Steroids 88 (2014) 77–82

Contents lists available at ScienceDirect

Steroids

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

# Steroid sulfatase mediated growth Sof human MG-63 pre-osteoblastic cells

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ARTICLE INFO

*Article history:*
Received 1 February 2014
Received in revised form 1 July 2014
Accepted 7 July 2014
Available online 17 July 2014

*Keywords:*
Bone
Dehydroepiandrosterone sulfate
Estrone sulfate
Osteoblast
Steroid sulfatase
Estrogens

ABSTRACT

Estrogen plays an important role in maintaining bone density. Postmenopausal women have low plasma estrogen, but have high levels of conjugated steroids, particularly estrone sulfate ($E_1S$) and dehydroepiandrosterone sulfate (DHEAS). Conversion of these precursors to active estrogens may help maintain bone density in postmenopausal women. The enzyme steroid sulfatase (STS) converts sulfated steroids into active forms in peripheral tissues. STS occurs in bone, but little is known about its role in bone function. In this study, we investigated STS activity and expression in the human MG-63 pre-osteoblastic cell line. We also tested whether sulfated steroids can stimulate growth of these cells. MG-63 cells and microsomes both possessed STS activity, which was blocked by the STS inhibitors EMATE and 667 Coumate. Further evidence for STS in these cells was provided by RT-PCR, using STS specific primers, which resulted in cDNA products of the predicted size. We then tested for growth of MG-63 cells in the presence of estradiol-17β, $E_1S$ and DHEAS. All three steroids stimulated MG-63 cell growth in a steroid-free basal medium. We also tested whether the cell growth induced by sulfated steroids could be blocked using a STS inhibitor (667 Coumate) or using an estrogen receptor blocker (ICI 182,780). Both compounds inhibited $E_1S$-induced cell growth, indicating that $E_1S$ stimulates MG-63 cell growth through a mechanism involving both STS and the estrogen receptor. Finally, we demonstrated using RT-PCR that MG-63 cells contain mRNA for both estrogen receptor alpha and estrogen receptor beta. Our data reveal that STS is present in human pre-osteoblastic bone cells and that it can influence bone cell growth by converting inactive sulfated steroids to estrogenic forms that act via estrogen receptor alpha or beta.



## 1. Introduction

Bone is constantly being remodeled in the adult skeleton by an integrated mechanism involving bone reabsorbing osteoclasts and bone forming osteoblasts. Together they maintain the structural integrity of the skeletal system throughout an individual's lifetime. Estrogenic steroid hormones (primarily estradiol-17β) act directly on bone tissue by decreasing bone turnover and by limiting osteoclast activity [1–3]. However, at the onset of menopause, decreased estrogen levels lead to increased osteoclast activity, paving the way for increased resorption of bone and decreased bone mass. This facilitates the development of osteoporosis and leads to an increased risk of bone fractures [4].

Post-menopausal women have low levels of circulating estrogens, but have high blood levels of inactive sulfated steroids, which can serve as precursors to more active estrogens by removal of the sulfate group. Desulfonation is catalyzed by the enzyme steroid sulfatase (STS), which converts sulfo-conjugated steroids into unsulfated forms [5]. Two such sulfated steroids, estrone sulfate ($E_1S$) and dehydroepiandrosterone sulfate (DHEAS), are present at considerable levels in peripheral circulation of postmenopausal women [6], and these can be transformed to active estrogens in osteoblast cells [7–9]. This involves the hydrolysis of $E_1S$ and DHEAS to estrone and dehydroepiandrosterone, respectively, via STS. Subsequently, several enzymes known to be present in bone can catalyze transformation of estrone and dehydroepiandrosterone to the potent estrogen 17β-estradiol and other active estrogens [6,10,11].

STS has been shown to be present in bone tissues [9,12] and bone cell lines. [6,7,13–15]. Furthermore, several osteoblast-like bone cell lines (HOS, U2OS and MG-63) have been shown to possess the other enzymes, aromatase and 17-beta-hydroxysteroid dehydrogenase, that are necessary to produce active estrogens from circulating androgens [7,13,14]. Also, STS present in MG-63 cells has been shown to use both $E_1S$ and DHEAS as substrates [8], demonstrating the potential of the STS enzyme to convert

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http://dx.doi.org/10.1016/j.steroids.2014.07.001
0039-128X/

these two prevalent circulating steroid precursors into active estrogenic steroid hormones in bone cells.

Little is known about the regulation of STS in bone. However, if a regulatory mechanism were deciphered, it could conceivably be used to increase STS activity in mature bone, increasing the local production of estrogen from circulating precursors. This could be a potential therapy for osteoporosis in post-menopausal women.

In this study, we examined STS activity and mRNA expression in the MG-63 pre-osteoblastic cell line using radioactive sulfated steroid conversion assays and RT-PCR. We also examined the effect of sulfated steroids on growth of MG-63 pre-osteoblastic cells, and the role of STS in this process, using steroid sulfatase inhibitors and an estrogen receptor blocker.

## 2. Methods and materials

### 2.1. Chemicals and reagents

[6,7-$^3$H] estrone-sulfate (49 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA). Radioinert steroids and 667 Coumate (STX 64) were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Liquid scintillation cocktail was Ultima Gold (Packard Instrument Co., Meriden, CT, USA). The steroid sulfatase inhibitor estrone 3-O-sulfamate (EMATE) was obtained from Dr. Pui-Kai Li (Ohio State University School of Pharmacy).

### 2.2. Cell culture methods

Human preosteoblastic MG-63 cells were purchased from ATCC (Rockville, MD). Cells were cultured in 100 mm tissue culture dishes (Beckton Dickinson, Franklin Lakes, NJ, USA) in 10–12 mL growth media containing 47.5% (v/v) Hams F12 nutrient mixture, 47.5% (v/v) Minimal Essential Medium (MEM) from Invitrogen (Grand Island, NY, USA), 5% (v/v) heat-inactivated fetal bovine serum from HyClone (Logan, UT, USA), and 10 mg/ml penicillin/streptomycin solution from HyClone. Media added to plates was changed every 2 or 3 days to ensure continuous nutrient supply. Basal media, used for cell growth assays, was 49.25% (v/v) Ham's F12 nutrient mixture, 49.25% (v/v) Minimal Essential Medium, 0.5% (v/v) charcoal-stripped, heat-inactivated fetal bovine serum (HyClone), and 10 mg/ml penicillin/streptomycin solution from HyClone.

### 2.3. Measurement of sulfatase activity of intact cells

MG-63 cells were seeded into Falcon 6-well tissue culture plates (Beckton Dickinson and Co.,) at a density of approximately 250,000 cells/well and incubated in growth medium overnight to allow them to adhere. After incubation, the medium was replaced with 2 ml of growth media containing $^3$H-estrone sulfate (100,000 dpm/ml) and radioinert estrone sulfate (1 μM) in the presence or absence of specific inhibitors, estrone 3-O-sulfamate (EMATE, 1 μM) or 667 Coumate (1 μM). After 18 h of incubation, 0.5 ml of medium was aliquoted into each of two 13×100 borosilicate glass tubes. Three millilitres of toluene was added to each tube for extraction of unconjugated steroids. The mixture was vortexed for 1 min and then centrifuged for 10 min to separate the aqueous and organic phases. Duplicate aliquots of 1 ml were removed from the organic phase (containing the unconjugated steroids) and transferred to scintillation vials, after which 5 ml of scintillation cocktail was added. Radioactivity was counted in a Packard Tri-carb scintillation counter at 50% efficiency for $^3$H. The conversion values obtained for all treatments were adjusted for spontaneous product formation by subtracting the value obtained for wells containing medium and $^3$H-estrone sulfate with no cells (18 h incubation). The experiment was repeated three times, with seven wells per experiment (two control wells, two EMATE treatment wells, two 667 Coumate treatment wells, and one 35 mm dish with media only).

### 2.4. Preparation of MG-63 microsomes

Pre-confluent MG-63 cells were scraped off the surface of the dishes and the cells were pelleted by centrifugation (1000 g for 10 min). Pellets were resuspended in 1:5 w:v in ice-cold Tris-sucrose buffer (50 mM Tris–HCl, 25 mM sucrose, pH 7.5) and homogenized using three 30 s bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). The nuclear fraction was pelleted by centrifugation at 2500 g for 10 min at 4 °C. The resulting supernatant was removed to Beckman ultracentrifuge tubes (11 × 60 mm) (Beckman Coulter Inc., Fullerton, CA) and centrifuged at 107,000 g for 1 h at 4 °C to yield the microsomal fraction. The resulting pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.5) at 1:2 original w:v. Protein concentrations of microsomes were determined by BCA assay (Pierce Chemical Co., Rockford, IL).

### 2.5. Measurement of sulfatase activity of MG-63 microsomes

$^3$H-estrone sulfate was diluted in Tris–HCl buffer (50 mM, pH 7.5) and 100 μl (100,000 dpm/ml) was added to the assay tubes. Radioinert estrone sulfate was dissolved in ethanol and then diluted into Tris–HCl buffer such that 100 μl would yield a concentration of 10 μM in the final assay volume. Estrone 3-O-sulfamate (EMATE) and 667 Coumate were also dissolved in Tris–HCl buffer. 100 μl of these solutions were added to the respective assay tubes to achieve the appropriate final concentration of inhibitor (1 μM). MG-63 microsomes were diluted with Tris–HCl buffer to achieve the final desired concentration of membranes (25–200 μg) in 100 μl of buffer (500 μl final volume). The assay tubes were pre-incubated for 5 min at 37 °C in a water bath. The assay was initiated by addition of the microsomes (100 μl) to the tubes containing the compounds. Control tubes with no inhibitor, tubes without microsomes (to control for spontaneous hydrolysis), and tubes with inhibitors at 1 μM concentrations were incubated simultaneously. After 30 min of incubation at 37 °C, 3 ml of toluene was added for extraction of unconjugated steroids. The samples were vortexed for 1 min and centrifuged at 2500 g for 10 min at 24 °C. Duplicate 1 ml aliquots were removed from the organic phase of the samples and added to 5 ml of scintillation cocktail. The aliquots were counted in a liquid scintillation counter (Packard Instrument Co.) for determination of product formation. The experiments were run three times, with duplicate tubes for each experiment.

### 2.6. BCA protein assay

The Pierce (Rockville, IL) BCA Protein Assay was used according to the manufacturer's instructions. A standard curve of bovine serum albumin was prepared with 50 mM Tris–HCl as the diluent. Duplicate concentrations (μg/tube) were 200, 150, 100, 75, 50, 25, 12.5 and 2.5. Duplicate tubes of MG-63 microsomal samples were prepared using 100 μl of microsomal suspension. Absorbances were read at 562 nm using a spectrophotometer (Thermo Fisher, Genesys 20; Pittsburgh, PA, USA).

### 2.7. RNA isolation

Total RNA from MG-63 cells was isolated using TRIzol® Reagent (Invitrogen Life Technologies) according to manufacturer's