REVIEW

Protein/amino-acid modulation of bone cell function

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Nutrients (protein, carbohydrates and fats) have traditionally been thought of as fuels simply providing the energy for cellular metabolic activity. According to the classic view, if nutrients are available, then anabolic pathways are activated, and if nutrients are not available, catabolic pathways are activated. However, it is becoming increasingly clear that nutrient effects on bone cells (stem cells, osteoblasts and osteoclasts) are complex, some nutrients promote bone formation, whereas others interfere with bone formation or actually promote bone breakdown. At an organ level, nutrient intake can suppress bone breakdown and modulate the activity of the calcium/vitamin D/parathyroid hormone axis. At a cellular level, nutrient intake can impact cellular energetics either through a direct mechanism (binding or uptake of the nutrient into the cell) or indirect (by elevating nutrient-related hormones such as insulin, insulin-like growth factor 1 or incretin hormones). It is also becoming clear that within a nutrient class (for example, protein), individual components (that is, amino acids) can have markedly different effects on cell function and impact bone formation. The focus of this review will be on one nutrient class in particular, dietary protein. As the prevalence of inadequate dietary protein intake increases with age, these findings may have translational implications as to the optimal dietary protein content in the setting of age-associated bone loss.

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Introduction

Although calcium and vitamin D are widely accepted as important nutrients for bone formation/maintenance, the impact of other nutrients on skeletal health is increasingly recognized. This recognition relates to our changing perception of the skeleton as mainly a calcium/phosphate reservoir serving a biomechanical function to an important endocrine/paracrine organ playing a role in the body’s overall energy balance.¹,² It should be kept in mind though that widespread nutritional excess is a relatively recent phenomenon. Although many current studies focus on excess-associated diseases such as obesity and diabetes mellitus, the skeleton evolved in the setting of caloric restriction or intermittent nutrient intake.

Therefore, from the nutrient/bone perspective, there are three different scenarios that could potentially impact overall skeletal health: (1) total caloric intake, irrespective of dietary composition will impact the balance between bone formation vs breakdown; (2) physiological dietary intake, carbohydrates, fats or proteins impact bone turnover in different ways; (3) pathophysiological dietary intake; excessive dietary intake of carbohydrates, fats or protein and associated conditions such as diabetes mellitus and obesity can negatively impact bone mass.

Caloric intake effects on bone are complex, fasting promotes bone breakdown,³ moderate caloric intake inhibits bone breakdown⁴ and increased intake of high-quality nutrients promotes bone formation.⁵ Caloric restriction effects on bone appear to be time dependent with short-to-medium-term restriction leading to a decrease in cortical bone mass;⁶ however, continued caloric restriction in rodents past 1 year leads to a preservation of bone mass compared with controls.⁷

Acutely, nutrient ingestion (for example, glucose and amino acids) predominantly suppresses bone breakdown by inhibiting osteoclastic activity.⁸ Chronically, however, continued nutrient...
ingestion could increase bone formation by modulating osteoblastic activity/mineralization.\(^8\)

In addition to macronutrients, a number of additional, trace elements, vitamins such as vitamin K and ions such as zinc have also been evaluated for potential roles in bone development and formation,\(^9,10\) but will not be discussed in detail here.

Although all macronutrients (dietary protein/fat/carbohydrate) impact bone turnover, this article will focus mainly on the effects of dietary protein on bone with only a brief discussion of the other nutrients.

**Dietary carbohydrates**

Carbohydrate effects on bone mass can be either through direct action on bone cells (osteoblasts and osteoclasts) or indirect by increasing hormones that lower blood sugar (for example, insulin and incretins), which themselves have effects on bone turnover.

Bone resorption by osteoclasts is dependent on acid secretion, which is an energy-dependent process as evidenced by the large number of mitochondria present in osteoclasts.\(^11,12\) Glucose increases bone resorption dose-dependently between 0.1 and 7 mm, whereas fatty acids and essential amino acids do not increase bone resorption,\(^12\) in fact some amino acids can inhibit osteoclastic resorption.\(^13\)

Osteoblast function is also modulated by glucose. An endocrine regulatory loop has been proposed where the osteoblast-related protein, osteocalcin, is released into the circulation and stimulates insulin secretion, which in turn promotes glucose utilization by osteoblasts and bone formation.\(^8\) Elevations of glucose lead to an increase in osteoblastic differentiation and mineralization. Immature osteoblasts have been reported to have insulin receptors and glucose transporters (GLUTs 1, 2 and 4) with GLUT 4 expression increasing with osteoblast maturity.\(^2\) Glucose appears to be the main substrate utilized by these cells and anabolic agents such as parathyroid hormone (PTH) increase aerobic glycolysis.\(^14\) Glucose in addition to serving as an important energy source for the osteoblast also aids this process by generating reactive oxygen species (ROS). Osteoblast mineralization is facilitated by the generation of ROS as evidenced by the fact that an excess of antioxidants such as N-acetyl-cysteine can inhibit mineralization.\(^15\) Interestingly, although dietary ingestion of proteins, fatty acids and carbohydrates all generate ROS, it is carbohydrates that generate the most ROS.\(^16,17\) Thus, from an integrative physiological perspective, an elevation of blood glucose would lead to the increased insulin secretion, which together with glucose promotes osteoblastic differentiation/mineralization. Increased glucose also increases osteoclastic activity, which increases undercarboxylated osteocalcin further promoting insulin release and synergistically promoting osteoblast differentiation (Figure 1). The control point to avoid this being a continuous positive feedback loop that resulted in hypoglycemia is serum glucose,\(^18\) as serum glucose drops insulin secretion is inhibited. Thus, osteoblastic mineralization would only occur when glucose was elevated and inhibited as blood glucose and insulin levels dropped. A corollary to this statement is that in situations when blood glucose and insulin remained high (DM type II) then increased bone mineralization could be observed.\(^19\)

In contrast to osteoblasts, mesenchymal stem cells (MSCs) are not greatly impacted by glucose unless it is in excess.\(^20\) With respect to MSCs, the data would suggest that protein/amino acids and fatty acids have a more important role that glucose in promoting stem cell proliferation (discussed below). Thus, the impact/role of nutrients on bone cell function would vary as these cells underwent proliferation vs differentiation.

Glucose also has a number of indirect effects on bone turnover. Multiple nutrient-related hormones have been implicated in meal-related inhibition of bone resorption including glucose-dependent insulinotropic peptide (GIP), GLP2 and insulin.\(^7,21,22\) In human studies, both glucose and protein were found to significantly decrease bone breakdown, whereas nutrient ingestion was not found to affect bone formation (as assessed by measuring osteocalcin levels).\(^23\) In another human study, in which oral glucose also significantly reduced bone breakdown (as assessed by decreased collagen fragments, C-telopeptide), this glucose-dependent suppression was abolished by a somatostatin analog. These findings suggest that somatostatin blocked the secretion of a nutrient-induced hormone, which was responsible for suppressing bone breakdown (that is, nutrient effects were indirect).\(^24\) Insulin infusion has also been found to modestly decrease bone breakdown (~30% of the decline in resorption markers that occurs post-prandially).\(^23\) Data from our laboratory and from others suggest that the acute suppression of bone turnover may be mediated in part by incretin hormones such as GIP and glucagon-like peptide 2 (GLP2).\(^7,21\) Our group has coined the term ‘entero-osseous’ axis to describe this close association between nutrient ingestion and bone turnover. In essence, during fasting, bone resorption occurs and the skeleton is a source of ions and minerals, and during times of nutrient

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**Figure 1** Nutrients impact multiple cell types in bones. Nutrients can modulate bone marrow stem cell function, osteoblast differentiation and osteoclast activity. Glucose has direct effects on osteoclast and osteoblasts, and indirect effects by increasing nutrient-related hormones such as insulin and incretin hormones. Amino acids and polyunsaturated fatty acids (n-3 PUFA in particular) can modulate MSC function.
ingestion, catabolic activity is decreased and anabolic activity increases related to the type and quantity of nutrients ingested.

**Dietary fat**
Similar to the negative effects of high glucose on bone, high-fat diets lead to lower bone mass, possibly through decreased differentiation of osteoblasts from MSCs, markers of bone formation are reduced as is the anabolic hormone insulin-like growth factor 1 (IGF-1). In addition, high-fat diets may increase osteoclastic bone resorption contributing to the loss of bone mineral density observed with these diets. High-fat diets are associated with an increase in inflammatory cytokines, which might have a role in subsequent bone loss. Overall, the net effect of obesity on bone health appears to be negative despite it being associated with higher bone mass. Evolutionarily obesity-related bone problems were not widespread until relatively recently. Much of the available research on the physiological roles of fats and fatty acids have in normal bone turnover has focused on polyunsaturated fatty acids (PUFAs), in particular comparing n-6 vs n-3 PUFA. In animal models, increasing dietary n-3 and decreasing n-6 PUFA probably has a beneficial effect on bone density and strength. In humans, there is limited clinical trial data to support a beneficial effect of n-3 PUFA. However, dietary n-3 PUFA (for example, eicosapentaenoic and docosahexaenoic acids) have anti-inflammatory properties, which could be beneficial for increasing bone mass.

In addition to the direct effects that lipids may have on bone cells, adipocytes also secrete many adipokines and cytokines that can also modulate bone turnover including leptin, adiponectin, visfatin, omentin and fibroblast growth factor 21. Effects of some of these adipokines on bone metabolism may be complex and for some like leptin may relate to whether the effects are central or peripheral. The rest of this discussion however will focus on protein effects on bone.

**Protein**
High-protein diets have long been thought to have a negative influence on bone mass. The rationale for this belief was that the high-protein intake would necessitate bone dissolution to buffer the acid load. Evidence provided for this idea was that high-protein diets induced an increase in urinary calcium, presumably a result of increased bone breakdown. However, recent studies have led us to change our perspective on the impact of dietary protein on bone mass. Low-protein diets (0.8 g kg⁻¹) are associated with low intestinal calcium absorption and thus induce secondary hyperparathyroidism with elevations in PTH and 1,25 dihydroxyvitamin D. High-protein diets (>2 g kg⁻¹) actually increase intestinal calcium absorption (the increased urinary calcium reflects absorption rather than resorption) and these subjects have low PTH and 1,25 dihydroxyvitamin D values. The impact of dietary protein on urinary calcium excretion increases in a linear manner between low- and high-protein intake such that on average every 50 g increase in protein intake results in a 1.6 mmol increase in 24-h urinary calcium excretion. Thus, average dietary protein intakes (representative of up to 50% of the US population) would not appear to have any impact on calcium or bone turnover. Interestingly, in addition to the direct effects of protein intake on skeletal mass, dietary protein may also modulate other anabolic signals for bone. In a study by Chevalley et al. where dietary protein and calcium intakes, physical activity and bone mineral content (BMC) were assessed in 232 pre pubertal boys, the anabolic effects of physical activity on BMC were enhanced in those subjects with higher median dietary protein intake. These beneficial effects of higher-protein intake and physical activity on BMC seem to persist and lead to beneficial microstructural changes in bone as assessed by high-resolution peripheral quantitative computed tomography (HR-pQCT).

In contrast, in studies where other dietary factors are controlled, low dietary protein intake has been consistently shown to be associated with both lower bone mineral density and higher rates of bone loss. Further, in a case–control study examining over 2500 male and female subjects divided according to their dietary protein intake (low to high quartiles), the adjusted odds ratio of having a hip fracture for subjects between 50 and 69 years of age consuming the highest percentage of dietary protein was only 0.35 (95% confidence interval: 0.21–0.59; P < 0.001). Thus, a high-protein diet was linked to a very significant drop in hip fractures in this age group. Dietary protein requirements increase with age and the prevalence of dietary protein insufficiency increases particularly in those over 70 years of age and in the institutionalized individuals. Further, an interesting study by Amman et al. showed that low dietary protein intakes could also impair the normal anabolic response on bone mass of PTH injections in a rodent model. This report would suggest that decreased dietary protein intake could potentially negatively affect the response of anabolic agents used in the treatment of osteoporosis.

There are also data that support a negative association between low total caloric intake and low bone mass. Thus, a caloric supplement (300 additional calories, predominantly fat and carbohydrate) was found to prevent bone loss but not to increase bone mass or stimulate bone formation in elderly (>70 years old) undernourished (body mass index ≤21) female patients.

A study by Elefteriou et al. provides molecular evidence supporting the importance of protein (amino acids) in bone formation. In this study, ATF4, a transcription factor that stimulates amino-acid uptake and collagen synthesis in osteoblasts, was not activated in patients with Coffin–Lowry syndrome (CLS, patients with RSK2 mutations). CLS patients have a skeletal phenotype (kyphoscoliosis, pectus carinatum, short stature and so on) and, at least in the CLS mouse models, the anabolic effects of physical activity on bone mass are reduced as is the anabolic hormone insulin-like growth factor 1 (IGF-1). In addition, protein ingestion also increases release of incretin hormones, which themselves have anabolic effects on bone.

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Direct Cellular Effects of Amino Acids

Studies going back to as early as 1920 identified a relationship between protein intake and calcium homeostasis, but the mechanism was not known. Dietary protein is important in the regulation of the parathyroid gland as evidenced by the elevation of parathyroid hormone in absence of adequate dietary protein.

The CaSR is a G-protein-coupled receptor expressed in the parathyroid cells that modulates PTH secretion in response to extracellular calcium concentration. However, the CaSR is known to be expressed in other tissues including the intestines and the kidneys, and has also been shown to bind L-amino acids. Aromatic amino acids serve as the most potent activators of these receptors (Table 1), though most amino acids besides basic and branch-chain amino acids elicit a response. Not only does amino-acid activation of the CaSR influence the parathyroid gland and PTH secretion but it also influences the rate of calcium urinary excretion. Earlier studies have shown that high-protein intakes lead to increased calcium excretion.

High plasma levels of amino acids result in the increased activation of basolateral CaSRs in the cortical thick ascending limb resulting in suppression of calcium reabsorption and increased calcium excretion. The influence of amino acids on renal calcium homeostasis further indicates the importance of amino-acid signaling.

The CasR has also been shown to be present in bone marrow cells in addition to cartilage and bone (osteoblasts) cells. However, other investigators have failed to find CasR messenger RNA (mRNA) expression in osteoblastic cell lines. Interestingly, even though these investigators failed to detect CasR expression in osteoblasts, they found that they functionally responded as if the CasR were present. Thus, in the preosteoblastic cell line MC3T3-E1, the addition of calcium, gadolinium, aluminum and neomycin (activators of CasR) increased DNA synthesis even though no CasR mRNA was detected in these cells. Thus, these authors proposed that a molecularly distinct but functionally similar extracellular calcium sensor was present in osteoblasts. In a follow-up paper, these authors found that osteoblasts deriving from CasR knockout mice were still able to respond to divalent cations, suggesting that additional mechanisms existed in these cells for sensing changes in extracellular calcium. These authors have also suggested that the GPRC6A receptor is the receptor that they have identified as being important as a divalent cation sensor because mice in which GPRC6A has been knocked out have impaired mineralization. Interestingly, the GPRC6A receptor may also be the osteocalcin receptor. Thus, in certain cells, individual amino acids could be binding to specific extracellular receptors and acting as a ‘hormone’ rather than as a nutrient. Amino acids can thus exert direct effects on several tissues where the CasR is expressed including the pancreatic islet and liver, and have a role in energy balance. In summary, the cellular mechanisms mediating these direct AA effects fall into three general categories (Figure 2): (1) calcium receptors: L-type amino acids have been shown to modulate several members of the class 3 G-protein receptor superfamily. Members of this family with amino acid binding include the CasR, the taste receptors T1R1 and T1R3, and member 6A of the G-protein-coupled receptor family C, GPRC6A. It has been suggested that the T1Rs (T1R1 and T1R3 or umami taste receptor) developed evolutionarily, as a means of detecting amino acids in the diet. Thus, sweet and umami taste receptors promote ingestion of beneficial nutrients, whereas many of the toxic compounds have a bitter taste. T1R knockout mice lose all appetite for amino acids (dietary protein); (2) CAT proteins (cationic amino-acid transporter): these transport proteins are widely expressed and belong to the solute carrier family 7 (SLC7) with four members CAT-1, 2A, 2B and 3. All these members have similar affinity for cationic L-amino acids, which are transported in a sodium-independent manner. Once taken up by the cells, amino acids are utilized in a variety of cellular processes and help to regulate cellular energy balance. The metabolism of these AA alters the AMP/ATP ratio. Adenosine 5’-monophosphate-activated protein kinase (AMPK) consists of three subunits (α-, β- and γ-subunits) and serves as a cellular ‘energy sensor’. When ATP levels drop (and AMP levels rise), AMPK is activated and serves to activate catabolic pathways and inhibit anabolic pathways. Thus, AA uptake by the cell results in an increase in ATP levels and an inhibition of AMPK; and (3) amino acids can also have indirect effects on amino acids by modulating the hormonal production of (for example, arginine stimulation of nitric oxide production) or because of the acid load changing the local pH. Although these effects do occur, they are not felt to be a major mechanism of AA action.

Effects of arginine and taurine

Although arginine is not in the earlier discussed category of aromatic amino acids, arginine has an impact on both osteoblastic differentiation and osteogenesis. First, arginine has been shown to increase IGF-1 in mouse osteoblast-like cells and may directly or indirectly increase bone formation by increased IGF-1 production. Treatment with arginine also increased alkaline phosphatase activity and collagen synthesis in these same cells. More recently, the role of arginine in shifting BMSC differentiation away from adipocytic differentiation and towards osteoblastic differentiation has been explored.

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**Table 1** Common amino acids

| Family         | Aliphatic         | Aromatic       | Basic            | Acidic        | Sulfur containing | Hydroxyl containing |
|----------------|-------------------|----------------|------------------|---------------|-------------------|---------------------|
| Alanine        | Tyrosine          | Arginine       | Asparagine       | Cysteine      | Serine            |
| Leucine (BCAA) | Tryptophan        | Histidine (aromatic) | Aspartic acid   | Methionine    | Threonine         |
| Isoleucine (BCAA) | Phenylalanine | Lysine         | Glutamic acid    |              |                   |
| Valine (BCAA)  |                   |                |                  |               |                   |
| Proline        |                   |                |                  |               |                   |
| Glycine        |                   |                |                  |               |                   |
rate of bone breakdown. Real-time PCR was used to analyze the response of various osteoclastic differentiation markers in response to amino acids with a focus on aromatic amino acids.

Early markers of osteoclast differentiation including vitronectin, calcitonin and carbonic anhydrase II were all found to be downregulated by aromatic amino acids, indicating that aromatic amino acids could inhibit bone resorption through this mechanism. Gene expression of the late markers of osteoclastic differentiation, matrix metalloproteinase 9 and cathepsin K was similarly downregulated. This indicates that the aromatic amino acids influence and likely suppress the attachment of osteoclasts and the formation of the resorption site resulting in the increase in bone mass seen with aromatic amino-acid supplementation.13

Amino-Acid Impact on MSCs in an Aging Model
Our laboratory also evaluated the effect of oxygen tension on MSC signaling pathways. Oxygen tension in the bone marrow niches in which the MSCs normally reside is low (1–3%). Under these hypoxic conditions that in fact more closely resemble normal ‘young’ physiologic conditions, stimulation with aromatic amino acids had little effect on MSC pro-survival markers (Akt, STAT3, CAV1 or NOTCH1). In contrast, if oxygen tension is raised (21%), aromatic amino acids such as tyrosine and tryptophan induce increased expression pro-survival proteins in MSCs. With aging, there is niche breakdown that leads to an increase in oxidative stress, suggesting that ‘normoxic’ conditions might be a more appropriate model for aged MSCs.79 Aromatic amino acids have been shown to function as antioxidants in physiologically relevant concentrations. These findings would support a protective role for aromatic amino acids against the increased oxidative stress in MSCs caused by aging.

Presumably under conditions of oxidative stress, these aromatic amino acids would be oxidized and result in an accumulation of their oxidation products in the marrow. A recent study by our laboratory examined the effect of these AA oxidation products on MSC function. In particular, tryptophan and its oxidations products were examined because it is the only amino acid to decline significantly (14%) with increasing age.80 This drop in tryptophan levels with aging has been associated with an increase in kynurenine levels, an oxidation product of tryptophan.81 We found that while tryptophan induced increased expression of pro-survival markers (Akt, STAT3, CAV1 or NOTCH1). In contrast, if oxygen tension is raised (21%), aromatic amino acids such as tyrosine and tryptophan induce increased expression pro-survival proteins in MSCs. With aging, there is niche breakdown that leads to an increase in oxidative stress, suggesting that ‘normoxic’ conditions might be a more appropriate model for aged MSCs.79 Aromatic amino acids have been shown to function as antioxidants in physiologically relevant concentrations. These findings would support a protective role for aromatic amino acids against the increased oxidative stress in MSCs caused by aging.

Summary and Research Questions
In summary, data from our lab and others support a role for distinct nutrient effects at different stages of cell differentiation. Glucose is an important nutrient for osteoclast activity and osteoblast differentiation. In contrast, protein and PUFA appear to also have a role in MSC proliferation and function. Because of the higher caloric and or nutritional quality of these nutrients, they serve as anabolic signals for bone growth (Figure 1). Further, as a nutrient, protein’s role goes beyond simple energy provision as the amino acids that compose dietary proteins serve a distinct role in several signaling pathways in the maintenance and protection of bone. As the roles of dietary
protein and amino acids in nutrition and osteogenesis are further defined, new therapeutic agents for the treatment of osteoporosis and specific dietary recommendations for prevention of bone loss may be discovered and established. The different subclasses of amino acids have varying effects in the body, particularly the aromatic amino acids and the branched-chain amino acids. The differing effects of the subtypes of amino acids may lead to observations about the differing effects of protein intake that varies according to the source of this protein.8,13 Thus, in addition to increasing the protein intake in the elderly, paying attention to the amino-acid composition of the dietary supplements may be relevant to the prevention of age-associated osteoporosis. Finally, the best way to administer dietary protein also needs to be clarified. In aging studies, intermittent fasting provides many of the benefits in as much as longevity as continuous caloric restriction. Historically, protein was generally ingested by humans in an intermittent rather than in a daily manner. In fact, if some amino acids are truly acting as ‘hormones’ and binding to extracellular receptors then providing amino acids in a continuous manner is more likely to lead to receptor downregulation and loss of a beneficial effect. Thus, further studies are needed to clarify the timing of dietary protein intake.

Conflict of Interest

The authors declare no conflict of interest.

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