decreased COX-2 gene expression in PFF-treated osteocytes by 2.3-fold (p=0.02; Figure 2). Twenty-four h incubation with oleuropein abolished the inhibitory effect of IL-1b on PFF-induced COX-2 gene expression (p=0.02; Figure 2).

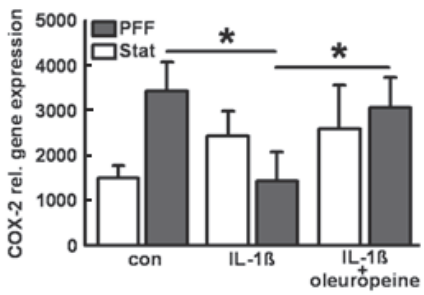

Figure 2. Effect of IL-1β and oleuropein on PFF-induced gene expression in MLO-Y4 osteocytes. Twenty-four h incubation with IL-1β decreased COX-2 gene expression. Values are mean ± SEM of relative gene expression obtained from 6 independent experiments. Stat, stationary control culture; PFF, pulsating fluid flow; OP, oleuropein; IL-1β, interleukin-1β. *Significantt effect of PFF, oleuropein or IL-1β, p<0.05.

We then investigated whether IL-1β and oleuropein affect the actin-cytoskeleton. Oleuropein at 10^{-8} M did not change the amount of F-actin per well (Figure 3). IL-1β decreased the amount of F-actin by ~1.2 fold (p=0.01), but addition of oleuropein could not prevent this IL-1β-induced decrease in f-actin fiber content (p=0.07). These data show that IL-1β affected the F-actin cytoskeleton, but that addition of oleuropein could not prevent this drop in F-actin content.

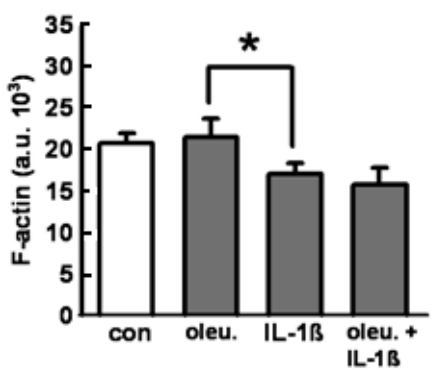

Figure 3. Quantification of F-actin in MLO-Y4 osteocytes. Values are mean ± SEM of 4 cultures from 5 independent experiments. *Significant effect of oleuropein, p<0.05.

DISCUSSION

Osteocytes regulate bone mass by dictating the balance between bone formation and resorption in response to mechanical loading[1]. Therefore, any factor affecting this osteocyte response to mechanical loading, in essence, is able to alter bone mass. One factor known to affect the response of bone cells to mechanical stimuli is IL-1β. Since oleuropein may reverse the effects of cytokines on bone cells, we hypothesized that the phenolic compound oleuropein counteracts the modulatory effects of IL-1β on the response of osteocytes to mechanical stimulation. We found that primary bone cells derived from osteoporotic patients produce less NO in response to PFF compared to bone cells from non-osteoporotic patients. We also found that IL-1β inhibits the PFF-induced up-regulation of NO production by MLO-Y4 osteocytes. These results are in accordance with findings by others[28]. Our findings show that the inflammatory cytokine IL-1β, which plays a role in the development of osteoporosis[29], modulates the mechanoresponse of bone cells to PFF.

The frequency spectra after hip loading *in vivo* in humans have been calculated[30], showing a range between 1-3 Hz (walking) up to 9 Hz (running). Daily mechanical loading-induced fluid shear stress *in vivo* has been predicted to range from 0.8-3 Pa[30]. Bone cells in vitro seem to be highly sensitive to shear stress of this order of magnitude. The stimulus of 0.7 ± 0.3 Pa at 5 Hz we applied during PFF-treatment is therefore based on known physiologic bone-loading stimuli. We showed that both primary human bone cells and MLO-Y4 osteocytes respond similarly to PFF in terms of increased NO production, indicating a physiological response of these bone cells to mechanical loading.

Oleuropein has been reported to increase NO production from LPS-challenged mouse macrophages via increased iNOS gene expression[24]. However oleuropein is also able to scavenge nitric oxide[31]. The scavenging capacity is explained by the fact that oleuropein is a catecholic compound. In catechols, each OH-group has a high electron-donating effect on the other OH-group. Weakening of the OH-bond by this electron donation facilitates transfer of a hydrogen atom to OH, converting the OH to water[32,33]. Oleuropein has also been shown to protect against bone loss in a rat model of inflammation-induced bone loss, likely via modulation of cytokines[22]. We showed that oleuropein did not counteract the inhibitory effects of IL-1β on PFF-stimulated NO production. These findings are not in line with the findings in the rat model of inflammation-induced bone loss, since oleuropein did not modulate the effects of IL-1β in our model.

Various studies have shown a key role for prostaglandin signaling in mechanotransduction. Exposure to mechanical stimulation rapidly increases COX-2 expression in osteoblasts and osteocytes[34-36]. We found that IL-1β decreased the PFF-induced COX-2 gene expression. This is in contrast with findings by others that cytokine treatment enhances COX-2 gene

expression[37]. However Sugiyama et al. (2012) recently reported that inhibition of COX-2 expression in mice does not affect the trabecular or cortical bone response to mechanical stimuli. Therefore we speculate that the IL-1β-induced decrease of PFF-stimulated COX-2 expression does impair the skeletal response to daily loading in women[38]. Bakker et al.[28] reported that 24 h of IL-1β treatment dose-dependently increased caspase 3/7 activity which indicates increased osteocyte apoptosis[28]. In our study we also treated the cells for 24 h with IL-1β to mimic inflammation *in vivo*, which might have caused osteocyte apoptosis, and decreased COX-2 gene expression. Polyphenols in olive oil rapidly inhibit p38 and CREB phosphorylation, which leads to downstream reduction in COX-2 expression[39]. Oleuropein counteracted the inhibitory effect of IL-1β on PFF-stimulated COX-2 gene expression.

Since the response of osteocytes to PFF depends on an intact cytoskeleton, we tested the effect of oleuropein on the F-actin content of osteocytes treated with or without IL-1β. Oleuropein alone did not affect the F-actin content in MLO-Y4 osteocytes. IL-1β decreased the amount of F-actin, while addition of oleuropein was not able to counteract the IL-1β-induced decrease in F-actin content. Others have found that oleuropein directly disrupts actin filaments in a cell-free assay, while we tested the effect of oleuropein on F-actin in intact cells. This might explain the unaffected f-actin content in our experiments. The nature and kinetics of the disruption is currently under investigation.

One must be cautious when interpreting these data, because many variables are involved in a chronic inflammatory response[15,16]. The absence of an effect of oleuropein on the decrease in F-actin content caused by IL-1β does not necessarily imply that oleuropein is not counteracting the negative effects of inflammation of bone mass, since in this study we focused on the effect of IL-1β , at one time point only. It is possible that oleuropein has an effect on TNF-a induced bone loss, or bone loss induced by other cytokines.

We conclude that oleuropein did not affect the IL-1b-modulation of mechanical loading-induced NO production in osteocytes, but it did counteract the negative effect of IL-1b on PFF-induced COX-2 expression. This suggests that oleuropein and IL-1b interact in the process of mechanotransduction via an effect on COX-2 expression.

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DISCLOSURES

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CHAPTER 7

GLUCOSE-DEPENDENT INSULINOTROPIC PEPTIDE DOSE DEPENDENTLY AFFECTS THE MECHANORESPONSE OF OSTEOBLASTS

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ABSTRACT

In response to mechanical stimuli mechanosensitive bone cells produce signaling molecules such as nitric oxide (NO) resulting in the adaptation of bone mass and structure to the demands of mechanical loading. A disturbed response of bone cells to mechanical loading can lead to osteoporosis and increased fracture risk. Since fracture prevention is needed the search for new therapeutic agents is ongoing. An interesting agent might be glucose-dependent insulinotropic peptide (GIP) that affects bone mass and bone micro-architecture, but its mechanism of action is still unclear. We aimed to determine whether GIP modulates the response of mechanosensitive osteoblasts to mechanical stimulation. MC3T3-E1 osteoblasts were treated for 2 h with or without 0.05, 0.2, or 0.5 nM GIP, and subjected for 1 h to pulsating fluid flow (PFF: 0.7±0.3 Pa, 5 Hz) or static control culture. The osteoblast response to PFF was assessed by quantifying NO-production. In the absence of GIP, PFF stimulated NO production by osteoblasts within 5 min. Treatment with GIP at all concentrations tested decreased the PFF-stimulated NO production by 1.5 to 5.5-fold, demonstrating that GIP affects the mechano-response of osteoblasts. Since GIP can be readily found in the circulation, it might reach the bone cells within the highly vascularized bones. An altered responsiveness of bone cells to mechanical stimuli *in vivo* likely leads to a change in the balance between bone formation and resorption, thereby altering bone mass and structure. We conclude that biological pathways affecting GIP might be interesting new potential targets for treatment of bone diseases such as osteoporosis.

Key words: osteoblasts, mechanical stimulation, PFF, GIP, nitric oxide.

INTRODUCTION

The lack of mechanical stimuli results in a rapid loss of bone mass[1,2] since bone adapts its mass and structure to the demands of mechanical usage[3,4]. In this process of mechanical adaptation of bone, the mechanical signals are received by the osteocytes within the bone matrix[5-7]. Osteocytes are the most prevalent bone cells; there are approximately 20 times more osteocytes than mature osteoblasts and osteoclasts combined. With their cell bodies located in lacunae and their long interconnected cell processes positioned in the canaliculi, osteocytes are uniquely positioned within the bone matrix[8]. Upon mechanical use, a slight deformation of bone occurs as a result of mechanical loading, which drives a flow of interstitial fluid through the canaliculi[9], resulting in shear stress on the osteocyte cell membrane[7,11]. In response to this fluid flow the osteocytes produce signaling molecules, such as nitric oxide (NO), which is essential for adaptive bone formation[12]. Osteocytes are terminally differentiated cells of the osteoblast lineage. It has been shown that osteoblasts also produce signaling molecules in response to mechanical stimuli, although osteoblasts are less sensitive to mechanical stimuli in the form of fluid flow than osteocytes[6].

Skeletal abnormalities such as osteoporosis can be seen as failure of the skeleton to maintain bone mass to withstand fracture[13]. Osteoporosis is primarily present in the elderly, and it is expected that the number of osteoporosis-related fractures will increase substantially in the near future[14]. Osteoporosis-related fractures lead to diminished quality of life, disability, discomfort, and sometimes even death. Thus, prevention of bone fractures is urgently required[14], and the search for new therapeutic targets is ongoing.

Food intake affects bone turnover [15,16]. This occurs via the well-known pathways involving vitamin D or parathyroid hormone[17], but there are indications that other mechanisms are also involved in the interaction between nutrition and bone homeostasis. Recently it has been suggested that hormones produced in the gastro-intestinal tract might play an essential role in bone homeostasis[18]. Glucose-dependent insulinotropic peptide (GIP) is a gut factor released by entero-endocrine K-cells in the duodenum and jejunum in response to carbohydrate and fat ingestion[19]. GIP increases collagen type I expression and alkaline phosphatase activity in various osteosarcoma cell lines[20], and GIP protects osteoblasts from going into apoptosis[21]. The role of GIP in the modulation of bone turnover has been studied using knockout mice models[22]. GIP-receptor knockout mice show a phenotype of decreased bone formation, smaller bones, decreased bone mass, and alterations in bone microarchitecture and biomechanical properties[23]. On the other hand, transgenic mice overexpressing GIP show increased markers of bone formation, and decreased markers of bone resorption[23]. Consistent with these findings is the observation that GIP overexpressing mice show increased bone mass[24]. These studies show that GIP can positively affect bone

formation, resulting in higher bone mass. In view of GIP's role in nutrient absorption, this published data suggests that GIP might play a role in linking nutrient ingestion to bone formation. Although there is a proven connection between GIP and mechanical stimulation[25], to date this connection has not been observed in bone. Therefore the aim of this study was to determine whether GIP modulates the response of mechanosensitive osteoblasts to mechanical stimulation. We subjected MC3T3-E1 osteoblasts treated with or without GIP (0.05, 0.2, or 0.5 nM) to mechanical loading by a pulsating fluid flow (PFF). The osteoblast response to mechanical loading was assessed by quantification of NO production.

MATERIALS AND METHODS

Osteoblast culture

MC3T3-E1 osteoblast-like cells were cultured in α-MEM medium (Gibco, Paisly, UK) supplemented with 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 µg/ml streptomycin (Sigma-Aldrich), 1.4 mM L-glutamine (Sigma), 9 mM β-glycerophosphate (Sigma-Aldrich), 1.9 mM ascorbic acid (vitamin C; Merck, Darmstadt, Germany), and 10% fetal bovine serum (FBS; Gibco) at 37°C, in a humidified atmosphere of 5% CO_2 in air. Medium was changed every 3-4 days. Upon confluence, cells were harvested using 0.25% trypsin and 0.1% EDTA in phosphate buffered saline (PBS), seeded at 2×10^5 cells per 75 cm^2 culture flask (Greiner Bio-One, Kremsmuenster, Austria), and cultured until the cell layer reached confluency again. Then cells were used for pulsating fluid flow experiments as described below.

Pulsating fluid flow

Two days before pulsating fluid flow (PFF) treatment, cells were harvested from the culture flasks and seeded onto polylysine-coated (50 µg/ml; poly-l-lysine hydrobromide, mol wt 15-30 × 10^4; Sigma) glass slides (size 2.5 × 6.5 cm) at 2×10^5 cells/glass slide, and cultured overnight in petri dishes with 13 ml culture medium containing 10% FBS. Then the culture medium was replaced by α-MEM medium containing 0.2% bovine serum albumin (BSA), antibiotics, ascorbate (Merck), and with or without 0.05, 0.2 or 0.5 nM GIP (Sigma-Aldrich). Cells were incubated with GIP for 2 h or 24 h before subjecting the cells to PFF or static control culture in the presence of GIP.

PFF was applied as described earlier[26]. Briefly, PFF was generated by pumping 13 ml of culture medium through a parallel-plate flow chamber containing the MC3T3-E1 osteoblast-like cells. Cells were subjected to a 5 Hz pulse with a mean shear stress of 0.7 Pa, pulse amplitude of 0.3 Pa, and a peak shear stress rate of 8.4 Pa/s[27]. Stationary control cultures were kept in a Petri dish under similar conditions, i.e. at 37°C in a humidified atmosphere

of 5% CO_2 in air. After 5, 10, 15, and 30 min of PFF or static control culture, medium was collected and assayed for NO concentrations.

Nitric oxide

NO was measured as nitrite (NO_2^-) accumulation in the medium using Griess reagent containing 1% sulfanylamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in non-conditioned medium were used as standard curve. Absorbance was measured at 540 nm with a microplate reader (BioRad Laboratories Inc.).

Statistical analysis

PFF data was obtained from 5 or more separate experiments. NO production was expressed as PFF-over-static control values. All data is expressed as mean ± SEM. NO production data was analyzed using Wilcoxon signed-rank test for paired observations. A p value less than 0.05 was considered significant.

RESULTS

Application of PFF for 30 minutes to MC3T3-E1 osteoblasts did not result in visible changes in cell shape or alignment of the cells in the direction of the flow. No cells were removed by application of the fluid shear stress regime, as assessed by visually inspecting the cultures before and after PFF treatment.

In the absence of GIP, bone cells kept under stationary control conditions produced small amounts of NO (1.22±0.18 nMole) during the 30 min of the experiment (Fig. 1A). PFF treatment rapidly stimulated NO production by MC3T3-E1 osteoblasts (Fig. 1A). Incubation with GIP (0.05-0.5 nM) for 2 h significantly decreased the amount of PFF-stimulated NO production by MC3T3-E1 osteoblasts by 1.5-5.5 fold compared to cells that were not treated with GIP (Fig. 1B). Twenty four h of GIP treatment had no effect on the PFF-stimulated NO production by MC3T3-E1 osteoblasts (data not shown).

To exclude the effects of basal variation in NO production, the PFF-treated-over-static control (T/C) ratios were calculated for each separate experiment. In the absence of GIP, PFF stimulated NO production by 5 to 14-fold (Fig. 2A). Incubation for 2 h with 0.05 nM GIP inhibited the magnitude of the NO response by MC3T3-E1 osteoblasts already at 5 min (Fig. 2B). This inhibitory effect of GIP on the magnitude of the NO response became significant for all tested concentrations of GIP after 30 min of PFF treatment.

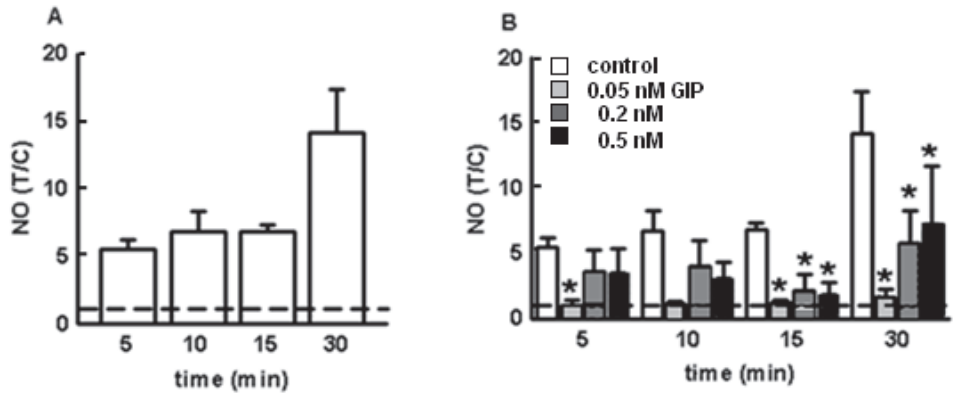

Figure 1. Effect of PFF on NO production by MC3T3-E1 osteoblasts. (A) PFF-stimulated NO production, and (B) GIP significantly inhibited the PFF-induced NO production by MC3T3-E1 osteoblasts during 30 min of PFF-treatment. Values are mean ± SEM of absolute NO values (5 independent experiments). Stat, stationary control culture; PFF, pulsating fluid flow; *Significant effect of PFF or GIP, p<0.05.

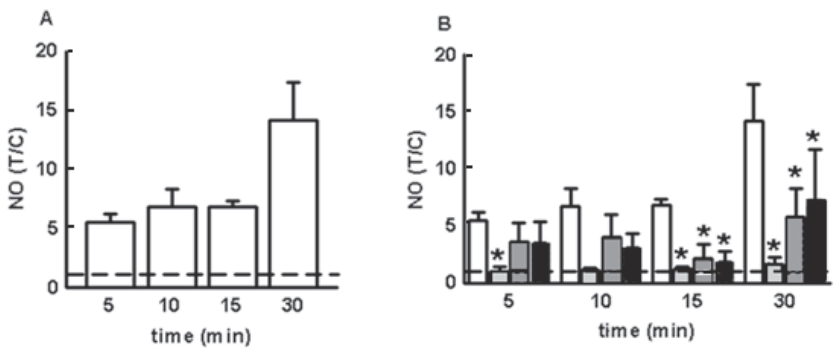

Figure 2. Mechanical loading by pulsating fluid flow increases NO production of MC3T3-E1 osteoblasts. A) GIP diminished the PFF stimulated NO-production. B) GIP significantly inhibited the PFF-induced NO production in MC3T3-E1 osteoblasts during 30 min of PFF-treatment. Values are mean ± SEM of PFF-treated over static-control ratios (T/C, of independent experiments). Dashed line, T/C=1 (no effect). GIP, glucose-dependent insulinotropic peptide. *Significant effect of GIP, p<0.05.

DISCUSSION

It is well documented that transduction of a mechanical stimulus by bone cells leads to NO production, an essential regulator of loading-induced bone formation[1,26,28]. Gut-hormones like GIP have also been suggested to be involved in bone metabolism[29,30]. The role of GIP in bone turnover has been investigated using transgenic mice models[23,24]. From these studies it can be deduced that GIP affects bone strength, likely due to alterations in skeletal

micro-architecture and biomechanical properties, and increased bone mass[23]. Although the number of studies on the role of GIP in bone biology is increasing, we were the first to test whether GIP has a direct effect on the response of bone cells to a mechanical stimulus. We found that GIP (0.05-0.5nM) reduces the magnitude of the NO response of osteoblasts to mechanical stimulation by PFF. These results are in accordance with the finding that GIP reduces the activity of nitric oxide synthase (NOS)[30], the enzyme responsible for NO production, in a mouse model of type 2 diabetes. It has been reported that GIP favors bone formation *in vivo*, which seems contradictory to our *in vitro* observations, since NO is essential for bone formation while we found a reduction in NO production in cells treated with GIP compared to cells treated without GIP. However, the concentration of GIP in the serum during fasting is 0.06 nM and after food ingestion 0.2 nM[31]. It might thus be more justified to compare PFF-stimulated NO production in the presence of 0.05 nM GIP to 0.2 nM or 0.5 nM GIP, in which case GIP shows a trend towards increasing the magnitude of the NO response to PFF (Fig. 2B). In addition, there are multiple other signaling molecules important for bone formation, e.g. Sost, PGE_2, VEGF, and BMP7. These molecules were not tested in this study, but they might have been affected by GIP as well. Our results show that a mechanical loading regime by PFF significantly decreased the NO production by MC3T3-E1 osteoblasts after GIP treatment.

We have used the MC3T3-E1 osteoblast-like cell line as a model to study whether GIP affects the response of bone cells to mechanical loading. The MC3T3-E1 cell line is well known for its mechano-sensitive properties[32,33]. We showed that MC3T3-E1 osteoblasts respond to PFF with increased NO production. The available MLO-Y4 osteocyte cell line needs to be grown in a-MEM, supplemented with 2.5% FBS and 2.5% calf serum. We were interested in the effects of a known concentration of GIP on the mechanoresponse of bone cells. Since basal GIP serum concentrations vary from 0.06 – 0.1 nm and reach 0.2–0.5 nm after food ingestion[21,34], we used BSA in the culture medium. The MC3T3-E1 osteoblasts adapt well to BSA in the culture medium, while MLO-Y4 osteocytes undergo apoptosis.

We showed that MC3T3-E1 osteoblasts respond to PFF. In addition, the applied mechanical stimulus (0.7 ± 0.3 Pa at 5 Hz) during the PFF regime was based on known physiologic bone-loading stimuli. Frequency spectra after hip loading *in vivo* in humans have been calculated[34], and range from 3 Hz (walking) to 9 Hz (running). The loading-induced fluid shear stress *in vivo* has been predicted to range from 0.8 to 3 Pa[28]. Bone cells *in vitro* are highly sensitive to shear stress of this order of magnitude, indicating a physiological response of these cells.

We found an inhibiting effect of GIP on the PFF-stimulated NO-production by MC3T3-E1 osteoblasts *in vitro*. Inhibition of NO production potentiates osteoclastic bone resorption *in vitro*[36], and high NO levels have been shown to be important for the prevention of

osteoclast recruitment and bone resorption[37]. However this study does not reveal whether NO production is the only parameter affected by GIP treatment, and therefore the effects of GIP *in vitro* on PFF-induced NO-production are not necessarily indicating a negative effect on bone mass *in vivo*.

In summary, our results show an effect of GIP on the PFF-stimulated NO-production of osteoblasts, suggesting an alterated mechano-response. A changed mechano-responsiveness *in vivo* may lead to a change in the balance between osteoblast and osteoclast activity, therefore affecting bone mass. We therefore conclude that biological pathways affecting GIP might be of interest as new potential targets in the treatment of bone diseases.

GRANTS

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DISCLOSURES

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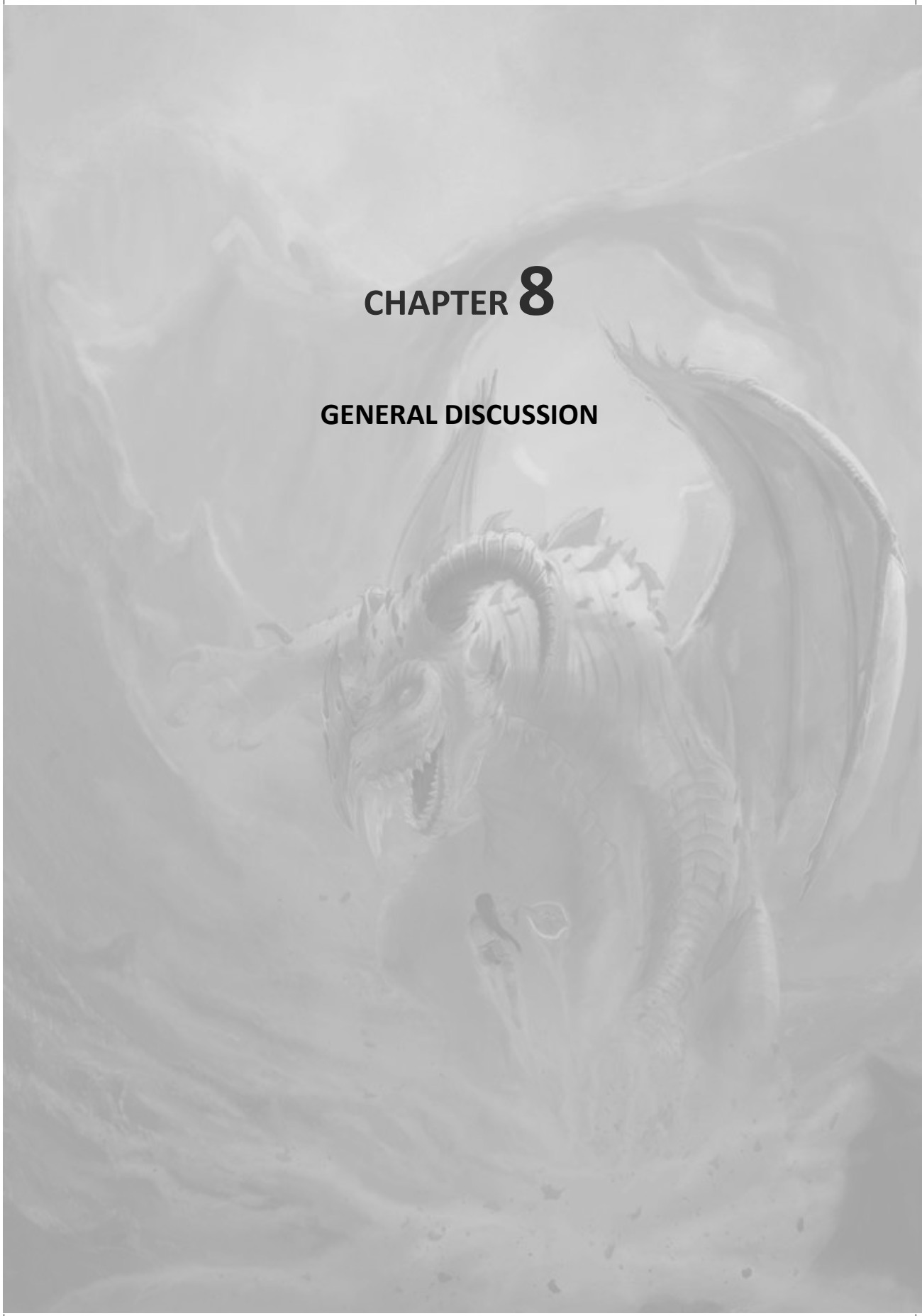

CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

In this thesis we have identified $1,25(OH)_2D_3$, sodium fluoride and oleuropein and GIP, to have an effect on the NO response of bone cells to mechanical loading. These results could contribute to a better understanding of osteoporosis. Skeletal abnormalities such as osteoporosis can be seen as the failure of the skeleton to maintain a proper bone mass and structure to withstand fracture upon mechanical loading[1]. Osteoporosis is primarily present in the elderly. Since populations age globally, it is to be expected that the incidence of osteoporosis-related fractures will rise substantially over the coming decades[2]. For Europe in 2000, there were an estimated 4 million fractures[3]. The costs of these fractures were estimated at € 31.7 billion, and are expected to increase up to € 76.7 billion in 2050[4]. In addition, osteoporosis-related fractures lead to diminished quality of life, disability, discomfort and death. Thus, prevention of these fractures is urgently required[5], and new therapeutic agents have been developed[6] for that purpose. For example denosumab, a fully human monoclonal antibody against receptor activator of nuclear factor κB ligand (RANKL), which regulates osteoclast differentiation, activity, and survival, is produced by osteocytes. Denosumab inhibits bone resorption and is approved for the treatment of women with postmenopausal osteoporosis[7]. Other examples of anti-osteoporosis drugs are antibodies against sclerostin and inhibitors of cathepsin K. In the absence of mechanical stimuli, osteocytes produce sclerostin, which inhibits osteoblastic bone formation[8]. The osteo-anabolic properties of monoclonal antibodies to sclerostin, such as AMG 785, have the potential to improve clinical outcomes in patients with osteoporosis[9]. The increasing number of osteoporosis patients, bringing along consequences concerning both the individual patient and society, asks for intervention. Since the current treatments have drawbacks, such as the inhibition of bone formation and the incidental occurrence of osteonecrosis, there is a cry for new therapeutic approaches. These new approaches might be found in the prevention of the development of osteoporosis, and it is to be expected that a 'change in lifestyle', in the form of a well balanced diet and daily exercise will contribute to the prevention of osteoporosis.

Physical activity, diet and bone health

Physical activity and diet are among the most commonly advocated lifestyle measures to improve skeletal health[10], and are supported by the European Food Safety Authority (EFSA). Nutrients like vitamin D and calcium have been recognized to have a beneficial effect on bone health for numerous years (EFSA, Panel on Dietetic Products, Nutrition and Allergies, 2010), but only recently other nutrients such as vitamin K and B vitamins have been fully recognized to contribute to bone health[11]. With regard to the contribution of physical activity, whereby the skeleton is exposed to mechanical loading, Wilhelm Roux

(1850-1924) proposed a concept of bone structure adapting to the mechanical demands, thereby optimizing the load-bearing function of the skeleton[12]. Today there is compelling evidence that high physical activity positively affects the skeleton by increasing the bone mineral content (BMC) and the bone mineral density (BMD)[13]. A striking example is found in athletes, where high load activities during training result in a 10–20% higher BMD compared to control subjects[14], and a 10–20% gain in BMC[15,16]. This increased BMC was not found in unloaded bones in the same athletes[17,18]. Moderate physical activity also contributes to skeletal health during human skeletal growth[19-21]. Bone mass at age 70 largely depends on peak bone mass[22]. Peak bone mass is reached between the age of 20 to 30 years[23]. Therefore physical activity during growth could be used as intervention against osteoporosis and/or fractures at later age[24]. Especially in the pre-pubertal and early peri-pubertal years, physical activity stimulates rapid bone apposition at bone surfaces[25,26], but this effect subsides after puberty[27,28].

It is known that the combination of frequent mechanical stimuli and nutrition affect the skeleton. Several interrelated conditions prevalent in athletes; A strict diet or even eating disorder, in combination with decreased endogenous estrogen, can finally result in a decrease in bone mass[29]. In athletes a delayed menarche is not rare, resulting in higher risk of failure to reach peak bone mass[30]. Despite the common eating disorder or at least the poor diet, and the disturbed hormonal balance, the total body mineral density/bone mineral content ratio of calcified matrix in female athletes was shown to be relatively high[31]. Thus somehow the negative factors caused by poor diet can be counteracted with high levels of physical activity.

Regardless the importance of diet and physical activity for bone, not much is known about which specific nutrients contribute to a gain in bone mass, and in which cellular pathways the mechanisms that are involved in nutrition and mechanical loading do overlap or interact. In this thesis we focused on the effects of nutrients on osteocytes, which are the mechanosensitive bone cells that regulate the activity of the bone forming osteoblasts and the bone resorbing osteoclasts, in response to mechanical signals. In theory, any factor (such as nutrients) that affects the response of osteocytes to mechanical loading will affect bone mass.

Mechanotransduction and bone remodeling

Bone is often thought of as a dead tissue, but the opposite is true. Bone is a combination of organic and an-organic matrix, which contains many living cells; osteoblasts, osteoclasts, osteocytes, and bone lining cells. Osteoblasts are the bone forming cells. They produce collagens[32] and non-collagenous proteins, and probably play a role in the initiation of calcification of the bone matrix. Osteoclasts demineralize the bone matrix and degrade the

collagen[33]. Together osteoblasts and osteoclasts repair micro damage caused by fatigue loading through targeted remodeling, a process believed to be orchestrated by osteocytes[34-36]. This process of bone remodeling leads to adaptation of bone to its mechanical demands [37,38].

Osteocytes are the bone mechanosensor cells and constitute 95% of all cells in bone. During bone formation, a subpopulation of osteoblasts undergoes terminal differentiation and become embedded within the bone[39]. By this time they are referred to as osteoid-osteocytes[40]. After mineralization of the bone matrix the entombed cells are called osteocytes. Osteocytes form an interconnected cellular network extending throughout the mineralized bone. Osteocytes are in contact with neighboring osteocytes via extended cell protrusions that make cell-cell contact possible[41]. The protrusions are positioned within the canaliculi in the calcified bone matrix, and they are surrounded by interstitial fluid[42]. It has been a matter of debate how mechanical loading by physical activity of intact bone is transduced into a signal that activates the osteocytes[43]. In the canalicular flow hypothesis by Pieckarski and Munro (1977)[44] it was proposed that the loads result in deformation within the bone matrix, thereby causing a flow of the interstitial fluid through the canalicular network[42,44], causing a shear stress on the osteocyte protrusions[42]. Osteocytes can be stimulated by relatively small fluid shear stresses acting on the membranes of their osteocytic processes. As a result of the shear stress, the glycocalyx shifts and activates kinases. In addition, fluid flow may cause a strain amplification in the osteocyte processes[45] or open stretch activated ion channels in the cell membrane[46]. The activation of kinases leads to the production of chemical signals. As a result of shear stress, osteocytes translate the physical signal into a biochemical signal. This process is called 'mechano transduction'. Subsequently osteocytes produce signaling molecules that modulate the activity of osteoblasts and osteoclasts.

Signalling molecules

Bone Morphogenetic proteins (BMPs) are key proteins in the regulation of cell-cell communication and differentiation[47,48]. Although BMPs are one of the most studied growth factors in bone regeneration[47], the role of BMPs in bone mechanotransduction had to be elucidated. In chapter 2, we show that a mechanical stimulus in the form of a pulsating fluid flow, significantly increases BMP7 gene and protein expression in human osteocytes. We found that in osteocytes basal BMP2 protein levels were significantly higher than basal BMP7 protein levels, but basal BMP2 protein levels were not altered in response to mechanical loading. BMP7 is specifically upregulated during mechanical loading induced bone remodeling. Since BMP7 is known to play a relevant role in pre-osteoblast maturation in bone[49], it seems logical that BMP7 is produced to promote pre-osteoblast growth and differentiation during mechanical stimulation. The upregulation of BMP7 as a result of a mechanical stimulus shown in chapter 2 thus indicates that osteocytes might control

osteoblastogenesis via loading induced-BMP7 production. This shows a novel role for BMP7 in the adaptation of bone to a mechanical stimulus. The upregulation of BMP7 in osteocytes by mechanical stimulation was likely mediated by the nuclear vitamin D receptor. Not much is known about the function of the vitamin D receptor in the pathways involved in mechanical loading. We went more in to the details of this receptor and its role in mechanical loading in chapter 3.

Another important early mediator of the response of osteocytes to mechanical loading is nitric oxide (NO), released by nitric oxide synthase (NOS), which mediates the induction of bone formation by mechanical loading *in vivo*[50,51]. In chapter 3 we show that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) stimulated NO production by MC3T3-E1 osteoblasts but abolished the effect of this mechanical stimulation on NO production, even in cells lacking the nuclear VDR. Therefore we suggested that it is likely the membrane VDR receptor that is involved in the modulation of NO after incubation with $1,25(OH)_2D_3$. The VDR is a member of a nuclear receptor subfamily which forms heterodimers with RXR and contains an AF-2 domain that undergoes ligand-induced conformational change. The regulation of gene expression by VDR is dependent of vitamin D responsive elements (VDREs) in the promoters of target genes. However, some vitamin D-regulated genes do not contain VDREs in their promoters and are thought to be under indirect regulation. Thus it seems that vitamin D/VDR affects gene expression by binding to VDREs, but also via regulation of other pathways[52]. One such pathway that is clearly regulated this way by nuclear receptors is the wnt pathway, which affects gene expression through β-catenin. Beta-catenin, is required for cell-cell adhesion and for regulation of gene expression in response to wnt signaling[53-55]. Specific residues in the AF-2 domain of the VDR can discriminate between its classical activity and its ability to interact with β-catenin[52]. Santos et al (2010) showed that PFF induces β-catenin stabilization and activation of the β-catenin signaling pathway mediated by amongst others NO, in osteocytes[56]. The role of the $1,25(OH)_2D_3$ and the VDR in the bone cell response to mechanical loading may interact with the Wnt/β-catenin pathway, but further research is needed.

The role of the cytoskeleton in mechanical sensing

Lately more evidence emphasizes the role of the cytoskeleton as a structure that is highly responsive to external physical and chemical stimuli[57]. The cytoskeleton is involved in mechanosensing and determines the material properties of the cell (i.e., cell stiffness). The effects of mechanical stress to an object largely depends on the material properties of that object. In bone, low magnitude (<10 με) and high frequency (10–100 Hz) loading can stimulate bone mass and strength while high loading rates increase bone mass and strength *in vivo*[58]. The production of signaling molecules by osteocytes in response to a fluid shear

stress (at 5 and 9 Hz) *in vitro* is correlated with the applied force stimulation[59-61]. Moreover osteocytes with round suspended morphology required lower force stimulation in order to show an increase in NO production, even though they were an order-of-magnitude more elastic, compared to flat adherent cells[62]. Apparently elastic osteocytes seem to require less mechanical force in order to respond than stiffer cells. This data indicates that differences in mechanosensitivity in osteocytes are related to the shape and material properties of the cell[62].

Fluoride and oleuropein both affect the cytoskeleton. Fluoride disrupts the cytoskeleton of protozoa[63], and F-actin in ameloblasts[64]. Oleuropein is known to induce cell rounding in different cell lines *in vitro* and in mice tumor cells *in vivo*. The effect of oleuropein on tumor cells was explained by an immediate effect of oleuropein on the actin cytoskeleton[65]. In chapter 4 and 5 we discuss the effect of fluoride and oleuropein on the response of osteocytes to mechanical stimulation. Having demonstrated that only fluoride has an effect on the amount of F-actin fibers and alignment of the fibers, we concluded that fluoride treatment results in changed osteocyte shape and mechanical loading-properties. We did not find the expected effect of oleuropein on the f-actin cytoskeleton of osteocytes, as has been described by others[65]. It has been reported that oleuropein rounded both normal and tumor cells, but the normal cell rounding was reversible after washing. Since in our experimental setup cells are not continuously exposed to oleuropein, this might be a plausible explanation for the absence of disruption of the cytoskeleton.

The effects of gut hormones on bone health

Upon food intake, the hormone Glucose-dependent insulinotropic polypeptide (GIP) is secreted from the intestinal K-cells, which has established insulin-releasing actions. However, the GIP receptor (GIPR) is widely distributed in peripheral organs, including bone tissue[66]. As GIPR is found on osteoblasts and osteoclasts, a physiological role for this hormone in bone remodelling has been suggested. GIP seems to increase bone density and exerts anabolic effect on bone in vitro: in osteoblastic cell lines, GIP dose-dependently prevents apoptosis and increases intracellular calcium levels, alkaline phosphatase activity and collagen type 1 mRNA levels[67]. Furthermore, postprandial activation of osteoclast GIPR inhibits bone resorption[68]. After meal ingestion, GIPR-knock-out mice had increased plasma calcium levels compared to wild-type animals. In addition, bone resorption markers decreased after oral and intravenous administration of nutrients in presence of GIP, suggesting GIP directly stimulates calcium uptake and bone formation by osteoblasts postprandially[68]. Lately evidence rises suggesting exercise may acutely modulate GIP action in humans[69]. In chapter 6 we describe the effect of GIP treatment on the mechanosensitivity of bone cells. We found that 2 h incubation with GIP increased osteoblast NO production upon mechanical

stimulation, suggesting that there might be a connection between exercise, activation of GIP action and the response of bone cells to this mechanical stimulus.

The ability of nutrients to affect the mechano-response of bone cells

The classic role of the vitamin D endocrine system is to stimulate calcium absorption in the intestine, thus maintaining normal calcium homeostasis and indirectly regulating bone mineralization[70]. $1,25(OH)_2D_3$ induces the differentiation of osteoblasts via sequential induction of cell cycle arrest[71], maturation of extracellular matrix and finally bone mineralization[72]. Rickets and osteomalacia are the diseases traditionally associated with severe vitamin D deficiency, defined as 25-hydroxyvitamin D levels below 25 nmol/l. Less severe degrees of vitamin D deficiency (25 and 50 nmol/l) and vitamin D insufficiency, (50 and 75 nmol/l), already impair gastrointestinal absorption of calcium and bone mineralization, contributing to the pathogenesis of osteoporosis in older people[73]. The importance of vitamin D for bone health and the prevention of osteomalacia and osteoporosis are well recognized. In a rat model of hind limb unloading, exogenous $1,25(OH)_2D_3$ not only prevented the bone loss but also increased the bone mineral density to greater than the baseline level (+7%). These results suggest that elevating circulating $1,25(OH)_2D_3$ levels presumably increasing calcium absorption can counteract bone loss induced by disuse or microgravity with its associated reductions in circulating $1,25(OH)_2D_3$ and decreased calcium absorption[74]. Our mechanical loading studies did show an effect of $1,25(OH)_2D_3$ on the mechanosensitivity of osteoblasts, in terms of NO production.

The fluoride anion substitutes hydroxyl in hydroxyl-apatite crystals, thereby changing the crystalline structure of bone tissue[75]. Fluoridated hydroxyl-apatite increases osteoblast cell attachment, proliferation and differentiation[76]. Fluoride also stimulates bone formation at cellular[77,78], tissue[79] and organ levels[80], leading to an increase in trabecular bone volume[79,81] in osteoporotic patients. Fluoride therapy on the osteopenic bone of the ovariectomized rat causes an increase of bone stiffness due to the presence of fluoroapatite. This introduces new properties in osteopenic rat bone: higher resistance to compression loading and a greater frailty to flexion loading[82]. In another study, the effect of increasing fluoride doses on the bone formation, microarchitecture, mineralization and microhardness of the A/J, SWR/J and 129P3/J mouse strains was assessed. Fluoride treatment had no significant effect on bone microarchitecture in these three strains[83]. In our study, we found that fluoride had an effect on NO production of mechanically stimulated osteocytes, and cellular f-actin content. We concluded that, based on these findings, fluoride might changes the biomechanical properties of the cell via changes in f-actin content, and thereby the response to mechanical stimuli. In vivo this would result in more brittle bone, caused by less mechanosensitive bone cells.

To the best of our knowledge, it is unknown whether oleuropein has the ability to alter the mechanical properties of bone cells. However few studies have shown that oleuropein alters the actin cytoskeleton[63,64]. Earlier we explained that the cytoskeleton is a major component in determining the mechanical response of the cells[62]. We found that oleuropein itself did not alter the mechanoresponse of osteocytes, nor did it alter the cellular f-actin content. Therefore the effect of oleuropein on COX-2 gene expression is not mediated via the cytoskeleton.

Conclusion

The increasing number of osteoporosis patients is accompanied with high costs and decreased quality of life. This asks for a new therapeutic approach in the near future. These approaches preferably need to be found in the prevention of the development of osteoporosis, and should be non-invasive. Suggested is a 'change in lifestyle', daily exercise and a balanced diet, possibly fortified with bone formation stimulating nutrients. Therefore it will be necessary to determine which nutrients enhance bone mass, amongst others via an increase in the sensitivity of bone cells to mechanical stimuli. In this thesis we have identified $1,25(OH)_2D_3$, sodium fluoride to have an effect on the response of bone cells to mechanical loading. However, the found effects had no extra effect on the amount of NO produced by osteocytes on top of the NO production in response to mechanical loading alone. Although conditions such as vitamin D toxicity and fluorosis are rare, they do occur at excessively high doses, and result into hypercalcemia and hyperphosphatemia[84], excessive osteoid formation and incomplete mineralisation[85,86] Intoxication can be iatrogenic, due to self medication or accidental with abuse of fortified milk product or contamination of common dietary constituents like table sugar or cooking oil. Therefore we recommend daily exercise and a balanced diet. Consumption of foods such as vitamin enriched milk products might be beneficial when the natural intake is not sufficient, but should not be needed when the diet is well balanced.

A better understanding of the pathways involved in mechano-transduction and regulating factors in daily nutrition are necessary to develop such strategies.The results described in this thesis contribute to a better understanding of interactions between bone, daily loading and diet, which might be of importance for the development of new strategies to prevent osteoporosis.

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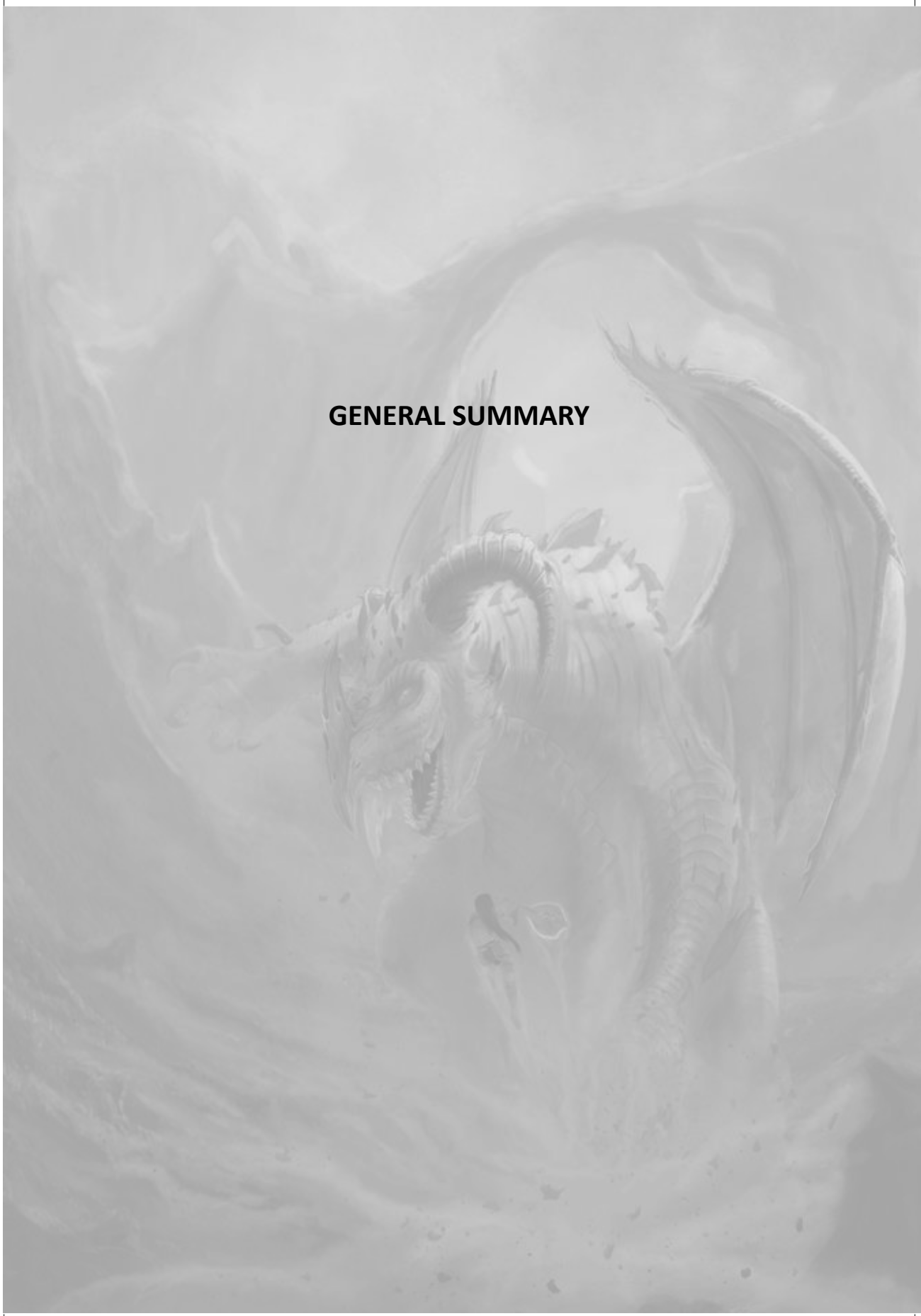


GENERAL SUMMARY

Bones adapt their mass and structure to the demands of mechanical usage in order to achieve an optimal resistance to mechanical failure with a minimum use of material. This process is known as functional adaptation of bone and occurs during bone remodeling. In the process of bone remodeling, osteoclasts resorb the bone, while osteoblasts deposit new bone. This interplay between osteoclasts and osteoblasts is tightly coordinated by the mechanosensing osteocytes. When osteocytes are mechanically loaded, they start to produce signaling molecules, such as nitric oxide (NO), that affects bone formation. In addition to mechanical loading, several nutrients are also able to affect NO production, but the combined effect of mechanical usage and nutrients on the production of NO by bone cells has not been elucidated.

This thesis focuses on the effect of nutrients on the response of mechano-sensitive bone cells to mechanical stimuli. The aim was to identify nutritional factors that alter the bone cell response to mechanical loading. We also assessed whether the combination of certain nutrients and mechanical stimuli can counteract the effects of inflammatory cytokines on mechanosensitive bone cells, since bone loss associated with systemic inflammatory diseases may involve elevated cytokines levels.

In this thesis the following scientific questions were addressed:

1. What are the pathways leading to the release of signaling molecules, specifically bone morphogenetic proteins, by bone cells in response to mechanical loading?

2. How do dietary components that are used as agents in osteoporosis treatment, such as 1,25-dihydroxyvitamin D_3 and fluoride, affect the response of osteocytes to mechanical stimulation?

3. Could nutrients such as oleuropein potentially counteract inflammation-induced bone loss by restoring the altered response of bone cells to mechanical stimuli in an environment of inflammation?

To seek answers to these questions we first assessed whether osteocytes produce BMPs in response to mechanical loading (**Chapter 3**). We demonstrated that mechanical loading by pulsating fluid flow (PFF) upregulates BMP7 gene and protein expression in osteocytes *in vitro*, likely via the vitamin D receptor (VDR). BMP2 gene and protein expression was not affected by PFF.

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) mediates bone mass. The genomic responses to $1,25(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), a steroid hormone nuclear receptor. In addition, $1,25(OH)_2D_3$ seems to mediate cellular responses via a variety of receptor types located within or near the plasma membrane. In **chapter 4** we aimed to determine whether $1,25(OH)_2D_3$ affects the production of NO by osteoblasts that are either or not mechanically stimulated by PFF. We expected $1,25(OH)_2D_3$ to stimulate NO production by osteoblasts via the VDR, rather than via rapid membrane receptor-mediated

mechanisms. We showed that $1,25(OH)_2D_3$ stimulates inducible NOS expression and NO production by osteoblasts in the absence of mechanical stimulation, likely via genomic VDR action. However the mechanical loading-induced NO production might be affected by $1,25(OH)_2D_3$ independent of genomic VDR action, since $1,25(OH)_2D_3$ diminished PFF-induced NO production in bone cells lacking the VDR.

The bone anabolic agent fluoride has been used for decades to enhance bone mass in osteoporosis, but it is known to disrupt the cytoskeleton. Since the response of bone cells to mechanical loading is mediated by the cytoskeleton, we investigated whether fluoride affects the response of bone cells to mechanical loading by PFF, and whether this is cytoskeleton-mediated. Fluoride inhibited the mechanical loading-stimulated NO production, and decreased the amount of the cytoskeletal component F-actin. Treatment of young hamsterds with fluoride resulted in more elongated, smaller osteocytes in interdental bone (**Chapter 5**). This suggests that fluoride inhibits the mechano-response of bone cells, which might occur via cytoskeletal changes.

The inflammatory cytokine interleukin-1b (IL-1b) reduces the response of osteocytes to mechanical stimuli. Phenolic dietary compounds counteract the effects of inflammatory cytokines. We showed that the phenolic compound oleuropein affects the response of IL-1b–treated osteocytes to mechanical stimulation by PFF. We also found that the NO response to mechanical loading was lower in human osteoblasts from osteoporotic patients than in control bone cells. In MLO-Y4 osteocytes, IL-1β reduced mechanical loading-stimulated NO production, which could not be reversed by oleuropein treatment. IL-1b also decreased F-actin content and COX-2 gene expression in response to mechanical loading Oleuropein prevented the effect on COX-2 expression, but not on F-actin content. Our results suggest that oleuropein may reduce the inflammation-induced bone loss *in vivo* by reducing the inhibitory effect of IL-1b on PFF-stimulated COX-2 gene expression (**Chapter 6**).

Glucose-dependent insulinotropic peptide (GIP) is a gut-hormone that is released by entero-endocrine K-cells in the duodenum and jejunum in response to carbohydrate and fat ingestion. GIP is known to have an effect on bone mass and bone micro-architecture, but its mechanism of action is unclear. In view of GIP's role in nutrient absorption, it might play a role in linking nutrient ingestion to bone formation. We aimed to determine whether GIP modulates the response of mechanosensitive osteoblasts to mechanical stimulation. Treatment with GIP decreased the mechanical loading-stimulated NO production by osteoblasts suggesting that GIP affects the mechano-response of osteoblasts (**Chapter 7**).

In this thesis we have identified $1,25(OH)_2D_3$, sodium fluoride, oleuropein and GIP to have an effect on the NO response of bone cells to mechanical loading. These results could contribute to a better understanding of osteoporosis. The increasing number of osteoporosis patients asks for new therapeutic approaches in the near future. These

approaches preferably need to be found in the prevention of osteoporosis, and should be non-invasive. Daily exercise and a balanced diet, possibly fortified with bone formation stimulating nutrients, might be such a new approach. Therefore a better understanding of the pathways involved in mechano-transduction and regulating factors in daily nutrition are necessary to develop such strategies. The results described in this thesis contribute to a better understanding of interactions between bone, daily loading and diet, which might be of importance for the development of new strategies to prevent osteoporosis.

ALGEMENE SAMENVATTING

Botten adapteren hun massa en structuur aan de eisen van de mechanische belasting om een optimale weerstand tegen mechanisch falen te bereiken met een minimale benutting van materiaal. Dit proces staat bekend als functionele adaptatie van bot en vindt plaats tijdens bot-remodellering. Tijdens het proces van bot-remodellering wordt het bot door osteoclasten geresorbeerd, terwijl osteoblasten nieuw bot aanmaken. Dit samenspel tussen osteoclasten en osteoblasten wordt strikt gecoördineerd door de drukgevoelige osteocyten. Wanneer osteocyten mechanisch belast worden gaan ze signaal moleculen produceren, zoals stikstofoxide (NO), dat de botvorming beïnvloedt. Zowel mechanische belasting als ook verscheidene voedingsstoffen kunnen de NO productie beïnvloeden, maar het effect van een combinatie van mechanische belasting en voedingsstoffen op de NO productie is nog onbekend.

Dit proefschrift richt zich op het effect van voedingsstoffen op de reactie van drukgevoelige botcellen op mechanische belasting. Het doel was om voedingsstoffen te identificeren die een verandering in de reactie van botcellen op mechanische belasting kunnen bewerkstelligen. We hebben ook onderzocht of de combinatie van bepaalde voedingsstoffen en mechanische belasting het negatieve effect van ontstekingsmediatoren op botcellen kan opheffen, omdat verhoogde concentraties van ontstekingsmediatoren mogelijk een rol spelen bij het bij botverlies geassocieerd met systemische ontstekingsziekten.

In dit proefschrift werden de volgende wetenschappelijke vragen aan de orde gesteld:

1. Welke biochemische routes leiden tot de afgifte van signaalmoleculen, in het bijzonder “bone morphogenetic proteins”, door botcellen in reactie op mechanische belasting?
2. Hoe beïnvloeden voedingscomponentenin het dieet die worden gebruikt ter behandeling van osteoporose, zoals 1,25-dihydroxyvitamin D_3 en fluoride, de reactie van osteocyten op mechanische belasting?
3. Hebben voedingsstoffen zoals oleuropeïne de potentie om botverlies veroorzaakt door ontstekingsreacties tegen te gaan door de veranderde reactie van botcellen op mechanische belasting bij ontsteking te herstellen.

Om deze vragen te beantwoorden hebben we eerst bepaald of osteocyten in reactie op mechanische belasting BMPs produceren (**Hoofdstuk 3**). We hebben aangetoond dat mechanische belasting in de vorm van een pulserende vloeistofstroom (“pulsating fluid flow” (PFF)), de gen- en eiwitexpressie van BMP7 in osteocyten *in vitro* verhoogd, vermoedelijk via de vitamine D receptor (VDR). PFF had geen effect op de gen- en eiwitexpressie van BMP2.

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) medieert de botmassa. De genomische reacties op $1,25(OH)_2D_3$ worden gemedieerd door de VDR, een steroïd hormoon kernreceptor. $1,25(OH)_2D_3$ kan waarschijnlijk ook cellulaire reacties bewerkstelligen via een aantal receptoren die gelegen zijn in of tegen het plasmamembraan. In **hoofdstuk 4** was het doel om aan te tonen dat $1,25(OH)_2D_3$ een effect heeft op de productie van NO door osteoblasten,

die al dan niet mechanisch belast werden met PFF. Wij verwachtten dat $1,25(OH)_2D_3$ de NO productie door osteoblasten zou stimuleren via the VDR maar niet via de snelle membraan receptor-gemedieerde mechanismen. We hebben aangetoond dat in de afwezigheid van mechanische belasting $1,25(OH)_2D_3$ de expressie van "inducible NOS" en de productie van NO door osteoblasten stimuleert, waarschijnlijk via de kernreceptor. Het is echter ook mogelijk dat de door mechanische belasting-geïnduceerde NO productie onafhankelijk van de VDR kernreceptor wordt beïnvloedt door $1,25(OH)_2D_3$, , omdat $1,25(OH)_2D_3$ de PFF-geïnduceerde NO productie door botcellen zonder deze VDR verminderde.

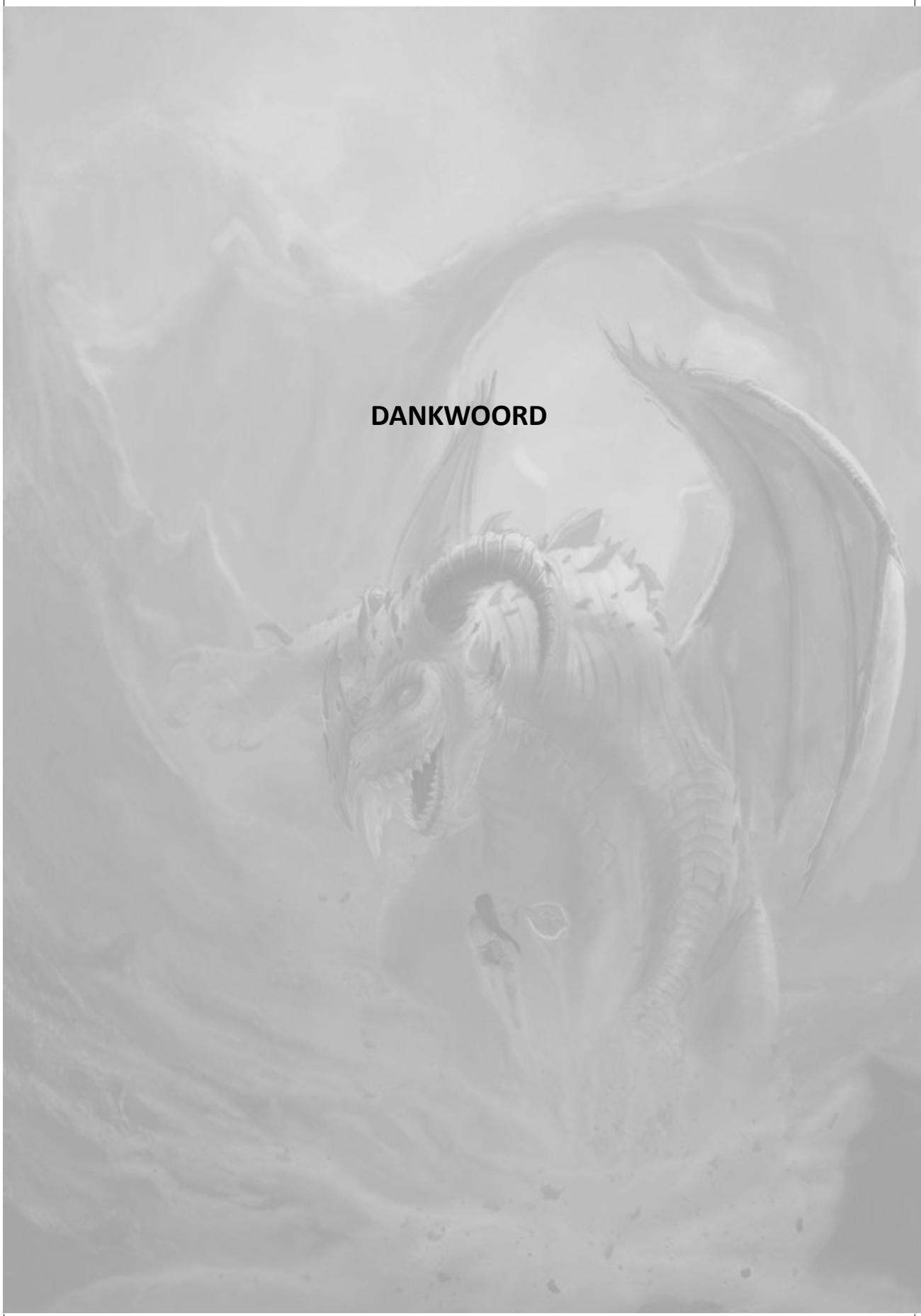
Het voor bot anabole agens fluoride is decennia lang gebruikt om de vorming van botmassa in osteoporose patiënten te stimuleren, maar van fluoride is het ook bekend dat het het cytoskelet verbreekt. Omdat de reactie van botcellen op mechanische belasting wordt gemedieerd door het cytoskelet, hebben wij onderzocht of fluoride de reactie van botcellen op mechanische belasting door PFF beïnvloedt, en of dit cytoskelet gemedieerd is. Fluoride inhibeerde de door mechanische belasting gestimuleerde NO productie, en verlaagde de hoeveelheid F-actine, een bestanddeel van het cytoskelet. In jonge hamsters die met fluoride waren behandeld waren de osteocyten in interdentaal bot langer en kleiner (**Hoofdstuk 5**). Dit suggereert dat fluoride de reactie van botcellen op mechanische belasting inhibeert, vermoedelijk door veranderingen in het cytoskelet.

Het inflammatoire cytokine interleukine-1b (IL-1b) verlaagt de reactie van osteocyten op mechanische belasting. In de voeding aanwezige fenolen kunnen de effecten van cytokines tegenwerken. Wij hebben aangetoond dat het fenol oleuropeïne de reactie van IL-1b behandelde osteocyten op mechanische belasting door PFF verandert. Ook hebben we gevonden dat de NO respons op mechanische belasting lager was in humane osteoblasten van osteoporose patiënten dan in controle botcellen. In MLO-Y4 osteocyten verminderde IL-1β de mechanische belasting-gestimuleerde NO productie, die niet worden hersteld door toevoeging van oleuropeïne. IL-1b verlaagde ook de hoeveelheid F-actine en COX-2 genexpressie in respons op mechanische belasting. Toevoeging van oleuropeïne voorkwam het effect op COX-2 genexpressie, maar niet op de hoeveelheid F-actine. Onze resultaten suggereren dat oleuropeïne het ontstekings-gemedieerde botverlies *in vivo* vermindert door het effect van IL-1b op de PFF-gestimuleerde COX-2 genexpressie te verminderen (**Hoofdstuk 6**).

Glucose-afhankelijk insulinotropisch polypeptide (GIP) is een darm-hormoon dat wordt afgegeven door entero-endocriene K-cellen in de twaalf-vingerige darm en de dunne darm na inname van koolhydraten of vet. Van GIP is bekend dat het een effect heeft op de botmassa en op de bot micro-architectuur, maar het werkingsmechanisme van GIP is onduidelijk. Met het oog op de rol van GIP bij de opname van voedingsstoffen zou het een wellicht een rol kunnen spelen bij de link tussen opname van voedingsstoffen en botvorming.

Ons doel was om te onderzoeken of GIP de reactie van mechanogevoelige osteoblasten op mechanische stimulatie beïnvloedt. Behandeling met GIP verlaagde de mechanische belasting-gestimuleerde NO productie door osteoblasten, hetgeen suggereert dat GIP de mechano-respons van osteoblasten beïnvloedt (**Hoofdstuk 7**).

In dit proefschrift hebben we aangetoond dat $1,25(OH)_2D_3$, fluoride, oleuropeïne en GIP een effect hebben op de NO productie door botcellen in respons op mechanische belasting. Deze resultaten kunnen bijdragen aan een beter begrip van osteoporose. Het stijgende aantal osteoporose patiënten vraagt om een nieuwe therapeutische aanpak in de nabije toekomst. Deze aanpak zal bij voorkeur gezocht moeten in de preventie van osteoporose en moet niet-invasief zijn. Dagelijkse beweging en een uitgebalanceerd dieet, waar mogelijk verrijkt met specifieke voedingsstoffen die de botvorming stimuleren, is zo'n mogelijke aanpak. Om nieuwe strategieën te ontwikkelen is meer kennis nodig van de biochemische routes die betrokken zijn bij de mechano-transductie, en van de regulerende factoren in het dagelijks dieet. De resultaten beschreven in dit proefschrift dragen bij aan een beter begrip van de interacties tussen bot, dagelijkse belasting en dieet, hetgeen van belang kan zijn voor de ontwikkeling van nieuwe strategieën ter voorkoming van osteoporose.



DANKWOORD

In den beginne, de eerste week, was promoveren spannend. Totdat bleek dat er die week niemand was om het project mee door te nemen, te brainstormen. Ik werd geplaatst in het kantoor van Astrid, die zelf op een super spannende vakantie was in Jordanië. Met wat oude proefschriften en toegang tot het wereld wijde web, werd ik geacht mijzelf in te lezen. Het onderzoeksvoorstel had ik niet, maar wel wat oude proefschriften. Ik heb mijzelf daar maar op gestort, en gelukkig was het toen bijna tien uur. De deur vloog open en een super enthousiaste Jolanda stormde binnen en vertelde dat bij OCB elke ochtend om 10.00 de koffie klaar stond. En op dat moment voelde ik mij dus zeer welkom. In de weken die volgden leerde ik langzaam aan iedereen kennen, en de meeste nieuwe mensen waren zeer vriendelijk en behulpzaam. Later die week was ook mijn eerste meeting met Jenneke, mijn waarde promotor. En vele meetings volgden. En deze meetings waren stuk voor stuk zeer bijzonder. Jenneke heeft mij in de afgelopen 5 jaar getracht veel dingen bij te brengen. Hoe dingen 'moeten' en 'horen'. Want Jenneke weet dat, Jenneke is de professor. En zegt ze er vaak geruststellend bij, 'jij kan dat ook niet weten, want je bent nog maar AiO'. En ik heb veel geleerd. Hoe dingen moeten, maar vooral ook, hoe dingen niet moeten. Presentaties werden uitvoerig geoefend, niet zelden tot in de late uurtjes, waarop meer dan eens door Jenneke werd gevraagd haar dan toch gewoon maar na te spreken. Maar het resultaat was er wel naar. Abstracts, publicaties, presentaties, ze klopten tot in de puntjes. Maar gelukkig kon ik ook Jenneke nog wat leren. Met hulp van Nina is ze bekend geworden met de meest verkochte cocktails, kent ze nu meer dan 3 smaakjes jenever, weet ze het 'kleine cool down cafe' blind te vinden en is ze nu een groot fan van Dries Roelvink. Ik kan dan ook alleen maar zeggen dat de afgelopen 5 jaar onvergetelijk waren, en dat Jenneke daar voor een groot deel verantwoordelijk voor is.

Mijn co-promotor Ellen is een compleet ander verhaal. Ellen werkte in Deventer, en was niet vaak in beeld, maar ze was altijd enthousiast, blij met nieuwe resultaten, en was vaak de aanzetster van nieuwe ideeën en experimenten. Ondanks de afstand, hield Ellen een flinke vinger in de pap. Regelmatig hadden we meetings waarbij Ellen steeds de kant van de nutriënten bleef benadrukken. Het was dan ook zeer leerzaam om samen met Ellen de review te schrijven. En Ellen hield ons met beide benen op de grond. Wij (van ACTA) hadden vaak het idee al klaar te zijn met het manuscript als Ellen nog met input kwam, waardoor het werk uiteindelijk naar een ander niveau werd gebracht. Zonder Ellen had dit proefschrift dan ook nooit zijn huidige vorm bereikt.

Astrid werd pas na 2 jaar officieel mijn co-promotor. Het was fijn om met Astrid te bomen over experimenten. Meer dan eens heeft ze me uit de wetenschappelijke put getrokken en verteld dat ik het allemaal wel kon. Dat was een mooi tegengeluid. Verder hebben we best veel lol gehad op NVCB meetings, girls only events, de Dries premiere, en Toronto. Ja, we zijn zelfs samen in Toronto geweest. Waar Astrid letterlijk 'to the rescue' kwam, en 'a girls

right to have meat balls' zeer dapper heeft verdedigd. Als ik alle avonturen beleefd met Astrid zou opschrijven zou dit een zeer ongeloofwaardig dankwoord worden, en dat is niet de bedoeling. Maar Astrid heeft een zeer groot aandeel in het feit dat ik het zover heb gebracht, en daarom alleen al is ze goud!

Een ander persoon die de afgelopen 5 jaar steeds weer om de hoek kwam kijken was Ruud. Ruud Schoemaker. Vanaf de eerste meeting was Ruud nauw betrokken bij het project. En ik was meteen onder de indruk van alle kennis over nutriënten die zich ophield in Ruud's hoofd. En niet alleen over nutriënten weet Ruud veel, je kunt ook nog eens gezellig kletsen over niet werk gerelateerde onderwerpen. Een ware verademing! En Ruud herkende zelfs psycho Bob in Jenneke's kantoor! Ruud, dank je wel voor al je hulp de afgelopen jaren!

Voor het schrijven van de verschillende publicaties kwam veelal hulp uit onverwachte hoek. De eerste paper die gepubliceerd werd had niet tot stand kunnen komen zonder de inbreng en input en wijze woorden van Ton Bronckers. De eerste keer dat ik Ton zag, noemde hij mijn NutriBone project het 'koetjeboe-project'. Gelukkig weerhield dat Ton er niet van om mij te helpen met de fluoride paper, en met het idee van de uitbreiding met de hamsterkaakjes te komen. En hij zorgde ook nog eens voor 2 studenten die de metingen konden doen (ik hoefde hen dan alleen maar te begeleiden). Ton, bedankt voor je hulp en je (mee)luisterend oor! De tweede paper over de effecten vitamine D op de NO productie van botcellen mede dankzij de hulp van Professor Geert Carmeliet uit Leuven tot stand gekomen. Hartelijk dank voor uw wetenschappelijke input en het beschikbaar stellen van de muizen. Ook wil ik Ingrid Stockmans bedanken voor het kweken en verzorgen van de dieren, en alle hulp die ik kreeg als ik weer naar Leuven kwam. Eerder noemde ik al mijn twee studenten, maar de heren hadden ook een naam. Seb Castelein en Joost Keverling-Buisman. Mijn eerste (en enige) echte studenten. Dank aan jullie beiden voor het werk wat jullie hebben verricht aan de metingen in de hamsterkaakjes. Ik hoop dat ik jullie iets heb kunnen leren, en jullie de wetenschap niet helemaal de rug zullen toekeren. Jullie eerste publicatie is in ieder geval al binnen. En ik heb van jullie geleerd dat je met stroop nog steeds meer vliegen vangt dan met azijn, slijmballen!

Nou vraagt u zich misschien af of ik de rest dan wel allemaal alleen heb gedaan. Wat denkt u zelf? Natuurlijk niet! De eerste week heb ik constant aan het handje van Jolanda en Cor gelopen. Als een jong angstig eendje liep ik de eerste weken over het lab. Bang om dingen stuk te maken, bang om de medische faculteit op te blazen, bang voor… Maar dankzij Cor en Jolanda kwam alles goed. Cor die mij de beginselen van fluid flow heeft bijgebracht, en Jol die mij wegwijs maakte met de lab etiquette (ruim je rommel op, schrijf overal duidelijk je naam op, vul iets bij als het op is, taart). Jol, dankjewel voor alle keren dat je voor me opkwam, bedankt dat je me uit de brand hebt geholpen met de laatste experimenten. Ook bedankt dat je me er regelmatig aan herinnerde dat ik wel 'een beetje

stupide was', je houdt de AiO's altijd lekker met beide beentjes op de grond. Maar vooral dank voor alle gezelligheid die je OCB brengt! Cor, jij ook bedankt voor alles wat je me op het lab hebt geleerd, voor je luisterend oor, en het delen van de Fokke&Sukke kalender, en de gezelligheid. Let it snow, let it snow, let it snow! Ook ben ik Dirk-Jan dankbaar voor alle vage stofjes die hij voor me besteld heeft, Marion wil ik bedanken voor haar luisterend oor en scherpe humor, en Don voor het leveren van de hamsterkaakjes.

Bijzondere dank gaat uit naar mijn paranimf Nina. We hebben samen het AMC overleefd, Sanquin onveilig gemaakt, OCB opgeschud, en hoe fantastisch is het dat we de komende twee jaar samen met Candida gaan spelen?! Dankzij jou was het nooit saai tijdens de lunch, tijdens congressen, presentaties en andere werk gerelateerde dingen die zonder jou als saai aangeduid zouden kunnen worden. Ik was supertrots dat ik jouw paranimf mocht zijn, en ben heel blij dat je mijn paranimf wilt zijn. Bedankt! Een andere bijzondere vriendin (en collega) die de afgelopen jaren net iets gezelliger maakte is Greetje. Samen op congres in Wenen (wat was ik blij dat je er was) met als hoogtepunt de high tea bij Haas und Haas. Dank je wel dat je er voor mij was, samen met Sas!

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De afgelopen 5 jaar stonden in teken van de promotie, maar de jaren ervoor waren van cruciaal belang om daar te komen. Mijn interesse in de biologie werd tenslotte op de mavo gewekt dankzij de altijd interessante lessen van meneer Kooij en meneer van Rij. Tijdens mijn studie biomedische wetenschappen werd heb ik verschillende stages gedaan, en die zijn niet allemaal even prettig verlopen. Toch heeft de minst fijne stage mij de kans gegeven een paar van de leukste vriendinnen die ik heb te leren kennen. Ro(mana), we zien elkaar niet veel, maar als we afspreken wordt er gelachen, gegeten en geroddeld. De Bananen, waarvan ook Nina en Greetje lid zijn, zijn niet compleet zonder Elise, Sarah en Marleen. Dank jullie voor jullie luisterend oor, en dank dat jullie er niet alleen voor mij zijn in goede tijden. Jullie zijn me dierbaar! Mijn laatste stage bij RBC op Sanquin heeft ervoor gezorgd dat ik verder ben gegaan in de wetenschap. Alle negatieve ervaringen met stages zijn hier gewoon uitgewist. Dus Arthur, Petra, Iris, Dave, Robin, Eric, Richard, Mya, Dirk en Herbert, Rosa en Thomas, bedankt!

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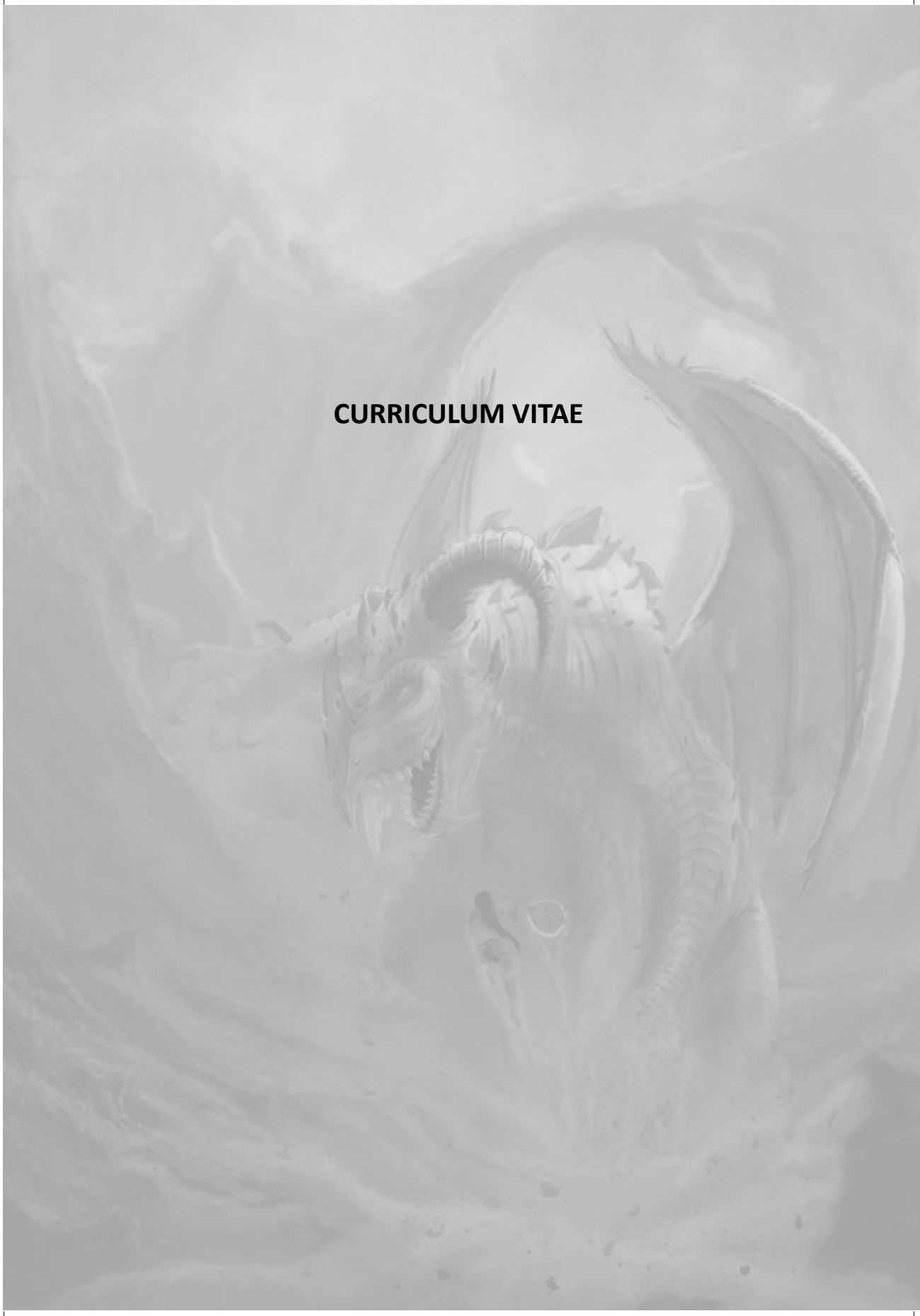
CURRICULUM VITAE



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Willems HME, van den Heuvel EG, Castelein S, Buisman JK, Bronckers AL, Bakker AD, Klein-Nulend J. (2011) Fluoride inhibits the response of bone cells to mechanical loading. Odontology 99:112-118.

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