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Effects of Nutrition and Alcohol Consumption on Bone Loss

Martin J.J. Ronis^{*}, Kelly Mercer, Ph.D., and Jin-Ran Chen, M.D., Ph.D.

Arkansas Children's Nutrition Center, 15 Children's Way, Little Rock, AR 72202, USA. Tel. 364-2785. MercerK@uams.edu

Department of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children's Nutrition Center, 15 Children's Way, Little Rock, AR 72202, USA. Tel. 501-364-2707. ChenJinran@uams.edu

Abstract

It is well established that excessive consumption of high fat diets results in obesity. However, the consequences of obesity of skeletal development, maturation and remodeling have been the subject of controversy. New studies suggest that the response of the growing skeleton to mechanical loading is impaired and trabecular bone mass is decreased in obesity and after high fat feeding. At least in part, this occurs as a direct result of inhibited Wnt signaling and activation of PPAR γ pathways in mesenchymal stem cells by fatty acids. Similar effects on Wnt and PPAR γ signaling occur after chronic alcohol consumption as the result of oxidative stress and result in inhibited bone formation accompanied by increased bone marrow adiposity. Alcohol-induced oxidative stress as the result of increased NADPH-oxidase activity in bone cells also results in enhanced RANKL-RANK signaling to increase osteoclastogenesis. In contrast, consumption of fruits and legumes such as blueberries and soy increase bone formation. New data suggest that Wnt and BMP signaling pathways are the molecular targets for bone anabolic factors derived from the diet.

Keywords

oxidative stress; redox; Wnt; bone morphogenic protein; PPAR γ , receptor activator of NF- κ B ligand; estradiol; mesenchymal stem cell; obesity; nonesterified free fatty acid; berries; fruit; soy; ethanol

Introduction

Although nutritional status and dietary factors including protein, fat, fruits, vegetables and alcoholic beverages are well known to play a key role in regulation of skeletal growth; attainment of peak bone mass and risk of osteoporosis, until recently, with the exception of studies on vitamins and minerals such as vitamin D and calcium, most studies of nutrition and alcohol on bone have been descriptive rather than mechanistic. This has changed in the past 2–3 years as new clinical studies have challenged the established paradigm that increased body weight protects against osteoporosis [1,2] and animal studies have identified molecular targets for the actions of non-traditional dietary factors on bone cells.

Basic studies using genetic approaches have revealed a central role for Wnt- β catenin and BMP signaling pathways in regulation of bone formation [3,4]; the importance of PPAR γ in

^{*}Corresponding author. M. Ronis Ph.D., Department of Pharmacology & Toxicology, University of Arkansas for Medical Sciences, Arkansas Children's Nutrition Center, 15 Children's Way, Little Rock, AR 72202, USA. Tel. 501-364-2796. RonisMartinJ@uams.edu.

lineage commitment of mesenchymal stem cells between osteoblasts and adipocytes [5] and for RANKL-RANK signaling in differentiation of osteoclasts [6]. In addition, the importance of reactive oxygen species and of redox status in bone cells in regulation of bone turnover and survival of osteoblasts, osteoclasts and osteocytes and interaction of redox systems with actions of sex steroids has now received wide recognition [7–9]. Increased understanding of the molecular basis of bone cell differentiation and survival has revealed new targets for the actions of alcohol and other nutritional and dietary factors on bone which are reviewed in this article.

Bone Quality, Adiposity and High Fat Diet

The effects of adiposity on bone are complex. Studies of lean and normal weight adults have supported the clinical dogma that increased mechanical force associated with increases in body weight result in increased bone mineral density and stronger bones [1]. However, in the past few years several studies of adult obesity and metabolic syndrome have suggested increased fracture risk and an inverse relationship between percent fat mass and bone mineral density when corrected for body weight [2,10, 11]. A new study by Dimitri et al. [12**] observed a similar inverse relationship between adiposity and bone mass and reported that pediatric obesity also substantially increases fracture risk. At least in part, it appears that lower trabecular bone mass reported in studies of obese children can be explained by the complex interplay between adipokines secreted by adipose tissue and bone [2]. Studies by Karsenty's group in genetically manipulated mice suggest that leptin, a hormone secreted by fat cells which increases with obesity, can act centrally via suppression of serotonin synthesis and release by the raphe nuclei and thus act on the hypothalamus to stimulate sympathetic tone and suppress bone accrual via adrenergic actions on osteoblasts to induce RANKL [13]. In addition there appear to be direct effects of adipose derived factors on bone cell turnover. Co-cultures of bone marrow stromal cells and intra-abdominal adipose tissue have revealed profound suppression of osteoblast differentiation [2]. Additional analysis of obese children by Dimitri et al. suggests that obese children also have increased bone resorption which correlated with reduced serum osteoprotegerin and have increased circulating concentrations of the soluble Wnt inhibitor Dkkopf [14*].

Although trabecular bone loss and increased bone resorption have been reported experimentally in mice made obese by feeding of high fat diets [15–17], interpretation of bone data from these models is complicated by increased body weight and thus increased mechanical loading in addition to the consequences of increased fat mass. In order to overcome this experimental limitation, recent studies in our laboratory have taken advantage of the precise control of caloric intake and diet composition afforded by using total enteral nutrition (TEN) in young rapidly growing prepubertal rats to increase adiposity while equalizing weight gain between groups fed diets with differing fat/carbohydrate ratios by adjusting caloric intake [18**]. In this model, we observed a dose-dependent reduction in tibial trabecular bone mineral density with increasing dietary fat content and increasing abdominal fat pad weight. Negative effects of high fat-driven obesity on bone mass were accompanied by decreases in bone formation and increases in bone resorption and by increasing numbers of fat cells in the bone marrow. Impaired differentiation of mesenchymal stem cells into osteoblasts and increased adipogenesis was associated with inhibition of Wnt- β catenin signaling both *in vivo* and *in vitro* in ST2 bone marrow stromal cells exposed to serum from high fat-fed compared to low fat diet-fed rats. Reduction in β -catenin mRNA and protein expression and decreased TCF/LEF-dependent transcription as measured by TOPFLASH was accompanied by a reciprocal increase in nuclear PPAR γ protein expression and activation of PPAR γ signaling as measured by TansAM™ and chromatin immunoprecipitation [18**] (Figure 1). A similar reciprocal pattern of changes in Wnt and PPAR γ signaling in stromal cells exposed to an artificial mixture of nonesterified

free fatty acids (NEFA) mimicking the concentration and composition of NEFA in the peripheral circulation attained after high fat TEN feeding. It is unclear if direct activation of PPAR γ signaling by NEFA or their metabolites occurs in mesenchymal stem cells or if this is secondary to impaired Wnt signaling since silencing of β -catenin with siRNA in the absence of fatty acids was able to significantly increase PPAR γ expression.

Other recent studies suggest a role for immune signaling and inflammation in the increased bone resorption observed in obesity and hyperlipidemia. Increased osteoclastogenesis in obese mice has been linked to reductions in levels of the anti-inflammatory cytokine IL-10 in bone marrow [16] and to increases in activation and expression of RANKL in T-lymphocytes after feeding mice high fat/high cholesterol atherogenic diets [17].

Alcohol-Induced Bone Loss

Like obesity, chronic alcohol consumption has complex direct and indirect effects on bone resulting in loss of BMD, impaired bone quality and increased risk of osteoporosis. For many years the focus of research in this area was related to inhibited bone formation. However, more recent studies in alcoholics have also provided definitive evidence for increased bone resorption in alcoholics [19*,20].

With regards to inhibition of bone formation, indirect actions of ethanol have been ascribed to effects on calcium homeostasis through actions on the parathyroid hormone (PTH)-vitamin D axis [21–23] and to disruption of in growth hormone (GH)-insulin-like growth hormone (IGF1) signaling [24,25]. Recent studies by Turner et al. [25] demonstrated impaired tibial growth and cancellous bone formation in ethanol-fed hypophysectomized-GH replaced rats which it was concluded might be mediated via skeletal resistance to GH. However, an alternative explanation for this data could be that the effects of EtOH are simply mediated via a GH-IGF1 independent pathway. Similarly, analysis of the effects of low dose PTH on alcohol-induced bone loss provided no evidence of interactions between alcohol and PTH and no effects on alcohol-mediated inhibition of mineral apposition rate or increase in marrow adiposity, even though PTH was able to blunt the decrease in mineralizing perimeter/bone perimeter in alcohol-treated rats [23].

Recent analysis of alcohol actions on the bone transcriptome by Callaci et al. using gene array approaches have revealed several important new molecular targets which may underlie inhibited bone formation and fracture repair [26**, 27*]. Using a rat model of binge drinking, these investigators revealed that acute ethanol exposure resulted in significant effects on genes involved in chemokine/cytokine and integrin signaling. Recent use of another mouse model of ethanol exposure in which uncoupled bone formation was produced during limb lengthening by distraction osteogenesis (DO) has confirmed earlier studies suggesting an important role for the cytokine tumor necrosis alpha (TNF α) in ethanol inhibition of osteoblastogenesis [28–30]. Ethanol and recombinant TNF α were able to block DO. In contrast, administration of a soluble TNF receptor blocker sTNFR1 was able to reverse alcohol inhibited osteoblastogenesis and the effects of ethanol and recombinant TNF α on bone formation could be blocked in TNFR1 knockout mice [28–30]. Interestingly, chronic alcohol also had suppressive effects on Wnt signaling. Wnt appears to represent a common target in alcohol and obesity-associated bone loss [18**, 26**, 31**] (Figure 1). Interestingly, significant effects on several pathways persisted after 30 d of alcohol abstinence, particularly on genes regulating the circadian clock system [27*]. Our laboratory has confirmed and extended data on interactions between ethanol exposure and skeletal Wnt-signaling using a rat TEN model [31**]. We have shown that suppression of Wnt- β catenin signaling and nuclear translocation of β catenin after ethanol treatment is dependent on development of oxidative stress in mesenchymal stem cells and can be reversed *in vivo*

and *in vitro* by treatment with the dietary antioxidant N-acetylcysteine (NAC), coincident with reversal of inhibition of bone formation. [31**, 32]. Similar to the skeletal phenotype in obesity and aging, alcohol consumption increased bone marrow adiposity and alcohol suppression of Wnt signaling coincided with increased PPAR γ signaling and increased adipogenesis. This was also reversed by NAC treatment. In addition to effects of ethanol on bone formation, we have also recently shown that ethanol promotes senescence in mature osteoblasts associated with inhibition of estrogen receptor signaling and induction of p53 and p21 [32*]. This also appears to be related to development of oxidative stress and in accordance with studies by other investigators [7–9], we have evidence that negative cross-talk between estrogen signaling and reactive oxygen species regulates osteoblast lifespan.

Since ethanol increases oxidative stress in bone cells, it is not surprising that in addition to inhibiting bone formation, bone resorption is also increased in alcoholics and in animal models which mimic binge drinking [19*, 33, 34]. Oxidative stress plays a key role in regulation of osteoclast differentiation through stimulation of RANKL expression in osteoblasts and their precursors and in T cells in bone marrow (Figure 1). Signaling through the RANKL-RANK signaling pathway is essential for differentiation of osteoclast precursors into mature osteoclasts and has recently been shown to be reversible by various antioxidants *in vitro* [9]. Our laboratory has demonstrated that ethanol treatment induces bone resorption and RANKL mRNA and protein expression in the bone of cycling female rats without effects of expression of osteoprotegerin. In addition, induction of RANKL by ethanol appeared to involve oxidative stress since it could be blocked by the antioxidant NAC [35**] and to require additional protein synthesis since induction could be blocked by co-treatment of osteoblasts with the protein synthesis inhibitor cyclohexamide [36]. Many different sources of oxidative stress have been suggested to result from cellular exposure to ethanol [37]. However, the major source of ethanol-induced reactive oxygen species in liver, cytochrome P450 CYP2E1, was found not to be present in osteoblasts [36]. Instead, 3 forms of the enzyme NADPH oxidase (Nox) Nox1, Nox2 and Nox4 which form superoxide from molecular oxygen, were found to be present in osteoblasts [35**]. Most Nox enzymes require activation by complex formation with a series of cytosolic phox protein co-factors and Rac1 in order to form superoxide [38]. However, Nox 4, is a constitutively active Nox form which has been shown to generate superoxide in parallel with its expression [39]. Expression of mRNA for all 3 Nox enzymes were significantly increased in osteoblasts after ethanol treatment. However, Nox4 was the most responsive. Inhibition of RANKL induction by ethanol *in vitro* in the presence of the pan-Nox inhibitor diphenyleneiodonium (DPI) suggested that ethanol-dependent osteoclastogenesis was Nox dependent [35**]. Induction of RANKL by ethanol appeared to involve ethanol metabolism to acetaldehyde since the effects of ethanol could be mimicked by acetaldehyde at a 100-fold lower concentration and since the alcohol dehydrogenase inhibitor 4-methylpyrazole was able to block ethanol-induced RANKL expression [36]. The increase in RANKL could be reversed by administration of estradiol to produce plasma levels similar to those observed during pregnancy [36]. We have observed increases in RANKL expression in osteoblasts and osteoblastic cell lines treated with ethanol *in vitro* and increased osteoclastogenesis in ethanol-treated osteoblast-osteoclast precursor co-cultures [35**, 36]. As demonstrated *in vivo*, estradiol was able to block RANKL induction by ethanol in osteoblasts *in vitro* and this appeared to be mediated via estrogen receptor (ER) signaling since the ER antagonist ICI 182,780 prevented the estradiol action [36]. Moreover, estradiol treatment appeared to act in a similar fashion to the antioxidant NAC by blocking ethanol-dependent formation of reactive oxygen species and was able to block ethanol-induced Nox4 expression [35**]. Signaling downstream from Nox-generated reactive oxygen species to increase RANKL expression appears to involve MAP kinase signaling and in particular, increased phosphorylation of ERK. This was observed 2–3 h following ethanol exposure of osteoblasts *in vitro* and was abolished by DPI, estradiol and NAC treatments. In addition, co-treatment

of osteoblasts with ethanol and the ERK inhibitor PD98059 prevented RANKL induction [36]. A transcription factor known to positively regulate RANKL expression and known to be an ERK target is signal transducer and activator of transcription (STAT)3. We have observed changes in STAT 3 phosphorylation in osteoblasts in parallel with changes in ERK phosphorylation after ethanol treatment *in vitro* and inhibition of ethanol-dependent STAT3 phosphorylation by estradiol, NAC and PD98059 [35**]. We have also prevented ethanol induced RANKL mRNA expression in osteoblasts directly by use of the STAT3 inhibitor AG490 (Ronis et al. unpublished). To confirm the importance of Nox-dependent oxidative stress on alcohol-induced bone loss *in vivo*, we have recently completed new studies in the cycling female rat TEN model in which we have prevented ethanol-induced bone resorption and elevation of Nox4 and RANKL mRNA and protein in bone by co-treatment with estradiol, NAC or DPI [40**].

Diet Derived Bone Anabolic Factors

In contrast to dietary factors such as fats and alcohol and nutritional states such as obesity which are linked to bone loss, it has been clear for some time that consumption of some types of fruits and vegetables can increase bone mineral density and improve bone growth and quality [41, 42]. Recent animal studies have demonstrated dramatic increases in bone mass associated with consumption of dried plums and berries such as blueberries and prevention or even reversal of sex-steroid deficiency and aging associated bone loss [43, 44*, 45**]. Our laboratory has demonstrated that diet supplementation with blueberry in growing rats resulted in substantially increased bone formation in both males and females and inhibited bone resorption associated with down-regulated RANKL expression [45**]. The bone anabolic actions of blueberry on osteoblastogenesis *in vivo* were replicated *in vitro* in ST2 stromal cell cultures exposed to serum from blueberry fed animals and were associated with activation of Wnt- β catenin signaling and increased phosphorylation of the MAP kinase p38 (Figure 1). Nuclear localization of β catenin in ST2 cells stimulated by serum from blueberry fed rats was inhibited by the p38 inhibitor SB 239063. Analysis of serum after blueberry feeding revealed substantial increases in the concentration several phenolic acid metabolites of polyphenol pigments generated during intestinal digestion. Interestingly, an artificial mixture of these phenolic acids mimicked the effects of blueberries on Wnt signaling and osteoblastogenesis indicating their potential in the prevention of bone loss. However, it remains unclear how phenolic acids activate p38.

Other foods such as soy protein have also recently received attention for potential protective effects against post-menopausal bone loss. Purified soy phytochemicals such as the weakly estrogenic isoflavones genistein and equol, which may be used as dietary supplements, have shown some promise in prevention of bone resorption and probably act in a SERM-like fashion to suppress RANKL-RANK signaling [9, 46]. However, dietary soy appears to be anti-estrogenic in bone when consumed in the presence of endogenous estrogens [47] and has little or no effect on aged bone [48]. In contrast, when consumed early in life, we have recently demonstrated that in addition to suppressing RANKL expression, soy has estrogen-independent bone anabolic effects [48, 49*]. Soy formula feeding increased bone mineral density in neonatal piglets relative to breast-fed piglets coincident with increased serum bone formation markers osteocalcin and bone specific alkaline phosphatase, increased osteoblast numbers and increased mineral apposition rate. In addition, serum from soy formula-fed piglets stimulated osteoblast differentiation *ex-vivo*. However, in this case the molecular target for bone anabolic effects appears to be bone morphogenic protein (BMP) signaling (Figure 1). Feeding soy formula increased expression of BMP2 mRNA and downstream signaling in bone through increased phosphorylation of ERK and Smad proteins to increase expression of the osteoblast differentiation factor Runx2 [49*]. Moreover, the BMP inhibitor noggin was able to block *ex-vivo* osteoblast differentiation stimulated by

serum from soy formula fed animals. What remains to be determined is which soy component is responsible for activation of BMP signaling. Interestingly, recent *in vitro* studies with hesperetin-7-O-glucuronide, the major circulating metabolite of hesperidin, a glycoside flavinoid found in high concentrations in citrus fruits and juices such as orange juice, have demonstrated increased osteoblast differentiation using rat calvarial cell cultures [50*]. Differentiation was stimulated at concentrations attainable *in vivo* after fruit juice consumption and as was the case with soy, was accompanied by evidence of enhanced BMP signaling including increased Smad phosphorylation and Runx2 expression. Surprisingly, this study suggests that conjugated phytochemicals may have biological activities on bone cells and in fact the authors report higher osteoblastogenic activity with the conjugate than with hesperetin aglycone. In addition, distribution studies suggested little entry of the conjugate into osteoblastic cells and active export. This implies action through an as yet uncharacterized cell surface receptor.

Conclusions

The recent explosion of molecular studies into the regulation of bone turnover and bone cell lifespan using genetically modified mice has led to a paradigm shift away from focus on sex steroid action to new mechanisms such as interactions of endogenous factors and xenobiotics with cellular redox systems and modulation of cellular concentrations of reactive oxygen species [7–9]. Oxidative stress/redox state appears to be a critical signal for normal bone growth and homeostasis but oxidative stress can be suppressed and antioxidant status elevated via nongenomic actions of sex steroids to prevent bone loss [7]. Recent studies suggest that nutritional states such as obesity and consumption of high fat diets produce a pro-inflammatory/pro-oxidative state in bone which results in inhibition of bone formation via impaired Wnt signaling and induced bone resorption via increases in the RANK-RANKL signaling pathway. In addition, circulating fatty acids may also directly inhibit bone formation through diversion of mesenchymal stem cell differentiation away from osteoblasts and into adipocytes as the result of PPAR γ activation. The effects of obesity/high fat on bone appear very similar to those of both aging and alcohol with common molecular targets all related to generation of oxidative stress. However, the sources of oxidative stress may be different. In aging, reactive oxygen species produced by mitochondria via the adaptor protein p66^{shc}, lipid products produced via the action of lipoxygenase and lipid peroxidation products such as hydroxynonenal have all been implicated in bone loss [7]. Our recent studies suggest that fatty acid metabolites also appear to play a role in bone loss in obesity. However, the major source of oxidative stress in bone following alcohol exposure appears to be superoxide generated as the result of induction of Nox enzymes. It appears that dietary factors can also have anabolic actions on bone via actions on Wnt and RANKL-RANK signaling pathways and it remains to be seen to what degree modulation of oxidative stress/redox systems are involved in the bone anabolic effects of fruits and vegetables. However, we and others have also recently identified additional pathways related to BMP signaling which appear to be involved in bone anabolic effects of soy and citrus fruits. Manipulation of early diet may be an important way of increasing peak bone mass and reducing the risk of osteoporosis later in life. In addition, these recent studies suggest that new bone anabolic products derived from dietary factors have the potential to prevent bone loss or even restore it during aging.

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Figure 1.

Ethanol (EtOH), nutritional status and dietary factors impact bone turnover and bone cell survival via common signal transduction pathways. BMP, bone morphogenic protein; E₂, 17 β -estradiol; MSC, mesenchymal stem cell; NEFA, nonesterified free fatty acids; Nox, NADPH oxidase; OB, osteoblast; OC, osteoclast; OJ, orange juice; OPG, osteoprotegerin; ROS, reactive oxygen species.