Comparative Biochemistry and Physiology, Part A 203 (2017) 152–158

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Effects of hyperglycemia on bone metabolism and bone matrix in goldfish scales

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ARTICLE INFO

Article history:
Received 11 June 2016
Received in revised form 8 September 2016
Accepted 14 September 2016
Available online 16 September 2016

Keywords:
Alloxan
Bone metabolism
Bone model
Diabetes
Fish scales
Glycation of matrix collagen
Hyperglycemia
Increased fracture rate
Type 1 collagen

ABSTRACT

Increased risk of fracture associated with type 2 diabetes has been a topic of recent concern. Fracture risk is related to a decrease in bone strength, which can be affected by bone metabolism and the quality of the bone. To investigate the cause of the increased fracture rate in patients with diabetes through analyses of bone metabolism and bone matrix protein properties, we used goldfish scales as a bone model for hyperglycemia. Using the scales of seven alloxan-treated and seven vehicle-treated control goldfish, we assessed bone metabolism by analyzing the activity of marker enzymes and mRNA expression of marker genes, and we measured the change in molecular weight of scale matrix proteins with SDS-PAGE. After only a 2-week exposure to hyperglycemia, the molecular weight of α- and β-fractions of bone matrix collagen proteins changed incrementally in the regenerating scales of hyperglycemic goldfish compared with those of euglycemic goldfish. In addition, the relative ratio of the γ-fraction significantly increased, and a δ-fraction appeared after adding glyceraldehyde—a candidate for the formation of advanced glycation end products in diabetes—to isolated type 1 collagen *in vitro*. The enzymatic activity and mRNA expression of osteoblast and osteoclast markers were not significantly different between hyperglycemic and euglycemic goldfish scales. These results indicate that hyperglycemia is likely to affect bone quality through glycation of matrix collagen from an early stage of hyperglycemia. Therefore, non-enzymatic glycation of collagen fibers in bone matrix may lead to the deterioration of bone quality from the onset of diabetes.

1. Introduction

Increased focus has recently been placed on the elevated fracture risk associated with type 2 diabetes (de Liefde et al., 2005; Vestergaard, 2007; Petit et al., 2010). However, its cause remains unclear. Fracture risk is intimately related to decrease in bone strength, which can depend on the bone mineral density (quantity) and the quality of the bone. The relationship between the density of a weight-bearing bone and type 2 diabetes has yet to be delineated. Patients with type 2 diabetes have significantly higher bone mineral density than healthy subjects (de Liede et al., 2005; Vestergaard, 2007; Yamaguchi et al., 2009; Oei et al., 2013). However, the hip bone mineral density was significantly lower in men with type 2 diabetes compared to men without diabetes (Yaturu et al., 2009). In contrast, another study found that there was no difference in mineral density of the tibia between men with and without type 2 diabetes (Petit et al., 2010). There is no scientific consensus that type 2 diabetes is associated with bone mineral density or bone metabolism. However, with respect to the quality of the bone, the possibility of altering bone matrix collagen properties by non-enzymatic glycation was identified in aging and diabetic subjects (Reiser, 1991; Saito et al., 2006).

Osteoblasts have been used in *in vitro* studies to investigate the effects of hyperglycemia on bone metabolism. However, the effects of high glucose concentration on bone mineralization have contradictory results: some studies reported inhibited mineralization (Balint et al., 2001; Zhang and Yang, 2013; Cunha et al., 2014), whereas others

Abbreviations: ALP, alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; AGEs, advanced glycation end products; rplp1, 60S acidic ribosomal protein P1.

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http://dx.doi.org/10.1016/j.cbpa.2016.09.010
1095-6433/

reported increased mineralization (Li et al., 2007; García-Hernández et al., 2012; Liu et al., 2015).

Bone is a dynamic tissue, and bone remodeling continues into adulthood to optimize mineral homeostasis and the strength of the bone. Bone remodeling consists of a close coupling of bone resorption by osteoclasts and bone formation by osteoblasts (Parfitt, 1982; Martin and Sims, 2005). In addition, many signaling molecules between osteoclasts and osteoblasts have been identified (Sims and Martin, 2014), highlighting the importance of cell-cell communication between osteoclasts and osteoblasts at the remodeling sites for bone metabolism. Thus, an *in vivo* model involving osteoblasts, osteoclasts, and bone matrix proteins is essential for assessing the effects of hyperglycemia on bone metabolism.

Teleost fish, such as goldfish (*Carassius auratus*), have cycloid scales with calcified tissue consisting of osteoblasts, osteoclasts, and bone matrix proteins (Bereiter-Hahn and Zylberberg, 1993). Teleost scales have been shown to be a good model for studying bone metabolism (Kitamura et al., 2010; Kitamura et al., 2013; Yano et al., 2013).

In the present study, to investigate the cause of the increased fracture rate in patients with diabetes, we evaluated the response of bone metabolism to high glucose concentration with insulin deficiency in goldfish scales collected from alloxan-induced hyperglycemic fish. We measured the enzymatic activity of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), and mRNA expression analyses of bone metabolism marker genes using goldfish scales. In addition, we detected the change in molecular size of bone matrix type 1 collagen, in relation to glycation of collagen, induced by high glucose concentration *in vivo* and *in vitro*.

2. Materials and methods

2.1. Animals

We used 123 adult goldfish purchased from a commercial source (Higashikawa fish farm, Yamatokoriyama, Japan). The goldfish were kept in an aquarium at 25 ± 0.5 °C. These goldfish were fed a commercial diet (42.0% protein, 11.0% fat, 2.0% fiber, 10.5% mineral and vitamin mixture) (TetraFin, Tetra Werke, Melle, Germany) twice a day, and were grown to an average body weight of 18.8 ± 3.0 g before use in this study. All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University (No. 93255).

2.2. In vivo study

2.2.1. Determination of the optimal dose of alloxan

Forty-seven goldfish were subjected to a 24 h fasting period prior to alloxan injection, anesthetized with 0.03% ethyl 3-aminobenzoate methanesulfonic acid salt (MS-222; Sigma-Aldrich, St. Louis, MO, USA), and weighed. Alloxan monohydrate (A4547, LKT laboratories, Saint Paul, MN, USA) was dissolved in ice-cold saline (0.58%), and injected immediately into the peritoneal cavity of 40 goldfish in incremental doses of 300, 400, 500, and 600 mg/kg-body weight. Similarly, seven control fish were injected with the vehicle control (0.58% ice-cold saline). The dosage of alloxan dose sufficient to induce insulin deficiency was determined from the results of immunohistochemistry of Brockmann bodies (Fig. 1A), insulin synthesis islet homologous tissues in teleost using anti-barfin flounder (*Verasper moseri*) insulin, and through the measurement of blood sugar level.

2.2.2. Immunohistochemistry of goldfish Brockmann body

Goldfish have anatomically discrete islet organs called Brockmann bodies. Eight alloxan-treated goldfish and two control (vehicle) goldfish were anesthetized and sacrificed after 5 days of receiving peritoneal injections. Brockmann bodies were removed from each goldfish and fixed with paraformaldehyde (4%, buffered neutral pH 7) for 4 h at 20 °C. Fixed Brockmann bodies were embedded in paraffin and sliced into sections for immunohistochemistry staining using primary antibody of anti-barfin flounder insulin-II (Andoh and Nagasawa, 2002).

2.2.3. Measurement of blood glucose concentration

2.2.3.1. Blood sugar level after eating. Fifty-one goldfish were subjected to a 24 h fasting period prior to eating. Blood samples were taken at 0, 1, 2, 3, 4, 6, 18, and 24 h after feeding the commercial goldfish diet. Goldfish were anesthetized with MS-222, and 100 μL of blood was collected from the vertebral vein using a 1 mL syringe with 30-gauge needle. Blood was transferred into a new tube after sampling. After the blood clotted, clots were centrifuged at 1500 ×*g* for 10 min, and serum from the supernatant was stored at −20 °C until glucose measurement was performed. The serum glucose levels were determined by the Mutarotase-glucose oxidase method using a commercial glucose measurement kit (Glucose CII-test Wako, Wako Pure Chemical Industries, Osaka, Japan).

2.2.3.2. Blood sugar level after alloxan administration. Blood sampling was performed 8 h and 24 h after alloxan administration using 18 goldfish. Additionally blood sampling was performed at 4 h after the first meal on day 5, day 7, day 10, and day 14 after the alloxan injection at a dose of 500 mg/kg body weight. The procedure for analyzing serum sugar level after alloxan administration was the same as described above.

2.2.4. Preparation of regenerated scales

One day prior to peritoneal injection, fourteen goldfish were anesthetized with MS-222 and thirty-six ontogenetic scales were removed from each goldfish. On day 0, fourteen goldfish were put on 24 h fasting prior to alloxan-injection, anesthetized with MS-222, and then weighed. Subsequently, goldfish were divided into two treatment groups. Seven goldfish in the alloxan-treated group were injected with 500 mg/kg of body weight dose of ice-cold alloxan monohydrate solution into the peritoneal cavity. The rest of the seven goldfish in the control vehicle group were injected with a relatively equal volume of ice-cold saline into the peritoneal cavity. On day 15, after intra-abdominal injection, goldfish were anesthetized again, and twenty regenerated scales were collected from each goldfish for measurement of enzymatic activities and mRNA expression analyses. These scales were divided into three groups: eight regenerated scales were transferred to a well in a 96-well microplate with 100 μL of an acidic buffer (20 mM tartrate in 100 mM sodium acetate, pH 5.3), another eight were transferred to a well in a 96-well microplate with 100 μL of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM MgCl2; 0.1 mM ZnCl2), and the remaining four regenerated scales were transferred to a 1.5 mL tube with 350 μL of lysis buffer (1.0% 2-mercaptoethanol-containing buffer RA1, NucleoSpin® RNA, TAKARA, Kusatsu, Japan). The regenerated scales in each microplate and 1.5 mL tubes were immediately frozen at −80 °C pending analyses of TRAP and ALP activities and mRNA expression. On day 21 after peritoneal injection, both the alloxan-treated group and vehicle group of goldfish were anesthetized again and sixteen regenerated scales were collected from each fish for analysis of the molecular size in bone matrix type 1 collagen. Noncollagenous soluble proteins and polysaccharides were removed from goldfish scale isolates as described previously (Pacak et al., 2011) with some modifications to the temperature.

2.2.5. Effect of hyperglycemia on TRAP and ALP activities

We quantified the enzymatic activity of each scale by measuring the enzymatic decomposition rate of the substrate and the planimetry of the scale surface, as described below.

2.2.5.1. Measurement of enzymatic decomposition rate of para-nitrophenyl phosphate (pNP-mEq/h). Regenerated scales, frozen with 100 μL of an acidic buffer without enzymatic substrate in a microplate were uniformly thawed at room temperature. An aliquot of 100 μL of an acidic