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Effects of parathyroid hormone on cortical porosity, non-enzymatic glycation and bone tissue mechanics in rats with type 2 diabetes mellitus

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ABSTRACT

Type 2 diabetes mellitus increases skeletal fragility; however, the contributing mechanisms and the efficacy of bone-forming agents are unclear. We studied diabetes and parathyroid hormone (PTH) treatment effects on cortical porosity (Ct.Po), non-enzymatic glycation (NEG) and bone mechanics in Zucker diabetic fatty (ZDF) rats.
Eleven-week old ZDF diabetic (DB) and non-diabetic (ND) rats were given 75 μg/kg PTH (1–84) or vehicle 5 days per week over 12 weeks. The right femora and L4 vertebrae were excised, micro-CT scanned, and tested in 3-point bending and uniaxial compression, respectively. NEG of the samples was determined using fluorescence. Diabetes increased Ct.Po (vertebra (vert): +40.6%, femur (fem): +15.5% vs. ND group, $p < 0.05$) but had no effect on NEG. PTH therapy reduced vertebral NEG in the ND animals only (−73% vs untreated group, $p < 0.05$), and increased femoral NEG in the DB vs. ND groups (+63%, $p < 0.05$). PTH therapy had no effect on Ct.Po. Diabetes negatively affected bone tissue mechanics where reductions in vertebral maximum strain (−22%) and toughness (−42%) were observed in the DB vs. ND group ($p < 0.05$). PTH improved maximum strain in the vertebra of the ND animals (+21%, $p < 0.05$) but did not have an effect in the DB group. PTH increased femoral maximum strain (+21%) and toughness (+28%) in ND and decreased femoral maximum stress (−13%) and toughness (−27%) in the DB animals (treated vs. untreated, $p < 0.05$). Ct.Po correlated negatively with maximum stress (fem: $R = -0.35$, $p < 0.05$, vert: $R = -0.57$, $p < 0.01$), maximum strain (fem: $R = -0.35$, $p < 0.05$, vert: $R = -0.43$, $p < 0.05$) and toughness (fem: $R = -0.34$, $p < 0.05$, vert: $R = -0.55$, $p < 0.01$), and NEG correlated negatively with toughness at the femur ($R = -0.34$, $p < 0.05$) and maximum strain at the vertebra ($R = -0.49$, $p < 0.05$).
Diabetes increased cortical porosity and reduced bone mechanics, which were not improved with PTH treatment. PTH therapy alone may worsen diabetic bone mechanics through formation of new bone with high AGEs cross-linking. Optimal treatment regimens must address both improvements of bone mass and glycemic control in order to successfully reduce diabetic bone fragility.
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1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder resulting from a combination of insulin resistance and insufficient insulin production. T2DM affects a number of organ systems including the skeleton, where fracture risk is increased in patients despite normal or even high bone mineral density (BMD) [1]. This implies that deficiencies in the bone tissue itself, rather than the amount of bone present, are the cause of reduced bone strength. The parameters that describe the mechanical behavior of bone can be divided into two categories. *Extrinsic properties* define the whole-bone mechanics (e.g. failure load and energy absorption), while *intrinsic properties* describe the mechanics of the bone tissue (e.g. failure stress and toughness) [2]. Bone mass, structure and intrinsic material properties all contribute to the extrinsic or whole-bone mechanics. Causes of reduced bone tissue mechanics in T2DM may include reduced bone turnover [3], increased porosity [4] and the accumulation of non-enzymatic collagen cross-links known as advanced glycation end-products (AGEs) [5]. These cross-links form spontaneously through non-enzymatic glycation (NEG), and are known to affect bone mechanics directly by reducing extrinsic [6] and

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intrinsic [7] properties, and indirectly by inhibiting osteoblast function [8,9], in particular in combination with high levels of glucose [10]. In diabetic rats, AGE cross-links have been shown to be associated with reduced maximum load and energy absorption capabilities [11]; however, no link to the intrinsic material properties has yet been demonstrated in this setting.

The male Zucker diabetic fatty (ZDF) rat has been established as a rodent model for T2DM, and recapitulates the obesity and insulin resistance observed in humans [12,13]. These animals have impaired bone mechanics due to reductions in bone mass, structure and tissue-level material properties [13,14]. While impaired osteoblast function is known to contribute to this [15], other factors such as collagen cross-linking, glycation and cortical porosity, have not yet been studied in this model.

Because of the negative impact of T2DM on bone structure and strength, pharmaceutical interventions for the treatment of osteoporosis have been explored as potential therapeutic agents for diabetes. Recently, the bone-anabolic agent sclerostin-neutralizing antibody (romosozumab) has been shown to improve BMD and bone microstructure, leading to augmented extrinsic mechanical properties [14]. We have recently examined the effect of PTH treatment in ZDF rats [16], and documented that diabetes-induced reduction of bone formation and structure could be restored. Nevertheless, full restoration of mechanics, which was also impaired with the diabetic condition, could not be achieved. In this same work glucose metabolism was unaffected by PTH [16], which may have resulted in new bone being formed with higher levels of AGEs cross-links. In order to explore the mechanisms by which PTH affects diabetic bone mechanics, we examined the tissue-level mechanical properties of these rats, which were treated intermittently with PTH (1–84). To study the potential mechanisms leading to altered tissue mechanics, we determined the porosity and AGE cross-link content in the bone tissue.

2. Materials and methods

2.1. Animals and PTH therapy

A total of 36 male rats (18 ZDF fa/fa and 18 ZDF +/+) were used for this study (Charles River Laboratories), and were given a high-fat, high-carbohydrate chow (Purina 5008). ZDF fa/fa rats spontaneously develop T2DM between the age of weeks 9 and 11, and the ZDF +/+ served as non-diabetic control. All invasive procedures were approved by the local Institutional Animal Care Committee.

At the age of 11 weeks, nine of the ZDF fa/fa and nine of the ZDF +/+ (half of each group) were administered vehicle (water), and the remaining were administered 75 μg/kg PTH (1–84) (Nycomed), administered s.c. 5 days per week for 12 weeks. This resulted in four groups of nine rats each: (1) 12 weeks of normal development, (2) 12 weeks of diabetes only, (3) 12 weeks of PTH treatment only and (4) 12 weeks of simultaneous diabetes and PTH treatment. At the end of the study, the rats were sacrificed under general anesthesia and the right femora (N = 9/group) and L4 vertebrae (N = 7/group) were excised, fixed in 4% paraformaldehyde (PFA) and stored in 70% ethanol.

2.2. Micro-computed tomography analysis

Samples from both sites were analyzed by micro-computed tomography (micro-CT) using a vivaCT 40 (70 kVp, 114 μA, 300 msec integration time, 1000 projections on 180° 2048 CCD detector array, cone-beam reconstruction, ScancoMedical). All scans were done at an isotropic voxel size of 10 μm. The entire L4 vertebra was scanned after removal of the endplates (1.5 mm from each side), and the spinal, transverse, and articulate processes with a precision sectioning saw (IsoMet 1000, Buehler) (Fig. 1A). The femur was scanned at the mid-diaphysis, centered halfway between the femoral head and distal condyles, and consisted of 10 slices (Fig. 1B). A semi-automated contouring method was used to isolate the cortical bone of the femoral mid-shaft, and an automated contouring method [17] was used to separate the cortical and trabecular bone in the vertebrae. The images were Gaussian-filtered ($\sigma = \text{supp} = 2$) and thresholded (24% of maximal grayscale value) for analysis. The tissue mineral density (TMD) was determined and the results have been published previously [16]. The cortical porosity was estimated from the mineral density values after application of the endosteal and periosteal contours using the following equation:

$$\text{Ct.Po} = (1 - \text{BMD}/\text{TMD}) * 100. \quad (1)$$

2.3. Biomechanical testing

The samples were removed from the ethanol and rehydrated in PBS for 2 hours prior to mechanical testing (Zwick). For the 3-point bend tests, the specimens were loaded at a rate of 0.05 mm/s to a preload of 2 N and held for 5 s before being loaded at a rate of 0.5 mm/s to failure. For the vertebral compression, the specimens were loaded at 2 mm/min to a preload of 0.5 N and then loaded at the same rate until failure. Force (N) and displacement (mm) at the upper support were recorded (Fig. 1C). In order to examine the tissue-level mechanics independent from geometrical differences, the force and displacement were converted to stress and strain. The elastic modulus, yield and maximum stress, yield and maximum strain, modulus of resilience and toughness of each sample were then determined using equations of mechanics of materials [18] (Fig. 1D).

2.4. Assessment of non-enzymatic glycation

Following the mechanical tests, a 2.5 mm thick section was cut from the proximal end of the break in the femoral mid-shaft for collagen analysis. The entire vertebral body was used. The specimens were flushed of marrow, demineralized and digested with papain collagenase (0.4 mg/ml in 0.1 mM sodium acetate buffer, pH 6.0, 16 h, 65 °C) [7]. AGE content was determined using an Infinite200 fluorescence plate reader (Tecan) with an excitation wavelength of 370 nm and emission of 440 nm, and normalized to a quinine sulfate standard. The amount of collagen in each specimen was determined from the hydroxyproline content, which was estimated by the absorbance of the digested samples against a hydroxyproline standard at the wavelength of 570 nm using a SpectraFluor Plus microplate reader (Tecan) [19].

2.5. Statistical analysis

To determine the effect of diabetes, therapy and the influence of diabetes on the therapeutic effect, a two-way ANOVA was performed with diabetes (yes/no), PTH treatment (yes/no) and diabetes crossed with PTH treatment as model effects. Unpaired t-tests were then applied to evaluate the individual group differences for all measured parameters. Results are given in mean ± SD. Associations between NEG or cortical porosity and biomechanical properties were determined using linear regression and are presented in terms of the correlation coefficient. A multiple regression model was used to study the independent effects of porosity and NEG parameter on bone mechanics. P-values of <0.05 were considered significant. All statistical analyses were performed using JMP software, version 9.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effect of diabetes and PTH treatment on cortical porosity

A significant effect of diabetes on Ct.Po was observed at both sites (Fig. 2, right) with group differences showing higher porosity in both the untreated (vertebra: +40.6%, p = 0.043, femur: +15.5%,