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Saxagliptin affects long-bone microarchitecture and decreases the osteogenic potential of bone marrow stromal cells

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ABSTRACT

Diabetes mellitus is associated with a decrease in bone quality and an increase in fracture incidence. Additionally, treatment with anti-diabetic drugs can either adversely or positively affect bone metabolism. In this study we evaluated: the effect of a 3-week oral treatment with saxagliptin on femoral microarchitecture in young male non-type-2-diabetic Sprague Dawley rats; and the in vitro effect of saxagliptin and/or fetal bovine serum (FBS), insulin or insulin-like growth factor-1 (IGF1), on the proliferation, differentiation (Runx2 and PPAR-gamma expression, type-1 collagen production, osteocalcin expression, mineralization) and extracellular-regulated kinase (ERK) activation, in bone marrow stromal cells (MSC) obtained from control (untreated) rats and in MC3T3E1 osteoblast-like cells. In vivo, oral saxagliptin treatment induced a significant decrease in the femoral osteocytic and osteoblastic density of metaphyseal trabecular bone and in the average height of the proximal cartilage growth plate; and an increase in osteoclastic tartrate-resistant acid phosphatase (TRAP) activity of the primary spongiosa. In vitro, saxagliptin inhibited FBS-, insulin- and IGF1-induced ERK phosphorylation and cell proliferation, in both MSC and MC3T3E1 preosteoblasts. In the absence of growth factors, saxagliptin had no effect on ERK activation or cell proliferation. In both MSC and MC3T3E1 cells, saxagliptin in the presence of FBS inhibited Runx2 and osteocalcin expression, type-1 collagen production and mineralization, while increasing PPAR-gamma expression. In conclusion, orally administered saxagliptin induced alterations in long-bone microarchitecture that could be related to its in vitro down-regulation of the ERK signaling pathway for insulin and IGF1 in MSC, thus decreasing the osteogenic potential of these cells.

1. Introduction

Dipeptidyl-peptidase-4 (DPP4) is a membrane-bound glycoprotein with proteolytic activity that is expressed in different cell types including T-cell lymphocytes, hepatocytes, gastrointestinal epithelial cells, osteoblasts and bone marrow stromal cells (MSC) (Matteucci and Giampietro, 2009; Stanley et al., 2006). In T lymphocytes, DPP4 participates in the immune response, and its inhibition suppresses mitogen-mediated cell proliferation (Kahne et al., 2000; Schon et al., 1985). The specific role of DPP4 in other cell types is less known.

DPP4 is also involved in insulin metabolism, since it shows proteolytic activity against various enteric incretins. Enteroendocrine cells stimulated by different nutrients present in the gut lumen secrete incretins, and these peptide hormones then stimulate pancreatic insulin production, in a mechanism that could account for 50% of post-prandial insulin secretion. However, incretins are not only insulinotropic, but have multiple additional effects such as lowering glucagon, increasing satiety and slowing gastric emptying. The plasmatic half-life of incretins is dependent on their proteolysis by extracellular DPP4. Selective DPP4 inhibitors (gliptins) have been developed, and are used as second-line drugs for the post-prandial control of glycaemic levels in patients with type 2 Diabetes mellitus (Gagliardino et al., 2008). Saxagliptin is a licensed gliptin that is metabolized by cytochrome P450, and whose metabolite is also an active DPP4 inhibitor (Ali and Fonseca, 2013).

An increasing body of evidence demonstrates the association of type 1 and type 2 Diabetes mellitus with bone abnormalities, including osteopenia, osteoporosis and/or an increased incidence of low-stress fractures, in what has been termed diabetic osteopathy (Janghorbani et al., 2007). These bone alterations are partly induced by a significant decrease in the material properties of diabetic bone tissue (i.e., bone quality) (McCarthy et al., 2013). Additionally, bone quality can secondarily be affected by antidiabetic

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pharmacological treatment (Molinuevo et al., 2010; Rzonca et al., 2004; Sedlinsky et al., 2011). Thus, thiazolidinediones (TZD) promote bone loss and fractures via peroxisome proliferator-activated receptor (PPAR)-gamma dependent mechanisms, whereas the biguanide metformin shows in vitro and in vivo osteogenic effects and can prevent in vivo anti-osteogenic actions of TZD.

The effect of gliptins on bone tissue has not been clearly established to date. In the ovariectomized female rat model of post-menopausal osteoporosis, sitagliptin was found to increase bone mineral density and trabecular volume, although in the same study inactivation of the DPP4 gene had no effect on bone phenotype (Kyle et al., 2011). In a recent meta-analysis, other authors have suggested an overall decrease of fracture risk in diabetic patients treated with DPP4 inhibitors (Monami et al., 2011). However, the specific effect of saxagliptin on bone metabolism has not been published to date, and to our knowledge no randomized controlled trials have been reported to specifically evaluate the effects of saxagliptin treatment on fracture incidence.

In the present study we have evaluated the in vivo effects of oral saxagliptin administration in young male non-type-2-diabetic rats on the microarchitecture of load-bearing bones, the in vitro actions of saxagliptin on MSC isolated from control (untreated) rats, as well as possible mechanisms of action involved.

2. Materials and methods

2.1. Animal treatments

Three-month-old male Sprague-Dawley rats (190–210 g body weight) were used. All experiments on animals were performed in conformity with the Guide for Care and Use of Laboratory Animals of the National Research Council of the National Academies (Guide, 2011). Approval for animal studies was obtained from the institutional accreditation committee (INIBIOLP's Animal Welfare Assurance No A5647-01). Animals were divided into two groups of 5 animals per group: untreated controls [C] and rats treated for 3 weeks with Saxagliptin (Bristol-Myers Squibb Company, USA) administered in drinking water (2 mg/kg/day) [S] (Fura et al., 2009; Tahara et al., 2009). At the end of all treatments blood samples were obtained, after which the animals were sacrificed by cervical dislocation under anesthesia.

2.2. Biochemical parameters

Serum was separated from non-fasting blood samples of all animals. Glucose, triglycerides, cholesterol, transaminases (ALT and AST), alkaline phosphatase (ALP), creatinine and urea were measured in serum samples by commercial kits (Wiener Laboratories, Argentina) with a 2300Plus automatic Chemical Analyzer (Metrolab, Argentina). Serum insulin levels were determined with a rat-specific ELISA kit from ALPCO. No significant differences were observed between groups C and S for all parameters evaluated (data not shown).

2.3. Histological and histomorphometric evaluation of long bones

Femoral bones were processed for histological and quantitative histomorphometric analysis (Molinuevo et al., 2010). Briefly, sections were stained either with haematoxylin–eosin (H–E) for routine evaluation; with Alcian Blue (pH=3) to evaluate the average height of the proximal cartilage growth plate; or with tartrate resistant acid phosphatase (TRAP) histochemistry to specifically identify osteoclasts in the associated primary and secondary spongiosa. Images were analyzed using the Image J program (www.macbiophotonics.ca/imagej) with a Microscope scale plugin. Ten sections were processed for every different stains and for each animal.

In the femoral metaphysis (2 mm distal from the proximal growth plate), relative trabecular bone volume was determined as the quotient between the area of trabecular bone and the total area (trabecular bone plus bone marrow cavities). Additionally in the same metaphyseal sections, osteoblastic density was evaluated by counting the number of lining osteoblasts per millimeter of trabecular bone surface (H–E); and osteocyte density was assessed by determining the number of osteocytes per square millimeter of trabecular bone (H–E). In sections corresponding to the primary and secondary spongiosa associated with the proximal growth plate, relative osteoclastic density was calculated as the percentage of positive TRAP area per square millimeter of trabecular bone (Sedlinsky et al., 2011).

2.4. Osteoblastic cell cultures and MSC isolation

MSC were obtained from animals of group C as we have described previously (Molinuevo et al., 2010). Briefly, bone marrow cells were collected by flushing femora and tibiae of the animals with Dulbecco's modified essential medium (DMEM)–10% fetal bovine serum (FBS) (Invitrogen, Buenos Aires, Argentina) under sterile conditions. For comparative purposes, in some experiments the MC3T3E1 mouse calvaria-derived preosteoblastic cell line was used instead of MSC.

In experiments designed to evaluate either the direct effect of saxagliptin on MSC and MC3T3E1 proliferation, or its possible modulation of the action and signaling pathways of growth factors present in FBS, sub-confluent cells were serum-starved overnight and then incubated with 1.5 or 15 μM saxagliptin and/or FBS (1%), insulin (5 ng/mL) or IGF1 (10^{-8} M) for 24 h (effects on cell proliferation) or 1 h (effects on signal transduction mechanisms). In other experiments, cells were cultured in an osteogenic medium (DMEM–10% FBS containing 25 μg/ml ascorbic acid and 5 mM sodium β-glycerol-phosphate) for a further 15 or 21 days, with or without the different doses of saxagliptin. In vitro doses of saxagliptin (1.5 and 15 μM) were chosen in view of the plasmatic concentrations of the drug plus its active metabolite that are attained in the rat after administration of 2.5 mg/kg saxagliptin (Fura et al., 2009).

2.5. Evaluation of osteoblastic differentiation

After submitting MSC or MC3T3E1 cells to 15 days of osteogenic induction, type 1 collagen production was evaluated by Sirius Red staining as previously reported (Molinuevo et al., 2010). Extracellular calcium deposits (mineralization nodules) were measured after 21 days of osteogenic differentiation using Alizarin S red staining (Molinuevo et al., 2010).

2.6. Western blot analysis

Bone cells, with or without a 15-day osteogenic induction and under the experimental conditions indicated above, were subjected to 12% SDS-PAGE. The following antibodies were used: Runx2 and osteocalcin for evaluation of osteoblastogenesis; PPAR-gamma to evaluate adipocytic commitment; and total extracellular-regulated kinases (ERK) or phosphorylated-ERK to evaluate insulin and IGF1 signal transduction pathways (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Results were normalized with an anti B-actin antibody (Sigma, St. Louis, MO, USA) (Kanazawa et al., 2008) and quantified using the gel plugin of MBF_Image J (http://www.macbiophotonics.ca).