Bone 55 (2013) 84–92

Contents lists available at SciVerse ScienceDirect

Bone

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

Original Full Length Article

17β-Estradiol positively modulates growth hormone signaling through the reduction of SOCS2 negative feedback in human osteoblasts

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ARTICLE INFO

Article history:
Received 17 December 2012
Revised 22 March 2013
Accepted 23 March 2013
Available online 6 April 2013

Edited by: J. Aubin

Keywords:
Estrogen
Proteasome
Bone cells
Silencing
STAT5
Ubiquitination

ABSTRACT

Recent evidence demonstrated an interplay between estrogens and growth hormone (GH) at cellular level. To investigate the possible mechanism/s involved, we studied the effect of 17β-estradiol (E2) on GH signaling pathways in primary culture of human osteoblasts (hOBs). Exposure of hOBs to E2 (10^{-8} M) 60 min before GH (5 ng/ml) significantly increased phosphorylated STAT5 (P-STAT5) levels compared with GH alone. E2 per se had no effect on P-STAT5. E2-enhanced GH signaling was effective in increasing osteopontin, bone-sialoprotein, and IGF II mRNA expression to a greater extent than GH alone. We then studied the effect of E2 on the protein levels of the negative regulator of GH signaling, suppressor of cytokine signaling-2 (SOCS2). E2 (10^{-11} M–10^{-7} M) reduced dose-dependently SOCS2 protein levels without modifying its mRNA expression. The silencing of SOCS2 gene prevented E2 positive effect on GH induced P-STAT5 and on GH induced bone-sialoprotein and osteopontin mRNA expression. Treatment with the inhibitor of DNA-dependent RNA synthesis, actinomycin-D, did not prevent E2 induced decrease of SOCS2, thus suggesting a non-genomic effect. E2 promoted an increase in SOCS2 ubiquitination. To determine if increased ubiquitination of SOCS2 by E2 led to degradation by proteasome, hOBs were pretreated with the proteasome inhibitor MG132 (5 μM) which blocked E2 reduction of SOCS2. These findings demonstrate for the first time that E2 can amplify GH intracellular signaling in hOBs with an essential role played by the reduction of the SOCS2 mediated feedback loop.

Introduction

The somatotrophic pathway is a complex process that regulates key aspects of growth and metabolism. Estrogens are responsible for the development of secondary sexual characteristics, and they play a major role by regulating both somatic growth and the preservation of bone mass [1]. After menopause, their deficit has deleterious effects on the skeleton. There is close interplay between estrogens and growth hormone (GH) in the regulation of growth and development. The increase in GH and estrogens at puberty activates a growth spurt, which results in gender-specific body composition. The regulatory action by estrogens on GH may occur at many levels: secretion, clearance, and action. In addition to estrogens' effect on GH secretion at the hypothalamic–pituitary axis, there is emerging evidence that estrogens may modulate GH action on target tissues. Orally administered estrogens reduce the metabolic action of GH in the liver [2] and influence responsiveness to GH replacement therapy in GH-deficient adults [3,4]. Hypogonadal children have reduced GH secretion, which is normalized by sex steroid therapy [5,6].

Additional interactions between hormones can occur in the cell, after receptor activation, through signaling cross-talk [7]. The binding of estrogens to their receptors leads to activation of the transcriptional machinery for initial hormone genomic activity. Nonetheless, a variety of cells respond rapidly to estrogens, making a genomic mechanism of action unlikely. For example, estrogens can regulate cell-signaling phosphorylation cascades, thereby leading to anti-apoptotic activity in calvaria cells [8]. The protein known as a modulator of non-genomic action of estrogen receptor (MNAR) was recently isolated. MNAR promotes ligand-dependent interaction between estrogen receptor α (ERα) and members of the Src family of tyrosine kinases [9]. ERα non-genomic action has been suggested to be involved in estrogen-induced osteo-protection.

The binding of GH to its specific receptor (GHR) in target tissues induces the phosphorylation of Janus kinase 2 (JAK2), with subsequent phosphorylation of the signal transducers and activators of transcription (STATs). STAT proteins then translocate into the nucleus, where they bind to specific DNA motifs within the promoter regions to initiate transcription of GH-responsive genes. GH activation

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http://dx.doi.org/10.1016/j.bone.2013.03.016

of the JAK/STAT pathway is negatively regulated by phosphotyrosine phosphatases (PTPs; SHP-1,2) [7] and the suppressors of cytokine signaling (SOCS) [10].

In hepatoma and breast cancer cells, estradiol (E2) inhibits GH-dependent activation of JAK2/STAT5 by increasing SOCS2 gene expression, thus reducing the synthesis of insulin-like growth factor 1 (IGF1) in target tissues [11]. In bone cells, the post-receptor interaction between estrogens and GH has not yet been elucidated, although it is generally acknowledged that both E2 and GH have well documented anabolic activity [10,12,13], and their relative insufficiency states are involved in bone loss in both males and females [14]. The aim of this study was to evaluate a possible role for E2 in the regulation of GH intracellular signaling in bone cells. For this purpose, we investigated the effect of E2 on the GH-induced JAK/STAT cascade in human osteoblasts (hOBs). Considering the widespread use of oral estrogens and estrogen-related compounds, a better understanding of the effects of sex steroid/GH interaction on cell signaling in bone will have implications for improving the management of patients with pituitary deficits and reduced bone mass.

Materials and methods

Drugs

Human recombinant growth hormone (GH), 17β-estradiol (E2), actinomycin D and MG132 were purchased from Sigma (Milan, Italy).

Human osteoblasts (hOBs) cultures

Human bone cell cultures were established by means of a modified version of the Gehron-Robey and Termine procedure [15] using trabecular bone samples obtained from waste material of female patients during orthopaedic surgery for degenerative diseases or traumatic fractures of the femoral neck requiring osteotomy. None of the patients (aged 68–81 yr) had any malignant bone diseases and all of them gave their written consent for the use of the waste material. The protocol was approved by the institutional ethical committee. No significant trend related to donor age was observed in any of the effects studied. Briefly, the trabecular bone was cut into small pieces and thoroughly washed with commercial standardized Joklik's modified MEM (Sigma, Milan, Italy) serum-free medium, to remove non-adherent marrow cells. The pieces were incubated with rotation at 37 °C for 30 min with the same medium containing 0.5 mg/ml type IV collagenase (Sigma, Milan, Italy), and collagenase digestion was stopped by the addition of Iscove's modified medium (IMDM, Lonza, Walkersville, MD, USA) containing 10% fetal bovine serum (FBS, Euroclone, Milan, Italy). Between eight and ten pieces from each patient were then placed in 25 cm^2 flasks and cultured in IMDM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml mycostatin, and 0.25 μg/ml amphotericin B until confluence; the culture medium was changed every 2–3 days. At the first passage the culture medium was changed to Dulbecco's modified essential medium (DMEM, Sigma, Milan, Italy) without phenol red and with 10% FBS and antibiotics as IMDM. The cell population was tested for alkaline phosphatase and osteocalcin production after treatment with 10^{-8} M $1,25(OH)_2D_3$ to ensure that the cells were endowed with osteoblast characteristics. Cells were used at first passage to reduce the possibility of phenotype changes.

Human fibroblasts were cultured in DMEM without phenol red and with 10% FBS and antibiotics as IMDM.

Real time PCR

The relative expression of osteopontin (OPN), bone sialoprotein (BSP), and IGF-II mRNAs was evaluated in hOBs. At confluence after 24 h of serum starvation, cells were treated for 6 h with GH (5 ng/ml) or E2 (10^{-8} M). For the combined treatment with the two hormones, E2 was added 60 min before GH. The relative expression of SOCS1, SOCS2 and SOCS3 mRNAs was evaluated 60 min after treatment with E2.

Total RNA from confluent hOBs was extracted using TRIzol reagent, according to the manufacturer's instructions (Invitrogen Life Technology, Inc., Paisley, UK). One microgram of total RNA was reverse transcribed to cDNA using oligodT primers and M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). Real-time PCR reaction using primer-probe sets validated and purchased as "Assay-on-Demand" from Applied Biosystems (Foster City, CA, USA) in singleplex PCR mix was performed in an ABI PRISM® 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) on 10 μg cDNA. Each gene expression was first normalized with β-actin content and the relative quantification treated/untreated was calculated with the $2^{-\Delta\Delta Ct}$ method. Three replicates were performed for each experimental point and experiments were repeated several times with cells obtained from different donors.

Western blot analysis

Phosphorylated-STAT5 (P-STAT5) and STAT5 protein levels were tested in confluent hOBs after 24 h of serum starvation and treated for 60 min with different doses of GH (0.1 ng/ml–20 ng/ml), or E2 (10^{-8} M), or E2 + GH (5 ng/ml). In this latter case E2 was added 60 min before GH. SOCS1, SOCS2 and SOCS3 protein levels were detected in 24 h serum starved hOBs after 60 min treatment with

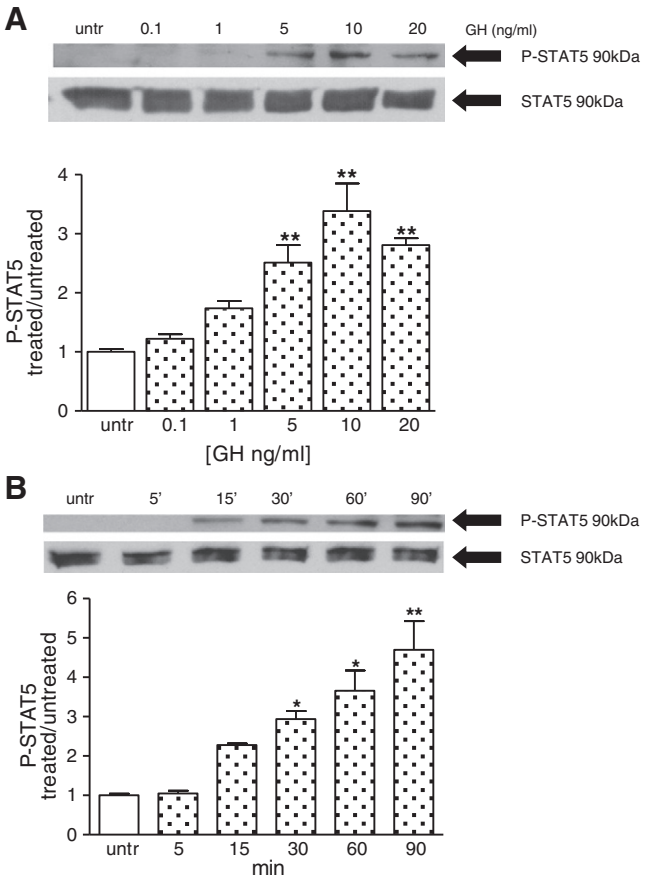

Fig. 1. A: STAT5 phosphorylation (P-STAT5) in response to increasing concentrations of GH (0.1–20 ng/ml) in hOBs after 60 min of treatment; representative Western blots and relevant quantification, as described in the Materials and methods section (n = 6). No changes in the total amounts of STAT5 were observed B: Time course of 5 ng/ml GH treatment on P-STAT5 (n = 3); $^{**}P < 0.01$, $^{*}P < 0.05$ vs. untreated cells (untr); Kruskal–Wallis with Dunn's test for multiple comparisons.