Vitamin A and Bone

Studies *in vivo* and *in vitro*

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Gothenburg, Sweden 2018

UNIVERSITY OF
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ISBN 978-91-7833-123-9 (PRINT)
ISBN 978-91-7833-124-6 (PDF)

Printed in Gothenburg, Sweden 2018
BrandFactory AB

Abstract

Background: Excess vitamin A is associated with decreased cortical bone and increased risk of fractures in humans. The aim of the present thesis was to assess the importance of vitamin A on the skeleton and bone cells in *in vivo* animal studies and mechanistic *in vitro* experiments. *In vivo*, we used clinically relevant doses of vitamin A to investigate its effects on bone after prolonged administration and on the anabolic bone response to mechanical loading. *In vitro*, we aimed to determine how retinoids affect inflammatory- and physiologically-induced osteoclast formation and how retinoids affect periosteal osteoclast progenitors.

Methods: *In vivo,* mice were fed diets containing clinically relevant doses of vitamin A for durations of 4 and 10 weeks and prior to and during 2-week mechanical loading of the tibia. *In vitro*, we investigated the effects of retinol on human monocytes and mouse bone marrow macrophages induced to form osteoclasts by physiological and inflammatory cytokines, and on periosteal cell cultures.

Results: *In vivo*, we found that clinically relevant doses of vitamin A are able to reduce cortical bone mass by means of increased resorption and to decrease the anabolic bone response to mechanical loading due to reduced bone formation. *In vitro*, our results indicate that all-trans retinoic acid (ATRA), the active metabolite of retinol, inhibits physiologically- and inflammatory-induced osteoclastogenesis, however, in mouse periosteal bone cell cultures, the addition of ATRA enhances osteoclastogenesis.

Conclusion: Our results demonstrate the importance of vitamin A status to bone health. Fortification of food with vitamin A and vitamin A supplementation should be re-examined as vitamin A status may be a risk factor for secondary osteoporosis, a disease of decreased bone mass and increased risk of fractures.

Keywords: vitamin A, retinol, osteoclasts, osteoblasts, cortical bone, osteoporosis

Sammanfattning på svenska

Benmassan i skelettet påverkas av en mängd hormoner, lokalt bildade signalsubstanser, gener och vår kost. Kliniska studier har funnit ett samband mellan nivåerna av A-vitamin i blodet och mängden benmassa och risken för fraktur. Många experimentella studier på möss och råttor har också visat att höga nivåer av A-vitamin leder till minskad benmassa och ökad frakturbenägenhet. Dessa experiment har emellertid använt mycket höga doser av A-vitamin under kort tid (en vecka) och är nödvändigtvis inte relevanta för de nivåer som människor normalt utsätts för. Vi har därför studerat hur kliniskt relevanta doser av A-vitamin under lång tid (4-10 veckor) påverkar skelettet hos möss. Vi fann att A-vitamin även i dessa doser minskar benmassan och att benen blir svaga. Mekanistisk kunde vi påvisa att antal bennedbrytande celler, så kallade osteoklaster, ökade på utsidan medan antal sådana celler minskade på insidan. I cellkulturer fann vi att A-vitamin stimulerar bildning av osteoklaster när celler från benhinnan på utsidan av benen odlas i cellkultur. När celler från benmärgen på insidan odlades i cellkulturer hämmades osteoklastbildningen. Varför A-vitamin har så olika effekter på in- och utsidan har vi ännu inte kunnat klarlägga.

När vi studerade hur A-vitamin påverkar bildning av ben kunde vi konstatera att A-vitamin minskar benbildning på utsidan vilket, tillsammans med den ökade nedbrytningen, förklarar varför benmassan minskar.

Eftersom belastning av skelettet är viktigt för benmassan studerade vi även hur A-vitamin påverkar den ökning av benmängden som kan åstadkommas när underbenet hos en mus mekaniskt stimuleras. Vi fann då att A-vitamin kraftigt hämmar denna bennybilding.

Våra fynd visar att doser av A-vitamin som kan erhållas hos människa har negativa effekter på benmassan. Med tanke på att många livsmedel är berikade med A-vitamin och genom att många kosttillskott innehåller stora mängder A-vitamin, är det viktigt att ytterligare studera hur A-vitamin påverkar benmassan hos människa. Det är också viktigt att i dessa studier även inkludera hur A-vitamin påverkar benmassan vid fysisk belastning.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Lionikaite V**, Gustafsson KL, Westerlund A, Windahl SH, Koskela A, Tuukkanen J, Johansson H, Ohlsson C, Conaway HH, Henning P, and Lerner UH.

Clinically relevant doses of vitamin A decrease cortical bone mass in mice

Journal of Endocrinology, 2018; 239(3): 389-402.

II. **Lionikaite V**, Henning P, Drevinge C, Shah FA, Palmquist A, Wikström P, Windahl SH, and Lerner UH.

Vitamin A decreases the anabolic bone response to mechanical loading by suppressing bone formation

Submitted.

III. **Lionikaite V**, Westerlund A, Conaway HH, Henning P, and Lerner UH.

Effects of retinoids on physiologic and inflammatory osteoclastogenesis *in vitro*

Journal of Leukocyte Biology, 2018; 1-13. Epub ahead of print.

IV. Henning P, **Lionikaite V**, Westerlund A, Conaway HH, and Lerner UH.

Retinoids enhance osteoclastogenesis in periosteal bone cell cultures

Manuscript.

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Content

Abbreviations

ALP	alkaline phosphatase
ATRA	all-trans retinoic acid
BMD	bone mineral density
BMM	bone marrow macrophages
CSF1/M-CSF	macrophage colony-stimulating factor
CTSK	cathepsin K
CTX	C-telopeptide of type I collagen
LPS	lipopolysaccharide
μCT	microcomputed tomography
NFATc1	nuclear factor of activated T-cells, cytoplasmic 1
OCN	osteocalcin
OPG	osteoprotegerin
pQCT	peripheral quantitative computed tomography
RAE	retinol activity equivalent
RALDH	retinal dehydrogenase
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
RAR	retinoic acid receptor
RBP	retinol binding protein
RDA	recommended daily allowance
RE	retinyl esters
RXR	retinoic X receptor
TNFα	tumour necrosis factor-α
TRAP	tartrate-resistant acid phosphatase
UTL	upper tolerable limit
VAD	vitamin A deficiency

1. Introduction

1.1 Vitamin A

Vitamin A is a name given to any compound possessing the biological activity of retinol. It is a fat soluble vitamin that is consumed in the diet and ingested as either preformed vitamin A (retinyl esters; RE) or provitamin A (carotenoids; β-carotene, α-carotene, and β-cryptoxanthin). Preformed vitamin A can be found in animal products and provitamin A carotenoids are present in vegetables such as carrots and spinach. Vitamin A has a vital role in cell differentiation, embryonic growth and development, immune function, vision, and bone growth and therefore, adequate consumption is necessary. The Recommended Daily Allowance (RDA) for vitamin A consumption in adults is 700μg retinol activity equivalent (RAE) per day for women, and 900μg for men. RAE is the preferred unit used to present total vitamin A and takes into account both preformed vitamin A and provitamin A carotenoids, for which the activity of vitamin A is less. Therefore, one RAE is equal to 1μg retinol, 12μg β-carotene, and 24μg α-carotene and β-cryptoxanthin[1]. The Upper Tolerable Limit (UTL), or the maximum amount that can be consumed without negative side effects is 3,000μg RAE/day[1]. These recommendations vary depending on age.

1.1.1 Metabolism

When ingested, vitamin A is absorbed by the small intestine and taken up by enterocytes, which are intestinal mucosal cells (Fig. 1). Retinyl esters are converted to retinol prior to uptake by enterocytes and then bound to cellular retinol binding protein (CRBP)[2]. Carotenoids may be directly absorbed by enterocytes, or converted to retinal followed by retinol and bound to CRBP[3]. Retinol is esterified with long-chain fatty acids and together with carotenoids[4, 5] incorporated into chylomicrons, transported by the lymphatics[6, 7], and released into the circulatory system. Over one third of dietary retinol is taken up by the liver and stored in hepatocytes[8], however, chylomicron remnants can also directly deliver retinoids to the target cell. In the liver hepatocytes, retinyl esters are hydrolysed into retinol and, if adequate retinol needs are met, can be further transported into the stellate cells, re-esterified, and stored[9]. Prior to mobilization from the

liver, retinyl esters are transported back to the hepatocytes as retinol, and after binding to retinol binding protein (RBP), can enter the circulation[10]. Transthyretin, a transport protein, carries retinol bound to RBP in plasma into the target cell[11, 12]. In addition to chylomicron remnants delivering retinoids, and retinol bound to RBP entering the target cell, other retinoids found in plasma, such as all-trans retinoic acid (ATRA), are bound to albumin and can also be taken up into the target cell[13] (Fig. 1).

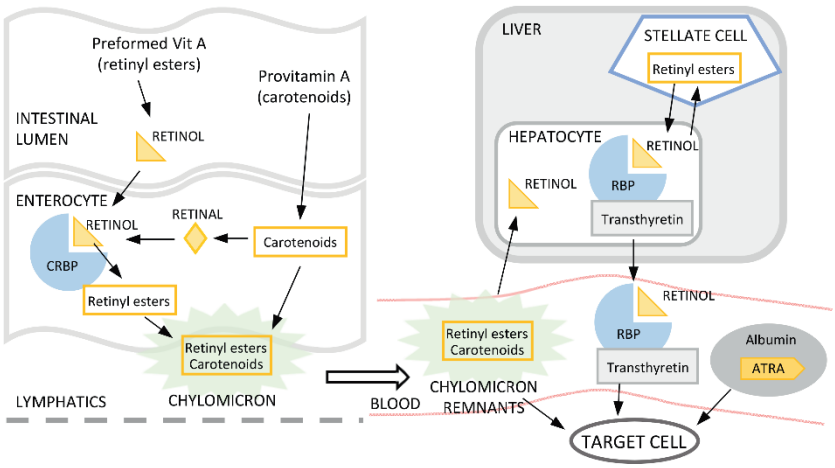

Figure 1: Vitamin A Metabolism. Vitamin A is consumed in the diet and absorbed in the intestine as carotenoids or retinyl esters. In the enterocytes, they are incorporated into chylomicrons and transported by the lymphatics. In the hepatocytes, retinyl esters are hydrolysed into retinol and can be further transported into the stellate cells for storage. Prior to mobilization from the liver, retinol is bound to retinol binding protein (RBP) and can enter the circulation. Chylomicron remnants, albumin bound retinoids, and RBP bound retinol can enter the target cell. Conaway *et al.,* 2013[14].

A membrane receptor, STRA6, facilitates the cellular uptake of retinol bound to RBP[15], while lipoprotein lipase is thought to facilitate the uptake of retinyl esters from chylomicrons (Fig. 2). After cellular uptake, alcohol dehydrogenase (ADH) oxidises retinol to retinal which is bound to CRBP. Retinal dehydrogenase (RALDH) oxidises retinal to ATRA, the biologically active metabolite of vitamin A. ATRA then binds to cellular retinoic acid-binding protein (CRABP) and can translocate to the nucleus[16].

1.1.2 Signalling

In the nucleus, ATRA primarily ligates to retinoic acid receptors (RARs) which exist in three different isoforms, RARα, RARβ and RARγ[17-22]. In addition to RARs, most cells also express retinoic X receptors (RXRs), of which there are also 3 subtypes; α, β and γ[23-25]. RARs and RXRs belong to a class of nuclear receptors and function as heterodimers[26]. RXRs can also heterodimerize with other nuclear receptors such as the vitamin D receptor (VDR)[26]. The RAR/RXR heterodimer binds to retinoic acid response elements (RAREs) and functions to regulate gene transcription (Fig. 2). In addition, ATRA can also bind to peroxisome proliferator-activated receptors (PPARs) α, β and γ, which can form heterodimers with RXRs[27]. These heterodimers also function as transcription factors activating PPAR response elements in target genes[28].

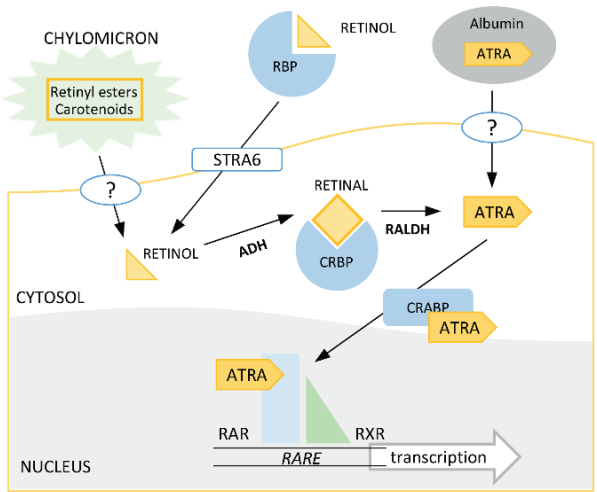

Figure 2: Cellular uptake and signalling. In the cytosol of target cells, alcohol dehydrogenase (ADH) converts retinol to retinal. Retinal bound to cellular retinol binding protein (CRBP) is oxidised by retinal dehydrogenase (RALDH) to all-trans retinoic acid (ATRA). ATRA bound to cellular retinoic acid-binding protein (CRABP) can enter the nucleus. ATRA ligates to retinoic acid receptors (RARs), which heterodimerize with retinoic X receptors (RXRs) and binds to retinoic acid response elements (RARE) regulating gene transcription. Adapted from Conaway *et al.*, 2013, Henning *et al.*, 2015[14, 28].

1.1.3 Deficiency

Vitamin A deficiency (VAD) can result from inadequate food intake, malabsorption, or chronic alcohol consumption[29-32]. Reduced food intake and malnutrition can impair the absorption of vitamin A due to low fat availability[29] and alcohol consumption can decrease hepatic vitamin A levels[31, 32] by competing with metabolizing enzymes such as ADH[33]. VAD results in impaired embryonic development such as functional defects of the lungs[34]. Due to the role of vitamin A in immune

function and response, VAD had been associated with increased risk of infectious diseases such as measles, thereby increasing morbidity and mortality risk in affected individuals[35, 36]. The most common and specific clinical effect of VAD is night blindness[37]. Vitamin A plays a major role in the production of rhodopsin, the light sensitive pigment in the eye. Rhodopsin is formed when 11-*cis*-retinal, a photosensitive derivative of vitamin A, is combined with the protein opsin[38, 39]. Xerophthalmia, abnormal dryness of the eyes, and complete blindness may occur if no treatment is sought[37, 40]. Vitamin A supplementation reverses these adverse effects of VAD and reduces the risk of mortality associated with the effects[41-47].

1.1.4 Excess

Excess vitamin A consumption is a potential threat in developed countries. It has been estimated that one third of the population of the United States ingests dietary supplements[48]. Supplements, whether single-ingredient or multimineral/ multivitamin, often contain over 100 percent of the daily value of one or more nutrients[49]. Besides athletes[50], the elderly (>65 years old) are the highest users of dietary supplements[48, 49]. In addition, consumption of foods rich in retinol (i.e. fish or animal liver) and fortification of foods with vitamin A can result in retinol levels approaching toxicity. Furthermore, retinoids are frequently used in skincare[51] and as drugs in preventing skin conditions such as psoriasis and acne[52-55] and the combination of these factors may increase the risk of excess vitamin A and potential hypervitaminosis A.

Serum retinol is the main method of determining vitamin A status in humans with physiological circulating levels of around 2-4μM[56, 57]. However, retinol levels in the serum are not reflective of vitamin A status unless there is a deficiency or surplus of the nutrient. Serum retinyl esters (RE) have been shown to be a more precise measurement of vitamin A status[58]. The normal physiological levels of retinyl esters in humans are in the range of 50-200nM[56, 59] and it has been suggested that levels over 200nM or exceeding 10% of total serum vitamin A (retinol and RE) may indicate excess vitamin A stores and potential vitamin A toxicity[56].

Symptoms of acute hypervitaminosis A include headache, dizziness, abdominal pain, nausea, and vomiting. Chronic hypervitaminosis A can result in changes to vision, dry skin, and bone pain. However, cessation of high vitamin A intake reverses these side effects.

1.2 Bone

The human skeleton can be divided into axial and appendicular skeleton. The axial skeleton is formed by the vertebral column, ribs, and skull, and appendicular skeleton consists of the limbs and other associated bones. The adult human skeleton is comprised of 206 bones. These bones provide a frame for support, movement, protection of vital organs, site of haematopoiesis, storage of minerals, and endocrine regulation. Bones are classified as either flat or long bones and are made of cortical and trabecular bone. Cortical bone makes up 80% of the skeleton and is hard compact bone found in the shaft of long bones and on the surface of all bones[60]. The fundamental unit of cortical bone is osteon, which is formed around Haversian canals that contain blood vessels and nerves. Trabecular bone can be found in the vertebra or at the end of long bones and comprises 20% of the adult skeleton[60]. Bone consists of a matrix made up of organic components, mainly type I collagen, and inorganic components, such as hydroxyapatite, calcium carbonate, and phosphate. It also contains a small amount on non-collagenous proteins. Bone is a dynamic tissue which involves three main cell types: the osteoclasts, which resorb bone, the osteoblasts, which form new bone, and the osteocytes, which sense the stress applied to bone. These cells drive bone modelling and remodelling. Bone modelling occurs during development and growth or in response to mechanical load. To maintain integrity of the skeleton, bone is constantly undergoing remodelling. The adult skeleton is fully replaced every 10 years[61].

1.2.1 Osteoclasts

Osteoclasts are bone resorbing cells. Mature osteoclasts are formed by fusion of mononucleated progenitor cells derived from myeloid hematopoietic stem cells (Fig. 3). The cytokine macrophage colony-stimulating factor (M-CSF/CSF1) and receptor activator of nuclear factor KB ligand (RANKL) are essential for the proliferation and differentiation of osteoclasts[62]. CSF1/M-CSF binds to colony-stimulating factor 1 receptor (CSF1R) on early osteoclast precursors. Interleukin-34 (IL-34) has also been shown to bind to CSF1R and promote monocyte proliferation[63, 64]. RANKL drives osteoclast differentiation, fusion, and maturation by binding to its receptor RANK, which is located on the surface of osteoclast progenitors. RANKL recruits adaptor protein tumour necrosis factor receptor–associated factor (TRAF6) which activates a number of crucial intracellular signalling pathways such as mitogen-activated protein kinase (MAPK)[65-69], PI3K/Akt[70-72], and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) through activation of

NF-κB and c-Fos[73-75]. The transcription factor NFATc1 is regarded as the master regulator of osteoclast differentiation[76]. Osteoclast progenitor fusion into multinucleated osteoclasts is facilitated by the transmembrane protein DC-STAMP[77].

Osteoclasts can also be formed by non-canonical, RANKL-independent, direct effects of pro-inflammatory substances acting on the osteoclast progenitor cells[78]. Pro-inflammatory cytokine tumour necrosis factor-α (TNFα) as well as bacterial products such as lipopolysaccharide (LPS) have been shown to be able to increase osteoclastogenesis[79-83].

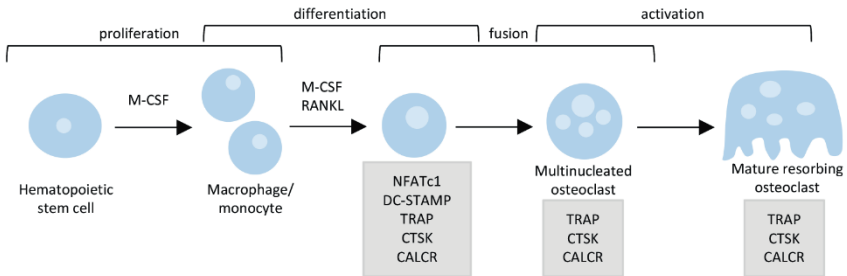

Figure 3: Osteoclastogenesis. Osteoclast progenitors stem from hematopoietic stem cells. M-CSF/CSF1 induces proliferation and survival of cells, and RANKL promotes differentiation of precursors by upregulation of osteoclastic transcription factors, with NFATc1 being the main driver. DC-STAMP is essential for fusion of cells into multinucleated osteoclasts. Markers of osteoclasts include the presence of TRAP and CTSK. Calcitonin receptor (CALCR) is also a marker of osteoclasts. It enables the binding of calcitonin and regulation of bone resorption[84, 85]. Adapted from Crockett *et al.*, 2011, Green *et al.*, 2016[86, 87].

To resorb bone, osteoclasts adhere to the bone surface via a sealing zone and the membrane adjacent to the bone surface becomes convoluted thus forming a ruffled border. Acidification of the resorption lacuna is necessary for dissolution of hydroxyapatite crystals. Carbonic anhydrase II provides the protons for acidification mediated by proton pump vacuolar-type H^+ ATPase (V-ATPase) located on the ruffled border[88]. A chloride-proton antiporter (ClC7) maintains electroneutrality. These actions result in secretion of HCl into the resorption lacunae, prompting an acidic pH of ~4.5[89], and resulting in dissolution of bone mineral. The organic component of bone, primarily type I collagen, is degraded by proteases secreted by osteoclasts such as cathepsin K (CTSK)[90-92]. Osteoclasts also secrete tartrate-resistant acid phosphatase (TRAP), which correlates with the rate of bone resorption and is indicative of the number of osteoclasts present[93, 94]. Bone degradation products, such as C-telopeptide of type I collagen (CTX), are endocytosed by the osteoclasts and transcytosed and released at the cell surface[95]. CTX in the serum can be used as a clinical marker of bone resorption[96].

1.2.2 Osteoblasts

Osteoblasts are bone forming cells that are derived from mesenchymal stem cells (Fig. 4). Differentiation is driven by the master transcription factors runt-related transcription factor 2 (RUNX2)[97, 98] and osterix[99]. The enzyme alkaline phosphatase (ALP) is a hallmark of active osteoblasts. Osteoblasts produce unmineralised bone matrix mainly consisting of type I collagen fibers but also of several other matrix proteins such as osteocalcin (OCN) and osteopontin. The matrix then becomes mineralised through the deposition of hydroxyapatite crystals. Osteocalcin secreted by osteoblasts can also regulate energy metabolism by enhancing insulin secretion and increasing insulin sensitivity, demonstrating that bone also has endocrine functions[100].

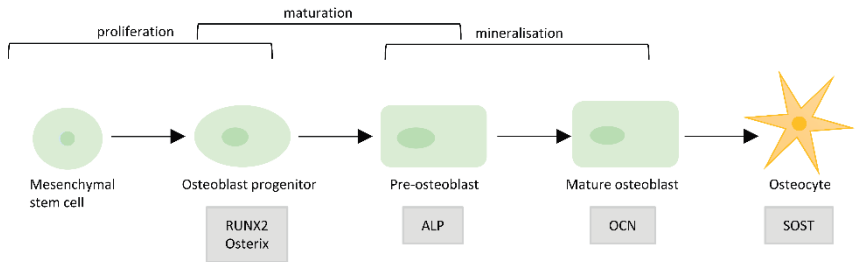

Figure 4: Osteoblastogenesis. Osteoblasts stem from mesenchymal stem cells. Expression of Runx2 and Osterix drives osteoblast differentiation. Alkaline phosphatase (ALP) is hallmark for active osteoblasts which secrete osteocalcin (OCN) as well as other proteins to the bone matrix. Mature osteoblast can then become embedded into the newly formed matrix where they become mechano-sensing osteocytes that express sclerostin (SOST). Adapted from Green *et al.*, 2016 and Crockett *et al.*, 2011[86, 87]

RANKL, the cytokine driving osteoclast differentiation, is expressed mainly by osteoblasts as well as bone marrow stromal cells, osteocytes, synovial fibroblasts, periodontal fibroblasts and certain lymphocytes[101]. The amount of RANKL available for RANK activation is dependent on osteoprotegrin (OPG), a decoy receptor also produced by the osteoblasts, epithelial cells, and B cells of the immune system, and on RANKL expression induced by a variety of hormones such as parathyroid hormone (PTH), and cytokines such as TNFα and interleukin-1 (IL-1)[102]. Thus, the RANKL/OPG ratio is rate limiting for osteoclastogenesis, and it is largely dependent on osteoblasts. This coupling of osteoblasts and osteoclasts indicates the importance of the two cell types working together.

1.2.3 Osteocytes

Osteocytes are the most abundant cell type found in bone[86]. These cells are derived from osteoblasts that embed into the newly formed bone matrix (Fig. 4). The embedded cells acquire a stellar shape with thin extensions that can connect with other osteocytes as well as cells on the bone surface. This osteocyte network senses mechanical forces being applied to the bone and transmits signals to influence the activity of the osteoblasts or osteoclasts[103, 104]. Sclerostin (SOST), a protein secreted by the osteocytes, is an inhibitor of bone formation. It binds to LRP5/6 receptors on the surface of osteoblasts inhibiting Wnt signalling which is important for formation of bone[105]. Osteocytes also possess endocrine capability by synthesising fibroblast growth factor 23 (FGF23), which regulates renal phosphate secretion[60]. Thus, osteocytes, along with the osteoclasts and osteoblasts, are important for building and maintaining bone mass through the processes of bone modelling and bone remodelling.

1.2.4 Bone Modelling

Bone growth and bone shape is modelled via independent resorption or formation[86, 101]. Bone tissue can be formed without prior resorption, or resorbed without subsequent bone formation. In bone modelling, longitudinal growth, as well as radial growth occurs[106]. The purpose of bone modelling is to alter bone shape to accommodate re-distribution of forces applied to the bone. This process involves modifications of both trabecular and cortical bone.

1.2.5 Bone Remodelling

Bone remodelling is the coupled interaction that occurs between the osteoclasts and osteoblasts[86]. A tightly regulated balance between the amount of bone being resorbed and the amount of new bone being formed is crucial to maintain bone mass. Damage or stresses applied to the bone are sensed by the osteocytes. The old or damaged bone is resorbed by the osteoclasts. To form new bone, osteoblasts at the surface of the bone lay down new matrix which later becomes mineralized. Some osteoblasts embed into the bone matrix and become osteocytes. The transition between resorption and formation, and the homeostasis between this coupled process, is crucial to maintaining a healthy skeleton. Disruption in the balance of bone resorption and bone formation can lead to bone diseases such as osteoporosis.

1.2.6 Osteoporosis

Osteoporosis is characterized as a disease of low bone mass. It is associated with increased bone resorption, exceeding the actions of bone formation and thereby resulting in low bone mass which leads to increased bone fragility[107-109]. Osteoporosis can either be primary or secondary. Primary osteoporosis is a progressive bone loss due to aging and can be influenced by genetic factors and/or changes in levels of sex hormones (i.e. oestrogen). Secondary osteoporosis can be caused by diseases (i.e. rheumatoid arthritis), medication (i.e. glucocorticoids), or lifestyle choices (i.e. smoking, heavy drinking, high vitamin A intake). It is estimated that one in two women, and one in five men will suffer from an osteoporotic fracture in their lifetime[110, 111], thus presenting a major economic burden. The most frequent sites of osteoporotic fractures include the vertebral body, hip, and distal forearm (wrist)[107, 109].

1.2.7 Inflammatory Bone Diseases

Inflammation is known to induce bone resorption in numerous rheumatic diseases. Arthritis is caused by inflammation of a specific skeletal site. The most common types of arthritis are rheumatoid arthritis (RA) and osteoarthritis (OA). RA is a chronic autoimmune disorder that effects the joints causing pain and swelling, and eventually causing destruction of synovial joints and leading to severe disability. Synovium of RA patients contains an abundance of pro-inflammatory cytokines such as TNFα and interleukins like IL-1 and IL-6 which are able to increase osteoclastic bone resorption[112], and thereby causing destruction of the cartilage and bone. OA is a progressive degenerative joint condition classified as a loss of articular cartilage within synovial joints. It is caused by repetitive use of joints. Both of these forms of arthritis increase in severity with age. Periodontal disease is another inflammatory condition. It is caused by a build-up of bacteria around the gums and teeth which causes inflammation in the area and eventually, can lead to loss of teeth. RA, OA, and periodontal disease all cause local inflammation which results in increased osteoclasts and bone resorption, and thereby bone loss.

1.3 Vitamin A Effects on Bone

Retinol and its derivatives are recognised as morphogens with important functions in early embryonic development. Defects in skeletogenesis can occur when retinol is lacking or present in excess[113]. Teratogenic effects of excess vitamin A

and vitamin A deficiency during embryogenesis results in mice with forelimb abnormalities as well as other impairments related to organ development[114-116]. In addition, vitamin A also affects post-natal maintenance of the skeleton, and due to increased vitamin supplementation[48], excess vitamin A and its effect on the skeleton is potentially becoming an increasing problem.

1.3.1 Humans

In humans, increased vitamin A intake and elevated serum retinol levels are associated with decreased bone mineral density (BMD)[117-119]. The highest decrease is observed at the femoral neck/hip, the primary indicator of cortical bone health status[117-119]. These decreases in BMD due to elevated vitamin A are associated with increased fracture risk at the hip[57, 117, 120]. Consumption of provitamin A β-carotene was not found to increase risk of fractures[57, 118, 120-122] due to a feedback mechanism suppressing the conversion of β-carotene to retinol[38]. In regards to trabecular bone, studies have demonstrated that individuals in the highest serum retinol or retinol intake quantile may decrease lumbar spine BMD by up to 14%[117-119], however, some studies observed no association in vitamin A and bone status[123-126]. Interestingly, a U-shaped relationship between retinol level and risk of hip fractures has been revealed, indicating that low and high levels of vitamin A may increase the risk of hip fractures[121, 127]. These data implicate retinol status as a risk factor for secondary osteoporosis and hip fractures.

1.3.2 Rodents

Rodent studies have also shown negative effects of vitamin A on the skeleton. By rapidly inducing hypervitaminosis A, either through the chow or by injecting retinoids, cortical bone loss in long bones of rats[128-134] and mice[131], which leads to a decrease in bone strength[130, 132] has been observed. Mechanistic studies in rodents have shown that hypervitaminosis A induces an increase in the number of periosteal osteoclasts[128, 129, 131, 132] and decrease in endocortical osteoclasts[131, 132], thereby increasing cortical bone resorption and resulting in thinner cortical bone. Furthermore, knockout mice lacking RALDH, the metabolic enzyme which converts retinal to the biologically active form ATRA, exhibit thicker cortical bone[135]. Collectively, these data illustrate the detrimental effect of excess vitamin A on cortical bone.

Vitamin A effects on trabecular bone *in vivo* have been inconsistent. Hypervitaminosis A resulted in decreased trabecular BMD in the long bones and vertebra[132, 136], an effect associated with both increased number of osteoclasts and decreased number of osteoblasts[136]. However, other studies have not been able to observe the same effects[130, 131, 133, 137], illustrating a gap in the understanding of retinol effects on trabecular bone.

The effect of vitamin A on osteoblasts and bone formation have been seldom investigated. Hypervitaminosis A has been shown to decrease mineralising surface[131, 134] and reduce the rate of bone formation on the periosteum[134]. These data suggest that vitamin A has a negative effect on osteoblasts located on the cortical bone.

1.3.3 Cells

In experimental *ex vivo* studies, bone organ cultures of mouse calvaria and rat tibia have shown that retinol stimulates osteoclast formation and bone resorption[129, 138-145]. Initial studies observed increased calcium release from the periosteum of the radius in rat[145] and mouse parietal bones[139] in response to retinoids. Further studies have continued to observe increased resorption with retinol in cultures of mouse calvaria[138, 140-143], rat calvaria[129], and rat tibia[144]. This enhanced resorption has been attributed to increased number of osteoclasts[138, 144, 145] and it has been suggested that this effect is mediated by RARα and due to the increase in RANKL[138]. These studies in organ cultured intact bones provide further evidence that vitamin A increases osteoclastogenesis and bone resorption.

In contrast to *ex vivo* organ cultures and *in vivo* rodent studies, *in vitro* cultures of osteoclast progenitors have shown that retinoids inhibit osteoclast formation. These studies have been performed in progenitor cells isolated from human blood monocytes[146] and bone marrow[147], rat[144, 148] or mouse[131, 149, 150] bone marrow, mouse spleen cells[149], and using a RAW264.7 mouse macrophage cell line[137, 146, 149, 150]. The mechanism for the inhibition of osteoclastogenesis by ATRA has not yet been elucidated, however it has been suggested ATRA decreases RANK expression[146] or that ATRA interferes with early steps in the intracellular cascade of signalling events downstream of activated RANK thereby decreasing the expression of NFATc1[137, 149, 150]. By the use of specific RAR agonists, RARα and RARγ have been implicated to mediate the inhibitory effect of ATRA[137, 149].

In vitro cultures of primary osteoblasts isolated from humans[134], mice[131], and rats[151], or pre-osteoblastic and mesenchymal cell lines[131, 134, 152-157] have indicated that retinoids are also able to affect osteoblasts, a response that has been observed to be dose dependent. An inhibitory effect of ATRA on osteoblasts has been observed at nanomolar concentrations, marked by decreased mineralisation in primary mouse osteoblast cultures[131, 134] and MC3T3-E1 mouse osteoblast precursor cell line[134, 152, 153]. These *in vitro* effects of retinoids on osteoblasts are similar to the observed periosteal effects *in vivo*[131, 134]. At micromolar concentrations, ATRA increased the expression and activity of ALP in primary rat calviaral culture[151] and cell line UMR-201[154] and in C3H10T1/2 mesenchymal stem cell line[155-157]. Thereby, retinoids at low concentrations appear to inhibit osteoblast differentiation, whereas high levels have a stimulatory effect *in vitro*.

Heterotopic ossification and fibrodysplasia ossificans progressiva (FOP) are conditions of abnormal endochondral bone formation in non-skeletal tissues. The use of a RARγ agonist in these conditions have been shown to inhibit the abnormal bone formation[158]. Palovarotene, a highly selective RARγ agonist, is already in phase III clinical trials for the treatment of FOP (Clementia Pharmaceuticals Inc.). These data indicate the significance of RARγ in the role of osteoblastic bone formation.

2. Aims

The overall aim of this thesis was to assess the importance of vitamin A on the skeleton and on bone cells in both *in vivo* animal studies and mechanistic *in vitro* experiments.

The specific aims are as follows:

I. To study how long term exposure to clinically relevant concentrations of retinoids affect bone at different skeletal sites

II. To study how retinoids, at a clinically relevant concentration, affect the anabolic response in bone to mechanical loading

III. To study how retinoids affect osteoclast formation *in vitro* using human and mouse osteoclast progenitors stimulated to form osteoclasts by different mechanisms

IV. To study how osteoclasts isolated from a periosteal bone model respond to retinoids

3. Methods

This section provides an overview of the methods used in the papers encompassing this thesis. For more detailed methodology, please refer to the respective papers. Please note that all experiments performed were approved by the local Ethics Committee.

3.1 Animals

For *in vivo* experiments (I, II), female C57BL/6 mice were used. Mice are biologically similar to humans, they can be genetically manipulated, have an accelerated lifespan, are cost-effective, and reproduce easily. Inbred strains of mice, such as C57BL/6, allow for genetic uniformity of each animal which enables reproducible results with smaller sample size. Similar to human, murine skeletal physiology consists of both modelling and remodelling of cortical and trabecular bone. Unlike humans however, bone acquisition and bone growth continues in mice after sexual maturity, which is around 6-8 weeks of age[159]. Peak bone mass in mice is obtained at 4-6 months of age[159-162], and the mean lifespan for C57BL/6 mice is up to two years. We used mice that were either 9 weeks (I) or 13 weeks (II) of age at the start of experiment. Weight loss was monitored as an assessment of health status.

3.2 Diets

Diets enriched with vitamin A were given *ab libitum* to mice throughout the duration of the experiments (I, II). These diets were modelled after the human RDA for vitamin A consumption which are 700-900µg RAE per day and upper tolerable limit (UTL) diet roughly 3-4 times higher at 3,000µg RAE/day[1]. In mice experiments, a balanced control diet contains 4.5µg retinyl acetate/g of chow (Teklad Global 16% Protein Diet, Harlan Laboratories Inc.). By using diets with increased concentrations of vitamin A by 4.5- and 13-fold, we aimed to mimic the fold increase observed in the human UTL diet, as well as a diet that may result from consuming UTL and further vitamin A supplementation. These diets contained either 20µg retinyl acetate/g chow (UTL), or 60µg/g chow (Supplemented

in I; Vitamin A in II). An additional hypervitaminosis A diet was also used for a short duration experiment. This diet contained 450µg retinyl acetate/g chow (I). All chow was repelleted to ensure the same texture. Food intake was not monitored as the food pellets were brittle, however, no significant decreases in body weights were observed suggesting that food intake and energy expenditure was not significantly changed between mice receiving control and vitamin A diets.

3.3 Mechanical Loading

Vitamin A effects on osteoblasts was observed using a model of rapid bone formation (II). Axial mechanical loading of the tibia is a non-invasive method of studying the osteogenic response in bone[163]. This method results in controlled loading of the whole bone, subjecting it to tension and compression occurring at the medial and lateral surfaces of the bone, respectively, with the adaptive response occurring at both cortical and trabecular bone[163-165]. The mechanical strain applied to the bones during the duration of the loading experiment is determined in a prior *ex vivo* experiment on post mortem intact mice, where the strain of the tibia is measured across a range of peak compressive loads. Linear regression analysis allows for calculation of the load required to achieve a chosen strain. The chosen strain is dependent on the magnitude of response desired and the age of mice[166]. *In vivo* tibial loading is administered to mice under anaesthesia. With the left tibia placed into loading cups, an axial load is applied through the knee joint for 40 cycles/day with 10 seconds rest between cycles on three alternative days/week for two weeks as common loading protocol for optimum increase of bone formation[163, 167]. The contralateral limb is used as a non-loaded control[164]. Slow decline in body weight of the mice throughout the duration of loading may be observed and could be due to stress as a result of loading and/or from repeated handling and anaesthesia.

3.4 Serum Analyses

Vitamin A status in humans is frequently determined via serum retinol analysis, with normal physiological levels ranging between 2-4µM[56, 57]. In mice, physiological serum retinol levels are around 1µM[168, 169]. However, serum retinyl esters have been shown to be a more precise measure of vitamin A status and for this reason, we analysed both serum retinol and retinyl esters (sum of retinyl linoleate, retinyl palmitate, retinyl oleate, retinyl stearate) (I). These analyses were car-

ried out by Vitas Analytical Services (Oslo, Norway) using high-performance liquid chromatography (HPLC). Serum analysis of bone formation and bone resorption markers were performed by commercially available enzyme-linked immunosorbent assay (ELISA) kits. Serum levels of CTX and TRAP, markers of bone resorption, and osteocalcin, a marker of bone formation, were assessed (I). Analysis of vitamin A status and bone markers in the serum provide an overview of the systemic bone effects occurring in response to vitamin A treatment.

3.5 Bone Analyses

Peripheral quantitative computed tomography (pQCT) and microcomputed tomography (μCT) were used to evaluate cortical and trabecular bone and their microstructural parameters (I, II). Both of these imaging techniques use a rotating x-ray around the specimen, giving rise to a 3D image and quantification. Tibia and femur were analysed via pQCT (I). μCT analysis provides better resolution compared to pQCT, however, this method is more time consuming. Just as pQCT, μCT allows for distinction between cortical and trabecular bone, however, μCT also enables better visualisation and quantification of the trabecular microarchitecture and network[170]. We used μCT for the analysis of the vertebral body (I) and tibia (II).

TRAP-stained sections of the femur were used to count the number of osteoclasts present on trabecular and cortical bone (I). Dynamic histomorphometry was used to measure the change in bone formation and mineralisation over time via two fluorescent labels. Calcein and alizarin, the most commonly used fluorescent labels, bind to calcium ions at the surface of newly mineralized bone[171]. This technique is used to quantify the bone formation occurring between two different time points[172] (I, II). Toluidine blue staining and immunohistochemical staining for detection of ALP positive cells allowed for visualisation of overall histology and bone formation (II).

Three-point bending analysis was used to determine mechanical bone strength of the tibia (I). Briefly, the tibia was placed on two support points and a load was applied at a third point located at the mid-diaphysis of the tibia. Information on the resistance of the bone (stiffness) and maximum loading force applied till breaking (i.e. bone strength) can be obtained with this method. These data represent the mechanical properties of the cortical bone.

Raman spectroscopy was used to characterize the organic and inorganic components of tibia (II). This technique uses a laser to measure the shift in the wavelength of light due to inelastic scattering. Results reveal details about the mechanical and chemical properties of bone and their response to vitamin A and/or loading. The degree of mineralisation of the bone is illustrated by the mineral-to-matrix ratio. The optimum amount of mineral for maximum strength of a collagen fibril is 30%[173], thus, increases in the mineral-to-matrix ratio are associated with increased risk of fractures[174]. Mineral crystallinity displays the degree of order and alignment of crystals and influences the hardness and density of the bone. Increases in mineral crystallinity can be mirrored by decreases in the carbonate-to-phosphate ratio, which is the ratio of carbonate substituted to hydroxyapatite crystals that occurs over time and is indicative of the age and maturity of the bone. Increased carbonate-to-phosphate ratio has also been associated with increased risk of fractures[175]. These three parameters contribute to the quality of the bone. The ability to use fixed and embedded specimens and even fluorescently labelled/stained tissue is an advantage of Raman spectroscopy.

3.6 Cell Culture

For *in vitro* experiments, cells were obtained from human peripheral blood, mice bone marrow, and mice calvaria. Human $CD14^+$ monocytes were isolated from human peripheral blood mononuclear cells (PBMCs)[176], which were obtained from blood of anonymous healthy donors at Sahlgrenska University Hospital (III). Wild type C57BL/6 mice were used for isolation of macrophages from the bone marrow (BMM) region adjacent to the endocortical bone (III)[177]. These methods provide a population of pure primary osteoclast precursor cells. Osteoclastogenesis in these cell cultures were stimulated with CSF1/M-CSF and RANKL or with CSF1/M-CSF and either TNFα or LPS from *E.coli.* For induction of osteoclastogenesis with LPS in $CD14^+$ monocytes and TNFα and LPS in mouse BMM, cells were initially primed with RANKL for 24-36 hours, after which the medium was removed and the cultures washed before new medium was added. The brief presence of RANKL was necessary to induce the formation of osteoclasts. In all cultures, the effects of vitamin A were assessed by adding ATRA and other retinoids with affinity to different RARs. Phagocytosis by human monocytes were examined by the uptake of FITC-labelled zymosan A. Overexpression of RANK in mouse BMM was induced by a lentiviral vector.

Periosteal cells were isolated from calvaria of new-born C57BL/6 mouse pups via enzymatic digestion (IV)[178, 179]. Cells were cultured on plastic or bone for a duration up to 25 days in media containing RANKL or ATRA or the combination of both.

In all *in vitro* experiments, osteoclasts were defined as TRAP positive cells with three or more nuclei.

Cell culture experiments can provide mechanistic explanations for observed effects *in vivo*, however, due to the isolated culture environment, they cannot completely reproduce the interactive environment observed in the body.

3.7 Gene Expression Analyses

Quantitative polymerase chain reaction (qPCR) is a sensitive method used for the assessment of the relative expression of a specific gene in cells or a tissue of interest. RNA is extracted from tissue and reverse transcribed into cDNA. The cDNA is then mixed with specifically designed primers and a fluorescent probe linked to a quencher complementary to the specific sequence of a target gene. During replication of the cDNA, the probe is degraded, releasing it from the quencher. The emerged emission of fluorescence is proportional to the amount of amplified product. Expression levels of osteoclastic and osteoblastic genes from cortical bone, trabecular bone, and cell cultures were quantified (I-IV). Cortical bone RNA was isolated from the diaphysis of the tibia flushed of bone marrow, and trabecular bone RNA was isolated from vertebral body, which is primarily trabecular bone with a very thin cortex. Expression of genes of interest were quantified as relative to housekeeping gene 18S. This method is not able to identify the specific cell type which expresses the gene and cannot distinguish between cells located on the periosteum or endosteum.

3.8 Western Blot

Western blot is a conventional method for the detection and quantification of specific proteins in a sample. First, the proteins are separated by molecular weight through gel electrophoresis, then transferred to a membrane, and by the use of antibodies, the specific protein of interests can be visualised as bands. A primary antibody recognises the target protein, and incubation in a secondary antibody,

which is labelled with a detection reagent (i.e. horseradish peroxidase; HRP), allows for detection of the location of the protein of interest by the signal/band it produces. The band thickness corresponds to the amount of protein present[180]. We extracted protein from cultures of human $CD14^+$ monocytes stimulated with RANKL or TNFα and analysed the protein expression of their respective receptors RANK and tumour necrosis factor receptor 2 (TNFR2) (III). The main advantages of this technique are its specificity and sensitivity however, it is semi-quantitative and relies on the availability of well-established and very specific antibodies.

3.9 Statistical Analysis

All statistical analyses were performed using GraphPad Prism. Statistical significance was defined as $P < 0.05$. Gaussian distribution was tested for key parameters in *in vivo* data and parametric tests were used throughout (I, II). Parametric and non-parametric statistical tests were used in *in vitro* experiments (III, IV). Unpaired Student's *t*-test was used when comparing two groups (I-IV). For comparison of 3 or more groups, one-way ANOVA followed by Dunnett's multiple comparison test versus control (I, IV) or Tukey's multiple comparison test was used (III, IV). Two-way ANOVA for interaction and/or Sidak's multiple comparison test was used to examine the influence of variables (time/bisphosphonate treatment/loading) on vitamin A (I, II). A linear regression model was also utilised for modelling the relationship between time and vitamin A dose (I) or strain and load applied (II).

4. Results

4.1 Paper I

Clinically relevant doses of vitamin A decrease cortical bone mass in mice

We investigated the effects of clinically relevant doses of vitamin A for a long duration in mice. Mice were fed control (4.5μg retinyl acetate/g chow), UTL (20μg/g), or supplemental (60μg/g) diet for 4 and 10 weeks, including an experiment involving injections of the bisphosphonate zoledronic acid, which inhibits bone resorption. An additional hypervitaminosis A (450μg/g) and supplemental diet experiment was performed for a duration of 8 days.

Main Results

- Serum retinol and RE levels increased with excess vitamin A in the chow
- Trabecular bone in the vertebra and tibia was not altered with UTL or supplemental diet after 4 or 10 weeks, nor in the femur after 8 days with supplemental or hypervitaminosis A diet
- Increases in vitamin A level time-dependently reduced cortical bone mineral content and periosteal circumference, which resulted in a trend of decreased bone strength
- Enhanced endocortical bone formation after 4 weeks of supplemental diet resulted in decreased marrow area and endocortical circumference at 10 weeks
- Bisphosphonate treatment abolished supplemental vitamin A diet effects on cortical bone
- Decreased periosteal bone formation was observed after 8 days of supplemental vitamin A diet, possibly contributing to the reduction in periosteal circumference
- Hypervitaminosis A diet increased periosteal osteoclasts and decreased endocortical osteoclasts after 8 days

Conclusion

Results obtained in the present study indicate that even clinically relevant doses of vitamin A consumed over a long period of time have a negative impact on the bone phenotype.

Discussion

In our experiments, 4 and 10 weeks of supplemented vitamin A diet resulted in RE levels which exceed the suggested RE threshold set for humans indicating potential vitamin A excess[56].

Rodent studies have suggested that hypervitaminosis A can decrease trabecular BMD[132, 136], however, others have not observed an effect[130, 131, 133, 137]. In the present study, we were not able to observe a change in trabecular bone phenotype, further strengthening the view that excess vitamin A does not affect trabecular bone, both with clinically relevant and hypervitaminosis A doses.

The severity of the observed cortical bone phenotype with supplemental diet increased with time. This suggests that consumption of increased vitamin A for a long duration is also detrimental to bone and may decrease bone strength. This observation is similar to the observed effects of hypervitaminosis A diet in rodents[130, 132] and to human associations of increased vitamin A intake and/or serum retinol levels increasing the risk of in hip/femoral neck fractures, which is primarily cortical bone[57, 117-120]. Zoledronic acid treatment abolished vitamin A-induced decrease in cortical bone, indicating that vitamin A effect is mediated by osteoclasts, as previously observed with hypervitaminosis A[128, 129, 131, 132].

Hypervitaminosis A has been shown to negatively affect bone formation by decreasing periosteal bone formation rate[134, 181] and/or decreasing osteoblast number[131]. In the present study, a transient effect on endocortical and periosteal bone formation was observed. Our observations suggest that reduced periosteal circumference caused by excess vitamin A may be due to increased bone resorption and decreased formation, and that the reduced endocortical circumference may be due to enhanced bone formation and reduced resorption.

While previous findings were obtained primarily using short term exposure to supraphysiological doses of vitamin A, our study indicates that extended exposure to levels only moderately exceeding the recommended norms lead to similar negative bone effects.

4.2 Paper II

Vitamin A decreases the anabolic bone response to mechanical loading by suppressing bone formation

We assessed the loading response in bone with prior and concurrent vitamin A treatment. Mice received control (4.5µg retinyl acetate/g chow) or vitamin A (60µg/g) chow for 6 weeks. In the last two weeks, mice underwent 6 bouts of axial mechanical loading of the tibia.

Main Results

- Vitamin A decreased the loading-induced increase in trabecular bone mass
- The loading-induced increase in cortical bone mass was reduced with vitamin A
- The loading-induced increase in periosteal and endocortical bone formation was decreased with vitamin A
- Vitamin A inhibited the increase in the expression of osteoblastic genes in cortical bone in response to loading
- In the presence of vitamin A, the quality of the bone was not improved by loading as assessed by Raman spectroscopy

Conclusion

Our novel findings indicate that the anabolic bone formation in response to mechanical loading is suppressed by increased vitamin A intake. This effect was mainly due to reduced periosteal osteoblast activity. Vitamin A negatively affected the enhancement of cortical and trabecular bone mass and quality in response to loading. These observations may have implications for the regulation of bone mass caused by physical activity and the risk of osteoporosis in humans.

Discussion

Vitamin A alone did not affect trabecular bone as reported in previous vitamin A studies (I)[131, 137, 182], however, it was able to suppress the magnitude of the loading-induced increases in trabecular bone mass. Vitamin A diet alone was able to decrease cortical bone in the present study. This response is similar to that observed in our previous study using the same dose (I)[182] as well as in hypervitaminosis A studies[128-134]. Vitamin A suppressed the loading gains in cortical bone parameters, suggesting that the bone-beneficial response to loading is decreased with excess vitamin A.

Mechanistically, vitamin A decreased the loading-induced increase in osteoblastic bone formation at the endocortical and periosteal bone sites, an effect mirrored by the reduced expression of osteoblastic genes. Periosteal decrease in bone formation has been reported previously with hypervitaminosis A[134, 181]. Endocortically, our observations that vitamin A alone increased osteoblast number and activity were consistent with our previous study (I)[182], as well as a study that showed increased osteoblastic gene expression in extracts from periosteum-free cortical bone containing bone marrow[132]. These data implicate that excess vitamin A may increase endocortical/marrow osteoblast activity and mineralisation. However, in the presence of loading, vitamin A supressed endocortical bone formation due to inhibition of the loading-induced gain in osteoblast activity.

Mechanical loading improves bone structure and strength, thereby decreasing fracture risk[183-185]. In contrast, hypervitaminosis A results in decreased bone strength and increased fracture risk[57, 119-121, 130, 132]. Vitamin A suppressed the loading-induced decrease in endocortical mineral-to-matrix ratio, indicating that no new bone was formed. Periosteally, vitamin A blocked the loading-induced decrease in carbonate-to-phosphate ratio and reversed the increase in mineral crystallinity indicating a poorer arrangement of mineral crystals in loaded vitamin A bones. The differences in the response to load endocortically and periosteally could be due to different strain levels at the two skeletal sites or to the different chemical and physical environments; endosteum to marrow interaction vs periosteum to muscle interaction.

We observed that a clinically relevant dose of vitamin A has a negative effect on osteoblasts when bone formation is induced by mechanical loading. The present novel results indicate that the loading response in bone is blunted by excess vitamin A, thereby, the bone growth that occurs in response to physical exercise may be impaired with excess vitamin A consumption.

4.3 Paper III

Effects of retinoids on physiologic and inflammatory osteoclastogenesis in vitro

Retinoids have been used as anti-inflammatory drugs for the treatment of numerous inflammatory diseases. In the present study, we investigated the effect of ATRA on osteoclastogenesis induced by RANKL (physiological) or LPS and TNFα (inflammatory) in both mouse bone marrow macrophages (BMM) and human osteoclast precursors ($CD14^+$ monocytes).

Main Results

- ATRA inhibits RANKL-induced osteoclast formation in human $CD14^+$ monocytes
- Inflammatory-induced osteoclastogenesis by TNFα and LPS was inhibited by ATRA in mouse BMM and human $CD14^+$ monocytes
- ATRA maintains RANKL- and TNFα-stimulated human $CD14^+$ monocytes at a macrophage phenotype by suppressing *IRF8*
- Overexpression of *Rank* in mouse BMM could not overcome inhibition of osteoclastogenesis by ATRA
- ATRA inhibition of TNFα-induced osteoclastogenesis is not due to decreased expression of TNFα receptors
- RARα agonist inhibited RANKL and TNFα-induced osteoclastogenesis, which could be reversed by a RARα antagonist

Conclusion

We demonstrate that ATRA can inhibit physiological and inflammatory osteoclastogenesis *in vitro* using osteoclast progenitors from mouse bone marrow and human blood. These data might help to explain why vitamin A can inhibit osteoclast formation on endocortical bone surfaces during physiological and pathological bone remodelling *in vivo*.

Discussion

In the present study, ATRA inhibited RANKL-induced osteoclastogenesis which may help explain why hypervitaminosis A treatment reduces osteoclast numbers at endocortical surfaces of bone[132]. This is in agreement with previous *in vitro* studies[131, 137, 146, 148-150, 186, 187].

In physiological osteoclastogenesis, the observed downregulation of *RANK* mRNA and decreased RANK protein expression by ATRA is similar to previous observations in $CD14^+$ monocytes[146]. Overexpression of *Rank* could not overcome the inhibition by ATRA in mouse BMM, indicating that ATRA can interfere with signalling pathways downstream of the RANK surface receptor. This notion is consistent with decreased expression of NFATc1 with ATRA in both physiologically- and inflammatory-induced osteoclastogenesis, an affect also observed in previous studies[137, 149, 150].

Intraperitoneal injections of ATRA can reduce the number of osteoclasts in mice with collagen-induced arthritis[187]. In the present study, ATRA inhibited osteoclast formation when induced with inflammatory compounds TNFα and LPS. ATRA inhibition of TNFα-induced osteoclastogenesis was due to inhibition of osteoclastogenic intracellular pathways.

Osteoclastogenesis is associated with downregulation of several transcription factors such as IRF8 which is believed to keep progenitor cells at monocyte/macrophage stage[188]. In the present study, downregulation of *IRF8* was suppressed by ATRA. Furthermore, we observed that $CD14^+$ cells actively phagocytosed zymosan, an effect abolished by RANKL-stimulation, but reversed by ATRA. These data indicate that ATRA maintains the osteoclast progenitors at a monocyte/macrophage state, thereby affecting differentiation, rather than proliferation.

We observed the importance of RARα in the inhibition of physiologically- and inflammatory-induced osteoclastogenesis in human progenitor cells. This observation was previously noted in mouse BMM[138], however RARα knockout mice have been reported to have no bone phenotype[137] and RARγ has been implemented in the inhibitory effect of ATRA[137].

Our principal findings demonstrate that ATRA inhibits RANKL-, TNFα-, and LPS-induced osteoclastogenesis in progenitors isolated from mouse bone marrow and human blood. This inhibition is due to the activation of RARα which inhibits differentiation of osteoclast progenitors by interfering with intracellular signalling pathways downstream of cell surface receptors.

4.4 Paper IV

Retinoids enhance osteoclastogenesis in periosteal bone cell cultures

Increased periosteal osteoclasts in response to hypervitaminosis A has been consistently reported in rodents[128, 129, 131, 132] and *ex vivo* organ cultures[138, 144, 145] and therefore, in the present study we aimed to assess the *in vitro* effect of retinoids on osteoclast differentiation in periosteal bone cell cultures.

Main Results

- ATRA alone does not stimulate osteoclast formation in periosteal bone cell cultures
- Osteoclast differentiation and bone resorption is enhanced by ATRA in prolonged RANKL stimulated cultures
- ATRA may increase the number of osteoclast progenitors present
- Macrophage genes were upregulated by ATRA
- Enhanced osteoclastogenesis by ATRA in periosteal cell cultures is not mediated via increased CSF1 (M-CSF) or IL-34

Conclusion

The present study demonstrates that ATRA, in the presence of RANKL, can increase the formation of mature bone resorbing osteoclasts in mouse calvarial periosteal bone cell cultures.

Discussion

Periosteal bone cell cultures contain both osteoclast and osteoblast progenitor cells[178]. We found that ATRA could not stimulate the mRNA expression of osteoclastic genes nor the formation of mature osteoclasts in periosteal bone cells cultured on plastic and bone discs. This indicates that the cells producing RANKL *in vivo* and in *ex vivo* organ cultures are not isolated during enzymatic digestion of the calvarial bone cells or that the cells become unresponsive to ATRA after isolation.

In prolonged cultures, RANKL-induced expression of osteoclastic genes was synergistically potentiated by ATRA. ATRA also synergistically potentiated RANKL-induced osteoclast formation when cells were incubated on bone discs. These findings may explain the increased number of osteoclasts seen on the periosteal surface after *in vivo* treatment with hypervitaminosis A[128, 129, 131, 132].

More osteoclasts were formed in cultures that had been pre-cultured with ATRA than in those pre-cultured in media without ATRA. This indicates that ATRA may have increased the number of osteoclast precursors in periosteal cell cultures. ATRA treatment also resulted in increased expression of the gene encoding for CSF1 receptor, indicating a potential mechanism by which ATRA stimulates osteoclast progenitor proliferation, an observation that has previously been reported in human osteoclast progenitors[146].

We also observed that ATRA increased expression of macrophage markers suggesting that ATRA increases the number of macrophages/osteoclast precursors in periosteal bone cell cultures, similar to what has been observed in cultures of $CD14^+$ monocytes and RAW264.7 murine cells[146]. CSF1/M-CSF and IL-34 bind to CSF1 receptor to promote osteoclast differentiation[63, 64]. CSF1/M-CSF and IL-34 protein levels were decreased by ATRA, thereby making it unlikely that an increase in osteoclast precursors observed after the addition of ATRA is due to CSF1/M-CSF or IL-34.

These results on periosteal cell cultures mimic the observed periosteal effects of vitamin A *in vivo*. It is important to note however, that *in vitro* periosteal cell cultures lack certain cell types such as osteocytes and immune cells, which may further contribute to the phenotype observed *in vivo* since both cells types are capable of expressing RANKL.

5. General Discussion

5.1 Osteoclasts

In vivo rodent hypervitaminosis A studies have illustrated the negative effect of excess vitamin A on bone. Numerous studies in both mice and rats have observed increased resorption and increased number of osteoclasts present on the periosteal bone of long bones (I)[128, 129, 131-133] and calvaria[181]. The methods of administration of vitamin A, and the dose used have all been different, however, results have consistently indicated the dramatic adverse effects of excess vitamin A on cortical bone due to osteoclasts. These results are derived from short duration toxic doses of vitamin A which seldom occurs in humans. Our clinically relevant dose of vitamin A also resulted in decreased cortical bone parameters such as periosteal circumference and bone mineral content, responses that were time dependent, indicating that the longer excess vitamin A is ingested, the more severe the bone phenotype (I)[182]. *In vivo* rodent studies are mainly performed on mice/rats still in their bone growing phase, thereby, results of vitamin A on bone may be influenced by bone modelling and remodelling. Vitamin supplementation is more common in the elderly[48, 49] therefore, further studies with vitamin A in old mice/rats which have reached bone homeostasis may be of significance.

The effect of clinically relevant dose of vitamin A was observed to be largely mediated by osteoclasts, since the use of a bisphosphonate inhibited the vitamin A effect on bone (I). The vitamin A effect observed on osteoclasts with clinically relevant or hypervitaminosis A doses are consistent with *ex vivo* bone organ cultures where the addition of retinoids results in increased periosteal bone resorption[129, 138-145] attributed to increased RANKL[138]. In cultures of periosteal cells from mouse calvaria, which contain a combination of osteoclast and osteoblast progenitors[178], we have also observed that the addition of RANKL and ATRA enhances osteoclastogenesis (IV). It is not yet clear if this effect is due to direct effect of ATRA on osteoclast progenitors or indirectly via effect on the osteoblasts present in the culture. This warrants further studies specifically isolating osteoclast progenitors from these periosteal cell cultures and/or *in vivo* studies excising periosteal and/or endocortical osteoclasts in rodents treated with excess vitamin A. Collectively, there is strong evidence from *in vivo*, *ex vivo*, and *in vitro* studies that retinoids are able to increase periosteal osteoclast formation and

resorption, thus mediating the decreased BMD observed in humans and increasing the risk of fractures.

Interestingly, endocortical osteoclasts decrease in response to hypervitaminosis A (I)[131, 132, 182], indicating an antagonistic effect of retinoids on osteoclasts at different skeletal sites. Cultures of osteoclast progenitors isolated from blood or bone marrow have resulted in inhibition of osteoclastogenesis by ATRA when stimulated by RANKL (III)[131, 146, 149, 189], or inflammatory compounds LPS and TNFα (III)[189]. These cultures of osteoclast progenitors are void of environmental influences found in bone marrow such as stromal cells, osteoblasts, and B and T cells[190]. Previous studies in crude bone marrow cell cultures, which contain the cells void in BMM cultures, also observed abolished osteoclast formation and resorption with ATRA, suggesting a direct action of retinoids on osteoclast progenitor cells[131, 149]. These cultures of osteoclast progenitors mimic endocortical osteoclasts as the bone marrow is adjacent to endocortical bone.

Mechanistically, the direct effects of ATRA on osteoclast differentiation has been suggested to be due to decreased expression RANK[146] or by a decrease in expression of NFATc1[149, 150]. Our studies suggested that ATRA inhibits via intracellular signalling independent of surface receptors, but concurs with studies associating with inhibition of NFATc1 (III)[189]. All in all, hypervitaminosis A effect on endocortical osteoclasts is similar to findings in *in vitro* cultures of endocortical osteoclasts isolated from blood and bone marrow where ATRA is able to inhibit osteoclastogenesis.

In contrast to the observed negative effects of excess vitamin A under physiological conditions, retinoids might have beneficial effects on osteoclasts in inflammatory conditions. ATRA plays a role in immune cell function[191-195] and therefore, retinoids are used as anti-inflammatory drugs in treatment of numerous inflammatory conditions[52, 54, 196-199]. Administration of retinoids has been shown to decrease inflammation and joint damage caused by arthritis in rodents[187, 200-202], and decrease the number of osteoclasts[187]. We have observed *in vitro* that ATRA is able to inhibit the formation of osteoclasts induced by inflammatory compounds LPS and TNFα. These data suggest that vitamin A may prevent inflammatory-induced secondary osteoporosis and that treatment with retinoid analogues may result in less negative bone side effects[203, 204].
RARs have been shown to mediate the direct effect of retinoids on osteoclasts. The expression of all subtypes of RARs, α, β, and γ, have been reported to be present in *in vitro* cell cultures, *ex vivo* organ cultures, and *in vivo* studies[138, 149, 189].

A pharmacological approach utilised in e*x vivo* and *in vitro* experiments have indicated the importance of RARα in mediating the effects of retinoids on osteoclastogenesis induced physiologically (III)[137, 138, 150, 189] and inflammatory (III)[189], however, RARγ has also been implemented[137]. Interestingly, using a gene deletion approach, knockout of RARα in mice resulted in no detectible bone phenotype[137], but RARγ knockout mice had reduced BMD and surprisingly, reduced trabecular bone mass[137]. The role of RAR subtypes in mediating the effects of retinol on bone needs to be further investigated. Culturing cells from knockout mice or cells where the specific receptors have been knocked down, or *in vivo* experiments with cell-specific receptor knockout mice fed hypervitaminosis A diets may provide further evidence for the roles and significances of the receptors.

5.1.1 Osteoclast Heterogeneity

The ability of vitamin A to enhance periosteal osteoclasts and reduce endocortical osteoclasts *in vivo* provides evidence for osteoclast heterogeneity. This is consistent with our observations *in vitro* where RANKL-induced osteoclastogenesis is inhibited by ATRA in cultures of pure populations of osteoclast precursors isolated from the bone marrow and blood (III) but stimulated in periosteal bone cell cultures, which contain osteoclast and osteoblast precursors (IV). The use of retinoids provides a method for studying osteoclast heterogeneity which may be attributed to numerous factors.

Osteoclast heterogeneity may be attributed to observed differences in osteoclast precursors and monocytes. Human and mouse osteoclast progenitor cells can differ in their ability to form osteoclasts and in their requirements for stimulatory cytokines[205-208]. Classical, intermediate, and non-classical are the three subtypes of human monocytes based on their expression of surface markers CD14 and CD16. Classical monocytes harbour the highest capacity to differentiate into osteoclasts in healthy individuals[209]. In inflammatory conditions intermediate and non-classical subtypes of monocytes increase in ability to form osteoclasts[210, 211]. Monocytes cultured *in vitro* have been isolated by the positive expression of CD14 on the cell surface[146, 189]. This cell population may consist of all three subtypes of monocytes and we have shown that ATRA can inhibit inflammatory- and physiologically-induced osteoclastogenesis in these cells (III).
Osteoclast differences may also be observed between different bones. Differences in proteolytic enzymes used for matrix digestion have been observed in long bones and calvaria, with mainly CTSK employed in long bone matrix degradation, and matrix metalloproteinases (MMPs) utilized by calvarial osteoclasts[212].

Bone-site specific differences in osteoclasts have also been observed in the expression of TRAP, where calvarial osteoclasts are reported to express more TRAP compared to long bone osteoclasts[213, 214]. TRAP expression has also been reported to differ between trabecular osteoclasts and osteoclasts located at the proximal epiphysis[215]. These data indicate bone-specific and bone site-specific osteoclast heterogeneity. In vitamin A studies *in vivo*, the observed negative effects on calvaria[181] and long bones (I)[128, 129, 131, 132, 182] did not differ and were a consequence of increased osteoclast numbers.

Osteoclast heterogeneity may also result from environmental differences. The presence of different cells may attribute to this. Periosteal and endocortical osteoclasts respond differently to vitamin A *in vivo*, as do cultures of pure progenitors (III) vs. periosteal cells containing osteoblasts and osteoclasts (IV). Studies using crude bone marrow cell cultures, which contain all environmental cells, indicate that ATRA effect is directly on osteoclast precursors[131, 149], and cultures of pure monocytes/macrophages strengthen this evidence as ATRA inhibits formation of osteoclasts (III)[189]. These models are reflective of the observed effect of vitamin A on endocortical osteoclasts. In regards to periosteal osteoclasts, in *in vitro* periosteal cell cultures, stimulation of osteoclastogenesis is observed by ATRA and RANKL (IV), and in *in vivo* hypervitaminosis A rodent experiments, an increase in periosteal osteoclasts has been reported (I)[128, 129, 131-133]. These periosteal osteoclast effects may be due to the direct effect on osteoclasts or indirectly via osteoblasts/other cells since periosteal cell cultures contain osteoblasts but lack other cells present *in vivo* such as osteocytes which may also contribute to the observed effects.

Environmental differences causing osteoclast heterogeneity may also pertain to the local levels of retinol present. Physiological levels of retinol in human serum range from 1-4µmol/L and increase with age[56, 57]. The concentration of ATRA, the biologically active form of vitamin A and the form most commonly used to study the effects of retinoids *in vitro*, is approximately 2-20nmol/L. Mice and rats have lower serum ATRA levels compared to humans[216, 217], and the concentrations of ATRA in tissues are higher than in the serum[217] since ATRA is produced locally. Concentrations of ATRA can also vary across regions of tissue. Significant differences have been reported between the upper and lower regions of rabbit rib growth plate, whereas the level of retinol was found to be similar in these locations[218]. Total extracts from rat femur and tibia illustrated retinol concentrations to be approximately 500-600nmol/g and to further increase with excess vitamin A diet[133]. However, the local concentrations of ATRA present in different

bone compartments under physiological conditions have not been investigated thoroughly and warrants further research.

5.2 Osteoblasts

In vivo, effects of hypervitaminosis A on osteoblasts has been seldom explored. Hypervitaminosis A and a clinically relevant dose of vitamin A have been reported to increase endocortical (I, II)[132, 182] and decrease periosteal (I)[131, 134, 182] osteoblast mineralisation and bone formation. Retinoid effect on osteoblasts *in vitro* has been shown to be dose-dependent, with inhibition of differentiation occurring at lower concentrations, and stimulation observed at high concentrations. *In vivo* studies using very high vitamin A doses with strong effects on osteoclasts might have resulted in an underestimation of the importance of osteoblastic effects. Under physiological conditions, we have observed that a clinically relevant dose of vitamin A is able decrease periosteal and increase endocortical bone formation (I). Furthermore, our novel findings indicate that consumption of an elevated level of vitamin A can decrease the bone-forming response to mechanical loading *in vivo* (II). This suppressed loading response with excess vitamin A is due to decreased osteoblastic bone formation on the periosteum, similar to previous rodent studies[131, 134, 182]. Furthermore, we have found that vitamin A alone changes the chemical and mechanical properties of bone at endocortical and periosteal bone surfaces (II). Periosteally, vitamin A alone increases crystallinity due to decreased substitution of carbonate to phosphate. Although this is positive for bone properties, the addition of loading abolishes this effect. Poor arrangement of mineral crystals is observed endocortically with vitamin A alone and the bone is less mineralised than controls. When loaded, increased endocortical mineral crystallinity is observed with both diets, however, the decrease in mineralisation observed with loading due to new bone formation in the control diet is absent with vitamin A diet. Therefore, in the presence of vitamin A, the addition of loading does not improve the quality of the bone (II). These novel data suggest that athletes taking supplementation containing vitamin A or its constituents must be aware of the restrictions in bone beneficial effects seen in response to exercise. This also applies to the exercising elderly which are already at a risk of osteoporosis. These data suggest that osteoblasts may be of more importance in vitamin A and bone status than previously acknowledged.

6. Conclusion

We have studied the role of vitamin A in determining properties of the skeleton and bone cells in both *in vivo* animal studies and mechanistic *in vitro* experiments. *In vivo*, we have found that prolonged exposure to clinically relevant concentrations of retinoids is able to decrease cortical bone parameters, an affect mediated by osteoclasts (I), similar to the observed effects of hypervitaminosis A. We also found that a clinically relevant concentration of vitamin A is able to affect osteoblasts after one week by increasing endocortical and decreasing periosteal bone formation (I). Furthermore, we have observed that 6 weeks of the same diet is able to suppress the anabolic bone response to mechanical loading by reducing bone formation, primarily at the periosteal bone site (II). We have not observed a trabecular bone phenotype with clinically relevant doses (I) or a hypervitaminosis A dose (I), however, we were able to observe a decrease in the loading-induced increase in trabecular bone with vitamin A (II). These data give further evidence that the detrimental effects of excess vitamin A on cortical bone is mediated by a combination of the effects on osteoclasts and osteoblasts and that excess vitamin A may have a negative impact on bone modelling induced by physical activity.

In vitro, we have found that ATRA is able to inhibit both physiological and inflammatory osteoclastogenesis in cultures of pure osteoclast precursors (III), data which may help explain the observed inhibition of endocortical osteoclasts in hypervitaminosis A *in vivo* (I) and may implicate retinol in preventing inflammatory-induced secondary osteoporosis. We have also found that ATRA is capable of potentiating physiologically-induced osteoclast formation in periosteal bone cells (IV), data which may explain why hypervitaminosis A enhances the number of periosteal osteoclasts (I).

Fortification of food with vitamin A and vitamin A supplementation should be re-examined as vitamin A status may be a risk factor for secondary osteoporosis.

Acknowledgements

First and foremost, I would like to express my deepest gratitude to my supervisors, without whom this work would have never been possible.

Dear Ulf, thank you for your knowledge, time, and support. Your constant positivity and excitement for science is inspiring and motivating. The knowledge I have gained under your supervision and your guidance has been invaluable.

Petra, you have gone above and beyond your duties as a supervisor to help me. Thank you for your patience and encouragement. I am in awe of your ability to always have the answers. Thank you for your support, enthusiasm, expert advice, and willingness to help.

Thank you Claes for being available to answer questions.

Thank you to all the co-authors, without whom this work would not have been achieved. Thank you all for your expertise and continuous assistance.

Thank you to the Euroclast supervisors and students. I have enjoyed our meetings and am proud of the work we have all accomplished.

Lastly and most importantly, thank you to my family for your unconditional love, support, and motivation.

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