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Characterization of steroid sulfatase in the MC3T3-E1 mouse pre-osteoblastic cell line

K.W. Selcer *, H.M. DiFrancesca

Department of Biological Sciences, Duquesne University, Pittsburgh, PA, USA

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ABSTRACT

Regulation of bone density is partly dependent upon steroid hormones, with estrogens playing an important role. Inactive conjugated estrogens may serve as precursors to active estrogens, especially in post-menopausal women, via steroid sulfatase, which converts conjugated estrogens into unconjugated estrogens. The purpose of this study was to characterize steroid sulfatase in the MC3T3-E1 mouse pre-osteoblastic cell line. Enzyme conversion assays were performed on whole MC3T3-E1 cells in culture and on microsomes prepared by differential centrifugation. ^{3}H-E_1S and ^{3}H-DHEAS were used as tracers, and radioinert E_1S and DHEAS were used as substrate. Whole cells and microsomes exhibited steroid sulfatase activity, which was blocked by the specific inhibitor estrone-3-O-sulfamate (EMATE). The K_m of steroid sulfatase in microsomes averaged 83 μM when using E_1S as substrate and 64 μM when using DHEAS. Western blotting of MC3T3-E1 microsomes for steroid sulfatase was performed, after SDS–PAGE, using an antibody generated against a peptide based on a conserved region of steroid sulfatase. Western blotting revealed three bands of cross-reactivity, ranging from 50 to 79 kDa. Reverse transcriptase polymerase chain reaction (RT-PCR), using specific primers, resulted in a single cDNA band of the expected size (100 bp) and sequence, indicating the presence of steroid sulfatase mRNA. Growth assays revealed that the MC3T3-E1 cells were stimulated by estradiol-17β, and also by estrone sulfate and DHEAS, revealing that the cells can use steroid sulfatase to produce active estrogens. Furthermore, growth of these cells in the presence of estradiol, estrone and estrone sulfate was inhibited by the estrogen receptor blocker ICI 182,780, indicating that stimulation of cell growth is mediated by the estrogen receptor. In our studies, four lines of evidence (enzyme activity, immunoassay, RT-PCR and growth assays) demonstrated the presence of steroid sulfatase in mouse MC3T3-E1 bone cells. The existence of steroid sulfatase in these pre-osteoblastic cells, along with the ability of sulfated steroids to promote their growth, suggest the possibility that this enzyme is involved in regulation of bone density in mice.

1. Introduction

The structural integrity of bone is dependent on a balance between the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Steroid hormones play important roles in the regulation of bone architecture [1–3]. Estrogens, in particular, appear to conserve bone density. They can act directly on bone, decreasing bone turnover and maintaining a balance between bone formation and bone resorption [2,4–8].

Conjugated steroids in blood may serve as precursors for active steroid hormones in a variety of tissues, including bone. Sulfated steroids, particularly estrone sulfate and dehydroepiandrosterone sulfate, are present at substantial levels in peripheral circulation [9]. Conversion of these precursors to active estrogens may provide the estrogen needed for maintenance of bone density [10–12].

Bone cells have been shown to contain all of the enzymes necessary for estrogen metabolism [9–15]. Dehydroepiandrosterone sulfate and estrone sulfate, both abundantly circulating steroid precursors, are converted to dehydroepiandrosterone and estrone, respectively, via steroid sulfatase. Dehydroepiandrosterone can then be converted to androstenedione by 3β-hydroxysteroid dehydrogenase – Δ5/Δ4 isomerase (3β-HSD). Androstenedione can be converted to estrone by aromatase or to testosterone by 17β-hydroxysteroid dehydrogenase. Dehydroepiandrosterone can also be converted to androstenediol by 17β-keto-reductase. Androstenediol is an androgen with estrogenic properties [16]. Estrone can be converted to the more potent 17β-estradiol by the enzyme 17β-hydroxysteroid dehydrogenase and testosterone can be converted to 17β-estradiol by aromatase. Thus, using pathways beginning with steroid sulfatase, bone cells have the ability to metabolize abundant steroid precursors, particularly dehydroepiandrosterone

* Corresponding author. Address: Department of Biological Sciences, **Duquesne University,** 600 Forbes Avenue, Pittsburgh, PA 15282, USA. Tel.: +1 412 396 5967; fax: +1 412 396 5907.
E-mail address: selcer@duq.edu (K.W. Selcer).

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sulfate and estrone sulfate, to biologically potent estrogens such as 17β-estradiol.

Steroid sulfatase is a microsomal enzyme that is classified as an aryl sulfatase C (EC 3.1.6.2). An aryl sulfatase C enzyme cleaves the sulfate group from sulfate esters of phenol- or 3β-hydroxysteroids [9]. This enzyme has been shown to be present in both human and rat bone [11,12,15,17,18]. Steroid sulfatase has been purified from mouse liver and partially characterized [19] and the gene encoding murine steroid sulfatase has been identified [20]; however, steroid sulfatase activity has not yet been demonstrated in mouse bone cells.

Herein we document for the first time the presence of steroid sulfatase in a mouse bone cell line, MC3T3-E1, using enzyme activity, immunoassays, and RT-PCR. We further demonstrate that sulfated steroids can promote growth of these bone cells.

2. Methods and materials

2.1. Chemicals and reagents

^{3}H-estrone sulfate (ammonium salt, [6,7-^{3}H[N]]-; 49 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). Radioinert steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Liquid scintillation cocktail was Ultima Gold (Packard Instrument Co., Meriden, CT). Estrone 3-O-sulfamate (EMATE) was kindly provided by Dr. Pui-Kai Li (Ohio State University School of Pharmacy).

2.2. Cell culture methods

Steroid sulfatase activity was assessed in the mouse pre-osteoblastic bone cell line MC3T3-E1. Cells were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media and reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). Growth medium, used for routine growth of MC3T3-E1 cells, was 90% (v/v) Dulbecco's Modified Eagle Medium containing 10% (v/v) heat-inactivated fetal calf serum (HyClone, Logan, UT), and 10 mg/ml penicillin/streptomycin (HyClone). Cells were routinely grown in Falcon 100 mm tissue culture dishes (Beckton Dickinson & Co., Franklin Lakes, NJ) in 12 ml growth medium. Basal medium, used for cell growth assays, was 99.5% Dulbecco's Modified Eagle Medium containing 0.5% (v/v) charcoal-stripped, heat-inactivated fetal calf serum (HyClone, Logan, UT), and 10 mg/ml penicillin/streptomycin (HyClone).

2.3. Cell growth assay

MC3T3-E1 cells were seeded into Falcon 24-well plates (Becton Dickenson and Co.) at a density of 25,000 cells/well and incubated in 1.0 ml of growth medium for 18 h to allow them to adhere. After incubation, the medium was removed, the cells were washed once with phosphate buffered saline (Sigma–Aldrich Co., St. Louis, MO), and basal medium was added. After 48 h, medium was changed to either basal medium alone (control) or basal medium containing treatments (10 μM estradiol-17β, estrone, estrone sulfate or dehydroepiandrosterone sulfate), with or without the estrogen receptor blocker ICI 182,780 (100 nM). Cells were then allowed to grow for a 4-day period. Medium was then removed and 200 μl MTT (dimethylthiazol tetrazolium bromide) (Sigma Chemical Co., St. Louis, MO) was added to the cells, after which they were incubated at 37 °C for 3 h. Following incubation, the MTT was completely removed and replaced with 1000 μl of acidic isopropanol. The plate was placed onto a plate shaker for 10 min to solubilize the membranes and then four 200 μl samples from each well were removed and placed into a 96-well microtiter plate and read at an absorbance of 595 nm using the BioRad Microplate reader (Model 3550). Absorbances were compared with a standard curve derived from plates with known numbers of cells (range of 5,000–500,000 cells).

The first experiment compared cell growth in the presence of estradiol-17β, estrone sulfate, or dehydroepiandrosterone sulfate (10 μM). The second experiment compared cell growth in the presence of estradiol-17β, estrone or estrone sulfate, in combination with the presence or absence of the estrogen receptor blocker ICI 182,780 (100 nM). All treatments were run in triplicate and each experiment was repeated three times.

2.4. Steroid sulfatase assay of intact cells

MC3T3-E1 cells were seeded into Falcon 6-well tissue culture plates (Beckton Dickinson and Co.) at a density of approximately 200,000 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 a specific inhibitor, estrone 3-O-sulfamate (EMATE, 1 μM). After 18 h of incubation, 0.5 ml of medium was aliquoted into each of two 13 × 100 borosilicate glass tubes. Three milliliters 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 experiments were repeated six times, with seven wells per experiment (three control wells, three treatment wells, and one 35 mm dish with media only).

2.5. Preparation of MC3T3-E1 microsomes

Pre-confluent cultures of MC3T3-E1 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).

2.6. Determination of steroid sulfatase activity of MC3T3-E1 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 50 μl would yield a concentration of 10 μM in the final assay volume. Estrone 3-O-sulfamate (EMATE) was dissolved in Tris–HCl buffer. This solution (100 μl) was added to the assay tubes to achieve the appropriate final concentration of inhibitor (1 μM). MC3T3-E1 microsomes were diluted with Tris–HCl buffer to achieve the final desired concentration of