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Regulation of the growth of mouse Leydig cells by the inactive stereoisomer, 17α-estradiol: Lack of correlation between the elevated expression of ERα and difference in sensitivity to estradiol isomers

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Abstract. We examined the effects of 17α-estradiol, the biologically inactive stereoisomer of 17ß-estradiol, on cell growth and cell cycle kinetics using the normalized mouse TM3 Leydig cells. A significant biphasic stimulatory growth response was observed by 17α-estradiol exposure with peaks at 1 pg/ml (157.13%) and 100 ng/ml (120.04%) ($p<0.05$). The growth stimulatory effects of 17α-estradiol were inhibited by tamoxifen. A significant decrease in cell cycle time of Leydig cells exposed to 17α-estradiol was observed in treated cells ($p<0.05$). RT-PCR analysis indicated that exposure to Leydig cells to 1 pg/ml and 100 ng/ml 17α-estradiol resulted in a 10- and 5-fold increases in the expression of ERα, respectively. Similar effects were observed with exposure to equivalent concentrations of 17ß-estradiol. Difference in sensitivity to stereoisomers of estradiol to growth response of Leydig cells did not correlate with the elevated expression of ERα. We conclude that the TM3 Leydig cells are sensitive to the non-typical estrogen, 17α-estradiol, presumably through the activation of ER-independent signaling transduction pathways.

Introduction

17α-Estradiol is a stereoisomer of 17ß-estradiol. It is not a biologically active estrogen *in vivo* (1). Also, in most of the cell culture models, it is estrogenically inactive (2). It has been shown that 17α-estradiol interacts with estrogen receptors (ERs), but with a very weak affinity compared to diethylstilbestrol or 17ß-estradiol. The order of affinity of estrogenic agonists toward ERα is DES >4-OH-tamoxifen >17ß-estradiol > coumestrol >estrone >17α-estradiol (3).

Recently, we have reported that TM3 Leydig cells are estrogen sensitive (4). However, the mechanism of the stimulation of cell growth of Leydig cells by estrogen is not clear. In order to determine whether estrogen-induced proliferation in TM3 Leydig cells occurs through a typical estrogen receptor, we examined the influence of an inert estrogen analogue, 17α-estradiol, on the growth of Leydig cells. Contrary to our expectation, we observed growth stimulatory effects by 17α-estradiol. To understand the mechanism, we measured the expression of ERα and ERß in cells exposed to different concentrations of 17α-estradiol or 17ß-estradiol. The findings revealed that difference in sensitivity to stereoisomers of estradiol to growth response of Leydig cells did not correlate with the elevated expression of ERα.

Materials and methods

Determination of cell growth. The source of chemicals and other products required for cell culture were the same as described previously (4). Prior to treatment, the Leydig cells were cultured using DMEM/F-12 medium supplemented with 5% horse serum and 2.5% fetal bovine serum. The cells were subcultured at a ratio of 1 to 200 during their late log phase growth. The cells (~500,000) were placed into a 75-cm^2 culture flask and allowed to attach for 24 h. The growth medium was replaced with serum-free medium and then treated with 17α-estradiol. The concentrations of 17α-estradiol tested from 10^{-6} to 10^{-12} g/ml. Tamoxifen was co-administered at a 1,000-fold ratio (17α-estradiol to tamoxifen: 10^{-12} to10^{-9}, 10^{-11} to10^{-8}, and 10^{-10} to10^{-7} g/ml). The exposure time for all assays was 72 h. Each trial was conducted in triplicate. The cells were lysed with 1X trypsin-EDTA and counted with a hemacytometer. Cell growth data were analyzed using the Student's t-test along with an analysis of variation. The α was set at 0.007 for the Student's t-test.

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Reverse transcription for cDNA synthesis. Total RNA was isolated using TRIzol® Reagent (Life Technologies, Grand Island, NY) following manufacturer's protocol. The quality of RNA samples was determined by RNA gel electrophoresis. cDNA was synthesized by using SuperScript® Preamplification System for first strand cDNA synthesis (Life Technologies, Grand Island, NY) following the manufacturer's protocol (4). Briefly, 2 μg of total RNA was treated with 2 units of amplification grade DNase I at room temperature for 15 min. cDNA was synthesized by reverse transcription at 42°C for 50 min in a final volume of 20 μl containing of 0.5 μg Oligo (dT), 200 units of SuperScript II reverse transcriptase, 2 μl of 10X PCR buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4), 2.5 mM $MgCl_2$, 125 μM of each dNTP, and 0.01 M DTT. The mixtures were then incubated with 2 units of *E. coli* RNase H at 37°C for 20 min to remove RNA.

PCR conditions for ERα, ERβ and β-actin. Oligonucleotide primers were synthesized in UAB Comprehensive Cancer Center Oligo Core Facility. We performed amplification of ERα, ERß and ß-actin as described previously (4) using the primer sequences of ERα (sense: 5'-CCG GGG AAG CTC TTT G-3'; antisense: 5'-AGA GAT GCT CCA TGC CTT TGT TAC-3'), ERß (sense: 5'-AAA GCC AAG AGA AAC GGT GGG CAT-3'; antisense: 5'-GCC AAT CAT GTG CAC CAG TTC CT-3') or ß-actin (sense: 5'-GTG GGC CGC TCT AGG CAC CA-3'; antisense: 5'-CGG TTG GCC TTA GGG TTC AGG GGG G-3'). In brief, each 25 μl of PCR mixture contained 0.5 μl of RT reaction mixture (cDNA), 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer, and 1 unit of AmpliTaq® DNA polymerase. Amplification was performed in a RoboCycler® (Stratagene, La Jolla, CA). After amplification, the PCR mixture was analyzed on a 1.5% agarose gel and detected by ethidium bromide.

Results

17α-Estradiol exposure to Leydig cells significantly induced growth of TM3 Leydig cells ($p<0.05$) (Fig. 1). The concentrations of 1 pg/ml and 100 ng/ml 17α-estradiol produced significant increases of 57% and 25% in cell growth, respectively ($p<0.05$). The growth response was biphasic with peaks at 1 pg/ml and 100 ng/ml 17α-estradiol. Inhibition of cell growth was observed with 1 μg/ml 17α-estradiol. We have recently reported that exposure to 1 pg/ml and 100 ng/ml diethylstilbestrol or 17ß-estradiol produced 86 and 148% or 25 and 12% increases in the growth of Leydig cells, respectively, compared to the growth of untreated cells (3). A comparison of 17α-estradiol-mediated growth stimulation observed in this study with our previous report (4) of diethylstilbestrol and 17ß-estradiol showed that 17α-estradiol is more potent growth stimulating agent than that of 17ß-estradiol.

Treatment of Leydig cells with tamoxifen alone up to 100 ng/ml had no effect on cell growth, although higher concentrations resulted in cytotoxicity. Coadministration of tamoxifen significantly blocked 17α-estradiol-mediated cell growth ($p<0.05$) (Fig. 2).

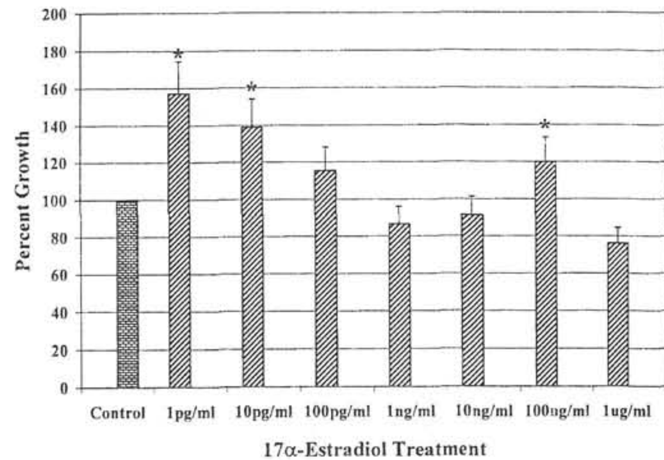

Figure 1. Induction of growth of Leydig cells exposed to 17α-estradiol. Cells were exposed to 7 different concentrations of 17α-estradiol and cell growth was measured after 72-h exposure (4). Each value is a mean of three experiments. Data are presented as percent growth (controls set at 100%). *Indicates significant difference from controls ($p<0.05$).

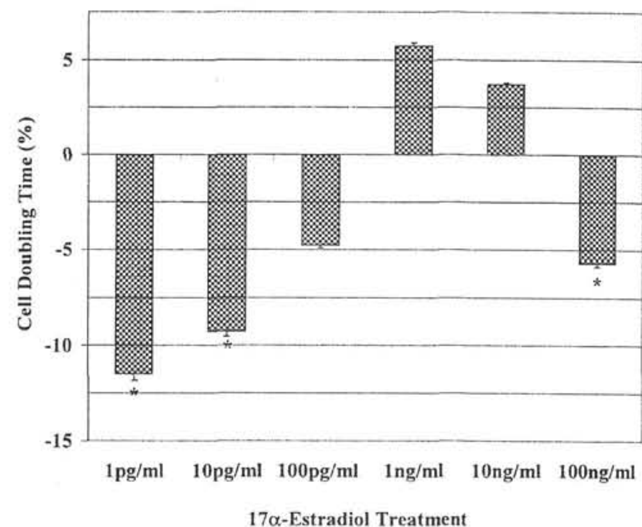

Figure 2. Reduction of Leydig cell doubling time by 17α-estradiol. Cells were exposed to 7 different concentrations of 17α-estradiol, cell growth was measured after 72-h exposure and cell doubling time was calculated (4). Each value is a mean of three experiments. Data are presented as percent growth (controls set at 100%). *Indicates significant difference from controls ($p<0.05$).

Cell doubling time was significantly reduced by 17α-estradiol treatment ($p<0.05$) (Fig. 3). The pattern of this response was inverse to that of the cell growth shown in Fig. 1. This suggests a strong correlation between 17α-estradiol-mediated cell growth and the reduction of cell doubling time.

We also measured the expression of the estrogen receptor ERα and ERß in Leydig cells exposed to 1 pg/ml and 100 ng/ml 17α-estradiol or 17ß-estradiol. We could not detect ERß transcripts in these TM3 Leydig cells. 17α-estradiol exposure resulted in a ~10-fold increase of ERα expression at 1 pg/ml and a ~5-fold induction at 100 ng/ml (Fig. 4). Similar effects on ERα expression were observed with exposure of

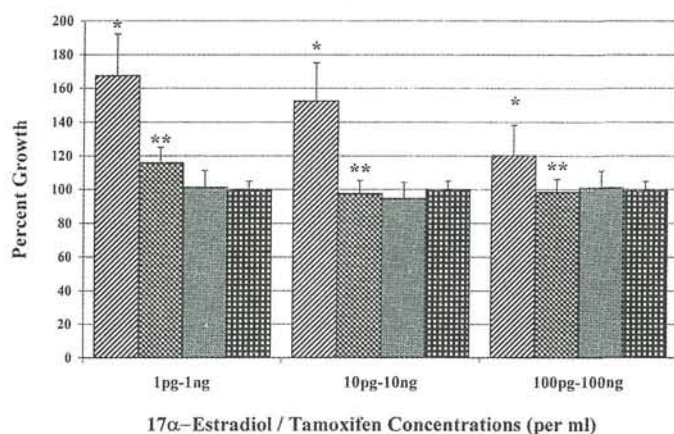

Figure 3. Inhibition of 17α-estradiol-induced growth of Leydig cells by tamoxifen. Cells were exposed to 3 different tamoxifen concentrations (1 ng/ml, 10 ng/ml, and 100 ng/ml), 3 different 17α-estradiol concentrations (1 pg/ml, 10 pg/ml, and 100 pg/ml), or 3 combination treatments (17α-estradiol + tamoxifen 1 pg/ml + 1 ng/ml, 10 pg/ml + 10 ng/ml, and 100 pg/ml + 100 ng/ml) for 72 h. Each value is a mean of three experiments. Data are presented as percent growth (controls set at 100%). *Indicates significant difference from controls ($p<0.05$) and **indicates a significant reduction of 17α-estradiol-induced growth of Leydig cells compared to 17α estradiol + tamoxifen ($p<0.05$). Striped, 17α-estradiol; Hatched, 17α-estradiol + tamoxifen; Dotted, tamoxifen; Checked, control.

Figure 4. Levels of ERα and ERß in Leydig cells exposed to 1 pg/ml and 100 ng/ml 17α-estradiol or 17ß-estradiol. TM3 Leydig cells were exposed to 1 pg/ml and 100 pg/ml 17α-estradiol or 17ß-estradiol for 72 h. RNA was isolated and RT-PCR analysis was carried out as described previously (4). Lane 1, DNA ladder; lanes 2-4, ERα, 2, untreated cells; 3, 1 pg/ml 17α-estradiol treated; 4, 100 ng/ml 17α-estradiol treated; lanes 6-8, ERß; 6, untreated cells; 7, 1 pg/ml 17α-estradiol treated; 8, 100 ng/ml 17α-estradiol treated; lanes 11-13, ERß; 11, untreated cells; 12, 1 pg/ml 17ß-estradiol treated; 13, 100 ng/ml 17ß-estradiol treated; lanes 15-17, ERß; 15, untreated cells; 16, 1 pg/ml 17ß-estradiol treated; 17, 100 ng/ml 17ß-estradiol treated; lanes 18-22, ß-actin; 18, untreated cells; 19, 1 pg/ml 17α-estradiol treated; 20, 100 ng/ml 17α-estradiol treated; 21, 1 pg/ml 17ß-estradiol treated; 22, 100 ng/ml 17ß-estradiol treated cells.

cells to equivalent concentrations of 17ß-estradiol (Fig. 4) and DES (4).

Discussion

Most of the estrogen sensitive cells do not respond to 17α-estradiol (1,2). Findings of this study revealed that 17α-estradiol stimulated the growth of TM3 Leydig cells in a biphasic manner; this effect was blocked by an antiestrogen, tamoxifen; and 17α-estradiol lowered cell cycle doubling time. Our finding is in agreement with previous observations indicating that 17α-estradiol increases the growth of breast MCF-7 and pituitary GH4C1 cells (2,5,6). The enhanced growth stimulatory potency of 17α-estradiol in Leydig cells raises the question of whether the stereo-specificity and thus, the sensitivity of the estrogen receptor in C7MCF7-173, GH4C1 or TM3 cells may be different than those estrogen sensitive cells not responding to 17α-estradiol.

Inhibition of 17α-estradiol's effect on cell growth by tamoxifen suggests that the stimulatory growth response by 17α-estradiol may occur presumably through an estrogen receptor pathway. We can rule out the involvement of ERß in this process, because we could not detect ERß transcripts in the TM3 Leydig cells. ERα is induced in these cells to the same extent by the equivalent doses (1 pg/ml and 100 ng/ml) of both 17α-estradiol and 17ß-estradiol. We have recently reported that 1 pg/ml and 100 ng/ml doses of 17ß-estradiol produce 25% and 12% increases in cell growth, respectively, over the control (4); whereas in this study we observed that equivalent doses of 17α-estradiol produced 57% and 25% increases in cell growth, respectively, compared to untreated cells. We did not observe the same pattern of changes in the expression of ERα in Leydig cells exposed to 17α-estradiol or 17ß-estradiol. This seems to suggest that change in ERα levels does not correlate with difference in sensitivity to stereoisomers of estradiol to growth of TM3 Leydig cells.

Comparison of the growth response of Leydig cells observed in this study with our previous findings of diethylstilbestrol and 17ß-estradiol (4) revealed that the order of growth stimulatory response of Leydig cells was diethylstilbestrol >17α-estradiol >17ß-estradiol. It is known that the interaction of 17α-estradiol with ER is very weak compared to that of 17ß-estradiol (2,3). It appears that the growth response of 17α-estradiol may be mediated through a non-classical ER-independent mechanism which is similar to that described in other tissues of neural crest origin. The growth stimulatory rank order observed in this study is very similar to the observations showing the ability of estrogenic compounds to interact with non-ER membrane receptor protein (diethylstilbestrol >17α-estradiol >17α-ethinyl estradiol >17ß-estradiol) (7). The biologically active effects of 17α-analogue is similar to 17ß-analogue of estradiol are also evident from the effects of these isomers on tyrosinase activity in skin melanocytes, prolactin production in monkey pituitary cells and relaxation of coronary vessels (6,8-10). The genomic mode of action is believed to represent the predominant effect of an estrogen. Recently, however, rapidly manifesting non-genomic effects have also been observed (7,8). The non-genomic effects seem to be mediated most likely by allosteric interaction of an estrogenic molecule with heterologous target structures such as membrane protein or via cell signaling networks. Our results of this study are compatible with the idea that 17α-estradiol causes growth stimulatory effects presumably by acting directly on the cell membrane, which, in turn, may activate ER-independent signal transduction pathways.

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