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Research Article

Calcium Intake is Associated to Changes in the Interplay between Bone, Pancreas and Fat Tissue in the Control of Glucose Homeostasis- Experimental Study

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Abstract

Background: Bone remodeling, insulin levels and fat mass interrelationship in glucose homeostasis control was evaluated in Weaning Normal (W) and Obese (O) rats fed High (H), Normal (N) or Low (L) Ca intakes.

Methods: Glucose, Cholesterol (Chol), HDL-Chol, Triglyceride (TGL), Ca, P, insulin, Osteocalcin (OCN) and collagen C-telopeptide (CTX), body composition, BMD, BMC, body Ca and P content, perigonadal plus retroperitoneal fat (PG+RP) and liver weight were determined and HOMA-IR calculated.

Results: WHCa reached the highest body fat, PG+RP and the highest CTX levels ($p<0.05$); WNCa had the lowest liver weight. WLCa reached the lowest body protein content ($p<0.05$) and the highest glucose, insulin and HOMA-IR ($p<0.05$). WLCa and WHCa had similar Chol levels but higher than WNCa; TGL increased and OCN decreased as dietary Ca content increased ($p<0.05$). OLCa presented the highest body fat, Chol and OCN levels but the lowest HDL-Chol levels ($p<0.05$); ONCa had the highest body protein percentage ($p<0.05$). OHCa had the lowest CTX levels ($p<0.05$). PG+RP, liver weight, glucose, insulin and HOMA-IR decreased as dietary Ca content increased ($p<0.05$). O groups reached higher adipose PG+RP fat, liver weight, glucose, insulin, Chol, TGL and HOMA-IR and lower OCN, CTX and body protein content than their matched-W groups ($p<0.05$).

Conclusion: The relative amount of dietary Ca to P may regulate energy metabolism and bone turnover, insulin and body fat interplay in glucose homeostasis control. However, the mechanisms differ in physiological conditions or in the presence of metabolic abnormalities of energy dysregulation such as obesity and T2-diabetes.

Keywords: Osteocalcin; Bone Resorption; Insulin; Body Fat

Abbreviations

OCN: Osteocalcin; BGP: Gamma-Carboxyglutamic Acid-Containing Protein; GLA: Gamma-carboxyglutamic acid; Ca: Calcium; InsR: Osteoblastic Insulin Receptor; OPG: Osteoprotegerin; RANK: Receptor Activator of Nuclear Factor-Kappa B; RANKL: Receptor Activator of Nuclear Factor-Kappa B Ligand; CNS: Central Nervous System; T2DM: Type 2 Diabetes Mellitus; BMD: Bone Mineral Density; CaI: Ca Intake; NCa: Normal Ca Diet; HCa: High Ca Diet; LCa: Low Ca Diet; BW: Body Weight; PG: Perigonadal Fat; RP: Retroperitoneal Fat; Pi: Inorganic Phosphorus; Chol: Total Cholesterol; HDL Chol: Higth Density Lipoprotein Cholesterol; TGL: Triglyceride; 25OHD: 25 hydroxyvitamin D; b-ALP: Serum Bone Alkaline Phosphatase; CTX: C-Terminal Telopeptide of Type I Collagen; tsBMC: Total Skeleton Bone Mineral Content; tsBMD: Total Skeleton Bone Mineral Density; BMD: Bone Mineral Content, SE: Standard Error; W: Wistar Rats; O: IIMb/β Obese Rats; PTH: Parathormone; 2ºHPT: Secondary Hyperparathyroidism; MS:Metabolic Syndrome).

Introduction

One of the energy metabolism regulations appears to occur through the interplay between bone, fat tissue and pancreas. According to literature, these organs maintain glucose homeostasis through the interaction between bone turnover markers, insulin and leptin levels.

Osteocalcin (OCN) is a non-collagenous protein secreted by osteoblasts/osteocytes. The negative charge of Gamma-carboxyglutamic acid (GLA) residues increases OCN Calcium (Ca)-binding properties, resulting in an association with hydroxyapatite in bone extracellular matrix. In clinical practice, OCN used to be used as a bone formation marker and more broadly, of bone remodeling. Bone matrix acidification performed by osteoclasts resorbing activity induces OCN bioactivation, which loses Ca affinity and is released into bloodstream. OCN bioactive form may regulate energy metabolism stimulating insulin synthesis and secretion by the pancreatic β cells, and insulin sensitivity and glucose utilization in peripheral tissues [1]. Transgenic OCN-deficient mice are fat, insulin resistant, glucose intolerant, and hyperlipidemic [2]. Ferron et al. demonstrated that OCN production and bioavailability are under insulin control [3].

Osteoblastic Insulin Receptor (InsR) is required for osteoblast survival, proliferation, and differentiation. Insulin signalling in

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osteoblasts regulates Runx2 expression, OCN production and decreases the expression of Osteoprotegerin (OPG). The latter increases osteoclastic activity. The acidic environment in the resorption lacuna increases OCN bioavailability [3]. Many factors regulate the positive feedback loop between the osteoblastic insulin signalling and OCN in pancreatic β cells.

Leptin plays an essential role on energy metabolism. Leptin secretion correlates positively with adipose tissue mass, and thereby it monitors overall energy availability. Leptin inhibits insulin secretion through a direct effect on pancreatic β cells and induces the indirect suppression of insulin signalling in osteoblasts *via* Central Nervous System (CNS) [4]. These observations establish the tight metabolic link between osteoblasts, pancreatic β cells and adipocytes Falta el punto.

Energy metabolism dysregulation is associated with intracellular lipid accumulation and excess of body adipose tissue storage that results in several comorbidities [5]. Type 2 Diabetes Mellitus (T2DM) is the most significant obesity-associated metabolic disorder, characterized by insulin resistance, hyperglycemia, dyslipidemia and alterations in hormonal signalling systems both, in CNS and peripheral nervous system [6]. Moreover, T2DM patients have an increased risk of bone fragility and fractures, regardless of having Bone Mineral Density (BMD) increase [7]. One of the main factors involved in T2DM-derived bone fragility would be bone quality deterioration due to bone turnover suppression [8].

There is no doubt about the negative effect of low Ca Intake (CaI) on bone mass. Furthermore, according to literature, it also appears to induce energy dysregulation by affecting insulin secretion and lipogenesis [9,10]. Our group evidenced the obesogenic effect of Ca insufficiency in rats fed a low Ca diet, which presented an increase in fat mass accumulation and a negative effect in lipid profile, but these alterations were more evident when animals were prone to obesity [11]. Moreover, epidemiological studies determined that obesity and T2DM incidence was likely to be inversely associated with the increase in dietary Ca [12].

The results of our previous paper led us to postulate that Ca amount supplied by the diet may mediate, at least in part, glucose homeostasis through changes in bone, pancreas and fat mass interplay. Besides, abnormalities in insulin secretion, fat mass accumulation and bone remodeling because of T2DM could additionally affect the possible effect of CaI on energy metabolism. On these bases, the present experimental report evaluated *in vivo* the interaction of bone remodeling, insulin levels and fat mass in glucose homeostasis in normal Wistar rats and in obese/T2DM rats fed three different dietary Ca contents. The results of this interrelationship were also compared between the two strains of rats.

Materials and Methods

Diets

Three experimental isocaloric diets were prepared according to American Institute of Nutrition Rodent Diets Recommendations settled in 1993 (AIN-93G) [13]. Diet composition was identical, except for Ca content. Normal Ca diet (NCa) contained 0.5% Ca, providing Ca requirement for rodents; High Ca diet (HCa) contained 0.9% Ca, exceeding Ca recommendations by 50% and Low Ca diet (LCa) contained 0.2% Ca, contributing by 40% of Ca recommendations (Table 1).

Table 1: Centesimal composition of the experimental diets (g/100g).

Diet	LCa	NCa	HCa
Energy (Kcal)	395	395	395
Proteins (g)[a]	17	17	17
Lipids (g)[b]	7	7	7
Ca-free salts mixture (g)[c]	3.5	3.5	3.5
Vitamins(g)[d]	1	1	1
Choline (g)[e]	0.25	0.25	0.25
Cellulose (g)	5	5	5
Dextrin[f]	to complete 100 g		
Calcium (Ca) (g)[g]	0.2	0.5	0.9
Inorganic phosphorus (g)[h]	0.4	0.4	0.4
Magnesium (g)[h]	0.051	0.051	0.051

All diets were prepared according to AIN93-G and they only varied in calcium content.

[a]Sodium caseinate (Lactoprot GMBH, Germany) containing 85.1% of protein and 0.095g% of Ca.

[b]Commercial soy oil. Molinos Rio de la Plata, Argentina.

[c]Ca-free salts mixture was prepared according to AIN93-G, except for Ca content.

[d]Vitamins was prepared according to AIN-93G that meet rat requirements during growth. Manufactured by the Department of Food Science School of Biochemistry, University of Buenos Aires (individual components from Sigma, Missouri, USA).

[e]Choline citrate 0.71% (food grade, Anedra, Argentina).

[f]Corn dextrin from corn refinery, provided by Food SA Argentina was added as carbohydrate (fibre) source to achieve 100 g of diet.

[g]$CaCO_3$ (food grade individual components, Anedra, Argentina), was added to obtain the required Ca concentration.

[h]Potassium phosphate monobasic and magnesium oxide (food grade individual components, Anedra, Argentina), were added to obtain the required P and Mg concentration, respectively.

Animals

Twenty-four male weaning IIMb/β rats were obtained from Lipid and Lipoprotein Laboratory, Clinical Biochemistry Department, Rosario National University, Argentina. IIMb/β rats develop obesity and T2DM from puberty onwards. They were obtained by genetic and environmental maladjustment and a high degree of inbreeding. Obese rats also develop hypertryacilglyceridemia without hypercholesterolemia, and their glucose intolerance progresses to T2DM and obesity-related hypertension [14,15]. Twenty-four male weaning Wistar rats were obtained from Nutrition and Food Sciences Laboratory Department, Pharmacy and Biochemistry Faculty, Buenos Aires University, Argentina.

All rats were housed in individual stainless steel cages and were maintained on a 12-h-light/-dark cycle in a temperature and humidity-controlled room (21±1°C and 55±10%, respectively).

Rats were maintained in keeping with National Institutes of Health Guide for Care and Use of Laboratory Animals. Bioethics Committees of Universities of Buenos Aires and Rosario approved the protocol.

Experimental design

The experimental design was previously published [11]. In brief, mothers were fed one of the three experimental diets from pregnancy

Table 2:

	OLCa	ONCa	OHCa	WLCa	WNCa	WHCa
BW T=21 (g)	50.1±5.5	48.7±6.2	49.0±5.3	44.5±5.8	44.2±3.4	45.5±5.8
BW T=60 (g)	284.6±8.7	235.1±9.1*	243.6±14.1*	197.6±2.6#	192.9±9.0#	199.3±21.8#
Food efficiency (g/g)	3.10±0.30	3.00±0.50	3.30±0.30	3.10±0.30	3.00±0.40	3.00±.03
Total skeleton BMC (g/100g BW)	0.64±0.20	1.44±0.30*	1.27±0.30*,**	0.71±0.30#	1.54±0.30*,#	1.34±0.20*,**,#
Body Ca content %	394±39	640.±20*	720±34*,**	494±34#	850±25*,#	917±55*,**,#
Body Pi content %	310±13	520±27*	532±22*	561±22#	728±30*,#	823±43*,**,#
Body Ca/Pi ratio	1.37±0.03	1.24±0.04*	1.35±0.03**	0.87±0.04#	1.17±0.03*,#	1.13±0.05*,#

Results were expressed as mean±SD (n=8).
Data were analyzed by ANOVA and Bonferroni as a post hoc test.
*p<0.05 O or W groups compared to LCa diet.
**p<0.05 O or W groups compared to NCa.
#p<0.05 W groups *vs*. O groups fed the same diet Ca content.

to weaning. Weanig male IIMb/β and Wistar pups (n=8 per group) continued feeding their maternal diet until post-natal day 50 (T=50).

Throughout the experimental period, rats were allowed to access deionized water and food ad libitum. Food consumption was recorded 3 times per week, until the end of the study.

Body Weight (BW) was registered at birth, at weaning (21 days) and thereafter twice a week until T=50. Relative capacity of a food source to contribute to weight gain, named food efficiency (g/g) was calculated according to the equation:

Food efficiency = Total food intake (g)/increase in BW (g).

At T=50, all animals were subjected to a whole-body densitometry *in vivo* under light anesthesia (ketamine hydrochloride 0.1 mg/100g BW and acetopromazine maleate 0.1mg/100g BW). Then, fasting blood samples were collected from tail vein, followed by sacrifice under CO_2 inhalation. Perigonadal (PG) and Retroperitoneal (RP) fat, and liver were removed and weighed, and body composition was determined.

Analytical procedures

Body fat and body protein contains were determined according to Association of Official Analytical Chemists methods as previously described [16].

Ca in diets, serum and body ashes was determined by atomic absorption spectrophotometry. Inorganic Phosphorus (Pi) in serum, diets and body ashes were evaluated according to Gomori's method. Glucose, total Cholesterol (Chol), Hight Density Lipoprotein Cholesterol (HDL Chol), and Triglyceride (TGL) levels were determined by conventional enzymatic methods. Insulin was determined by Enzyme Immunoassay (ELISA) (Rat/Mouse Insulin Kit, Millipore, Billerica, MA, USA). Insulin resistance degree was determined by Insulin Resistance index [HOMA-IR = (insulin × glucose)/22.5] [17]. 25 hydroxyvitamin D (25OHD) was assayed by a competitive protein binding method (Diasorin, Stillwater, MN, USA), 9% intra-assay coefficient of variation. Bone alkaline phosphatase (b-ALP) was measured by a colorimetric method (Boehringer Mannheim, Germany) after bone enzyme isoform precipitation with wheat-germ lectin. OCN (ng/mL) and C-terminal Telopeptide of type I collagen (CTX) (ng/mL) were measured by ELISA (Rat-osteocalcin and Rat-laps, respectively Osteometer BioTech, Herlev, Denmark), 6% intra-assay variation coefficient. OCN/b-ALP ratio was calculated.

Densitometry

Total skeleton Bone Mineral Content (tsBMC) and Bone Mineral Density (tsBMD) were evaluated using a whole body scanner with a specifically designed software for small animals (DPX Alpha, Small Animal Software, Lunar Radiation Corp. Madison WI), as previously described. In brief, all rats were scanned using an identical scan procedure. Precision was assessed by measuring one rat five times with repositioning between scans, on the same day and on different days [18]. Bone Mineral Content (BMC) and BMD coefficients of variation were 3.0 and 0.9% respectively.

Statistical methods

Results were expressed as mean ± Standard Error (SE). Data were analyzed using 2-way analysis of variance ANOVA, and Bonferroni multiple comparisons. The linear association was analyzed by Pearson's correlation coefficients r and multivariate linear regression. Statistical analyses were performed using SPSS for Windows 19.0 (SPSS, Inc. Chicago, IL). A value of P below 0.05 ($p<0.05$) was considered significant.

Results

Effect of feeding different dietary Ca content

Wistar (W) rats: Independently of dietary Ca content, no significant differences were observed in BW among W groups, neither at weaning nor throughout the experiment. Food efficiency showed similar results in the three W groups (Table 2).

WHCa presented the significantly highest BW-adjusted body fat and PG+RP ($p<0.05$), while no significant differences were observed between WLCa and WNCa. WNCa presented the significant lowest liver weight ($p<0.05$), while no differences were observed between WLCa and WHCa. WLCa presented the lowest BW-adjusted body protein content, but only reached statistical significance versus WNCa ($p<0.05$); no significant differences were observed between WNCa and WHCa (Table 3).

WLCa presented the significant highest glucose, insulin and HOMA-IR levels ($p<0.05$). These parameters were also significantly higher in WHCa *vs*. WNCa ($p<0.05$). WLCa and WHCa reached similar Chol levels, which were significantly higher than in WNCa ($p<0.05$). No differences in HDL-Chol were observed among groups.

Table 3:

	OLCa	ONCa	OHCa	WLCa	WNCa	WHCa
Glucose (g)	252±28	170±15*	148±12*,**	139±9#	97±12*,#	105±7*,**,#
Insulin (mg/dL)	6.87±2.37	4.07±0.81*	1.92±0,7*,**	1.30±0.54#	0.14±0.01*,#	0.44±0.05*,**,#
HOMA-IR	77.6±14.1	32.0±7.5*	10.3±6.1*,**	4.1±0.5#	0.6±0.4*,#	1.3±0.8*,**,#
Chol (mg/dL)	115±11	88±9*	86±11*	68±4#	52±7*,#	71±4**,#
HDL-Chol (mg/dL)	15.1±0.9	28.5±0.3*	27.3±1.8*	28.0±3.4#	27.9±4.6	28.3±3.1
TGL (mg/dL)	287±54	241±35*	332±29*,**	75±4#	86±8*,#	112±10*,**,#
Body protein % of BW	14.8±0.9	17.3±0.5*	15.8±0.5**	18.9±0.4#	20.4±0.7*,#	19.1±0.8#
Body fat % of BW	15.9±0.6	13.7±0.8*	14.2±0.8*	11.7±0.7#	10.8±0.5#	14.3±0.7*,**
PG+RP/BW	4.81±0.04	4.33±0.07*	3.12±0.01*,**	1.16±0.03#	1.24±0.09#	2.70±0.15*,**,#
Liver weight % of BW	5.48±0.04	5.21±0.05*	4.81±0.07*,**	4.21±0.01#	4.09±0.05*,#	4.35±0.11**,#

Results were expressed as mean±SD (n=8).
Data were analyzed by ANOVA and Bonferroni as a post hoc test.
*p<0.05 O or W groups compared to LCa diet.
**p<0.05 O or W groups compared to Nca.
#p<0.05 W groups *vs.* O groups fed the same diet Ca content.

Table 4:

	OLCa	ONCa	OHCa	WLCa	WNCa	WHCa
sCa (mg/dL)	10.8±0.8	10.5±0.3	9.7±0.2*,**	8.3±0.3#	8.6±0.2#	8.9±0.6#
sPi (mg/dL)	8.7±0.2	8.8±0.2	8.8±0.1	8.7±0.1	8.2±0.3	7.7±0.4*,#
b-ALP (IU/L)	107±14	160±4*	115±15**	85.5±7.3#	70.8±2.2*,#	79.7±4.0**,#
Total ALP	895±58	775±53*	750±66*	619±67#	333±38*,#	442±43*,**,#
OCN (µg/mL)	582±44	399±16*	368±26*	917±29#	800±49*,#	730±56**,#
sCTX (mg/mL)	82.1±3.6	79.7±6.1	69.6±7.4*,**	105.9±7.8#	94.0±6.0#	122.8±12.9*,**,#
OCN/b-ALP	5.65±1.12	3.06±0.60*	4.62±1.39*,**	12.8±0.85#	11.72±1.12#	8.01±0.86*,**,#
25OHD (ng/mL)	21.8±2.4	19.0±2.7	20.5±1.1	13.9±2.2#	12.4±2.8#	14.5±1.7#

Results were expressed as mean±SD (n=8).
Data were analyzed by ANOVA and Bonferroni as a post hoc test.
*p<0.05 O or W groups compared to LCa diet.
**p<0.05 O or W groups compared to NCa.
#p<0.05 W groups *vs.* O groups fed the same diet Ca content.

WHCa reached the significantly highest TGL levels ($p<0.05$), while WNCa had levels significantly higher than WLCa ($p<0.05$) (Table 3). WNCa presented the significant lowest ALP levels, while WHCa presented a significant lower level than WLCa ($p<0.05$) (Table 4).

Regardless of the dietary Ca content, no significant differences in 25OHD (ng/dL) were observed among the three W groups. WLCa showed the lowest Ca level and WHCa showed the significant lowest Pi level ($p<0.05$). WNCa presented the significant lowest b-ALP levels ($p<0.05$), while WLCa only showed a tendency to have higher levels than WHCa ($p=0.055$). OCN decreased significantly with the increase in dietary Ca content ($p<0.05$). The OCN/b-ALP ratio decreased as dietary Ca content increased, but only WHCa reached statistical significance as compared to the other two W groups ($p<0.05$). WHCa had the significant highest CTX ($p<0.05$), while WLCa only showed a tendency to have higher levels than WNCa ($p=0.061$) (Table 4).

Body Ca and Pi contents increased significantly with the increase in dietary Ca content ($p<0.05$). WLCa showed the significant lowest body Ca/Pi ratio and tsBMC ($p<0.05$), while no significant differences were observed between WNCa and WHCa (Table 2).

WNCa showed a significant correlation between OCN and glucose levels ($r=0.77$; $p<0.0001$); however, this correlation was not observed in WLCa or WHCa groups.

IIMb/β obese (O) and diabetic rats: No differences in BW were observed at weaning. Thereafter, OLCa presented the significant highest BW throughout the entire study ($p<0.05$), while OHCa group presented a tendency to reach higher values than ONCa, at the end of the experience ($p=0.067$). Irrespectively of the dietary Ca content, no differences in food efficiency were observed among the three O groups studied here (Table 2).

OLCa presented the significant highest BW-adjusted body fat ($p<0.05$), while no differences were observed between ONCa and OLCa. PG+RP/BW and liver weight percentage decreased significantly as the dietary Ca content increased ($p<0.05$). OLCa and OHCa had similar body protein percentages, which was lower as compared to ONCa ($p<0.05$) (Table 2).

Glucose, insulin and HOMA-IR levels decreased significantly as the dietary Ca content increased ($p<0.05$). OLCa presented the significant highest Chol and the significant lowest HDL Chol ($p<0.05$); no significant differences were observed between ONCa and OHCa. OHCa presented the significant highest TGL levels ($p<0.05$), and

OLCa had a significant higher level than ONCa ($p<0.05$) (Table 3).

No differences in 25OHD or Pi levels were observed, independently of the dietary Ca content. OHCa showed the significant lowest serum Ca ($p<0.05$), while no significant differences were observed between the remaining two groups. OLCa had the significant highest b-ALP and ONCa the significant highest b-ALP level ($p<0.05$); no significant differences in both parameters were observed between the two remaining groups. OLCa presented the significant highest OCN, while ONCa and OHCa showed similar concentrations. ONCa presented the significant lowest OCN/b-ALP ratio ($p<0.05$), while OHCa presented a significant lower ratio than OLCa ($p<0.05$). OHCa presented the significant lowest CTX levels ($p<0.05$), while no significant differences were found between OLCa and ONCa (Table 4).

Body Ca content increased significantly as the dietary Ca content increased ($p<0.05$). OLCa presented the significant lowest body Pi content ($p<0.05$); no significant differences were observed between ONCa and OHCa. ONCa had the significant lowest body Ca/Pi ratio ($p<0.05$), while no significant differences were observed between OLCa and OHCa. OLCa presented the significantly lowest tsBMD ($p<0.05$) while ONCa reached a value significantly higher than OHCa ($p<0.05$) (Table 2).

Independently of the dietary Ca content, OCN and glucose levels did not correlate in the three groups of O rats.

Comparative effect of the diet Ca content between both strains of rats: Independently of the dietary Ca content, O rats presented a higher BW than W rats, but only OLCa *vs*. WLCa reached statistical significance at weaning. At the end of the study, O groups reached a significant higher BW than their non-obese counterparts ($p<0.05$). No significant differences in food efficiency were observed between O and W groups, in spite of the dietary Ca content (Table 2).

OLCa and ONCa reached a significantly higher BW-adjusted body fat than WLCa and WNCa, respectively ($p<0.05$), while no differences were observed between OHCa and WHCa. Independently of the dietary Ca content, O groups presented significant higher adipose PG plus RP fat pads and liver weight and a significant lower body protein content than their matched-W groups ($p<0.05$) (Table 3).

Independent of the diet Ca content, O groups presented significant higher glucose, insulin, Chol, TGL and HOMA-IR than their matching part in WCa groups ($p<0.05$). OLCa had HDL-Chol significantly lower than WLCa, while no differences were found between the other two remaining Ca groups (Table 3).

Independently of the dietary Ca content, O rats presented significantly higher Ca, 25OHD, total ALP and b-ALP levels than their W-counterparts ($p<0.05$). OHCa presented significantly higher Pi levels than WHCa ($p<0.05$), while no differences were observed between the other two remaining groups. Obese rats presented significantly lower levels of OCN, CTX and, OCN/b-ALP ratio than their corresponding WCa groups ($p<0.05$) (Table 4).

Irrespectively of the dietary Ca content, total body Ca and Pi content were significantly lower and body Ca/Pi ratio was significantly higher in O rats than in their corresponding WCa groups ($p<0.05$). WNCa and WHCa presented a tsBMC significantly higher than ONCa and OHCa, while OLCa presented a significantly higher value than WLCa ($p<0.05$) (Table 2).

Discussion

The results of the present report strongly suggest that the relative amount of dietary Ca and Pi (different Ca/Pi ratio) regulates both, energy metabolism and relationship between bone turnover, insulin levels and body fat accumulation in glucose homeostasis control. However, the impact of diets appears to differ in diabetes and obesity versus normal physiological conditions. Such differences were determined by comparing ordinary Wistar rats with genetically modified rats. The IIMb/β rats were obtained from a high degree of inbreeding of Wistar rats [14,15]. For this reason, obese and diabetic rats appear to be an optimal energy metabolism dysregulation model to evaluate Ca intake effect in the interplay between bone, pancreas and fat tissue in glucose homeostasis.

Effect of the normal Ca diet in the interplay between bone and body fat for glucose homeostasis regulation

Recent studies suggested that Ca might be important for activity of various non-skeletal tissues. Ca modulates fat metabolism and many physiological functions related to glucose homeostasis, including insulin resistance [9].

WNCa was control group related to physiological conditions and nutritional requirements adequacy. Results of this group showed a strong association between glucose and OCN levels, suggesting a close linkage between bone remodeling, insulin levels and body fat mass in glucose homeostatic control. The mechanism responsible includes effect of insulin signalling in regulation of glucose uptake and osteoblast proliferation rate. Insulin stimulates collagen, b-ALP and OCN production and inhibits OPG synthesis, which decreases OPG/RANKL ratio stimulating osteoclastic activity [19]. Bone resorption induces OCN bioactivation and its release into serum from bone extracellular matrix [20]. Bioactive OCN, as osteoblastic hormone, stimulates pancreas β-cell proliferation, insulin secretion and insulin sensitivity, both, in mice and humans [21]. To avoid hypoglycemia, fat tissue produces leptin, which blocks osteoblastic insulin signalling through CNS [22].

Genetically modified strain of rats used here spontaneously develops obesity and insulin resistance. Obese growing rats fed normal Ca diet presented hyperglycemia, hyperinsulinemia and signs of fatty liver such as an increase in total ALP levels, liver weight and abdominal fat [23]. ONCa also showed a low OCN/b-ALP ratio, that along with abdominal fat excess and lower BMC as compared to WNCa rats might account for impairment in osteoblast differentiation. Adipose tissue secretes cytokines and fatty acids that may have induced differentiation of mesenchymal precursor cells to adipocytes, in detriment of osteoblast differentiation [24]. Early stages of osteoblast differentiation express b-ALP, while OCN is expressed in mature osteoblasts. Then, changes in OCN/b-APL ratio indicate osteoblast maturation disorders that. according to literature, may be involved in diabetic fracture risk [25].

Previous studies reported that bone turnover correlates inversely with fasting insulin and visceral adiposity [26, 27]. In addition, it was also suggested that in presence of adiposity, low OCN impairs insulin tolerance [28, 29]. These metabolic disarrays were observed in

obese rats fed normal Ca diet studied here. Indeed, ONCa presented insulin resistance and low bone turnover rate; the latter evidenced by lower OCN and CTX than control. Obesity was also associated to high Parathormone (PTH) levels, main regulator of bone turnover [30]. In the present report, PTH was not evaluated; however, ONCa showed higher calcemia than control, suggesting certain degree of secondary hyperparathyroidism (2°HPT) that, in presence of low bone turnover, seems contradictory. Nonetheless, ONCa also showed hyperlipidemia that according to a previous report, could induce PTH resistance, independently of 25OHD levels [31]. In this regard, excess of fat tissue increases the release of certain cytokines, such as resistin which links obesity to T2DM. Resistin is involved in development of other resistances than insulin resistance, including leptin and PTH resistances [32]. The latter might also contribute to bone turnover decrease in ONCa group.

According to the present results, we hypothesized that obesity along with insulin resistance and the possible presence of other resistances may affect whole-body glucose homeostasis, inducing changes in the interplay between bone, pancreas and fat mass. This mechanism may explain the lack of association between OCN and glycemia levels in ONCa rats.

Effect of the low Ca diet in the interplay between bone and body fat in the control of glucose homeostasis

WLCa rats showed a typically pre-diabetic condition, i.e., elevated levels of insulin and slight hyperglycemia. They also showed a high HOMA-IR index and high hypercholesterolemia without hypertriglyceridemia. These signs strongly suggest glucose homoeostasis impairment, presence of insulin resistance and a certain degree of dyslipidemia. The mechanism responsible for these findings is thought to be related to low dietary Ca without changes in Pi contain that led to low Ca/Pi ratio, which affects Ca absorption [33]. Relative excess of Pi, especially in combination with low Ca intakes, may reduce passive shift of Ca by forming insoluble salts in intestinal lumen [34]. Low Ca absorption negatively affects bone metabolism as evidenced in the present report. Increase in bone turnover could be responsible for the bone mass decrease observed in WLCa. In addition, low Ca availability may decrease Ca^{2+} influx to β-cell, affecting insulin secretion and signalling, and GLUT-4 activity [35]. Insulin secretion impairment leads to hyperglycemia and eventually causes diabetes, while glucose transport disability into adipocytes, along with inability to suppress cell lipolysis, place hepatocytes under great metabolic stresses and favor insulin resistance and T2DM development over time [36]. In this regard, some observational and control studies have shown an inverse association between Ca status and insulin resistance as well as risk of T2DM or Metabolic Syndrome (MS) [10]. These changes induced by low Ca intake may explain the loss of association between OCN and glucose levels.

WLCa also showed a small accumulation of abdominal and body fat and a quite important decrease in lean body mass, suggesting the onset of a possible shift in energy away from fat stores. A longer period of study would have possibly evidenced higher alterations in fat metabolism and obesity, at expenses of impairing protein synthesis. It is important to take into account that presence of insulin resistance affects carbohydrates, lipids and proteins metabolism. Defects in intracellular signalling result in several metabolic abnormalities. Muscle is a major site of insulin resistance and changes in intracellular signalling pathway-depending on insulin initiate body protein loss [37]. Insulin resistance in skeletal muscle has been linked to fat accumulation, which could be significant in long term and could explain the fact that Ca inadequacy in early life may be most remarkable and predispose to and/or program individuals to an increased susceptibility to obesity and MS later in life [38,39].

Low Ca intake exacerbated established diabetic condition of obese strain of rats. It had been previously reported that rate of fatty acid uptake in muscle is markedly increased, while ability to oxidize fatty acids is decreased, in insulin-resistant skeletal muscle of animals with obesity and/or T2DM [40]. Both factors appear to be causal for accumulation of intracellular lipid intermediates, indicating that insulin resistance not only increases risk but also severity of T2DM. In this regard, the present report showed that all parameters of glucose and energy metabolism dysregulation, i.e., glucose and insulin levels, insulin resistance, dyslipemia, liver weight and excess of abdominal fat accumulation were higher in OLCa than in the other O rats. This effect may account for the great lean body mass reduction of OLCa [41]. Moreover, insulin resistance and obesity decreased HDL-Chol, which is associated with increased risk of atherosclerosis [42].

As mentioned, presence of obesity and insulin resistance could have produced certain degree of PTH resistance [43] that may be responsible for bone remodeling decrease as compared to control rats. However, among obese groups, low Ca availability led to OLCa rats to have a slight increase in bone turnover and subsequent decrease in bone mass.

Effect of the high ca diet in the interplay between bone and body fat in the regulation of glucose homeostasis

High Ca intake also affected glucose homeostasis in both strains of rats. WHCa evidenced signs of insulin resistance, i.e., hyperglycemia, elevated insulin levels and elevated HOMA-IR, but to a lesser degree than those observed in WLCa.

WHCa presented reduced Pi that may indicate a relative insufficiency of P intake. In this regard, it was previously suggested that an increase in Ca intake without the corresponding increase in P could increase risk of P insufficiency [44]. Several reports suggested that low bioavailability of P might affect glucose metabolism. *In vitro* studies demonstrated that pancreatic islets of phosphate-depleted rats had low ATP levels, elevated cytosolic Ca and impaired insulin secretion capacity [45]. The imbalance between extracellular and intracellular β cell Ca^{2+} pools results in diminished cellular responsiveness to insulin, increasing insulin resistance and glucose levels [35]. Clinical studies showed that low serum Pi were associated to insulin resistance and elevated blood glucose levels in healthy non-diabetic conditions, independently of body fat percentage [46]. Glucose intolerance and insulin resistance have been also documented in several diseases characterized for hypophosphatemia (hypophosphatemia rickets, adult-onset hypophosphatemia osteomalacia and renal Pi leak) [47].

Conversely, other authors have suggested that hyperglycemia *per se* could affect Pi status, directly or indirectly. High glucose levels induce depolarization of renal brush border membrane for Pi, leading to a decrease in intracellular Pi levels and hyperphosphaturia. However, hyperglycemia could also affect Pi status stimulating

peripheral Pi uptake by insulin [46,48]. Indeed, during hyperglycemic-hyperinsulinemic conditions, high amounts of glucose enter into muscle and fat tissues (insulin-sensitive tissues); high Pi amount is required because glucose is metabolized by phosphorylation. This mechanism may lower serum Pi levels. Many investigators have found decreased concentrations of serum Pi in poorly regulated diabetic patients [49].

Adiposity was also reported to be inversely related to plasma Pi levels [45]. Low availability of Pi may hinders insulin phosphorylation having deleterious effects in phospholipids and hepatic fat accumulation [50]. A positive association was observed between low Pi levels and BW increase, which was largely attributed to increase in adipose tissue and impairment of in nitrogen retention [45]. The WHCa showed dyslipemia, *i.e.,* hypercholesterolemia and hypertriglyceridemia, along with more severe changes in energy metabolism than those observed in WLCa, including increase in liver weight. These findings suggest the increase in lipid storage *via* concerted modulation of lipogenic and lipolytic processes (upregulation of lipogenesis). This effect could be responsible for the increased accumulation of body fat mass, especially abdominal fat pads, at expenses of a poor reduction in lean mass.

WHCa rats showed low Pi levels that along with high CTX levels, suggest a certain degree of 2°HPT. High levels of PTH promote Ca influx into adipocytes and enhances lipogenesis and obesity [51], associated to impairment in glucose tolerance and to decrease in insulin sensitivity [52]. Low availability of Pi could also affect energy metabolism *via* CNS through ATP production, in particular hepatic ATP [53].

Despite high CTX levels, WHCa showed low OCN and a decrease in OCN/b-ALP ratio that in presence of fat mass excess may account for impairment of osteoblast differentiation, which is reinforced by the lower bone mineral mass as compared to WNCa.

As mentioned before, Ca is important for insulin synthesis, secretion and functionality while P is important for insulin phosphorylation capacity and signalling. Insulin increases glucose uptake in muscle and fat, and inhibits lipolysis, contributing to the drop in glucose and insulin levels [54]. The OHCa presented Ca and Pi levels between reference range, suggesting a better control of Pi-Ca homeostasis that may account for the improvement in glycemic indexes and fat metabolism. Indeed, according to literature lower liver weight and decrease in visceral fat mass accumulation without changes in lean mass may be the result of a better control of glucose metabolism and a low hepatic fat accumulation [55]. In spite, OHCa showed a low bone remodeling and low OCN release, which may explain the lack of association between glucose and OCN levels.

The weaknesses of this study using a growing rodent model would be relatively short duration of the experience. Further studies should be conducted to confirm whether same results are observed in adult animals. Another limitation might be that PTH and leptin, which could clarify presence of PTH and leptin resistance, were not measured. However, the strength was to evaluate and compare effect of habitual Ca intake on interrelationship between bone, pancreas and fat tissue in glucose homeostasis control, in normal physiological conditions and/*versus* animals with a predisposition to develop obesity and T2DM.

Conclusion

These evidence that bioavailability of Ca and/or Pi is associated to the relationship between bone turnover, adiposity and insulin resistance in glucose homeostasis control. However, effects on glucose homeostasis appear to differ in physiological conditions and in presence of metabolic abnormalities of energy dysregulation such as obesity and T2DM.

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