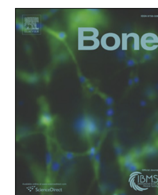
Bone 90 (2016) 99–106

Contents lists available at ScienceDirect

Bone

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



Full Length Article

High fat-fed diabetic mice present with profound alterations of the osteocyte network

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ARTICLE INFO

Article history:
Received 22 March 2016
Revised 28 May 2016
Accepted 11 June 2016
Available online 13 June 2016

Keywords:
Type 2 diabetes
Osteocyte
Osteocyte network
Image analysis

ABSTRACT

Diabetes mellitus is considered to be an independent risk factor for bone fragility fractures. Reductions in bone mass, observed only with type 1 diabetes mellitus, as well as modifications of bone microarchitectures and tissue material properties are landmarks of diabetes-related bone alterations. An interesting feature observed in type 2 diabetes mellitus (T2DM) is the augmented concentration in circulating sclerostin. This observation prompts us to hypothesize that modifications of osteocyte network and perilacunar mineralization occur in T2DM. As such, the aims of the present study were to ascertain by quantitative backscattered electron imaging, confocal microscopy and image analysis, modifications of perilacunar tissue mineral density, osteocyte morphology and osteocyte network topology in a mouse model of high fat-induced type 2 diabetes. As compared with lean control animals, diabetic mice exhibited a significant 48% decrease in perilacunar mineralization heterogeneity although mean perilacunar mineralization was unchanged. Furthermore, in diabetic animals, osteocyte volume was significantly augmented by 34% with no change in the overall number of dendrite processes. Finally, the network topology was profoundly modified in diabetic mice with increases in the mean node degree, mean node volume and hub numbers whilst the mean link length was reduced. Overall, it appeared that in diabetic animals, the dendritic network exhibited features of a scale-free network as opposed to the single-scale characteristic observed in lean controls. However, it is important to ascertain whether diabetic patients exhibit such modifications of the osteocyte network and whether anti-diabetic drugs could restore normal osteocyte and network parameters, thereby improving bone quality and protecting against fragility fractures.

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1. Introduction

Diabetes mellitus is now considered to be an independent risk factor for bone fragility fractures [1,2]. Epidemiological studies indicate that type 1 diabetes mellitus (T1DM) is associated with greater fracture risk than type 2 diabetes mellitus (T2DM) [3,4]. Whilst patients with T1DM often have modestly reduced bone mass and areal bone mineral density [3–5], T2DM patients present with normal or slightly elevated values for these parameters, even when normalized for body mass index [4,6]. Paradoxically, T2DM is associated with an augmentation in the frequency of fragility fractures [3,7,8]. The possible explanation for this elevated fracture occurrence may reside in T2DM-related complications (reduced muscle quality, retinopathy, nephropathy, poor balance and increased falls), poor glycemic control and anti-diabetic medications that may compromise bone quality [9], i.e. an ensemble of structural and material properties that are very important for bone strength [10].

Alterations of bone structural properties have been reported in T2DM after high-resolution peripheral quantitative computed tomography assessment at peripheral skeletal sites. It appeared that although trabecular microarchitecture was not markedly altered in T2DM, cortical porosity and cortical pore volume was dramatically augmented in this patient group [11–13]. Bone material properties are also altered in T2DM. An elegant study from Farr and colleagues using the OsteoProbe® microindentation device reported lower bone material strength index in post-menopausal T2DM patients as compared with controls [14]. Recently, we also provided evidence that in type 2 diabetic mice, bone strength at the tissue level was decreased together with reductions in collagen orientation and enzymatic collagen cross-linking plus augmentation in collagen glycation as compared with age- and sex-matched controls [15]. Reductions in enzymatic collagen cross-linking have also been reported by others [16,17]. High levels of advanced glycation end (AGE) products are likely to be implicated as they have detrimental effects on the bone matrix by increasing collagen

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http://dx.doi.org/10.1016/j.bone.2016.06.008
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stiffness and also modifying osteoblast activity [18–20], which might contribute to the low bone turnover observed in T2DM [4].

Other evidence for abnormal bone physiology in T2DM concerns higher circulating levels of sclerostin [21–23]. Sclerostin is generated almost exclusively by bone-embedded osteocytes [24,25]. Osteocytes are by far the most abundant cells in bone and organized themselves as a complex communication network by establishing numerous contacts with other osteocytes, blood vessels and cells at the bone surface through dendritic processes. Higher circulating sclerostin in T2DM may suggest modifications of osteocyte behavior and/or of the osteocyte network. However, although little is known about the organization of the osteocyte network in T2DM, it is plausible that the osteocyte network could be compromised in this condition, thereby participating in the observed fragility of bone.

The aims of the present study were to (i) evaluate whether the high-fat fed (HFD) diabetic mouse model exhibits high circulating sclerostin levels and compromised bone strength as observed in T2DM patients, (ii) ascertain whether modifications of osteocyte morphology and tissue mineral density around osteocyte lacunae occur in this mode of T2DM and (iii) investigate the topology of the osteocyte network in the HFD-diabetic mice.

2. Materials and methods

2.1. Generation of the HFD diabetic mouse model

Sixteen male NIH Swiss mice (mouse strain Hsd:NIHS) were purchased from our animal supplier (Envigo RMS Ltd., Blackthorn, UK) and housed individually in an air-conditioned room at 22 ± 2 °C with 12 h light:12 h dark cycle. This mouse strain and the number of animals per group was based on previous studies performed in our lab to successfully generate a mouse model of type-2 diabetes mellitus in response to a high fat diet [26]. These animals had free access to drinking water and standardized rodent chow (10% fat, 30% protein, 60% carbohydrate; percent of total energy 12.99 kJ/g; Trouw Nutrition, Cheshire, U.K.). In order to induce type 2 diabetes, eight mice ($n = 8$) were randomly switched to a high fat diet (45% fat, 20% protein, 35% carbohydrate; percent of total energy 26.15 kJ/g; Special Diet Service, Essex, U.K.) at 8 weeks of age. Lean control and high fat fed mice were sacrificed at 32 weeks of age. Data on body weight, bone mineral density and samples for determination of non-fasting plasma glucose, insulin and sclerostin concentrations were collected the day prior to sacrifice. Glucose tolerance (18 mmol/kg bw, *i.p*, 18-h fast) and non-fasting insulin sensitivity tests (25 U/kg bw, *i.p*) were performed at the end of the study, as previously described [27]. All experiments were performed according to UK Home Office Regulations (UK Animals Scientific Procedure Act 1986) and were approved by the Institutional animal care and use committee at the University of Ulster.

2.2. Dual X-ray absorptiometry

Bone mineral density (BMD, g/cm^2) and percentage of total fat were measured by a Lunar PIXImus dual-energy X-ray absorptiometry (DEXA) scanner (Inside Outside Sales, Wisconsin, U.S.A.). Mice were anaesthetised with isoflurane and sodium pentobarbital (10 μl/g bw) and then placed on the specimen tray. The animals were exposed to low energy X-rays (80/35 kV) and high-resolution pictures (0.18 × 0.18 mm pixel size) were generated. BMD was analyzed in three distinct regions for all mice: (1) the whole body excluding the head, (2) a tibial region of interest that was defined as the maximal scan area fitted within a portion of the tibia including both trabecular and cortical bone, and (3) a lumbar region that was defined as the maximal scan area fitted within a portion of the lumbar vertebrae including both trabecular and cortical bone. Calibration of the DEXA machine was performed using the phantom provided by the manufacturer at regular intervals.

2.3. Assessment of metabolic parameters

Blood samples were obtained from the cut tips of the tail of conscious mice and collected into fluoride coated microvette blood tubes (Sarstedt, Germany). The samples were immediately centrifuged using a microcentrifuge (Beckman Instruments, Galway, Ireland) for 3 min at 13,000 *g*. The separated plasma was aliquoted into 500 μl Eppendorf tubes and kept at −20 °C prior to analysis.

Plasma glucose concentrations were determined using 5 μl samples by the glucose oxidase method using an Analox GM-9 analyzer with Analox instruments glucose reagent. The analyzer was calibrated with 8 or 25 mmol/l glucose standards. Plasma insulin concentrations were determined by a modified dextran-coated charcoal radioimmunoassay (RIA) as previously described [28].

For glucose tolerance tests, pre-injection blood collections were taken (0 min) in 18 h fasted mice before intraperitoneal administration of glucose (18 mmol/kg bw). Blood was then collected at 15, 30 and 60 min post-injection and plasma samples were stored at −20 °C prior to assessment of glucose and insulin as described above. For insulin sensitivity tests, blood glucose levels were recorded using an Ascencia glucose meter (Bayer Contour) at time = 0 min in fed mice and at 30 and 60 min after intraperitoneal injection of bovine insulin (25 U/kg bw in 0.9% saline).

2.4. Circulating sclerostin levels

Plasma circulating sclerostin levels were determined by an in-house ELISA. Briefly, 100 μl plasma was mixed with 100 μl 0.1 M carbonate buffer (pH 9.5) and deposited overnight in covalent binding plate (Nunc CovaLink®, Thermofisher Scientific, Villebon sur Yvette, France). The next day, wells were washed with 0.1 M Tris buffer and blocked with 10 g/l bovine serum albumin at room temperature. The presence of sclerostin was detected using the goat anti-sclerostin antibody (ref AF1589, R&D Systems Europe, Abingdon, UK). The concentration of sclerostin was determined by comparison with a standard curve generated with recombinant mouse sclerostin (ref 1589-ST, R&D Systems Europe). The minimum detectable concentration was 10 pg/ml.

2.5. Three-point bending

Three-point bending experiments were performed on femurs. At necropsy, femurs were harvested, cleaned of soft tissue and stored in 70% ethanol until use. The storage period in 70% ethanol was less than a month. Short-term storage in 70% ethanol has been shown to have no negative effects on the mechanical properties [29,30]. Prior to mechanical testing, femurs were rehydrated in saline for 24 h at 4 °C. Measurements were done with an Instron 5942 (Instron, Elancourt, France) as reported previously [31]. The load-displacement curve was acquired with the Bluehill 3 software (Instron). Ultimate load, ultimate displacement, stiffness and total absorbed energy were computerized.

2.6. Quantitative backscattered electron imaging (qBEI)

Quantitative backscattered electron imaging (qBEI) was employed to determine the tissue mineral density distribution around osteocyte lacuna. Femurs were embedded undecalcified in poly (methylmethacrylate) at 4 °C as previously reported [32]. Polymethylmethacrylate blocks were cross-sectioned with a diamond saw at the midshaft of femurs, polished with graded silicon carbide paper followed by diamond particles on polishing cloths as previously reported [33]. Observations of bone-embedded blocks using a scanning electron microscope running in high vacuum (pressure $< 2.10^{-4}$ Pa) often resulted in cracks appearing around osteocyte lacuna that accumulate electron charges altering the backscattered signal. To overcome this problem, bone blocks were observed in an EVO LS10 environmental scanning electron microscope (Carl Zeiss Ltd., Nanterre, France) running at a pressure of 50 Pa. The

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