Journal of Steroid Biochemistry & Molecular Biology 154 (2015) 237–244

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

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

Osteoprotective effects of estrogen membrane receptor GPR30 in ovariectomized rats

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ARTICLE INFO

Article history:
Received 28 April 2015
Received in revised form 6 July 2015
Accepted 7 July 2015
Available online 14 July 2015

Keywords:
Estrogen
GPR30
Osteoporosis
Ovariectomy
Bone

ABSTRACT

G protein-coupled estrogen receptor 30 (GPR30) is expressed in bone tissue. However, little is known regarding the function of GPR30 in postmenopausal osteoporosis. In this study, we examined the effects of GPR30 on ovariectomy (OVX)-induced osteoporosis in rats, including the effects on proliferation, differentiation, and expression of proteins in osteoblasts. Administration of G1 (35 μg/kg, ip, 3 times/week for 6 weeks), a specific agonist of GPR30, prevented OVX-induced increase in bone turnover rate, decrease in bone mineral content and bone mineral density, damage to bone structure, and aggravation of bone biomechanical properties. In addition, G1 did not affect uterine weight in the OVX rats. Osteoblasts isolated from calvarias from newborn rats were used to explore the underlying mechanisms. G1 (150 pM) promoted proliferation and differentiation of osteoblasts through a positive feedback of GPR30, which then activated the PI3K-Akt, ERK, and CREB pathways. G15 (750 pM), a specific antagonist of GPR30, reversed the above effects initiated by G1 treatment. In conclusion, activation of GPR30 protected bones against osteoporosis in OVX rats and exerted no untoward effect on the uterus. We suggest that GPR30 can be used as an effective therapeutic target for the prevention and treatment of postmenopausal osteoporosis.

1. Introduction

Estrogen is critical in bone metabolism. Bone resorption and turnover are significantly elevated in postmenopausal women. This phenomenon leads to a condition called "primary osteoporosis", in which patients with this condition possess lower bone mass and a higher fracture risk. Estrogen replacement therapy (ERT) and selective estrogen receptor modulators (SERMS) are today still considered to be valuable additions to the treatment of osteoporosis [1]; and due to its diverse action on a wide variety of organs, ERT has the capacity to improve the quality of life for most postmenopausal women. ERT thereby still constitutes a first-line choice for the prevention of bone loss and fracture in the early postmenopausal period for up to 5 years. However, these analyses have also highlighted the negative impact on breast cancer risk and adverse effects on the cardiovascular system. The general lack of anti-fracture efficacy of SERMS with respect to non-vertebral fractures limits their use in women who are at high risk of osteoporotic fracture [1].

Estrogen needs to bind to its receptors to function. To date, at least three estrogen receptors (ERs), namely, ERα, ERβ [2,3], and G protein coupled estrogen receptor 30 (GPR30) [4], are identified. These ERs are widely distributed in the body, including in the skeletal, cardiovascular, and reproductive systems. Thus, when osteoporosis patients are treated with exogenous estrogen for an

Abbreviations: ALP, alkaline phosphatase; BMD, bone mineral density; BV/TV, ratio of trabecular volume/bone total volume; CA, cortical area; CD, trabecular connectivity density; CTX-I, C-telopeptide of type I collagen; ERT, estrogen replacement therapy; GPR30, G protein-coupled estrogen receptor 30; IP, inner perimeter; MT, mean thickness; OC, osteocalcin; OP, outer perimeter; OVX, ovariectomy; S-Ca, serum calcium; SERMS, selective estrogen receptor modulators; S-P, serum phosphorus; Tb.Th., trabecular thickness; Tb.Sp., trabecular separation; TRACP, tartrate-resistant acid phosphatase.

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http://dx.doi.org/10.1016/j.jsbmb.2015.07.002
0960-0760/

extended period, side effects arise, including endometrial hyperplasia and uterine and breast tumors [5]. GPR30-mediated rapid signaling events are sensitive to pertussis toxin, implying that the GPR30 couples to particular members of the G protein superfamily [6,7]. Depending upon the duration, magnitude, and subcellular localization of ER kinase (ERK) activation, this kinase pathway controls or modulates a plethora of cellular responses, including proliferation, migration, differentiation, and cell death [8,9]. Because of the wide distribution and multiple signaling pathways involved in the activation of ERs, researchers have recently attempted to optimize the unique characteristics of different ERs so as to improve efficacy and reduce side effects in ERT.

Previous studies suggest a possible role of GPR30 in bone metabolism [10]. However, these studies focused primarily on GPR30 gene knock-out animals, which may not completely represent the wild-type. Moreover, the mechanisms underlying these phenomena are still unclear. Thus, in the present study, we used G1 (a specific agonist of GPR30) in an OVX rat model in order to uncover the relationship between GPR30 and bone metabolism; and then used G1 and G15 (a specific antagonist of GPR30) on osteoblasts isolated from calvaria of newborn rats so as to explore the possible underlying mechanisms involved.

2. Materials and methods

2.1. Animals and treatments

Thirty-two 3-month-old nulliparous female SD rats were purchased from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, Shaanxi, China). After a 7-day adaptation period in an open animal feeding room at $23 \pm 2\,^{\circ}C$ and $60 \pm 5\%$ humidity, with natural light, standard nutrition pellets (Ca 0.9%, P 0.7%), and clean water, the rats were randomly divided into 2 groups. Rats were then anesthetized with pentobarbital sodium (Bioszune, USA, 40 mg/kg, ip). First, 8 rats were sham-operated and treated with vehicle (BETIS olive oil, Torres Y Ribelles, S.A., Spain, 1.5 mL/kg, ip, 3 times/week) as control (the "sham" group), whereas the remaining 24 rats were bilaterally ovariectomized [11] and randomly divided into 3 groups with 8 rats per group. Four weeks after surgery, the 24 ovariectomized rats were treated either with vehicle (olive oil, 1.5 mL/kg, ip), E2 (35 μg/kg, ip), or G1 (35 μg/kg, ip; Cayman, USA) every other day for 6 weeks. Body weight was recorded weekly during the experimental period. At the end of the 6th week, rats were anesthetized, and blood was collected from the inferior vena cava; uteri were removed and immediately weighed; and femurs were dissected and stored in 40% formaldehyde solution at room temperature until structural analysis and biomechanical testing. All animals were treated according to the principles and procedures contained in the NIH Guide for the Care and Use of Laboratory Animals. The procedures were approved by the Institutional Ethics Committee of the Fourth Military Medical University.

2.2. Serum chemistry assays for bone metabolism

Serum calcium (S-Ca), phosphorus (S-P), and alkaline phosphatase (ALP) concentrations were measured using commercial kits (Zhongshengbeikong Biotechnology Co., Ltd., Beijing, China) and analyzed with an automatic biotechnology analyzer (7160, Japan). Serum osteocalcin (OC) levels were determined with radioimmunoassay (RIA) kits (Beijing Huaying Biotechnology Research Institute, China) using a gamma counter (r-911, Industrial Company of University of Science and Technology of China). Serum tartrate-resistant acid phosphatase (TRACP) and C-telopeptide of type I collagen (CTX-I) levels were determined with ELISA kits (Elabscience Biotechnology, Wuhan, China).

2.3. Bone microarchitecture assessment by micro-computed tomography

Bone microarchitecture of the distal femur was scanned by eXplore Locus SP Pre-Clinical Specimen micro-computed tomography (micro-CT) (GE Healthcare, USA). Reconstruction and 3D quantitative analyses were then performed using the desktop micro-computed tomography system. In the femora, scanning regions were confined to the distal metaphysis, extending proximally 2.0 mm from the proximal tip of the primary spongiosa for cancellous bone and proximally 12.0 mm from the center of fossa intercondylica for cortical bone. The 3D indices in the defined region of interest (ROI) were analyzed, including bone mineral density (BMD), relative bone volume over total volume (BV/TV, %), trabecular thickness (Tb.Th.), trabecular separation (Tb.Sp.), and connectivity density (CD) of the cancellous bone; and also BMD, mean thickness (MT), cortical area (CA), inner perimeter (IP), and outer perimeter (OP) of the cortical bone. The operator conducting the scan analysis was blinded to the treatments associated with the specimens.

2.4. Measurement of bone biomechanical strength

The mechanical properties of the left femurs were determined by a three-point bending test. The biomechanical quality of the left femoral diaphysis was determined using a CMT4204 material testing machine (Shenzhen Skyan Power Equipment Co., Ltd., Shenzhen, China) at a speed of 2 mm/min. Briefly, the left femurs were thawed at room temperature for 1 h and then placed in the material test machine with two support points separated by a distance of 20 mm. Ultimate load (Max Load), energy absorbed to failure (Fracture Energy), ultimate stress (Max Stress), and elastic modulus were obtained, and the bone load–displacement curves were simultaneously plotted using the aforementioned software. The entire femur length was measured with a Vernier caliper before machine testing. The inner/outer long/short diameters of the bone fracture were measured by the same Vernier caliper after machine testing.

2.5. Cell culture

Primary osteoblasts were isolated from the calvarias of newborn SD rats and cultured with Dulbecco's modified Eagle's medium (DMEM/low glucose, HyClone, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) at $37\,^{\circ}C$ in a humidified incubator supplied with 5% CO_2 in compressed air. The medium was replaced every 3 days. The purified osteoblasts were plated at an appropriate concentration in different media for different experiments. For example, 1×10^4 cells/mL was plated in a 24-well plate (0.5 mL/well) in DMEM/low glucose containing 10% FBS for ALP staining; 2×10^5 cells/mL in a 24-well plate (0.5 mL/well) in DMEM/low glucose containing 10% FBS, 50 mg/L L-ascorbic acid (Shiny Bio, China), and 10 mmol/L β-sodium glycerophosphate (Regal Bio, Shanghai, China) for calcium nodule alizarin red S staining; 2×10^4 cells/mL in 96-well plate (0.1 mL/well) in DMEM/low glucose//phenol red-free (HyClone, USA) containing 10% FBS for MTT (Sigma, USA) assay; 2×10^4 cells/mL in 24-well plate (0.5 mL/well) in DMEM/low glucose//phenol red-free containing 10% FBS for ALP activity assay; and 2×10^5 cells/mL in 6-well plate (1 mL/well) in DMEM/low glucose//phenol red-free containing 10% FBS for Western blotting analysis.

2.6. Osteoblast identification

Osteoblasts were identified by ALP staining (BCIP/NBT ALP Color Development Kit, Beijing Leagene Biotechnology Co., Ltd.)