The biphasic stimulation of proliferation of Leydig cells by estrogen exposure

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Abstract. In this study, we have examined the influence of diethylstilbestrol (DES) and 17ß-estradiol on the proliferation of TM3 Leydig cells, a normalized mouse cell line. Cells were treated with seven different concentrations (1 pg-1 µg/ml) of DES or 17ß-estradiol, and cell growth was measured at 24-, 48-, and 72-h periods. DES treatment resulted in a significant ($p<0.05$) stimulation of cell proliferation. We observed two independent peaks of cell proliferation, one at 1 pg/ml DES (186.87%) and the other at 100 ng/ml DES (248.23%). Cytotoxicity was noted at all time periods with 1 µg/ml DES treatment. 17ß-estradiol treatment resulted in a significant stimulation of cell proliferation ($p<0.05$) with a trend similar in response to that of DES treatment, as peak proliferation was noted with 1 pg/ml 17ß-estradiol (125.27%) and 10 ng/ml 17ß-estradiol (138.31%). Based on these data, it appears that DES is more mitogenic in these Leydig cells compared to 17ß-estradiol. Furthermore, for the first time, we detected that both DES and 17ß-estradiol were able to stimulate proliferation of Leydig cells in a biphasic fashion. Cell cycle kinetic analysis revealed that cell entry into the S-phase was higher in the DES treated cells compared to the controls, and doubling times of DES exposed cells were significantly reduced ($p<0.05$). Co-administration of tamoxifen at a concentration 1000-fold higher than either DES or 17ß-estradiol resulted in complete inhibition of cell proliferation. Analysis of expression of ERα and ERß by RT-PCR in untreated Leydig cells, as well as Leydig cells exposed to 1 pg/ml DES, revealed that the transcripts of ERα and ERß were not detectable even after 40 cycles of amplification. A 100-ng/ml dose of DES induced ERα expression by 20-fold. These data suggest that estrogen exposure-mediated increases in cell proliferation, coupled with the decrease in cell cycle time, may allow greater accumulation of DNA damage to occur in the testicular target cells compared to untreated cells under normal cell cycle control. In addition, an unidentified estrogen receptor may be responsible for the mitogenic activity of estrogens at low levels.

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Introduction

Estrogen was once thought to be chiefly a female sex hormone. However, it is now being realized that estrogen plays a critical role in male reproductive tissues. Recently, high levels of 17ß-estradiol have been observed in spermatogonia and sperms (1). Estrogen at pharmacological levels produces adverse effects in the male reproductive organs in both humans and research animals. For example, estrogen exposure in humans typically induces testicular atrophy (2,3). The strongest evidence for testicular atrophy comes from pathological reports from postoperative transsexual patients who received chronic estrogen treatment (4,5). Both studies showed that there is a marked narrowing of the seminiferous cords, reduction of Leydig cells to undetectable levels, and loss of typical spermatogonia. In mice and hamsters, estrogen exposure has been shown to produce testicular tumors and initiate unscheduled DNA synthesis (6-8). Estrogen dependent tumors of the testis in animals and humans have also been reported (9-19). Many estrogen sensitive tumors have been reported to secrete estrogen, suggesting a self-proliferative role for estrogen in the etiology of hormonal carcinogenesis (9,12,20,21).

The mechanism of estrogen-induced testicular carcinogenesis is not clear. In this study, we have examined the influence of diethylstilbestrol (DES) and 17ß-estradiol on cell proliferation and on cell cycle kinetics of TM3 cells, a normalized mouse Leydig cell line, as well as the expression of estrogen receptor α (ERα) and ß (ERß) by DES. DES is a potent synthetic estrogen, whereas, 17ß-estradiol is the most common naturally occurring estrogen. Highly novel findings emerged from this study that both DES and 17ß-estradiol induced a biphasic stimulatory response in cell proliferation and reduced cell cycle time.

Materials and methods

Chemicals. Trypsin-EDTA (1X) and Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) (1:1) mixture containing 1.2 g/l sodium bicarbonate, 15 mM HEPES, and 3.5 g/l glucose, was purchased from Life Technologies, Grand Island,

NY. DES, tamoxifen, horse serum, fetal bovine serum, DMSO, and propidium iodide were purchased from Sigma Chemical Company, St. Louis, MO. TM3-Leydig cells (CRL-1714) were purchased from ATCC, Rockville, MD. Corning® 75-cm² culture-flasks were purchased from Fisher Scientific, Pittsburgh, PA.

Cell culture preparation. Prior to treatment, the cells were cultured using DMEM/F-12 medium supplemented with 5% horse serum and 2.5% fetal bovine serum. The growth medium was changed once every 2 to 3 days. Cultures were sub-cultured at a ratio of 1 to 200 during their late log-phase growth.

Cell growth assay. The cells (≅500,000) were placed into a 75-cm² culture flask and allowed to attach for 24 h. The growth medium was replaced with serum-free medium and then treated with either DES or 17ß-estradiol. The DES and 17ß-estradiol concentrations tested ranged from 10^{-6} to 10^{-12} g/ml. Tamoxifen treatment consisted of three different concentrations (10^{-7}, 10^{-8}, and 10^{-9} g/ml). DES and tamoxifen were co-administered at 1000-fold ratio DES to tamoxifen (10^{-12} to 10^{-9}, 10^{-11} to 10^{-8}, and 10^{-10} to 10^{-7} g/ml). A total of three different exposure times were used for DES proliferation trials (24, 48 and 72 h) and one for 17ß-estradiol and tamoxifen trials (72 h). Each trial was conducted in triplicate. The cells were lysed with 1X trypsin-EDTA and counted with a hemato-cytometer.

For analysis of cell cycle kinetics, the cells were treated using the same methodology. Once the cells were collected, they were prepared for propidium iodide staining by alcohol fixation (22). The freshly stained cells were then measured for cell cycle kinetics by flow cytometry; the data collected were analyzed by ModFit® V2.0.

RNA isolation. Total RNA was isolated using TRIzol® Reagent (Life Technology, Grand Island, NY) following manufacturer's protocol. RNA concentration and $A_{260/280}$ ratio was determined with a UV-spectrophotometer. The quality of RNA samples was determined by RNA gel electrophoresis.

Reverse transcription for cDNA synthesis. cDNA was synthesized by using SuperScript® Preamplification System for first stand cDNA synthesis (Life Technology, Grand Island, NY) following manufacturer's protocol. 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, 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. Negative control was prepared by omitting reverse transcriptase. An aliquot of the reaction mixture was used for PCR reaction.

Oligonucleotide primers. Oligonucleotide primers used in PCR were synthesized in UAB Comprehensive Cancer Center Oligo Core Facility.

RT-PCR for ß-actin. Specific primer sequences (sense: 5'-GTG GGC CGC TCT AGG CAC CA-3'; antisense: 5'-CGG TTG GCC TTA GGG TTC AGG GGG G-3') were used (23). The PCR conditions consisted of: a 25 µl of PCR mixture that contained 0.5 µl of RT reaction mixture (cDNA), 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer, and 1 unit of AmpliTaq® DNA polymerase. The reaction mixture was overlaid with 25 µl of mineral oil. DNA amplification was performed in a RoboCycler® (Stratagene Cloning Systems, La Jolla, CA). The reaction mixture was denatured at 94°C for 4 min, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C. The PCR was completed by a final extension cycle at 72°C for 10 min. After amplification, the PCR mixture was analyzed on a 1.5% agarose gel and detected by ethidium bromide. The size of the amplified product was 245 bp long.

RT-PCR for ERa and ERß. The expression of estrogen receptor subtypes, ERα and ERß was assayed by RT-PCR technique 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') and ERß (sense: 5'-AAA GCC AAG AGA AAC GGT GGG CAT-3'; antisense: 5'-GCC AAT CAT GTG CAC CAG TTC CT-3') (24,25). The PCR was performed under the following PCR conditions: 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 units of AmpliTaq DNA polymerase. The reaction mixture was overlaid with 25 µl of mineral oil. DNA amplification was performed in a RoboCycler. The reaction mixture was denatured at 94°C for 4 min, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 62°C for ERα and 70°C for ERß, and 1 min of extension at 72°C. The PCR was completed by a final extension cycle at 72°C for 10 min. After amplification, the PCR mixture was analyzed on a 1.5% agarose gel and detected by ethidium bromide staining.

Statistical analysis. Cell proliferation 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 as this allows for direct comparison of each sample concentration to trial's control value. The resulting overall α is 0.05, which is also the value used in the analysis of variation. Flow cytometry data were analyzed by ModFit V2.0 cell cycle kinetics.

Results

Effect of estrogen on Leydig cell proliferation. DES exposure to Leydig cells significantly altered cell growth. Exposure to Leydig cells to DES concentrations from 1 pg/ml to 100 ng/ml for 24-, 48-, and 72-h resulted in significant ($p<0.05$) increases in cell proliferation with the exception of 1 ng/ml at 48 and 72 hours and 10 ng/ml at 24 h (Fig. 1). Maximal increases of proliferation by DES were observed with 72-h treatment. We observed two peaks of cell proliferation, one at 1 pg/ml DES (186.87%) and the other at 100 ng/ml DES

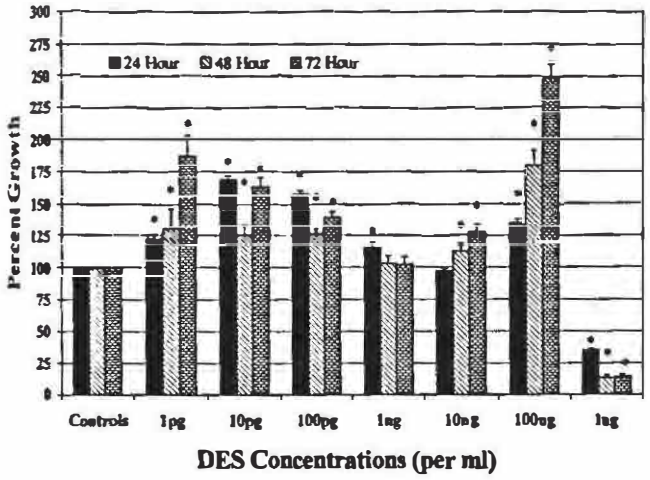

Figure 1 Stimulation of TM3 Leydig cell proliferation by DES. Data are presented as percent growth with controls set at 100%. Results from 24-, 48-, and 72-h treatment times are presented for 7 different DES concentrations. *Indicates treatments significantly different from controls ($p<0.05$) using the Student's t-test. Error bars (T) represent standard deviations.

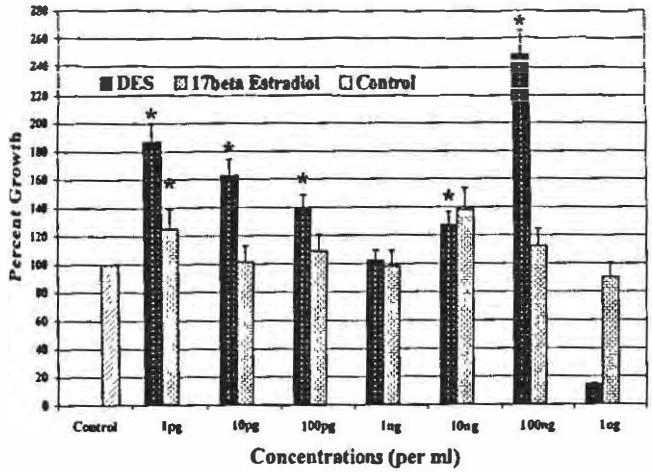

Figure 2 Induction of cell proliferation by estrogen exposure. Data are presented as percent growth with controls set at 100%. Results for 72-h treatment time are presented for 7 different concentrations. *Indicates treatments significantly different from controls ($p<0.05$) using the Student's t-test. Error bars (T) represent standard deviations.

(248.23%). The exposure of Leydig cells to 1 µg/ml DES resulted in a decrease in cell proliferation at all three time periods. Our data reveal two independent stimulatory peaks of cell proliferation in response to DES exposure, one at a physiological dose and another at a sub-pharmacological dose, while cell death occurs at microgram levels.

17ß-Estradiol treatment also induced cell growth in similar fashion. Maximal increases in proliferation were observed at 1 pg/ml and 10 ng/ml. A 17ß-estradiol-mediated decrease in cell proliferation was not observed at 1 µg/ml. A comparison of the DES and 17ß-estradiol data is shown in Fig. 2. With the exception of a slight dose shift (10 ng

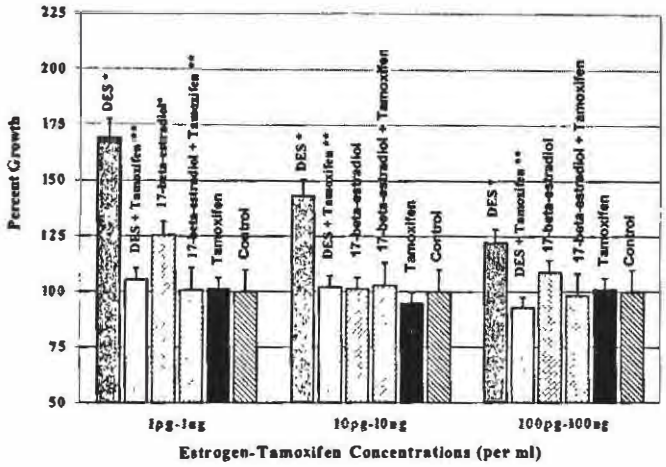

Figure 3. Inhibition of estrogen induced Leydig cell proliferation by tamoxifen. Data are presented as percent growth with controls set at 100%. Results from 72-h treatment time are presented for 3 different tamoxifen concentrations (1 ng/ml, 10 ng/ml, and 100 ng/ml), 3 different estrogen concentrations (1 pg/ml, 10 pg/ml, and 100 pg/ml), and 3 combination treatments (estrogen/tamoxifen 1 pg-1 ng/ml, 10 pg-10 ng/ml, and 100 pg-100 ng/ml) *Indicates treatments significantly different from controls ($p<0.05$) using the Student's t-test. **Indicates a significant reduction of DES-induced proliferation of Leydig cells ($p<0.05$) using the Student's t-test. Error bars (T) represent standard deviations.

17ß-estradiol vs. 100 ng DES), the response between the two estrogens was remarkably similar. However, the magnitude of cell stimulation in the presence of DES was higher than that of 17ß-estradiol.

Tamoxifen treatment of Leydig cells had no effect on cell proliferation when administered alone up to 100 ng/ml. Higher concentrations of tamoxifen decreased cell growth (data not shown). Co-administration of tamoxifen completely blocked DES and 17ß-estradiol-induced proliferation when administered at a 1000-fold greater concentration than that of the test estrogens (Fig. 3). Co-treatment of 1 ng-1 µg/ml DES or 17ß-estradiol with tamoxifen could not be evaluated due to tamoxifen's cytotoxicity when administered at concentration of 1 µg/ml and higher.

Cell cycle kinetics. DES increased entry of the cells into the S-phase ($p<0.05$) (Fig. 4). Maximal number of cells in S-phase were observed at 1 pg/ml (26.64%) followed by 100 ng/ml (24.31%) and 10 ng/ml (18.26%) DES when compared to the controls (10.04%) ($p<0.05$) (Fig. 5). The cell cycle kinetic analysis also revealed that doubling times of DES-exposed cells were significantly reduced compared to untreated cells (Fig. 6).

Estrogen receptor analysis. To understand the mechanism by which estrogens might have stimulated cell growth and increased S-phase entry, we examined the influence of stilbene estrogen on the estrogen receptor subtypes, ERα and ERß. DES concentrations of 1 pg/ml and 100 ng/ml, shown above to give maximum increases in cell proliferation, were used for their influence on estrogen receptor expression Analysis of expression of ERα and ERß by RT-PCR in

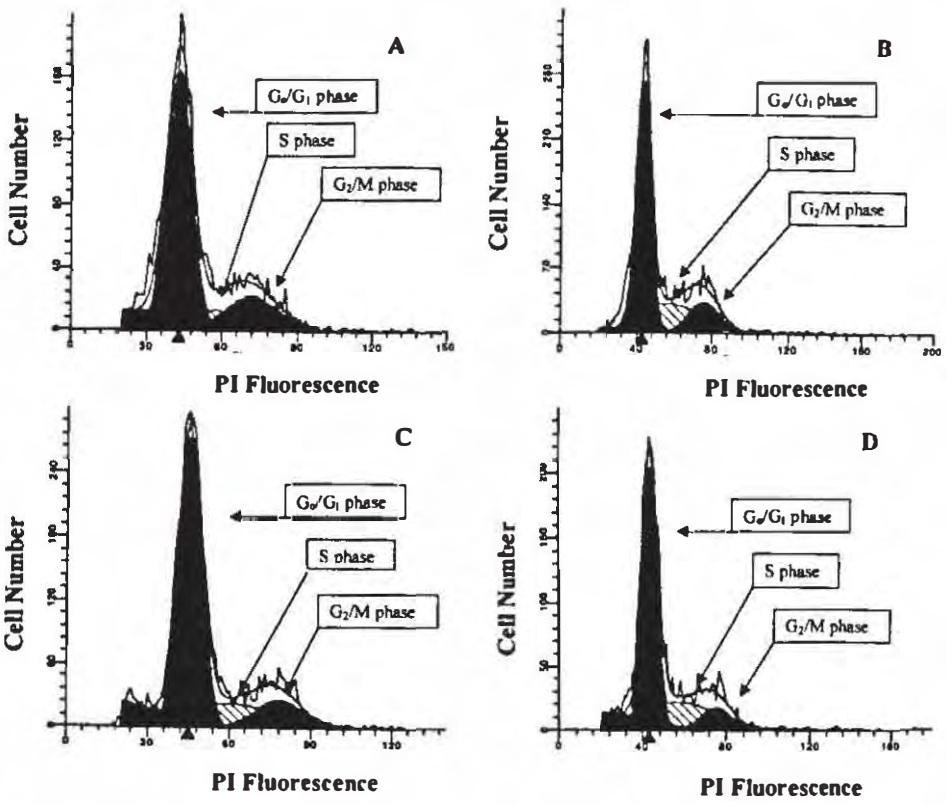

Figure 4 The effect of DES treatment on the cell cycle kinetics of TM3 cells was determined by flow cytometry. Cells were treated with either 1 pg/ml, 10 ng/ml, or 100 ng/ml DES 72 h prior to staining with propidium iodide. ModFitLt V2 0 was used to complete the analysis of cell cycle kinetics. A, Control. B, 1 pg/ml DES; C, 1 ng/ml DES; D, 100 ng/ml DES.

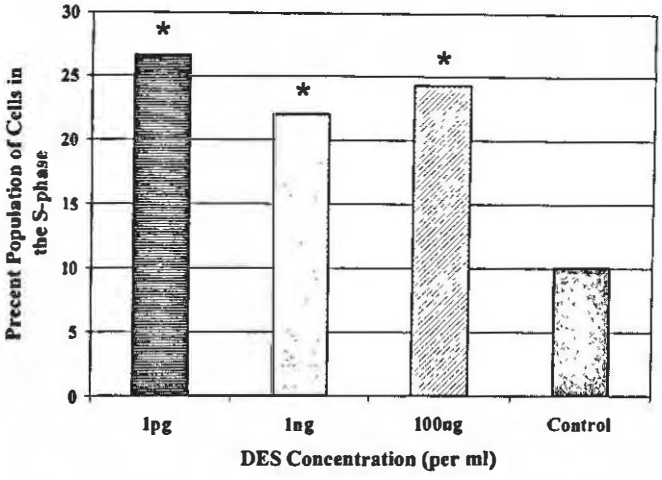

Figure 5. Induction Leydig cell entry into S-phase by DES. Results are from a 72-h treatment time at 3 different DES concentrations. *Indicates treatments significantly different from controls ($p<0.05$) using the Student's t-test.

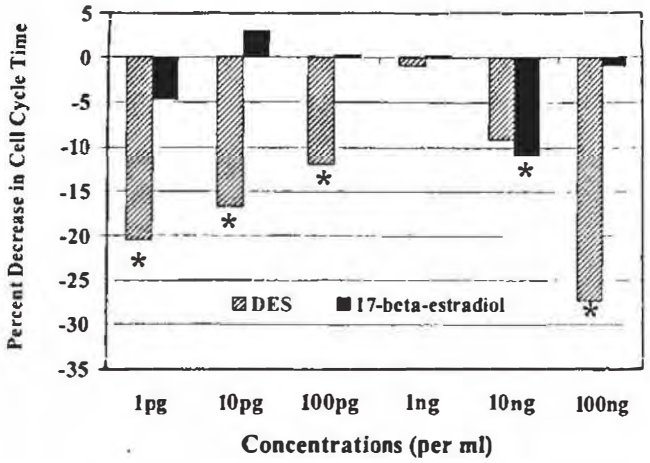

Figure 6. Reduction of Leydig cell cycle time by estrogens Data are presented as percent growth with controls set at 100%. Results from the 72-h treatment time for 6 different concentrations. *Indicates treatments significantly different from intra-trial controls ($p<0.05$) using the Student's t-test. Error bars (T) represent standard deviations

untreated Leydig cells revealed that the transcripts of ERα and ERß were not detectable even after 40 cycles of amplification (Fig. 7). A 100-ng/ml dose of DES, shown above to produce maximum cell proliferation, induced ERα expression by 20-fold. A 1-pg/ml dose of DES, also shown above to produce significant cell proliferation, did not induce the expression of ERα. Both 1 pg/ml and 100 ng/ml doses of DES failed to induce expression of ERß. These results suggest that the stimulatory response of Leydig cells at nanogram levels of DES is presumably mediated through the

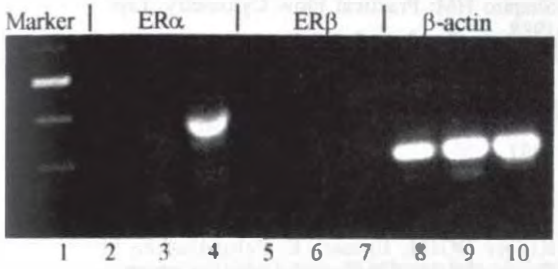

Figure 7. Expression of estrogen receptors ERα and ERß. The results shown here are from RT-PCR analysis of TM3 Leydig cells after DES exposures of 1 pg/ml and 100 pg/ml as well as sham treatment of the control. Lane 1, DNA ladder. ERα lanes 2-4, (2), control; (3), 1 pg/ml DES; (4), 100 ng/ml DES. ERß lanes 5-7, (5), control; (6), 1 pg/ml DES; (7), 100 ng/ml DES. ß-Actin lanes 8-10, (8), control; (9), 1 pg/ml DES; (10), 100 ng/ml DES.

estrogen receptor α. The stimulation of cell proliferation by picogram level of DES is regulated by an estrogen receptor subtype other than that of ERα and ERß.

Discussion

We report for the first time that exposure to stilbene estrogen or natural estrogen stimulates cell proliferation of normalized testicular Leydig cells. Previous studies have failed to demonstrate the influence of estrogen on normalized Leydig cells, presumably due to the use of estrogens in non-effective concentrations. It is clear from this study that not all doses of DES or 17ß-estradiol result in increased growth of Leydig cells. For example, 1 ng/ml DES had no significant effect on cell growth, whereas 1 pg to 100 pg/ml and 10 to 100 ng/ml doses produced stimulation of cell proliferation and cell death at microgram levels. The estrogen-induced growth curve of Leydig cells is also novel, as this is the first report of the apparent biphasic regulation of cell growth by estrogens.

The mechanism of biphasic response by both physiological and sub-pharmacological doses of DES and 17ß-estradiol is not clear. The involvement of ERα in cell proliferation is well recognized, whereas the ERß involvement is questionable. The results of the RT-PCR analysis of the estrogen receptor expression coupled with the tamoxifen data suggest that a novel estrogen receptor may be present in this cell line. Treatment with a 100-ng/ml dose of DES induced the expression of ERα. The same dose of DES failed to induce expression of ERß. This concentration of DES also produces maximum increases of cell proliferation. Based on these findings, it appears that this dose of DES may be increasing cell proliferation via the ERα pathway. The other dose of DES, 1 pg/ml, which also produces maximum cell proliferation, failed to induce expression of either ERα or ERß. However, this dose-mediated stimulation of cell proliferation is inhibited by tamoxifen. Thus, it appears that stimulation of cell proliferation at low picogram dose of DES may not be mediated through ERα and ERß pathways. This argues in the favor of involvement of a new estrogen receptor pathway other than that of ERα and ERß in the stimulation of proliferation of Leydig cells by estrogen. Estrogen availability may be another possible explanation for the biphasic effect. A plasma membrane estrogen receptor that may mediate an accumulation of higher intra-cellular concentrations of estrogen, which then contributes to the overall cell growth response, cannot be ruled out (26).

Since DNA repair is time dependent, any reduction of cell cycle time, as observed in this study, may result in a reduction of DNA repair capacity (27,28). A significant decrease of cell doubling time in response to stilbene estrogen may potentially lend to loss of DNA repair capacity. When this potential decrease in DNA repair capacity is coupled with the endogenous errors associated with DNA replication, a greater accumulation of errors may occur compared to cells under normal cell cycle control. This potential estrogen-mediated decrease in DNA repair activity, in combination with the ability of estrogen metabolites to produce oxidative damage, may be responsible for estrogen induced genomic instability (29,31).

In summary, we have demonstrated that normalized mouse Leydig cells, TM3, are estrogen responsive for both growth and perturbation of cell cycle kinetics. In addition, our data suggest the presence of a new estrogen receptor subtype that may be responsible for estrogen-induced stimulation of proliferation of Leydig cells at picogram levels.

Acknowledgements

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References

1. Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS and Lubahn DB: A role for oestrogens in the male reproductive system. Nature 390: 509-512, 1997.
2. Crisp TM, Clegg ED, Cooper RL, Wood WP, Anderson DG, Baetcke KP, Hoffmann JL, Morrow MS, Rodier DJ, Schaeffer JE, Touart LW, Zeeman MG and Patel YM: Environmental endocrine disruption: an effects assessment and analysis. Environ Health Perspect 106 (Suppl. 1): 11-56, 1998.
3. Venizelos ID and Paradinas FJ: Testicular atrophy after oestrogen therapy. Histopathology 12: 451-454, 1988.
4. Sapino A, Pagani A, Godano A and Bussolati G: Effects of estrogens on the testis of transsexuals: a pathological and immunocytochemical study. Virchows Arch (A) 411: 409-414, 1987.
5. Gooren L: The neuroendocrine response of luteinizing hormone to estrogen administration in heterosexual, homosexual, and transsexual subjects. J Clin Endocrinol Metab 63: 583-588, 1986.
6. Bacon R and Kirkman H: The response of the testis of the hamster to chronic treatment with different estrogens. Endocrinology 57: 255-271, 1955.
7. Fowler KA, Gill K, Kirma N, Dillehay DL and Tekmal RR: Overexpression of aromatase leads to development of testicular leydig cell tumors: an *in vivo* model for hormone-mediated testicular cancer. Am J Pathol 156: 347-353, 2000.
8. Racine RR and Schmid BP: DNA-damaging potential of diethylstilbestrol evaluated in the germ cell unscheduled DNA synthesis assay. Environ Mutagen 6: 211-218, 1984.
9. Clegg ED, Cook JC, Chapin RE, Foster PM and Daston GP: Leydig cell hyperplasia and adenoma formation: mechanisms and relevance to humans. Reprod Toxicol 11: 107-121, 1997.
10. Catala Bauset M, Girbes Borras J, Carmena-Ramon R, Real Collado J and Gonzalez-Bayo E: Gynecomastia and Leydig cell tumor. An Med Interna 14: 131-134, 1997.

11. Assi A, Sironi M, Bacchioni AM, Declich P, Cozzi L and Pasquinelli G: Leydig cell tumor of the testis: a cytohistological, immunohistochemical, and ultrastructural case study. Diagn Cytopathol 16: 262-266, 1997.
12. Nishizawa Y, Yamamoto T, Terada N, Fushiki S, Amakata Y and Nishizawa Y: Effects of antiallergic drugs on the proliferation of estrogen-sensitive mouse Leydig cell line. Anticancer Res 16: 1241-1245, 1996.
13. Bercovici JP, Kerlan V, Monguillon P, Fournier G and Nahoul K: Mid-term and long-term outcome in Leydig cell estrogen tumors. Ann Endocrinol 55: 181-184, 1994.
14. Li JJ and Li SA: Estrogen carcinogenesis in hamster tissue: a critical review. Endoc Rev 11: 524-531, 1990.
15. Li JJ, Gonzalez A, Banerjee S, Banerjee SK and Li SA: Estrogen carcinogenesis in the hamster kidney: role of cytotoxicity and cell proliferation. Environ Health Perspect 101 (Suppl. 5): 259-264, 1993.
16. Li JJ, Li SA, Oberley TD and Parsons JA: Carcinogenic activities of various steroidal and nonsteroidal estrogens in the hamster kidney: relation to hormonal activity and cell proliferation. Cancer Res 55: 4347-4351, 1995.
17. Gelety TJ and Judd KL: Menopause: new indication and management strategies. Curr Opin Obstet Gynecol 4: 346-353, 1992.
18. Okada H: Inhibitory effect of a new androstenedione derivative, 14α-hydroxy-4-androstene-3,6,17-trione on aromatase activity of human uterine tumors. J Steroid Chem 36: 517-521, 1990.
19. Birnbock H and von Angerer E: Sulfate derivatives of 2-phenylindoles as novel steroid sulfatase inhibitors: an *in vitro* study on structure-activity-relationship. Biochem Pharmacol 39: 1709-1713, 1990.
20. Valensi P, Coussieu C, Killian E, Tran-Quang N, Attali JR and Sebaoun J: Leydig cell tumor estrogen secretion: suppression by a gonadotropin releasing hormone agonist. J Endocrinol Invest 14: 583-589, 1991.
21. Yamamoto T, Li I, Uchida N, Nishizawa Y, Yamaoto R, Terada N, Tani-Guchi H, Matsumoto K and Sato B: Stimulation by estrogen and progesterone of the *in vivo* growth of transformed murine Leydig cells at the limited early phase of growth. Anticancer Res 12: 1339-1345, 1992.
22. Shapiro HM: Practical Flow Cytometry. Liss, New York, NY, 1988.
23. Snedekar SM, Brown CF and Di Augustine RP: Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary gland ductal morphogenesis. Proc Natl Acad Sci USA 88: 276-280, 1991.
24. Zhai J, Lanclos KD and Abney TO: Estrogen receptor messenger ribonucleic acid changes during leydig cell development. Biol Reprod 55: 782-788, 1996.
25. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson JA: Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93: 5925-5930, 1996.
26. Moats RK II and Ramirez VD: Electron microscopic visualization of membrane-mediated uptake and translocation of estrogen-BSA: colloidal gold by hep G2 cells. J Endocrinol 166: 631-647, 2000.
27. Friedberg EC, Walker GC and Siede W: DNA Repair and Mutagenesis. American Society for Microbiology, Washington, DC, 1995.
28. Beckman WC, Newbold RR, Teng CT and McLachlan JA: Molecular feminization of mouse seminal vesicle by perinatal exposure to DES: altered expression of messenger RNA. J Urol 151: 1370-1378, 1994.
29. Chen Y, Shen L, Zhang F, Lau SS, van Breemen RB, Nikolic D and Bolton JL: The equine estrogen metabolite 4-hydroxy-equilenin causes DNA single-strand breaks and oxidation of DNA bases *in vitro*. Chem Res Toxicol 11: 1105-1111, 1998.
30. Butterworth M, Lau SS and Monks TJ: Formation of catechol estrogen glutathione conjugates and gamma-glutamyl transpeptidase-dependent nephrotoxicity of 17beta-estradiol in the golden Syrian hamster. Carcinogenesis 18: 561-567, 1997.