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Synthetic 19-nortestosterone derivatives as estrogen receptor alpha subtype-selective ligands induce similar receptor conformational changes and steroid receptor coactivator recruitment than natural estrogens

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Abstract

The binding of estradiol (E_2) to estrogen receptors (ER) is followed by conformational changes resulting in coactivator or corepressor recruitment that influences gene transcription. A series of synthetic A-ring reduced 19-nortestosterone-derived progestins has the capacity to selectively bind and activate transcription through the ERα. Herein, the molecular mechanisms involved in ER subtype-selective interactions of these compounds as assessed by their effects upon both ERα and ERβ structural conformation and their ability to induce recruitment of steroid receptor coactivator-1 (SRC-1) to ERα were investigated. The results demonstrated that all synthetic A-ring 3β,5α-tetrahydro-reduced derivatives of 19-nortestosterone induced an ERα trypsin digestion pattern similar to that seen with E_2, without effects upon ERβ. In addition, these compounds had the ability to recruit SRC-1 to the ligand-binding domain of ERα similar to E_2. Our data indicate that A-ring 3β,5α-tetrahydro-reduced 19-nortestosterone-derived progestins behave as selective ERα agonists with ligand–receptor structural and functional responses similar to those induced with natural E_2.

Keywords: Estrogen receptors; Selective ligands; Progestins; Coactivators; Estrogen action

1. Introduction

Estrogens by interacting with nuclear transcription factors known as intracellular receptors modulate the expression of target genes [1]. The estrogen receptors (ERs), ERα and ERβ, belong to the steroid and thyroid hormone receptor superfamily [2], which upon ligand-binding and activation interact with specific DNA sequences and stimulate gene transcription [3]. It has been demonstrated that ligand-induced activation of steroid receptors involves the dissociation of receptor-associated heat shock proteins, as well as changes in the conformation of the receptor [4]. This hormone-induced change in the receptor structure can be detected by limited proteolysis, which produces, depending on the receptor, a prominent proteolytic resistant fragment encompassing the ligand-binding domain [4]. In as much as ligand-dependent protection from proteolysis is a general phenomenon for all steroid receptors, it can be used as screening assay to identify and characterize agonist or antagonist properties of new or established natural and synthetic ligands. In this regard, the estrogenic activity

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of 19-nortestosterone-derived progestins has been well established [5]. Indeed, we have shown that enzymatic A-ring reduction of 19-nortestosterone derivatives such as norethisterone (NET), levonorgestrel (LNG) and gestodene (GSD) to their corresponding tetrahydro-reduced metabolites results in compounds with significant estrogenic activities [6–8]. Among the compounds studied, the 3β,5α-reduced metabolites of NET, LNG and GSD were able to bind to the estrogen receptor (ER), with an affinity lower than estradiol, and induced activation of gene transcription by a mechanism involving ERα, but not ERβ. This ERα specific effect might be of use not only in identifying ligand requirements for ER selectivity, but in the process of developing new potential selective agonist/antagonist ligands for ER subtypes.

In this study we have examined the relative agonist/antagonist estrogenic properties of several 19-nortestosterone-derived progestins in terms of their abilities to change the conformational structure of ER by limited trypsinization and to recruit coactivator proteins.

2. Material and methods

2.1. Reagents

Non-radioactive estradiol (E_2), progesterone (P_4) and trypsin (type III) were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture medium was purchased from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from Hyclone Laboratories, Inc. (Logan, UT). The antiestrogen ICI 182,780 was purchased from Zeneca Pharmaceuticals (Wilmington, DE) and unlabeled RU486 (RU 38486, mifepristone) was a gift from Roussell Uclaf (Romainville, France). Authentic NET (17α-ethynyl-17β-hydroxy-4-estren-3-one) and LNG (13β-ethyl-17α-ethynyl-17β-hydroxy-4-gonen-3-one) were kindly provided by Schering Mexicana, S.A. (Mexico D.F., Mexico) and GSD (13β-ethyl-17α-ethynyl-17β-hydroxy-4,15-gonadien-3-one) from Schering AG (Berlin, Germany). Synthesis of the corresponding 5α-dihydro (5α-NET, 5α-LNG and 5α-GSD), and the 3α,5α- (3α,5α-NET, 3α,5α-LNG and 3α,5α-GSD) and 3β,5α- (3β,5α-NET, 3β,5α-LNG and 3β,5α-GSD) tetrahydro derivatives, including the description of their corresponding physical and spectroscopic constants have been previously described [6,9]. All other solvents and reagents used were of analytical grade.

2.2. Plasmids

The expression vectors for hPR_B (pCR3.1-hPR_B), ERα (pCR3.1-hERα) and ERβ (pCR3.1-hERβ; aa 1-530) for in vitro transcription and translation were described previously [10–12]. The parent vector, pCR3.1 was obtained from Invitrogen (Carlsbad, CA). Plasmids for the mammalian two-hybrid expression plasmids pBIND (GAL4) and pACT (VP16), and the GAL4 responsive reporter plasmid (pG5*luc*) were obtained from Promega Corp. (Madison, WI). The construction of the pBIND-hSRC-1e plasmid encoding the GAL4 DNA binding domain fused to the amino-terminus of SRC-1e was previously described [13]. The VP16-ERα vector encodes the VP16 activation domain linked to the amino-terminus of the ERα ligand-binding domain (LBD) in the pACT vector [14].

2.3. In vitro translation and partial protease digestion

The in vitro synthesis of recombinant ERα, ERβ or PR_B using T7 RNA polymerase and the T7 TnT-coupled transcription-translation kit (Promega Corp., Madison, WI) has been previously described [15,16]. Briefly in vitro translation reactions were carried out in rabbit reticulocyte lysates according to the manufacturers instructions in the presence of L-[^{35}S]methionine (>1000 Ci/mmol, Amersham, Buckinghamshire, UK) using 1 μg of plasmid for human ERα (pCR3.1-hERα), ERβ (pCR3.1-hERβ) or PR_B (pCR3.1-hPR_B) expression plasmid. In vitro translated receptor was incubated in the presence of various concentrations (10^{-6} to 10^{-8} M) of the corresponding steroid at room temperature for 10 min or the vehicle alone (0.1% ethanol) as control. Partial proteolytic digestion was performed by incubation with trypsin at a final concentration of 50 μg/ml for 10 min at room temperature. About 1–2 μl of the various reactions were mixed with 5 μl SDS loading buffer and denatured at 100 °C for 5 min. The samples were analyzed on a 10% SDS-PAGE [17], and visualized by autoradiography.

2.4. Cell culture and transfections

HeLa cells were maintained in culture at a density of 2.75×10^5 cells/well/6-well plate and transfected as previously described [14]. Briefly transfections were performed using FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN) and 1.0 μg pACT-hERα, 0.1 μg pBIND-hSRC-1e and 1.0 μg pG5luc. The plates were incubated for 12 h at 37 °C in 5% CO_2. After incubation, media were replaced with phenol-red free DMEM-HG (Invitrogen) containing the compounds of interest at various concentrations (10^{-6} to 10^{-10} M). Dimethyl sulphoxide (DMSO) or ethanol alone (EtOH) were used as vehicles. After 12 h incubation, cells were harvested and cell extracts were obtained using lysis buffer (Promega Corp.). Luciferase activity in cell extracts was measured by using a Dual-Luciferase® Reporter Assay System according to the protocol provided by the manufacturer (Promega Corp.). Statistical significance was determined using two tailed *t*-test.

3. Results

3.1. Protease resistance of ligand-activated progesterone receptor (PR)

[^{35}S]methionine-labeled PR was incubated in the presence of P_4, A-ring reduced or non-reduced NET, LNG and