Bone 59 (2014) 7–13

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Protein quality affects bone status during moderate protein restriction in growing mice

Emilien Rouy [a,b,*], Laurence Vico [c], Norbert Laroche [c], Valérie Benoit [b], Brigitte Rousseau [b], François Blachier [a], Daniel Tomé [a], Anne Blais [a]

[a] UMR 914 INRA-AgroParisTech, Nutrition Physiology and Ingestive Behavior, 75005 Paris, France
[b] Yoplait France, 92641 Boulogne-Billancourt, France
[c] INSERM U1059/LBTO, Université Jean Monnet, Université de Lyon, 42023 Saint-Étienne, France

ARTICLE INFO

Article history:
Received 5 July 2013
Revised 16 October 2013
Accepted 16 October 2013
Available online 24 October 2013

Edited by: Rene Rizzoli

Keywords:
Protein undernutrition
Protein quality
Bone turnover
Bone architecture
Parathyroid hormone

ABSTRACT

Adequate protein intake during development is critical to ensure optimal bone gain and to attain a higher peak bone mass later on. We hypothesized that the quality of the dietary protein is of prime importance for bone physiology during moderate protein restriction. The target population was growing Balb/C mice. We compared two protein restricted diets (6% of total energy as protein), one based on soy (LP-SOY) and one based on casein (LP-CAS). For comparison, a normal protein soy-based control group (NP-SOY) and a low protein group receiving an anabolic daily parathyroid hormone (PTH) 1-34 injection (LP-SOY + PTH) were included in the protocol. After 8 weeks, LP-SOY mice had reduced body weights related to a lower lean mass whereas LP-CAS mice were not different from the NP-SOY group. LP-SOY mice were characterized by lower femoral cortical thickness, bone volume, trabecular number and thickness and increased medullar adiposity when compared to both the LP-CAS and NP-SOY groups. However, the dietary intervention had no effect on the vertebral parameters. The negative effect of the LP-SOY diet was correlated to an impaired bone formation as shown by the reduced P1NP serum level as well as the reduced osteoid surfaces and bone formation rate in the femur. PTH injection in LP-SOY mice had no effect on total weight or lean mass, but improved all bone parameters at both femoral and vertebral sites, suggesting that amino acid deficiency was not the primary reason for degraded bone status in mice consuming soy protein. In conclusion, our study showed that under the same protein restriction (6% of energy), a soy diet leads to impaired bone health whereas a casein diet has little effect when compared to a normal protein control.

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Introduction

Protein represents a critical factor for development and bone health in humans and rodents. Insufficient protein intake leads to growth retardation during early life stages and poor bone quality as an adult [1–4]. When casein is used as the sole protein source in adult rodents, a diet with 15–20% of energy as protein meets protein needs but a diet containing 2.5% of energy as protein reduces bone formation [5–7]. The influence of protein on bone metabolism has for the large part been related to the capacity to provide essential amino acids for the synthesis of the bone collagen matrix. Protein restriction is also associated to an inhibition of the insulin like growth factor 1 (IGF-1), an anabolic factor for bone and muscle [8,9] and an overexpression of IGF-1 in the osteoblasts could counteract for a part the effect of protein restriction [6].

Another factor associated to protein is the presence of protein-related bioactive compounds acting on bone physiology. Indeed, in the case of milk protein, casein-derived phosphopeptides were shown in vitro to exert positive effects on osteoblasts [10,11] and to increase calcium absorption in growing rats [12,13], although their effects in humans remain unclear [14]. Similarly, a positive effect of a soy-based diet on bone physiology has been reported in relation to the presence of isoflavone phytoestrogens that could act as selective estrogen receptor modulators (SERMs) [15]. Other non-identified anabolic factors in soy may also act on bone as suggested by a study using isoflavone-free soy protein extracts [16].

The present study intends to compare the effects of a casein-based diet and a soy-based diet on bone accumulation during moderate protein restriction in a growing mouse model. For this purpose, three groups of growing mice were fed either a control soy-based normal protein diet (protein making up 20% of total energy intake), a soy-based protein restricted diet (6%) or a casein-based protein restricted diet (6%). In addition, a soy-based protein restricted (6%) sub-group received a daily parathyroid hormone (1-34 PTH) injection. Intermittent PTH treatment is well known for its osteoanabolic effect on bone.

* Corresponding author at: AgroParisTech, 16 Rue Claude Bernard, 75005 Paris. Fax: +33 144081858.
E-mail addresses: emilien.rouy@gmail.com, emilien.rouy@agroparistech.fr (E. Rouy).

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http://dx.doi.org/10.1016/j.bone.2013.10.013

Interestingly, one of the proposed mechanisms of action of PTH is through IGF-1 [17] which is impaired during protein restriction [18].

Materials and methods

Animals

Eight week-old Balb/C mice (Harlan) were housed at 22 °C under a 12/12 h light cycle. Initially at five animals per cage for the first week of habituation, they were then moved to individual cages for another week. During the two weeks of habituation, the mice were fed a standard AIN-93 M diet containing 20% of total energy as soy protein. The soy protein used in our study did not contain any phytoestrogens in order to avoid interference with bone metabolism. The design of this study was approved by the Animal Ethics Committee of INRA Jouy-en-Josas and AgroParisTech (study 12/006).

At ten weeks old, the mice were divided into four groups of fifteen. One group stayed on the 20% soy protein diet as a normal-protein control (NP-SOY group). Two groups were shifted to a low protein diet containing only 6% of total energy as soy protein (LP-SOY group) or 6% total energy as casein (LP-CAS group). The last group was shifted to the 6% soy protein diet and was also treated for five days per week with a subcutaneous injection of 40 μg/kg of 1-34 PTH (Sigma-Aldrich, France) as an anabolic control (LP-SOY + PTH group). The restriction level was set to 6% as it is a moderate protein restriction and it has previously been shown that soy and casein have a different effect on calcium metabolism at this level [19]. To maintain an equal caloric intake the protein was replaced by starch and sucrose in the 6% protein diets (Table 1). Due to the protein leverage effect, mice under low protein diet have higher energy intake than mice under a normal protein diet [20]. To avoid the protein leverage in our study, we pair-fed all the groups with the NP-SOY group. Every day, the food consumption of the NP-SOY group was weighted and was used to determine the amount of diet for the 3 other groups. As all the diets were isocaloric, the pair-feeding ensuring that energy intake was similar in all the groups. The three groups not receiving PTH were injected daily with vehicle. After 60 days, the mice were anesthetized with isoflurane, blood was drawn by cardiac puncture and the mice were immediately decapitated to ensure death.

Body composition

We measured the body composition (fat mass and lean mass) at the beginning and the end of the study by dual energy X-ray absorptiometry (DEXA), using a Lunar PIXImus densitometer (DEXA-GE PIXImus). The stability of the device was controlled by a measurement of a phantom before each session. The mice were anesthetized by isoflurane inhalation during the measurement. Analysis of the images was performed with the software provided with the device (Lunar PIXImus v2.10), using auto-thresholding. On the day of sacrifice, the following organs were weighed: liver, uterus, spleen, kidneys and interscapular brown adipose tissue (BAT).

Table 1
Composition of the diets.

Ingredients (g/kg diet)	NP-SOY	LP-SOY	LP-CAS
Soy protein[a]	183	51	0
Casein [b]	0	0	51
Corn starch[c]	584	698	698
Sucrose[d]	95	114	114
Soybean oil[e]	40	40	40
Alpha cellulose[f]	50	50	50
AIN93M mineral mix[a]	35	35	35
AIN 93 M vitamin[g]	10	10	10
Choline[h]	2.3	2.3	2.3
Total energy (kcal/kg)	3660	3628	3651

[a] MP Biomedicals.
[b] Terra Lacta.
[c] Cargill.
[d] CristalCo Pro.
[e] Lesieur.
[f] Prat Dumas.
[g] Sigma-Aldrich.
[h] Jefo.

Bone mineral density and microarchitecture

In vivo areal bone mineral density (BMD, g/cm^2) measurements were performed at the beginning and the end of the study to quantify the evolution of BMD over time. BMD was measured for total skeleton, femur and spine on the same PIXImus DEXA scans as the one used for body composition described above.

A micro computed tomography scan (μCT, VivaCT40, Scanco Medical AG) was also used to evaluate microarchitecture and final volumetric BMD in the femur and spine. 30 slices at the femoral diaphysis were used to evaluate the cortical thickness (Ct.Th, mm), cortical porosity (Ct.Po, %), bone area (B.Ar, mm^2) and marrow area (Ma.Ar, mm^2). The following trabecular bone parameters were evaluated on 150 slices of the L2 vertebrae and 60 slices of the secondary spongiosa of the distal femur: bone volume over tissue volume (BV/TV, %), connection density (Conn.D, %), structure model index (SMI), degree of anisotropy (DA) and trabecular thickness (Tb.Th, mm), number (Tb.N, mm^{-1}) and spacing (Tb.Sp, mm). The settings used for both vertebral and femoral trabecular reconstruction were the following: sigma 1.5, support 2, threshold 260. For cortical bone, the reconstruction parameters were: sigma 0.8, support 1, threshold 260.

Bone histomorphometry

Each mouse received a subcutaneous injection of 25 mg/kg of tetracycline hydrochloride (Sigma-Aldrich) seven days before euthanasia and a second injection two days before euthanasia for measurement of bone formation by double labeling.

Sixty days after the beginning of the study, mice were euthanized and the left femurs were collected, fixed in 10% paraformaldehyde and dehydrated, first in absolute ethanol, then in acetone. The distal femoral metaphysis was embedded at 4 °C in methylmethacrylate [21] and sliced longitudinally (9 μm) with a dedicated microtome (Leica SM2500E). Slices were stained with a modified Goldner trichrome for determination of bone surface over bone volume ratio (BS/BV, mm^{-1}), trabecular thickness, osteoid surface (OS/BS, %). Adipocyte volume was also measured and normalized for marrow volume. Tartrate-resistant acid phosphatase (TRACP) staining was performed for the measurement of osteoclast surface (Oc.S/BS, %). Twelve micrometer thick slices were left unstained for measurement of tetracycline labeling parameters: single labeled surface (sL.S/BS, %), double labeled surface (dL.S/BS, %) and mineral apposition rate (MAR, μm/d). BS/BV was measured with image analysis software (Explora Nova, Bone, v3.50), and used for the normalization of cellular parameters. Osteoid surface, tetracycline labelings and osteoclast parameters were measured semi-automatically with a SummaGraphics (SummaSketch) tablet and image analysis software (EIP-LBTO Morphométrie, v2.10) [22]. Bone formation rate (BFR/BS, μm^3/μm^2/d) was calculated as MAR × (dLS/BS ± sLS/BS/2).

Biochemical analysis

Fasting blood was collected by intra-cardiac puncture on the day of sacrifice. N-terminal propeptides of type I procollagen (PINP) and C-terminal crosslinking telopeptides of type I collagen (CTx) were measured by enzyme immunoassay (EIA) according to the instructions of the manufacturer (Immunodiagnostic Systems). Total IGF-1 level was determined by enzyme-linked immunosorbent assay (ELISA) after inactivation of the IGF binding proteins according to the instructions of the

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